Zbigniew Brzozka
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Cardiac Cell Culture Technologies

Microfluidic and On-Chip Systems

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Zbigniew Brzozka
The Chair of Medical Biotechnology,
Faculty of Chemistry
Warsaw University of Technology
Warsaw
Poland

Elzbieta Jastrzebska
The Chair of Medical Biotechnology,
Faculty of Chemistry
Warsaw University of Technology
Warsaw
Poland

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I dedicate this book to my husband Mariusz, for his understanding, boundless faith and endless support…

Ela

Preface

Nowadays, one of the main branches of microfluidic development is cell engineering. A number of the devices for the cell cultivation, *Organ-on-a-chip*, lysis, single-cell analysis, and cell-based toxicity tests are being reported. A variety of the structures that can be created leads to obtaining the devices more closely mimicking the in vivo environment than classic cell culture. The microfluidic devices can mimic the in vivo environment at various levels of its organization. It is expected that research on microfluidic in vivo-like systems will effect in the evaluation of methods that can replace animals in different fields of biomedical research.

The application of the microfluidic systems creates a possibility to revolutionize the methods applied in the cell biology research. Compatibility of the microchannels to dimensions of the cells as well as the ability to control parameters of the cell microenvironment makes them an attractive tool for biological research. Cells and their internal components have physical dimensions of microns so tools developed with a microscale technology are invaluable and well suited for manipulation, testing, and probing in microfluidic environment. Typical single processes as growth, treatment, selection, lysis, separation, and analysis are successfully realized in the microdimensional systems. The *Lab-on-a-chip* devices have a great potential for improving sensitivity and complexity of experiments, where studies of cellular growth and responses to external factors are conducted. The analytical devices integrated with cell culture create a possibility for continuous monitoring of cell behavior and biochemical processes as well. The usage of microscale analytical systems would have a great impact on cell biology knowledge as microfluidic devices have potential to improve the sensitivity and complexity of cellular experiments.

The heart is one of the most important organs and performs a principal task in the organism providing a blood through the vascular bed. Since cardiovascular diseases (CVDs) are known to be the main cause of mortality in humans, there is a huge interest in development of novel therapies for myocardial dysfunction. There are a number of proposed approaches; however, a big hope has been placed in stem cell therapies.

Chapter 1 is an overview of basics of the microfluidic systems for cellular application, presenting important parameters of the microdevices, which have the greatest impact on the cell behavior.

The main advantage to using the microsystems is their ability to imitate in vivo conditions which are missing in static macroscale cell cultures. In the next chapter, the materials which find applications in *Lab-on-a-chip* devices for cellomics, their properties, microfabrication techniques, and examples from the literature were described.

Organ-on-a-chip systems are novel platforms, which imitate the key functions of a living organ, including specific microarchitectures, cell-cell and tissue-tissue interactions, and extracellular communication. Microtechnology offers the possibility of creating more complex, multi-organ platforms known as *Body-on-a-chip* or *Human-on-a-chip*. Such integration allows to conduct research on inter-tissue and inter-organ interactions as well as human metabolism simulation, which plays a key role in studies on toxic and dose-related effects of novel therapies.

The next two chapters introduce two crucial application fields of this volume: characteristics and engineering of stem cells and reconstruction attempts of heart, the most "mystic" of human organs.

Stem cells widely used in the studies aiming to understand and also control differentiation of various cell types as well as to design the therapeutic strategies allowing to treat various degenerative diseases and to regenerate damaged tissues and organs. Current review focuses on the selected studies aiming at their efficient derivation and application in cellular therapies.

We summarize recent advances in therapies of the heart and methods that could be used to enhance their efficacy in clinical application.

Heart-on-a-chip systems are specific types of *Organ-on-a-chip* systems. The aim of the fabrication of such systems is to develop an in vivo-like cardiac model, in which the investigation of cardiac cell processes, as well as the elaboration of new therapies for heart failure, will be possible.

Based on the properly designed microsystems, it is possible to perform rapid drug screening and analysis of the effects of electrical stimulation. Thanks to this, new mechanisms and cardiac cell functions can be discovered and can consequently be useful in regenerative medicine.

In the last chapter, we present cardiac cell culture microtechnologies based on stem cells—the microsystems utilized for stem cells (SC) differentiation into cardiomyocytes (CMs). Various types of SC differentiation performed in *Lab-on-a-Chip* systems are presented in this chapter, including cardiogenesis using biochemical, physical, and mechanical stimulations. Finally, we summarize the research focused on heart regeneration using *Lab-on-a-chip* systems and we outline future perspectives for the microsystems usage for SC differentiation into CMs.

The chapters of this volume were written by well-recognized experts in stem cells and their applications in therapies, in heart diseases, and their therapies based on stem cells. Some of the chapters were written by our research team long-term expertise in *Lab-on-a-Chip* technology or related areas. It was important for us that each chapter gives an overview of the state of the art in the corresponding field.

This was achieved by including a relevant number of references, pointing out a reader where further information can be found, especially in such an interdisciplinary field as *Lab-on-a-Chip* technology. Therefore, we hope that the reader may find the extensive and up-to-date literature lists at the end of the chapters.

Warsaw, Poland

Zbigniew Brzozka
Elzbieta Jastrzebska

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Contributors

Karolina Archacka Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Karolina Anna Bednarowicz Institute of Human Genetics Polish Academy of Science, Poznan, Poland

Edyta Brzoska Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Zbigniew Brzozka The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Magdalena Bulka The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Michal Chudy The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Maria A. Ciemerych Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Areta M. Czerwinska Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Artur Dybko The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Iwona Grabowska Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Ilona Grabowska-Jadach The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Elzbieta Jastrzebska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Dominika Kalinowska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Anna Kobuszewska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Kamil K. Kowalski Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Maciej Kurpisz Institute of Human Genetics Polish Academy of Science, Poznan, Poland

Sandra Skorupska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Patrycja Sokolowska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Aleksandra Szuplewska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Katarzyna Tokarska The Chair of Medical Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

Malgorzata Zimowska Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Abbreviations

AAVS1	Adeno-associated virus integration site 1
ADMSCs	Adipose-derived mesenchymal stem cells
ADSCs	Adipose-derived stem cells
ALK4/5/6	Anaplastic lymphoma kinase
Ang-1	Angiopoietin-1
ASCs	Adult stem cells
Bcl-2-B	Cell lymphoma 2
Bcl-xL-B	Cell lymphoma-extra large
bFGF	Fibroblast growth factor
BMCs	Bone marrow cells
BMSCs	Bone marrow stromal cells
cECM	Cardiac tissue-derived extracellular matrix
CLYBL	Citrate lyase beta-like
CMs	Cardiomyocytes
COC	Cyclic olefin copolymer
CPC	Cardiac cell progenitors
CsA-NP	Cyclosporine A-nanoparticles
CSCs	Cardiac stem cells
cTnT	Cardiac troponin T
CVD	Cardiovascular disease
Cx43	Connexin43
CXCR4	Chemokine receptor type 4
DMOG	Dimethyloxalylglycine
EBOs	Embryoid bodies outgrowths
EBs	Embryoid bodies
ECM	Extracellular matrix
ECV	Effective culture volume
EHT	Engineered heart tissue
EPCs	Endothelial progenitor cells
ERK1/2	Extracellular signal-regulated kinase 1/2

ESCs	Embryonic stem cells
GCP-2	Granulocyte chemotactic protein
GSK3	Glycogen synthase kinase 3
GSK3β	Glycogen synthase kinase 3 beta
HA	Hyaluronic acid
HDAC	Histone deacetylase
hESC-VCs	Human embryonic stem cell-derived vascular cells
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1α
HLA-DR	Major histocompatibility complex antigens of class II
Hmox-1	Heme oxygenase-1
HSCs	Hematopoietic stem cells
iPSCs	Induced pluripotent stem cells
KLF4	Kruppel-like factor 4
LOC	Lab-on-a-chip
LV	Left ventricular
LVEF	Left ventricular ejection fraction
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP9	Metalloproteinase 9
MRF	Myogenic regulatory proteins
MSCs	Mesenchymal stem cells
NF-κB	Nuclear factor kappa-light-chain-B (transcription factor)
Oct-4	Octamer-binding transcription factor 4
PC	Polycarbonate
PCLA	Polycaprolactone acid
PDGF	Platelet-derived growth factor
PDMS	Poly(dimethyl siloxane)
PF	Polyethylene glycol fibrinogen
PGA	Polyglycolic acid
PGAC	Polyglycolic acid cloth
PGS	Polyglycerol sebacate
PI3K	Phosphoinositide 3-kinase
PIPAAm	Poly N-isopropyl acrylamide
PKB/Akt	Protein kinase B
PKG1α	Protein kinase G1 α
PlGF	Placental growth factor
PMMA	Poly(methyl methacrylate)
POC	Point-of-care
PS	Polystyrene
PUMA-p53	Upregulated modulator of apoptosis
SAV	Surface area to volume
SCs	Stem cells
SD	Serum deprivation
SDF1-α	Stromal derived factor 1α

SeV	Sendai viral system
SKMs	Skeletal myoblasts
SMCs	Smooth muscle cells
SOX 2	Sex-determining region y-box 2
TALENs	Transcription activator-like effector nucleases
TGF-β	Transforming growth factor-β
TMZ	Trimetazidine
TNFR	Tumor necrosis factor receptor
VEGF	Vascular endothelial growth factor
VPA	Valproic acid

Chapter 1
Introduction

Elzbieta Jastrzebska and Zbigniew Brzozka

Microfluidics is a quite mature technology, but its medical applications for disease diagnosis and personal therapy studies are still insufficient. The usage of the microsystems for such applications should be supported by elaboration of new diagnostic methods/models because the currently applied ones exhibit many drawbacks. One of the biggest disadvantages of in vitro tests on cell cultures is an oversimplified model, i.e., a cell monolayer. Moreover, the existing protocols for in vitro tests consist of many steps, from preparation of biological material for the assay to the final determination, and are time-consuming. Standard methods require the application of the advanced, expensive instruments, and large amounts of expensive reagents. An application of the microanalytical devices for this purpose seems to be a promising approach/solution to all mentioned problems, and it can increase the experimental throughput.

The development of the microdevices allows for research carried out with three-dimensional (3D) biological model, for example, multicellular spheroids. The 3D arrangement is the factor that affects the cellular phenotype in vivo, including cellular interactions with other cell types, other systemic factors, and extracellular matrix. In situation when there are no other cells, which they could interact with or communicate, cells cultured in vitro do not behave in the same way as components of organs. In a cell monolayer, there is a lack of essential interactions present in vivo, i.e., 3D structure, direct cell-to-cell junctions, or paracrine signaling, which cause the inability of referring results obtained to the drug effect on living organism. Experiments performed on 3D biological model would be more reliable and more similar to the in vivo conditions than those carried out with cells culture in a form of monolayer.

E. Jastrzebska · Z. Brzozka (✉)
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: brzozka@ch.pw.edu.pl

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The main factors that stimulated the rapid development of the *Lab-on-a-Chip* devices are the economic considerations (reducing the costs of analysis, reagent consumption) and the requirements of the new areas as diagnostics in the patient's home, DNA analysis, rapid screening tests. A close cooperation between interdisciplinary research groups from many areas is essential to develop new microtechnologies, which could be useful in medical diagnostics, clinical chemistry, pharmacology, proteomics, metabolomics, biology, tissue engineering, etc. Miniaturization of the diagnostic tools and devices allows, among other things, the transfer of the complex analysis performed in clinical laboratories to non-laboratory environment. It promotes the introduction and dissemination of the concept of *Point-of-care*. One of the most prominent research fields in modern science is research work concerning the application of the integrated systems *Lab-on-a-Chip* in cell and tissue culture and engineering.

Organ-on-a-chip systems, which simulate organ functions, are increasingly developed. Such microfluidic systems are used to investigate cell-cell and cell-extracellular matrix (ECM) interactions as well as to perform cytotoxicity assays of various drugs. Lab-on-a-Chip systems for mimicking of organs such as: liver, skin, lung, kidneys, or breast are presented in the literature. The microsystems used to simulate the vascular system and heart tissue functions (*Heart-on-a-chip* systems) are also fabricated. They play an increasingly important role in biomedical sciences. It results from the fact that cardiovascular diseases (CVDs) are the most frequent causes of death over the world. There are many types of treatments and therapies of heart diseases. However, development of new methods for the improvement of heart functions is still needed. In vivo-like cardiac models created in *Heart-on-a-chip* systems can be used to develop new cardio-therapies. Two approaches of *Heart-on-a-chip* systems are proposed: the microsystems, which mimic a beating heart tissue and the microsystems, which mimic a whole vascular system. The developed microsystems can be used for investigation of cardiac cell processes as well as the elaboration of new therapies for heart failure. The usage of the microsystems for cardiac cell engineering have many benefits resulted from miniaturization, e.g., similar microstructure dimensions to cell dimensions, a laminar flow, high surface-to-volume (SAV) ratio, and effective culture volume (ECV). However, it should be underlined that conditions, which are specific for heart cells can be created in microscale. Features such as: dynamic condition, electrical field, stretching, hydrogels, and nanofibrous are used to mimic a native myocardium. Additionally, heart cell culture in the microsystems is often used to simulate heart diseases and investigate heart regeneration using stem cells (SCs).

Technology of *Lab-on-a-Chip* is an object of interest to companies in the following areas: laboratory diagnostics, pharmaceuticals, biotechnology, nanotechnology, medical devices, biocompatible polymer materials, bioinformatics, and genetic testing. It will also be the object of interest of the companies engaged in programs to discover and develop new drugs and biomarkers, microarray manufacturers and equipment *Lab-on-a-Chip*, antibodies, enzymes and primers, research teams in the following areas: bioinformatics and cancers and clinical practitioners.

Chapter 2
Microfluidic Systems

Sandra Skorupska, Elzbieta Jastrzebska, Michal Chudy, Artur Dybko and Zbigniew Brzozka

2.1 Introduction

Microfluidic technology has a rapidly growing meaning in many fields of science. It results from advantages which offer microtechnology with comparison to conventional analysis methods. Small volumes of reagents, low power consumption, flexibility, and adaptability to different experimental conditions and purposes these are some of benefits. Additionally, the microfluidic systems are easy to use and have a great potential for automation as well as they are compatible with a commercially available laboratory equipment. They are characterized by less material consumption, less waste production as well as saving time and costs (Zhang et al. 2016).

Analytical analysis using microdevices was performed in 1979 for the first time. Terry et al. created a miniature gas chromatograph made of silicon wafer (Terry et al. 1979). After that, many researchers put an impact on developing miniaturized separation devices for gas as well as liquid chromatography. There was also a rapid progress in high -throughput on-chip capillary electrophoresis systems for several purposes, for instance separation amino acids or deoxyribonucleic acid (DNA) analysis (Harrison et al. 1993; Woolley and Mathies 1994). The researchers started to expand applications of the microsystems for new purposes: inorganic synthesis (Lee et al. 2008), proteomic analysis (Dodge et al. 2006), or cell cultures (Leclerc et al. 2003). Nowadays, the microdevices are widely used in chemistry, biology, and pharmacology. In microscale, there is a possibility to carry out inorganic synthesis (for instance quantum dots) or determine a concentration of analyte (for instance uric acid) (Grabowska et al. 2008). The researchers developed the microsystems for molecular biology purposes (for instance sequencing DNA

S. Skorupska (✉) · E. Jastrzebska · M. Chudy · A. Dybko · Z. Brzozka
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: sskorupska@ch.pw.edu.pl

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(Gan et al. 2014)) and enzymology (Reif et al. 2010). Moreover, there are many microdevices for cellular applications such as cytotoxicity tests (Becker et al. 2017), research on single cell (Altinagac et al. 2016), cell metabolism study (Lee et al. 2016), or effectiveness of new therapy (Ahn et al. 2016; Jastrzebska et al. 2016a). The microfluidic systems for cell cultures will be widely discussed in this chapter.

Nowadays, in vitro cell cultures are used in many fields of science such as molecular biology, biotechnology, analytical chemistry, and biosensors. They also play an essential role in pharmaceutical industries. In vitro cell cultures are used to design models of native tissues and cell-based assays for drug screening and investigation on cell response to external factors. Furthermore, they fulfill the 3Rs rule (i.e., replacement, reduction, refinement), which requires minimalizing a number of tests on animals, propagating alternative methods such as in vitro cell cultures. Conventional in vitro cell cultures are performed as two-dimensional (2D, monolayer) model on a flat surface. In an organism, cells are exposed to multiple signals, which are changing in time and space, such as nutrients, growth factors, ions, and biochemical signals. Furthermore, there are also presented direct cell–cell and cell–extracellular matrix (ECM) interactions. Conventional in vitro cell culture do not provide proper environment to mimic state that occurs in living organisms what certainly influences on the cellular response. This can cause differences in drug efficiency between in vitro research and human responses. Moreover, analyzes of cells in macroscale have several important drawbacks. First of all, such studies require a great number of cells and large volumes of reagents. Additionally, a single cell analysis is complicated and time-consuming in macroscale. The microfluidic systems for cell engineering (*Cell-on-a-chip*) are one of the solutions, which can improve current biological techniques or develop a novel method to investigate cellular interactions and responses to external factors. First microsystems were based on cell sorters and detection molecules secreted by the cells (Fu et al. 1999). The microsystems for 2D and three-dimensional (3D) cultures as well as analysis of cell lysis, proliferation, viability, migration, and interactions were developed in the next years. Advanced in vivo-like culture models for investigation of various cell types are being developed nowadays.

The microfluidic systems for cell culture have several advantages. Firstly, benefits from physiological point of view of using microfluidic systems for cell culture will be considered. The organism is built by billions of cells that communicate each other by many networks. These cells are organized in functional units such as tissue, organs, and organs' systems. The nutrients are delivered to cells by a complex network of blood vessels. The nutrition occurs at the cellular level what correspond to the microscale. Due to unique properties and broad possibilities of modification of the microdevices, it is possible to mimic natural cells' environment. Micrometer-sized chambers where cells are seeded and narrow channels with laminar flow of medium correspond to nature physiological environment. Furthermore, it is possible to create a model of interaction between different kinds of cells representing various tissues and organs by culturing them in one microdevice. That kind of the microsystems is called *Organ-on-a-chip* or *Human-on-a-chip* (Cho and Joon 2017; Jastrzebska et al. 2016b). They allow to

communicate between distant cells, what is difficult or even impossible in macroscale. Such systems provide more information about cells response to many external factors. Moreover, they can be used in personal medicine to study individual responses to drugs to establish the most effective treatment.

Secondly, cell cultures in vitro can be realized in static as well as dynamic conditions. In both, nutrients are delivered through microchannels where the fluid flow is always laminar. The mass transport occurs only in diffusion way. This feature makes appropriate conditions for many applications such as cells separation, immunoassays, and various analyzes on cells. Furthermore, it is well known that cells communicate each other by many signals such as autocrine, paracrine, and endocrine factors. It is possible to manipulate cell–cell interactions by the formation of gradient concentration of solutions in a static cell culture or applying various values of a flow rate under perfusion conditions. Moreover, dynamic cell culture enables the imitation of physiological processes, for instance by mechanical stimulation of cells. Due to a wide range of materials that can be used to produce the microdevices and various geometry of the designed channels and chambers, the interactions between cells and ECM can be investigated. It is a huge step to better mimicking physiological environment. Furthermore, it is possible to make 3D cell culture what truly imitate in vivo conditions (Cho and Joon 2017; Khademhosseini and Langer 2016).

At last, economical advantages of using the microfluidic systems for cell culture are also important. The microfluidic systems operate on very small volumes of fluids. Therefore, less volumes of reagents are consumed and also the amount of chemical wastes is reduced. Moreover, the reaction time is shortened, due to the fact that there is large surface-to-volume ratio (SAV) in a microsystem. There are various methods of production of the microdevices—some of them are not complicated and do not require advanced equipment. Currently, there is a wide range of materials for microdevices' manufacturing, which are inexpensive and commonly used (for instance poly(dimethyl siloxane)—PDMS). Furthermore, it is possible to make several tests simultaneously using a single microdevice. It is a first step to make high-throughput platform for biological and pharmaceutical research. Moreover, many different analyzes can be integrated on a single device, what also reduces costs and minimizes a risk of mistakes made by an operator during analytical process (Bhise et al. 2014; Cho and Joon 2017).

There are no doubts that the microfluidic systems have a great application potential in many fields. Due to their unique properties they become more and more popular. Currently, the microdevices are mostly used by academic researchers but it is desired to commercialize the microsystems to make faster analyzes in medical laboratories. Recently, there are some companies that offer standard and customized microdevices for biological as well as analytical applications (Halldorsson et al. 2015). The most important parameters of microfluidic systems will be discussed below.

2.2 Microfluidic Systems for Cellomics

2.2.1 Materials and Sterilization

Materials used for the fabrication of the microdevices for cell cultures should have specific properties. Various types of materials utilized for cell engineering and techniques of microstructure fabrication in these materials are presented in details in Chap. 3. Here, we shortly discuss this aspect. First of all, the materials used for developing *Cell-on-a-chip* systems should be non-toxic and biocompatible. The first microfluidic systems were made of silicon as a substrate. Nowadays, glass and polymers are most commonly used (Minerick and Swalm 2008). Glass has several advantages in comparison to silicon. Firstly, it is transparent, what allows to observe analysis in a real time. Additionally, glass is a hydrophilic material, which provides proper conditions for cell adhesion (for 2D culture). Moreover, glass can be easily bonded to another material. Polymers such as: poly(methyl methacrylate) (PMMA), polylactic acid (PLLA), polycarbonate (PC), and poly(dimethyl siloxane) (PDMS) are widely used for fabrication of the microsystems for cell cultures. PDMS is the most popular of them. It is transparent and non-toxic to the cells. Furthermore, PDMS is permeable to gases what allows their exchange. Different geometries of microchambers and microchannels can be designed for the flow regulation. PDMS can be bonded to materials such as silicon, glass, and other polymers. A great advantage of PDMS is also low price and a simple method of microstructure fabrication (Hashimoto et al. 2013). However, PDMS is a hydrophobic material, which impedes adhesion of the cells. This is advantage for 3D cell cultures and disadvantage for 2D cultures. In this case, surface modifications such as gas-phase processing and chemical modification of PDMS are needed. The most popular methods are plasma treatment, ultraviolet (UV) irradiation, and introducing different proteins, i.e., poly-L-lysine, gelatin, fibronectin, and collagen (Zuchowska et al. 2016).

The microfluidic systems for cell cultures need to be sterilized to ensure aseptic conditions and avoid microbiological contamination (Kakac et al. 2010). The most widely used method of sterilization is autoclaving. It is characterized by a simplicity and a high efficiency. This technique is used for sterilization in both macroscale and microscale. Despite the several advantages, autoclaving can not be applied to sterilize the microdevices very often. There is a risk of the destruction or the geometrical deformation of microchambers and microchannels, because of their very small dimensions. There are several different sterilization techniques that can be used with the microfluidic systems. The exposition of a microdevice to UV light as well as oxygen plasma treatment is widely used. The aseptic conditions in the microdevices are being achieved by flushing it with ethanol, hypochlorite, or sodium hydroxide. It is really important to remove chemicals after sterilization by rinsing a microdevice with phosphate buffer and then with culture medium.

2.2.2 Geometry

The microdevices have several advantages and one of the most important of them is a possibility to manipulate of the microstructure geometry. Due to this feature, it is possible to create appropriate conditions for many different cell cultures as well as various assays. The conditions in the microfluidic systems are characterized by several unique properties. Firstly, the ratio of cell volume to medium volume is generally greater than one. Therefore, the scale of the microstructures corresponds with natural environment of cells. This phenomenon contributes to create conditions that better mimic the in vivo environment. Moreover, small dimensions of microchambers require a lower number of cells to single experiment and also lower volume of reagents. The SAV ratio is another important feature of microsystems' geometry. SAV ratio is high in the microsystems, and therefore, the transport of molecules (e.g., gases and nutrients) by diffusion is more effective. It is very significant feature, due to the fact that cells require continuous gas exchange such as oxygen and carbon dioxide. Furthermore, the control of temperature in the microdevices is also facilitated by high SAV ratio, because of effective heat transport. Temperature of cells environment plays a key role in cell culture and has influence on their morphology and viability. In the microfluidic systems, it is possible to realize uniform thermal conditions and precise temperature control. On the other hand, high SAV ratio contributes to rapid liquid evaporation if the environment is not properly humidified. PDMS, which is most commonly used material for microdevices fabrication, is permeable to gasses. Due to the fact that, the microfluidic systems contain very small amount of fluids, uncontrolled liquid evaporation is dangerous and may lead to changes in cellular microenvironment. In this case, the cellular response could be modified and the results of the experiments could be unreliable. Furthermore, high SAV ratio can contribute to nonspecific adsorption of proteins. The culture medium comprises a wide range of proteins, which play an important role in cellular nutrition and have huge impact on cellular growth. The protein adsorption results in inhomogeneous cellular environment. It can cause differences in cellular growth conditions, and therefore, the cellular response could be altered. This problem can be solved by modification of the microstructure surface with hydrophilic compounds (e.g., poly(ethylene oxide)—PEO) (Wu et al. 2010). Walker and coworkers introduced a new indicator of cell environment in the microfluidic systems—an effective culture volume (ECV). It indicates a cell ability to regulate their microenvironment (Fig. 2.1). The ECV takes into account the influence of mass transport, rate of diffusion, and level of protein adsorption on the surface of microstructures. The ECV is smaller in microscale than in macroscale, and therefore, cells can better control their microenvironment in microscale (Walker et al. 2004).

In the microfluidic systems, cell cultures could be carried out in static (with periodic medium exchanges) or perfusion conditions (with constant medium flow). The most common problems during cell cultures are: how often culture medium has to be changed in a static culture and how to determine a proper flow rate in a

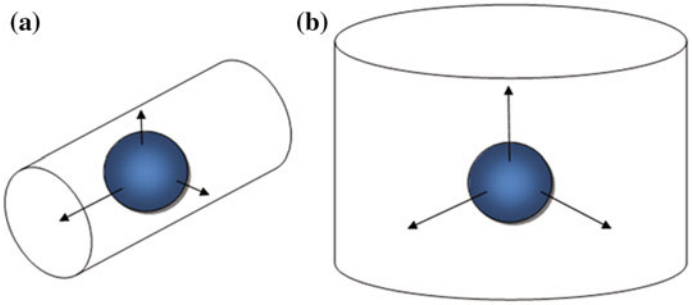

Fig. 2.1 Scheme of effective culture volume (ECV) of a cell: **a** in microsystem—small ECV; **b** in macroscale—large ECV

perfusion culture. To resolve these issues there are another two parameters describing cell culture in microscale: an effective culture time (ECT) and critical perfusion rate (CPR). ECT helps to adjust the time interval between media changes, and CPR indicates appropriate perfusion system to cell culture in the microfluidic systems (Young and Beebe 2010).

2.2.3 Mass Transport

2.2.3.1 Laminar Flow

There are two main types of fluid flow: laminar and turbulent. First one is characterized by calm fluid flow in parallel layers. Due to a high order of the motion of the molecules, there is no any mixing process except diffusion. On the other hand, the turbulent flow describes the chaotic movement of the molecules that contributes to lateral mixing (Whitesides 2006). The type of flow is determined by the value of the Reynolds number (Re) (see Eq. 2.1):

$$\mathrm{Re} = \frac{udp}{\mu} \tag{2.1}$$

where: Re—the Reynolds number, u—fluid velocity, d—a diameter of capillary, ρ—density of the fluid, μ—dynamic viscosity of fluid (Walker et al. 2004).

If the Reynolds number is lower than 2000, the fluid flow is always laminar. In in vivo environments, predominantly laminar flows occur. Therefore, it is important to mimic that condition in research carried out in vitro. Due to small dimensions of microchannels in the microfluidic systems there are very low Reynolds numbers (usually Re < 10) what determines the laminar flow. There are no turbulences and the mass transfer occurs only by diffusion. It was difficult to create that conditions in macroscale; therefore, the fabrication of the microfluidic systems is a huge step to

better mimicking natural cell environment. Different cellular microenvironments even in a single microsystem can be created thanks to the laminar flow. However, it should be noted that laminar flow can be inconvenience in cases where mixing is required (Wu et al. 2010).

2.2.3.2 Diffusion

Diffusion is a phenomenon that describes the molecule movements from regions of higher concentration to regions characterized by a smaller amount of molecules. The flux of molecules is characterized by the first Fick's law of diffusion (Eq. 2.2):

$$J = -D\frac{\mathrm{d}C}{\mathrm{d}x} \tag{2.2}$$

where: J—flux of molecules, D—diffusion coefficient, C—concentration of molecules, x—position.

Diffusion dominates over other transport mechanisms only on short distances. This condition occurs in microscale where the mass transport takes place only in this way. Macroscale cell culture with huge volumes of media requires stirring to deliver nutrients to every cell and to distribute the metabolites and waste. Due to the fact that diffusion is only present in cell cultures carried out in microscale, less homogenous conditions of molecules are also observed (Husain et al. 2010).

2.2.3.3 Gas Transport

In vitro cell cultures are carried out under appropriate conditions, such as temperature and gas concentrations. Usually, the microfluidic systems are stored in a special equipment, such as incubators, where proper conditions are maintained. However, there are some microdevices with an additional equipment for temperature regulation (Tian and Finehout 2008). It is also necessary to provide oxygen and remove carbon dioxide from cellular environment. The regulation of concentration of gasses has an influence on cells metabolism and viability. Gas exchange depends on a microdevice geometry, construction material, cell type, and cell density. The gas concentration is mainly regulated by the usage of external devices (Meyvantsson and Beebe 2008). Workstations and specialized chambers have been used to study cell behavior in different levels of gasses till now. They suffer a several disadvantages such as ability to generate only single uniform concentrations of gasses, slow balance, and difficulties in live-cell microscopic observations. Nowadays, more and more microsystems have been developed to allow control of gasses levels in cell culture. The microfluidic systems seem to be a great platform for exploring the effect of gasses concentrations on the cellular responses. They are used for creation a native cell environment and investigating the mechanism of different diseases as well. The microdevices are based on diffusion from fluid,

mixing or using chemicals producing gasses directly on chip. They allow to control the cell environment including the generation of gradients of gasses. The microfluidic systems are high throughput and compatible with cell imaging platforms, which allow to generate multiple conditions with rapid equilibration times (Brennan et al. 2014).

2.2.4 Mixing

Mixing is required in many chemical and biochemical analyzes. It is very important to ensure homogenous conditions that can be repeated every time in every kind of experiments. In macroscale, mixing can be done using simple equipment, such as magnetic stirrers or vortex. In microscale, the mixing is not easy to obtain because there is laminar flow, where mass transport is achieved by the diffusion. To obtain mixing in the microdevices two main strategies have been developed: passive and active mixers. The first is performed based only a microstructure geometry, second one needs an external energy/factor to work.

2.2.4.1 Passive Mixing

The simplest examples of passive mixing are T- or Y-shaped microchannels where the mixing occurs only due to diffusion. A more advanced passive mixer divide main stream into several narrower where the diffusion process is faster. Then every single flux is merged into a common microchannel (Jeon et al. 2000). To obtain disorderly advection multiple changes in microchannel geometries (Fig. 2.2a, b) or some barriers in the mixers are used. Advection is efficient even at low Reynolds number and may cause fluid re-circulations (Novotny and Foret 2017). There are also 3D mixers, which contain C-, L-, or F-shaped microchannels to make rotational motion of flow on several levels (Liu et al. 2000). The next kind of mixers is based on Coanada effect (Fig. 2.2c). They used the phenomena that fluids flowing from a narrow channel to large space are attached to the curved surface and simultaneously they produced counter-flow what contributes to advection (Hong et al. 2001). Another kinds of passive mixers are vortex micromixers (Fig. 2.2d). They consist of one chamber and several tangential inlets. Due to the geometry, the influx of fluid makes rotations in a chamber and improves the diffusion (Lin et al. 2005).

2.2.4.2 Active Mixing

There are several kinds of active mixers, in which different principles of physics have been used. Firstly, pressure perturbation is used to achieve pulses in the fluid flow and then some deformation in a flux. For instance, the chaotic mixing in the

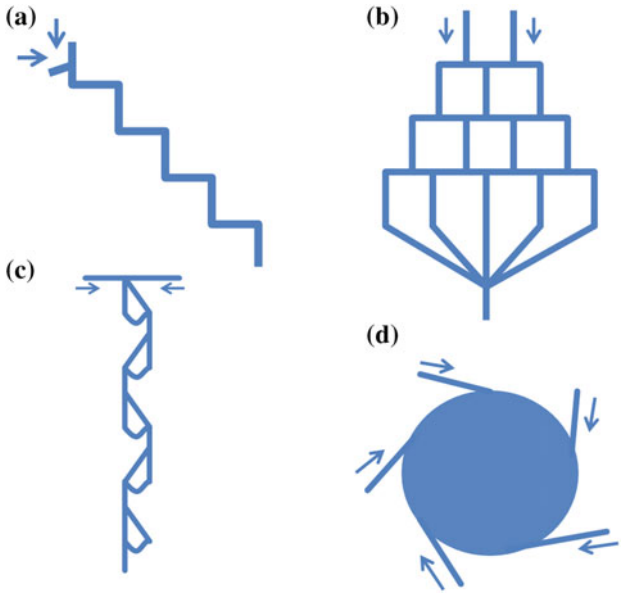

Fig. 2.2 Examples of passive mixers: **a** a zigzag mixer, **b** a microfluidic network, **c** a coanda-effect mixer, **d** a vortex micromixer

main microchannel was accomplished by the application the pressure between opposite channels (Niu and Lee 2003). Another kinds of mixers utilize absorption of acoustic waves by fluid what induces disorderly flux (Fig. 2.3a). This phenomenon was utilized in a microdevice based on trapped air bubbles, which were exposed to acoustic waves. This generated oscillations on the air/liquid surface and finally mixing of the fluids (Ahmed et al. 2009). Third kind of active mixers is actuated by an external magnetic field, which causes oscillations of magnetic microparticles placed in a fluid (Fig. 2.3b). For this purpose, magnetic particles or ferrofluids can be used. The motion of the particles leads to chaotic mixing of fluid (Wang et al. 2007; Oh et al. 2007). Lorentz force can also be used to actuate mixing in the microsystems (Fig. 2.3c). It can be done by subjecting conductive fluids to an external magnetic field, what promotes fluid flow and enhances fluid mixing (Bau et al. 2001).

2.2.5 Concentration Gradient Generators (CGGs)

Many biological and chemical analyzes require an application of a concentration gradient generator (CGG) to examine different phenomena such as chemotaxis or cell stress. There are two main types of gradient generators developed in the microdevices:

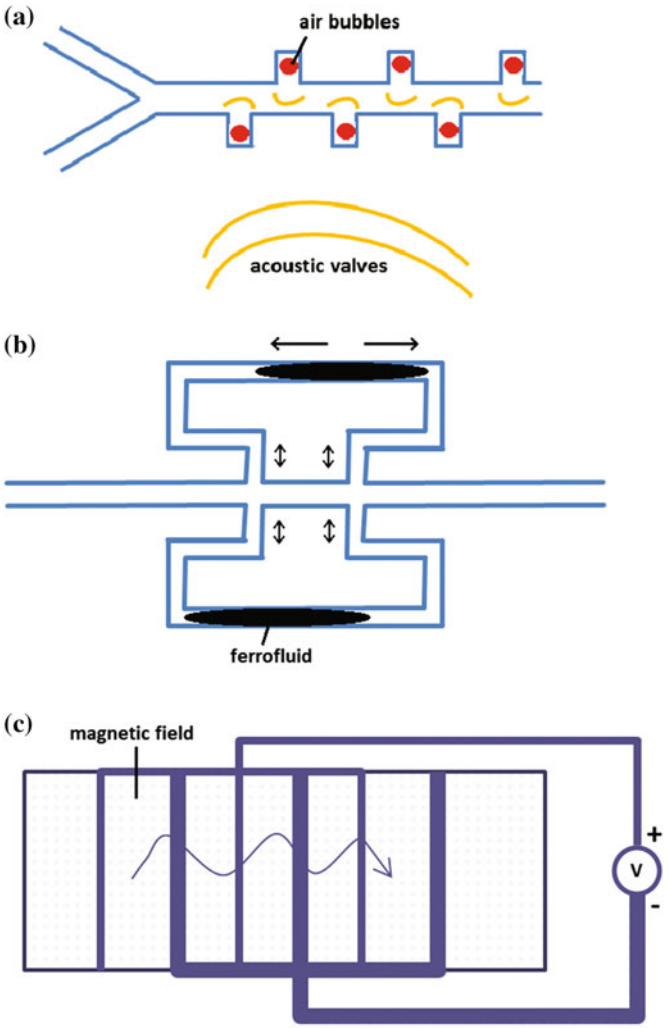

Fig. 2.3 Examples of active mixers: **a** a mixer utilizing absorption of acoustic waves, **b** a mixer with magnetic microparticles oscillating under external magnetic field, **c** a mixer utilizing Lorentz force

- flow-based gradient generator—a fluid flow is used to improve mass transport by convective contribution and advection,
- diffusion-based gradient generator—without a fluid flow, mass transport depends on the diffusion.

Both of them are commonly used in biological research because they allow mimicking of natural cellular environment (Fig. 2.4).

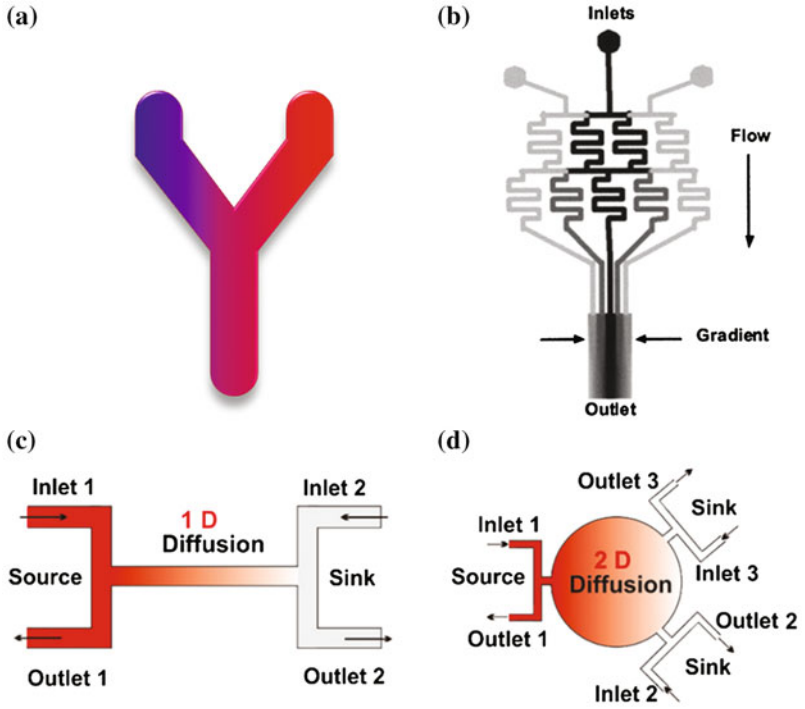

Fig. 2.4 Scheme of different concentration gradient generators (CGGs): **a**, **b**—flow-based gradient generators, **c**, **d**—diffusion-based gradient generators. **b**–**d**: Reprinted with permission from Nguyen et al. 2013. Copyright 2013 Elsevier

Flow-based gradient generators use a direct contact between two flowing streams. The flow velocity increases the diffusion process and generates the advection or convection. In a simple geometry, like T- or Y-shaped microchannels, the gradient region is not homogeneous. To make uniform gradient region a different networks of microchannels are used where streams are separated and mixed repeatedly. These kinds of gradient generators are particularly interesting because dynamic conditions that accurately mimic the natural cellular environment can be obtained. Furthermore, the usage of flow-based gradient generators gives possibility to monitor cellular responses under shear stress.

Diffusion-based gradient generators are based on the diffusion as only one mechanism of mass transport. Usually, a region of the diffusion is connected to several symmetric inlet microchannels with different concentrations of solutions. There are many geometric approaches to develop this kind of gradient generators. An application of diffusion-based gradient generators makes possibility to mimic natural static cellular environment. In such a type of CGG, no shear stress is generated. Therefore, they are mostly used to study: migration of cells at gradient

concentration, pharmacodynamic drug effects, and cellular response to different chemical and biochemical factors.

Flow-based and diffusion-based gradient generators are attractive and powerful tools for making appropriate conditions for wide range of analysis in the microfluidic systems. It is possible to create a cellular environment similar to in vivo and investigate a cellular response to various external factors what was impossible in macroscale. It is especially important for drug industry as well as for understanding the cellular behavior from biological point of view (Nguyen et al. 2013; Oliviera et al. 2016).

2.2.6 Shear Stress

Shear stress is present everywhere, where the fluid flow is applied (Fig. 2.5). This factor is especially important for perfusion cell cultures carried out in the microfluidic systems. It was reported that shear stress can enhance or inhibit cell proliferation, but it is dependent on shear stress value and cell type. To determine shear stress value for 2D perfusion cell culture carried out in the microsystems it is possible to use some equation:

$$\tau = \frac{6\mu Q}{h^2 w} \tag{2.3}$$

where: τ—shear stress, μ—viscosity, Q—flow rate, h—chamber height, w—chamber width.

Several methods are used to obtain the value of shear stress, which is proper for cell culture (e.g., low value of fluid velocity and high depth of culture microchambers are used). Thanks to that culture microenvironment is created, in which affective nutrient delivery and a low concentration of the secreted factors are obtained. Micropillars and microwells are also used for mechanical cell protection. On the other hand, a high level of shear stress is used to examine its influence on some biological aspects such as cell adhesion or function of protein (Kim et al. 2007). Some researchers investigated the effect of fluid flow and shear stress on cell proliferation, changes in cytoskeleton rearrangement, vessels formation, and ability

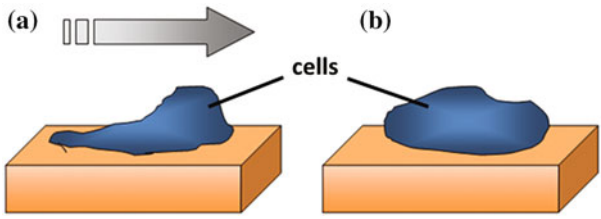

Fig. 2.5 Scheme indicating the shear stress: **a** perfusion cell culture—shear stress is present, **b** static cell culture—no shear stress

of tumor cells to metastasize. Shear stress reduces the number of new sprout vessels formed by endothelial cells. It is a simulation of cancer angiogenesis model. Although further research is needed, this is a huge step toward understanding the phenomenon of cancer metastasis (Song and Munn 2011).

2.3 Perfusion and Static Cell Cultures

The development of the microsytems created new opportunities to improve the biological studies. For instance, it has been possible to create a perfusion cell culture, which better mimics natural cellular environment in vivo. There are two different ways to carry out cell cultures performed in the microsystems: under static or perfusion conditions (Fig. 2.6). Both of them have some benefits and limitations.

Static cell cultures are simply to carry out and widely used and in biological research. The experiments are usually carried out in multi-well plates or Petri dishes but also in the microsystems. The cells are seeded in the culture microchamber, and the medium is exchanged periodically. This kind of cell culture is not preferred to long-term analysis due to the possibility of contamination. Additionally, in this type of culture there are periodic changes in cellular environment due to medium replacement processes. It can cause differences in cellular response. Furthermore, even small alteration in cells environment can affect the cell physiology. Therefore, it is very important to provide stable conditions during all steps of cell-based research.

Perfusion cell culture has several advantages compared with the static culture. Firstly, it is more appropriate for long-term analysis due to sterility resulting from less manual interventions. Furthermore, it is possible to continuously provide nutrients and remove waste what makes cellular environment more stable. It has positive influence on cell physiology and their response to external factors. On the other hand, perfusion cell culture could impede cell-to-cell communication due to

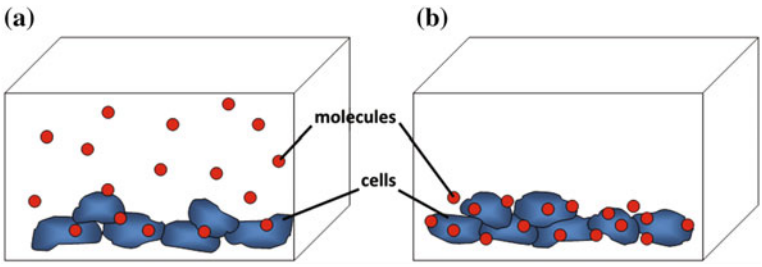

Fig. 2.6 Scheme of perfusion (**a**) and static (**b**) cell cultures: **a** nutrients are continuously provided and waste removed—cellular environment is stable, **b** culture medium with nutrients and waste is periodically changed—cellular environment is changed in time and space

elution of relay molecules. Even that, most of the researches used perfusion conditions because they better mimics natural cellular environment. The cells can be continuously fed and can be exposed to shear stress. Moreover, specific physical environment can be created such as gradient concentrations. These facilities allow to make in vivo-like conditions. Despite many advantages of perfusion cell culture over static cell culture both of them are equally popular and willingly used in scientific research. Choosing a cell culture method depends on the purposes and assumptions of the study and should be carefully thought out (Wu et al. 2010).

2.4 Monolayer and Spatial Cell Cultures

Cell cultures are commonly used in biological research instead of animals and tissue cultures due to easy handling and lower costs. There are two main techniques to carry out cell cultures: two-dimensional (2D, monolayer) and three-dimensional (3D, spatial) cell cultures. Scheme of most popular cultures types formed in the microsystems are shown in Fig. 2.7. The most important question is: does in vitro cell culture can mimic thoroughly the in vivo conditions?

Cellular assays such as drug research are mainly carried out in 2D cultures. In this case, the cells are seeded as a monolayer on the surface. 2D cell culture is a simple model in terms of both the manual operations and cellular behavior. It is well known that standard monolayer is not quite good model of the in vivo environment due to the absence of several biological factors such as signaling molecules (hormones, cytokines, etc.), cell–cell and cell–ECM interactions. All of these agents could affect on cell proliferation, viability or responding to external factors. Furthermore, cells in 2D cultures are exposed to more homogeneous conditions—in vivo the concentrations gradient is often present. For instance, in a tumor, there is cellular heterogeneity due to differences in mass transport. Therefore, there could be differences between cell responses in 2D and 3D cultures.

3D cell cultures better mimic in vivo conditions than monolayer. There are different methods of spatial cell cultures in the microfluidic systems, e.g., cell multilayers, spheroids, hydrogels, scaffolds, bioprinting (Costa et al. 2016; Sung and Beebe 2014; Tomecka et al. 2018; Zuchowska et al. 2017). 3D culture model is really promising due to the fact that it creates appropriate cell culture conditions and

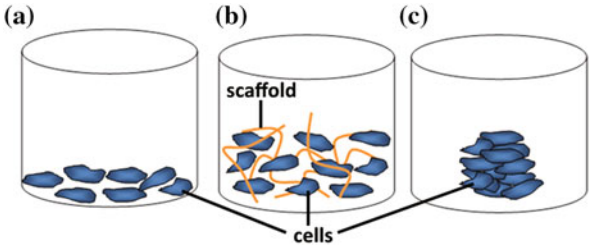

Fig. 2.7 Scheme of different cell cultures performed in the microsystems: **a** two-dimensional monolayer **b** spatial by usage of hydrogels **c** spatial—spheroid model

gives really good approximation of cellular environment in vivo. 3D cell culture allows to create ECM, which has a huge impact on cell behavior. Mechanics and dynamics of microenvironment affect cell viability and resistance to external factors. Moreover, in spatial cell culture, cell–cell interactions and signaling molecules are presented. ECM usually contains collagen, glycoproteins, and polysaccharides. To create ECM in the microsystems, most often are used hydrogels: natural forms and their derivatives such as collagen, chitosan, or alginate. Synthetic hydrogels, for instance poly(ethylene glycol) (PEG)-based, are also widely used due to their biocompatibility. Natural and synthetic hydrogels have some advantages and disadvantages. Firstly, natural hydrogels are components of native extracellular matrix what lead to their bioactivity and provide many adhesion sites. On the other hand, their mechanical properties are complicated and not explained at all. Furthermore, the compositions of them are various due to their different natural sources. Synthetic hydrogels are more reproducible and their chemical composition is well determined. Oppositely, there could be a problem with their bioactivity and adhesion. Sometimes, it is necessary to make additional treatment to promote cell adhesion and proliferation on this kind of materials (Wu et al. 2017). It is worth mentioning that there are also non-scaffold-based 3D cell cultures, named spheroids. Spheroids are microsized aggregates of cells. They are widely used as a model of solid tumor in many cancer drug research (Zuchowska et al. 2017).

Possibility to create 3D cell culture allows to take an attempt to mimic organ microarchitecture. *Organ-on-a-chip* systems, which contained cells simulating organ-level physiology, are developed in recent years. In such systems, different conditions of tissues such as spatiotemporal chemical gradients, mechanical forces, strains, and fluid shear stresses can be created. To design *Organ-on-chip* systems, it is necessary to take into account the following issues (Sung and Beebe 2014; Wu et al. 2017):

- Spatial (scaffold/hydrogel) material

First of all, material used for spatial arrangement of the cells should be biocompatible. It cannot affect on cell viability and morphology. The interaction between spatial material and cells can not change the cellular response to external factors. Furthermore, mechanical stability of material should be known to assure appropriate cell microenvironment. It is important to consider permeability of spatial material to enable migration of molecules.

- Microstructure geometry

Geometry and dimensions of culture microstructures are very important to make proper conditions to cell growth. ECV value should be selected in this way that the cells are able to control culture microenvironment. Furthermore, the microstructure should be specifically designed for each tissue culture. The proper geometry of the microchambers and microwells should be fabricated, when non-scaffold 3D cell cultures (spheroids) are performed in the microsystems. Microstructure should provide possibility to form single spheroid in one microstructure.

- Delivery of fluid flow

Fluid flow is an essential factor which mimics in vivo conditions. Continuous flow regulates the cell-to-cell signaling and also cell-to-ECM interactions. Fluid flow is responsible for delivery of the nutrients and removal of the waste as well. Additionally, it influences cell proliferation (mechanical stimulation).

- Compatibility with analytical method

It is necessary to establish analytical method that could be used to determine cell function in the designed microfluidic system. It is desired to develop the microdevices, which allow high-throughput analyzes.

2.5 Conclusions

The microfluidic systems are more and more popular in chemistry, biotechnology as well as molecular biology. They are also widely used in cell engineering. The microfluidic-based assays for cell study have several advantages compared with macroscale processes. They make a possibility to create more in vivo-like culture environment. This contributes to better mimicking conditions present in a living organism. The microfluidic systems for cell culture are promising tools for drug researches and cytotoxicity studies. It is desired to use them not only in scientific research but also commercially for analytical tests and personal medicine. The microfluidic systems are high-throughput platforms for drug testing which are characterized by better prediction of the human response to external factors. There are some limitations which impede practical applications of the microdevices. Firstly, potential user should be trained in the use of tools and the procedure of analyzes should be simple and clear. Secondly, the results from analyzes must be easy to interpret—the validation and careful verification are needed. Thirdly, receiving results should be fast and high throughput with using widespread analytical techniques. Taking everything into account, the most important challenge for widespread applicability of the microfluidic systems is to simplify the using of them.

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Chapter 3
Lab-on-a-chip Systems for Cellomics—Materials and Technology

Dominika Kalinowska, Katarzyna Tokarska, Ilona Grabowska-Jadach, Artur Dybko and Zbigniew Brzozka

3.1 Construction Materials for Microsystem Fabrication

Various materials such as silicon, glass, polymers, and biomaterials find applications in microfluidic systems for cellomics (Chen et al. 2010; Nieto et al. 2015; Schepers et al. 2016; Verhulsel et al. 2014). The choice of the material for cell engineering is dependent on the specific application of the microsystem. There are many applications involving cell culture studies: cell trapping, counting or sorting, cell lysis and fusion, cultivation, and analysis of the cells. The latter applications require some conditions to be met. In particular, the applied materials must be non-toxic and biocompatible. They should enable gas exchange to provide sufficient oxygen supply. Also, material transparency is desirable in order to observe cell morphology during the experiment or use methods of analysis which allow for optical detection. The material should exhibit appropriate surface properties. The surface of a device needs to be hydrophilic for adherent cells. In turn, to create aggregates and spheroids culture surface should be more hydrophobic, what improves cell–cell interactions. Additionally, the sterilization of a device should be possible and simple. The processing techniques are required to be cheap, easy, and fast. There are not many materials fulfilling each of these criteria at the same time. Therefore, hybrid *Lab-on-a-chip* (LOC) devices are often developed, combining two or more types of materials in one chip (Ziolkowska et al. 2011). The first material used in *Lab-on-a-chip* devices was silicon. It was adapted from *MEMS* (microelectromechanical systems) to chemical and medicine field. Silicon

D. Kalinowska (✉) · K. Tokarska (✉) · I. Grabowska-Jadach · A. Dybko · Z. Brzozka
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: dkalinowska@ch.pw.edu.pl

K. Tokarska
e-mail: ktokarska@ch.pw.edu.pl

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micromachining techniques were very well developed, and therefore, they were employed in the fabrication of other materials, e.g., glass (Fiorini and Chiu 2005). Silicon application for microdevices cell culture is limited because of its lack of transparency (Petersen 1982). However, some elements of *Point-of-care* (POC) devices may be built from this material (Hosseini et al. 2017).

3.1.1 Glass

Glass is one of the materials used to construct Lab-on-a-chip systems. There are many types of glasses, such as soda lime, quartz, Pyrex (Corning 7740), and Foturan used in the microfluidic devices. In spite of the fact that glasses have slightly different features depending on their composition, generally this group of materials exhibits the following properties, which enable them to be applied in biology. Glass is biocompatible, transparent, and a chemically inert insulator with a low auto-fluorescence level (Noori et al. 2008). Because of these qualities, it may be applied in *Lab-on-a-chip* devices with optical detection methods (Krawczyk et al. 2007). Moreover, glass is hydrophilic thus enabling the cells to be adhered to the surface, often without additional surface modification (Sasaki et al. 2001). In manufacturing, the ability to permanently bond glass to various materials such as another glass slide, metals, silicon is important (Allen and Chiu 2008; Kaneda et al. 2012; Schmidt 1998). However, this material also has some disadvantages. Primarily, glass is not gas permeable and should not be used for cell culturing itself (Lou et al. 2014). Additionally, the microfabrication techniques of glass are time-consuming and expensive (Rodriguez et al. 2003).

Taking into account the advantages and disadvantages of using this material, glass remains a popular material for cell culture studies, especially when it is used with polymers. The applications of the glass-based microfluidic systems are often limited to two-dimensional (2D) models and include: cell adhesion and interaction studies, cell capture, cell sorting, cell culture, toxicity assays, and drug screening. For instance, Chen et al. (2010) constructed a microdevice for cell–cell interaction and cell surface adhesion studies. A glass surface was partially modified with poly (ethylene glycol)-terminated oxysilane (PEG-oxysilane) or with fibronectin in order to prevent or promote cell adhesion, respectively. The microchannels were embedded in poly(dimethyl siloxane) (PDMS). Another example of glass-based microdevice application is capturing circulating tumor cells. For example, Nieto et al. (2015) presented a microsystem, in which glass surface was modified with an epithelial cell adhesion molecule (EpCAM) antibody to allow tumor cells (HEC−1A) to be captured. Cell cultures and cytotoxicity assays based on monolayers were also performed in the microsystems with glass substrate (Hattersley et al. 2012). Another approach for the usage of glass-based microsystems is their utilization for the evaluation of photodynamic therapy (PDT) effectiveness (Jastrzebska et al. 2015; Lou et al. 2014). There is only a few scientific groups, which have undertaken research in this field. Lou et al. (2014) described a

microfluidic device for cell culturing and control of parameters such as photosensitizer concentration, oxygen level, light influence, and PDT effectiveness. The device consisted of three layers, where glass layer placed in the middle was used for cell adhesion and culture. The next layer was built from a PDMS gas permeable membrane to control the concentration of oxygen. The last one layer called as a filter layer consisted of: the concentration gradient generators (CGG) to control a flow rate and the light filters to control the light intensity (Lou et al. 2014). Beside sodium glass, a Pyrex borosilicate glass is also used to fabricate the microsystems for cellomics. For example, the microphysiometer based on a Pyrex covered with a PMMA layer was applied for long-term cell culture and measurement of cell metabolism (Weltin et al. 2014).

3.1.2 Polymers

Polymeric materials applied in *Lab-on-a-chip* microdevices exhibit the following properties in comparison with glass: low costs of production, rapid fabrication methods, and easy integration with different materials. Other properties (optical, mechanical, and chemical) may vary depending on the polymer type. Generally, polymers may be divided into three groups: thermosetting (duroplastic), elastomeric, and thermoplastic (Noori et al. 2008). The properties of these polymers are summarized in Table 3.1. Thermosetting materials are not used as a substrate in the microfluidic devices but may be applied in the microfabrication process as resist materials for lithography, e.g., photoresist SU-8 (Becker and Gartner 2008). Elastomers are flexible polymers. PDMS belongs to this group and is commonly used for the fabrication of microfluidic systems. Its properties and applications will be discussed in the next section in detail. Thermoplastic polymers (plastics) are generally cheap, the modification of their surface is possible, and most of them are biocompatible and transparent. Plastics used in the microfluidic systems are: poly (methyl methacrylate) (PMMA), polystyrene (PS), polycarbonate (PC), cyclic olefin copolymer (COC) (Van Midwoud 2012). However, their application in LOC devices is limited in comparison with PDMS. The main reason is the fact that the fabrication techniques for thermoplastics require more expensive and time-consuming equipments, which are good for commercial applications but are not effective during the optimization process on the research stages of studies (Young et al. 2011).

3.1.2.1 Polystyrene

Polystyrene is the most commonly used material for cell cultures in macroscale, e.g., tissue culture plastic flasks and plates. PS is a cheap and commercially available material (Young et al. 2011). This material is biocompatible and transparent. Therefore, PS is more and more applied for development of the microsystem

Table 3.1 Selected properties of most popular polymers applied in *Lab-on-a-chip* systems for cellomics

Polymer	*Glass transition temperature* T_g [°C]	Contact angle	Oxygen gas permeability [$cm^3 \cdot mm/m^2 \cdot day \cdot atm$]	Biocompatibility	Optical transmissivity		References
					VIS	UV	
PDMS	−125 − −122	∼110°	52.5	Excellent	Excellent	Excellent	Tsao and DeVoe (2009)
PS	92−106	∼83°	145	Excellent	Excellent	Poor	Massey (2003)
PMMA	100−122	∼80°	5.8	Excellent	Excellent	Good	Tsao et al. (2007)
PC	140−148	∼93°	92	Excellent	Excellent	Poor	McKeen (2016)
COC	90−180	∼95°	63.5	Excellent	Excellent	Good	Sunkara et al. (2011)

PDMS poly(dimethyl siloxane), *PS* polystyrene, *PMMA* poly(methyl methacrylate), *PC* polycarbonate, *COC* cyclic olefin copolymer

used for cell culturing. Low oxygen permeability is the main obstacle of this material. However, Chan et al. (2014) proposed a polystyrene-based microfluidic device for long-term perfusion cell cultures with three-dimensional (3D) interconnected mesoporous walls. These mesoporous structures were created to increase gas exchanging. Also, the relatively hydrophobic PS surface was turned into hydrophilic after treatment with oxygen plasma for improved attachment and viability of cells in the culturing chamber. The small pieces of PDMS were used on inlet and outlet of channels to avoid leakage. The device was successfully used for long-term C3A human hepatoblastoma cells culture. Another solution which utilizes PS for microdevice fabrication was proposed by Tazawa et al. (2016). The authors presented a microdevice for cell cultivation without a CO_2 incubator. The device consisted of two PS layers: One was a cover, and second was a base for the cell culture. Polytetrafluoroethylene (PTFE) valves were applied to enable medium exchange, and an indium tin oxide (ITO) heater was installed to maintain appropriate cell culture temperature conditions. Additionally, an air bubble trap was built from a glass and silicone tube to avoid bubble contamination. The device enabled a RF/6A 135 vascular endothelial cell to be cultured in one week. Application of PS may also be useful in developing a microfluidic device for cryopreservation of cells in the adhered state. Kondo et al. (2016) described a microsystem that consisted of commercially available PS dish bonded with a PDMS cover layer. The channel structure enabled uniform cell seeding in the cell chamber. The HeLa, NIH3T3, MCF-7, and PC12 cell lines after cell adhesion were cryopreserved in −80 °C for 36 h. After 10 min of thawing at room temperature and 1 h of cell washing with a fresh medium, the cells in the microdevice were ready for tests to be performed.

3.1.2.2 Poly(Methyl Methacrylate)

Poly(methyl methacrylate) is another example of plastics used for microfluidic system fabrication. PMMA is transparent in the visible and (ultraviolet) UV region and exhibits low auto-fluorescence as well. It is also biocompatible and easy to sterilize. These features make PMMA a good material for the substrate of cell cultures. However, this material is not often used for fabrication of LOC devices for cellomics. There are a few examples of the microsystems made of PMMA. They were utilized for monitoring of cell behavior in a perfusion cell culture, long-term cell cultures as well as examination of cell–cell interactions using the co-culture model (Cheng et al. 2008; Stangegaard et al. 2006). Cells such as CL-1 lung cancer cells, macrophages, and osteoblasts MG-63 were investigated in these microsystems.

3.1.2.3 Polycarbonate

Polycarbonate used for microfluidics is low-cost and transparent material. This polymer also exhibits mechanical resistance, low adsorption of moisture, and a low

level of auto-fluorescence. It is hydrophobic, so it can be used for cell cultures after surface modification. The PC microfabrication techniques are cheap and easy. On the other hand, PC absorbs UV radiation and is not resistant to certain organic solvents (Ogonczyk et al. 2010). In the literature, there are some examples of PC-based microdevices used for cell cultures. However, the most often only some elements of the microsystems, e.g., membranes, are built from PC. Altmann et al. (2008) presented a 3D perfusion microsystem for cultivation hepatocytes in a co-culture. The microsystem was based on a polymeric chip with cubic micro-containers structured using microinjection molding. The fabrication process also involved solvent welding a perforated PC membrane to the back of the chip. Another approach was described by Vereshchagina et al. (2013). The authors developed a microdevice for a membrane-supported cell culture in various flow regimes. The microdevice was based on a PMMA chip and PC track-etched membranes. The sealing was obtained using adhesives. The microdevice was used for long-term culturing of HL60 myeloid leukemia cell line and screening antitumor agent mitomycin C.

3.1.2.4 Cyclic Olefin Copolymer

Cyclic olefin copolymer (COC) is biocompatible and has good optical properties such as transparency in the visible and UV region and a low auto-fluorescence level. Therefore, there are some examples in the literature of the application of this polymer to develop microsystems for cellomics (Gel et al. 2014). COC is most often used as a microporous membrane, which is integrated with others materials. For example, a microdevice, based on a COC microporous membrane, was developed for cell cultivation and mimicking of the barrier tissue structure in bone marrow microvascular niche.

3.1.2.5 Poly(dimethyl siloxane)

Poly(dimethyl siloxane) is one of the most commonly used materials for the fabrication of microfluidic devices (Becker and Gartner 2008). It was chosen for its low cost, transparency, gas permeability, high repeatability, flexibility, rapid and cheap fabrication techniques, and the methods for adhering it to glass and other materials (Ni et al. 2009). It is highly hydrophobic material. For monolayer culture it is obstacle, whereas for aggregate and spheroid cultures it is advantage. PDMS also absorbs small organic molecules like drugs or proteins on its surface and may leach un-cross-linked oligomers into the solution. Therefore, surface treatment is often required but after this PDMS may return its hydrophobicity (Rigat-Brugarolas et al. 2015; Zuchowska et al. 2016). The applications of PDMS in the microsystems for cellomics include among others: cell trapping, sorting and counting, cell lysis and fusion, single-cell analysis, cell cultivation and toxicity assays and drug screening. The PDMS-based microdevices may be used for cell culture in

monocultures and co-cultures, in 2D and 3D culture models as well. Various examples of the PDMS-based microsystems have been presented in the literature. They were applied for investigation cells coming from various organs. Below, we presented some examples of such LOC systems.

Cell trapping and capture of cells such as white blood cells (WBCs) and circulating tumor cells (CTCs) can be tested in PDMS-based microsystems (Fan et al. 2015). A microfluidic system for isolation and detection of CTCs is shown in Fig. 3.1. The device consisted of three layers: the top chamber for cell capture, PDMS-based microfiltration membrane (PMM), and bottom chamber for waste. The membrane was bonded with PDMS top and bottom layers with manufactured microchannels. The device was used to separate cancerous lung cells from peripheral blood (Fan et al. 2015).

Cell sorting is the next application of PDMS-based microsystems (Liu et al. 2016a; Song et al. 2015). Various types of the cells can be sorted in the microsystems depending on, e.g., their morphology, origin, function, and size. For example, a continuous-flow chip for sorting stem cells and their differentiation progenies based on dielectrophoresis was presented by Song et al. (2015). The microsystem was constructed from a PDMS layer with patterned microfluidic channels as well as a glass substrate with gold electrodes. The device allowed human mesenchymal stem cells (hMSC) to be separated from their progenies (osteoblasts).

The next application of PDMS-based microsystems is cell lysis and fusion (Hoefemann et al. 2012; Hung and Chang 2012; Sakamoto et al. 2016). Hoefemann et al. (2012) presented a microdevice for cell sorting and mechanical single-cell lysis using BubbleJet technology. The microsystem consisted of a top PDMS layer with microfabricated channels and a bottom glass wafer with titanium microheaters to generate bubbles. In order to sort the cells, the bubbles were generated in the separate chamber connected to the main channel where the single cells were

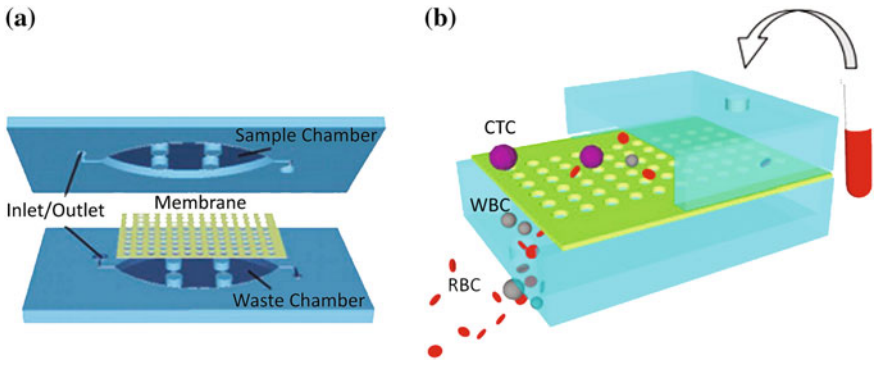

Fig. 3.1 Scheme of the microfluidic device integrated with microfiltration membrane (PMM). **a** Lateral and **b** sectional views. Reprinted with permission from Fan et al. (2015). Copyright 2015 Elsevier

flowing one by one. For cell lysis, bubbles were generated in the main channel. The device enabled hydrodynamic focusing of L929 mouse fibroblasts and polystyrene beads, their sorting and lysis of the cells while simultaneously being observed through the microscope. Sakamoto et al. (2016) described a microfluidic chip for cell fusion and tracking fused cells. It consisted of a PDMS layer with fabricated channels bonded with a PS dish with a pattern of aluminum electrodes. The chip was used to align cells through dielectrophoresis and individually fuse mouse embryonic fibroblasts (MEF) and embryonic stem (ES) cells using pulse voltage.

The PDMS-based microdevices are mainly used for cell culturing, toxicity, and drug screening assays. There are many examples of the microsystems applied for 2D and 3D cultures of the cells coming from various organs (e.g., heart, lung, kidney, liver) (Chen et al. 2016; Choucha-Snouber et al. 2013; Hegde et al. 2014; Rodriguez-Rodriguez et al. 2012; Zuchowska et al. 2016). Both cancer and non-malignant cells are investigated in such microsystems. Drugs, tested on such culture models, are delivered to the cells as solutions or nanocarriers (Fede et al. 2015; Grabowska-Jadach et al. 2016; Jastrzebska et al. 2016; Liu et al. 2016b; Wang et al. 2015). For instance, Hegde et al. (2014) developed a microfluidic device for long-term hepatocyte cultures. The microsystem consisted of two chambers formed by two layers of PDMS between which the porous membrane was inserted. In the bottom chamber, cells were cultured while embedded in a collagen gel and in the top chamber cells were cultured in a flow. The device construction enabled the two types of cultures to be compared. Choucha-Snouber et al. (2013) demonstrated a microfluidic chip for a liver–kidney co-culture. The device was composed of two interconnected culture chambers to allow medium perfusion between the hepatic chamber and kidney chamber. It was built from two layers of PDMS bonded together. To allow cell adhesion, the inner surface of the chambers was coated with fibronectin. A microsystem for toxicity studies of quantum dots (QDs) was presented by Grabowska-Jadach et al. (2016). The device was made of a PDMS layer with fabricated microchannels bonded with a thin glass slide. It enabled different concentrations of the examined nanoparticles to be obtained using a CGG. The viability of cells and QDs accumulation was determined using fluorescent staining and microscopic observations. Another approach to the topic of nanoparticle toxicity studies was demonstrated by Fede et al. (2015). The authors described a microdevice for toxicity evaluation of gold nanoparticles under continuous perfusion of the medium. The device was built from PDMS with a single linear channel bonded with cover glass. Before cell culturing, it was coated with fibronectin. The cellular uptake of nanoparticles and cell viability was assessed using microscopy. Not only mono- but also co-culture of the cells has been investigated in the microsystems made of PDMS. For example, the microsystem consisted of two layers of PDMS was used for toxicity assays on co-culture of three cell lines: L-2 rat lung epithelial cell line, and primary hepatocytes and adipocytes from mouse fibroblast 3T3-L1 (Nakayama et al. 2014).

3.2 Materials for Three-Dimensional (3D) Cell Cultures

As was mentioned in above sections, the microsystems can be successfully used for both 2D and 3D cell cultures. 2D culture is a simplification of in vivo, while 3D culture models better reflect the conditions in a human organism (Ramaiahgari et al. 2014). Therefore, spatial arrangement of the cells is often modeled in the microsystems for cellomics. Additionally, the utilization of microfluidics for 3D cell cultures is a good solution due to the occurrence of flow and chemical gradients and the introduction of different architecture of these devices which better mimic the conditions in native tissues. Two groups of 3D cell culture models: scaffold-free and scaffold-based may be obtained in the microsystems. The first group mainly consists of spheroids, which are good models of some tumors (Sun et al. 2014; Ziolkowska et al. 2012). Because PDMS is a hydrophobic material, spheroids are most often created in PDMS-based microsystems. 3D culture models are also obtained inside of the microsystems using additional materials, e.g., nanofibers or hydrogels (Carvalho et al. 2015; Pagano et al. 2014; Tomecka et al. 2018; Zhong et al. 2013). In this case, scaffold-based culture models are obtained. One of the tasks, which such models fulfill, is mimicking the extracellular matrix (ECM) (Geckil et al. 2010). Biomaterials used for creation of scaffold-based culture should have properties such as biocompatibility, biodegradability, and the possibility to interact with the cells. Characterization of materials utilized for the creation of scaffold-based culture models as well as examples of their application in the microsystems is presented in the next sections.

3.2.1 Hydrogels

Hydrogels are 3D cross-linked hydrophilic water-insoluble polymers which exhibit similar properties as native ECM (Van Duinen et al. 2015). Hydrogels based on hydrophilic, biocompatible polymers, and peptides are suitable for cell cultures because of their properties of in situ gel formation from aqueous solutions. Moreover, their application in the microfluidic devices prevents shear stress from medium perfusion (Carvalho et al. 2015). Hydrogels have the ability to absorb a high volume of water because of their porousness. They possess poor mechanical properties, like ECM; however, it may be adjusted, e.g., by changing the hydrogel composition. Natural hydrogels (e.g., collagen, Matrigel, hyaluronic acid-HA, fibrin, fibronectin), derivatives of natural materials (e.g., alginate and chitosan), and synthetic hydrogels (e.g., poly(ethylene glycol)—PEG) are used in the microsystems (Bichsel et al. 2012; Buchanan et al. 2014; Jang et al. 2015; Tomecka et al. 2017; Trkov et al. 2010; Verhulsel et al. 2014). Synthetic materials generally have better mechanical properties, whereas natural materials better mimic physiological conditions. Therefore, the combination of natural and synthetic polymers is also

applied to increase the biological (e.g., hydrophilicity), biophysical (e.g., porosity), and mechanical (e.g., stiffness) properties of these scaffolds (Geckil et al. 2010).

3.2.1.1 Natural Hydrogels

Collagen-based scaffolds are often used for cell cultures in the microsystems (Buchanan et al. 2014; Pagano et al. 2014; Verbridge et al. 2013). It can result from the fact that the 3D structure of this material correlates to the in vivo microenvironment of tissues (Carvalho et al. 2015). Collagen is biocompatible and biodegradable material. Collagen I has the tripeptide (Arg-Gly-Asp) in its structure, which has the ability to bond to the surface receptors of cells. Scaffolds from this material can be obtained in the microsystems using different methods. For example, Buchanan et al. (2014) developed a microdevice, which consisted of a transparent fluorinated ethylene propylene (FEP) tube capped with PDMS. Inside the tube, collagen I hydrogel comprised of cylindrical microchannels was prepared leaving a hollow cylindrical microchannel in the center of the tube to allow the medium to flow. The device was used for 3D co-culture of a human breast carcinoma cell line (MDA-MB-231) and telomerase-immortalized microvascular endothelial cells (TIME) with the possibility to observe the cells and analyze their viability. A microsystem with three parallel microchannels (one central channel filled with collagen and two side channels where the medium flowed) is the next approach to create collagen-based scaffolds. Pagano et al. (2014) designed such a type of a microdevice. Stem cell culture under the constant gradients of bone morphogenetic protein 2 (BMP-2) and cell migration as the effect of gradients were analyzed in this microsystem.

Matrigel, the next type of hydrogel, is very similar to native ECM due to its composition and structure. It is composed of growth factors and basic proteins present in cell membranes. It is biocompatible and enables cell adhesion. Additionally, it has the ability to vascularize faster than other natural hydrogels (Li and Guan 2011). Its properties may be simply adjusted as well. However, Matrigel is an animal-derived product and therefore it lacks the peptide motifs of humans. It may result in the contamination of growth factors, differences in endotoxins levels, and the possible occurrence of undefined substances (Carvalho et al. 2015). Despite some limitations, Matrigel is successfully used to create spatial arrangement of the cells inside of the microsystems (Jang et al. 2015; Markov et al. 2012; Shin et al. 2011). For example, Jang et al. (2015) constructed a 3D cell culture microdevice for oriented growth of cells. The device was made from PDMS with three fabricated microchannels. The channels were separated by an array of hexagonal pillars which functioned to fill each channel independently. A continuous fluid flow across the bulk gel was applied during gelation process. It was observed that the seeded rat primary cortical neurons and mouse neural stem cells were orientated according to the flow direction (Fig. 3.2).

Fibrinogen is a large glycoprotein presented in plasma. It plays a role in blood clotting, fibrinolysis, interactions between cells and matrix, and wound healing.

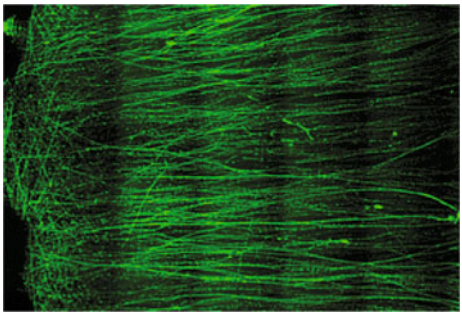

Fig. 3.2 The immunofluorescence image of axon inside hydrogels in a microfluidic device indicates the hydrogel structure. Axons derived from rat (E17–18) grew randomly without flow across the Matrigel, while axons were aligned in flow-induced hydrogels. Reprinted with permission from Jang et al. (2015). Copyright 2015 American Chemical Society

Fibrin hydrogels are widely used for 3D models of cell cultures due to their biocompatibility as well as nano- and macrofibrous architecture which imitates ECM (Song et al. 2014). Fibrin-based hydrogels are formed in the microsystems by the polymerization of fibrinogen with thrombin and calcium ions. Fibrinogen-based scaffolds can be utilized for studies of cell–cell interaction, cell migration, vasculogenesis, and cytotoxicity (Moreno-Arotzena et al. 2015; Trkov et al. 2010; Whisler et al. 2014). For instance, Trkov et al. (2010) presented a co-culture microsystem for cell–cell interaction studies between hMSC and human umbilical vein endothelial cells (HUVEC). The device's construction enabled the distance between two cell populations encapsulated in fibrin hydrogel to be controlled. The device was composed of two parallel main microchannels connected with side microchannels of different lengths.

Hyaluronic acid (HA) is biocompatible and biodegradable glycosaminoglycan which is a component of native ECM (Collins and Birkinshaw 2013). HA contains some functional carboxylic acids and alcohols which may be used in hydrogel production by cross-linking. Cell growth and differentiation of HA spatial culture can be investigated in the microsystems. For instance, Lee et al. (2014) presented an interesting microfluidic chip with HA hydrogel. The device was built from two PDMS layers with microfabricated channels between which a polyurethane (PU) nanofiber membrane was inserted. The hydrogel was prepared in the chamber based on the PU membrane with acrylation of hyaluronic acid. Glioma cell alignment and migration after cell exposure with matrix metalloproteinase's influence (MPP) and vascular endothelial growth factor (VEGF) was monitored in this microsystem. It should be noted that HA hydrogel can be applied either alone or in combination with other types of hydrogels. Dickinson et al. (2012) developed a microsystem in which fibronectin and HA were utilized. Fibronectin improves vascular network formation, whereas HA enables breast tumor development. The device was used for the co-culture of endothelial colony forming cells (ECFCs) and breast cancer cells (BCCs) as well as studies of tumor angiogenesis mechanisms.

Alginates, the next type of biomaterials, are naturally derived polysaccharides. The gelation of aqueous solutions of alginates occurs thanks to the cations such as Ca^{2+}, which bind between alginate chains. However, mammalian cells do not have the ability to specifically interact with alginate polysaccharides hydrogels, and therefore, alginate hydrogels promote minimal protein adsorption (Rowley et al. 1999). Despite this fact, they are widely applied as scaffolds mimicking ECM (Kobayashi et al. 2013; Yamada et al. 2012). An interesting example has been presented by Yamada et al. (2012). They developed a platform for forming 3D restiform hepatic microorganoids which consisted of primary rat hepatocytes and feeder cells (Swiss 3T3 cells). The alginate hydrogel fibers incorporating cells were created in a PDMS-based microfluidic device (Fig. 3.3). It enabled the oxygen concentration influence hepatocytes function studies to be performed. The formation of organoids occurred after a few days of cell culture. Additionally, there was a possibility to recover the restiform aggregates from the device by enzymatically digesting the alginate matrix. Hydrogels can init and improve spatial arrangement of the cells, whereas spheroids indicate self-organization of 3D culture. It was investigated that hydrogel-based microdevices can also be used for creation and culture 3D spheroids. Sabhachandani et al. (2016) proposed a microsystem for formation, imaging, and analyzing alginate hydrogel spheroids for osmotic studies and drug screening. To form spheroids in the microsystem, the alginate droplets containing cells were generated by introducing mineral oil with surfactant to the different inlets. The droplets were gelated in situ in incubation array. MCF-7 human breast carcinoma cells were encapsulated in a monoculture as well as in a co-culture with HS-5 bone marrow stromal fibroblast cells. They formed spheroids which were cultivated for 14 days.

Chitosan, a natural polymer with unique chemical properties, has the ability to form thin and transparent films. It also serves as a matrix for biomolecules, nanoparticles, or cells (Koev et al. 2010). Chitosan found applications in microfluidics as a hydrogel, mostly in addition to other natural hydrogels (Yu et al. 2014). For example, Tan and Desai (2003) used the collagen–chitosan matrix to develop a model system mimicking the in vivo vascular structure. The microsystem for building a 3D heterogenous multilayer structure with HUVECs, smooth muscle cells (HUV SMC), and human lung fibroblasts (IMR-90) was developed.

3.2.1.2 Synthetic and Hybrid Hydrogels

Despite the wide applications of natural hydrogels, they have some limitations such as a narrow range of physical properties, limited ability to control the matrix stiffness, and a lack of reproducibility between different batches of the material (Song et al. 2014). Therefore, synthetic hydrogels are more and more applied into microfluidic devices. PEG hydrogel is mainly used in microfluidics. PEG hydrogels are biocompatible, water soluble, and they have their own adjustable properties as well. However, due to the lack of bioactive molecules PEG hydrogels should be modified (e.g., arginyl-glycyl-aspartic acid (tripeptide)—RGD, matrix

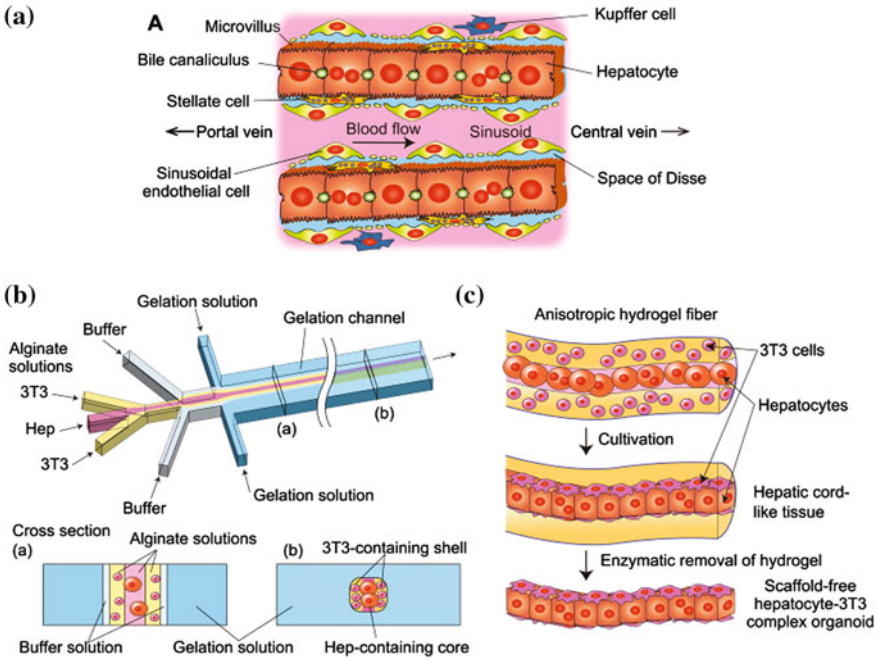

Fig. 3.3 Scheme of the liver microstructure and the fabrication procedure of the hepatic cord-like microorganoid by using the microfluidic system and the hydrogel microfiber. **a** Structure of the hepatic cord in the liver lobule. **b** The microfluidic system for fabricating sandwich-type alginate hydrogel microfibers that incorporate hepatocytes and 3T3 cells. Lower images (a) and (b) correspond to those shown in the upper image. **c** The formation process of hepatocyte-3T3 complex microorganoids in the microfiber. Reprinted with permission from Yamada et al. (2012). Copyright 2012 Elsevier

metalloproteinase—MMP sensitive sequence) or integrated with biopolymers (e.g., collagen, fibrinogen) (Song et al. 2014). Below, we describe a few examples of the microsystems, in which this material was used to create 3D culture models. PEG hydrogel was applied for encapsulation and further 3D culture of the trapped CTC cells (Bichsel et al. 2012). For this purpose, the microchannels of PDMS/glass microdevice were filled with a commercially available PEG hydrogel (Fig. 3.4). This type of a hydrogel can be degraded by enzymes secreted by cells what enables to test 3D cell invasion in a tumor model.

Li et al. (2014) presented ex vivo encapsulation of primary hepatocytes in 3D microstructures. PEG acrylate gel used in experiments was integrated with adhesive peptides RGD. In turn, Schepers et al. (2016) presented a microsystem, in which the cells were encapsulated in a PEG diacrylate hydrogel using a droplet generator. The microdevice consisted of an array of C-shaped traps for robust loading with encapsulated cells. A 3D culture of the hepatocytes with stable hepatic function and metabolic activity in mono- and co-cultures was obtained in the microdevice.

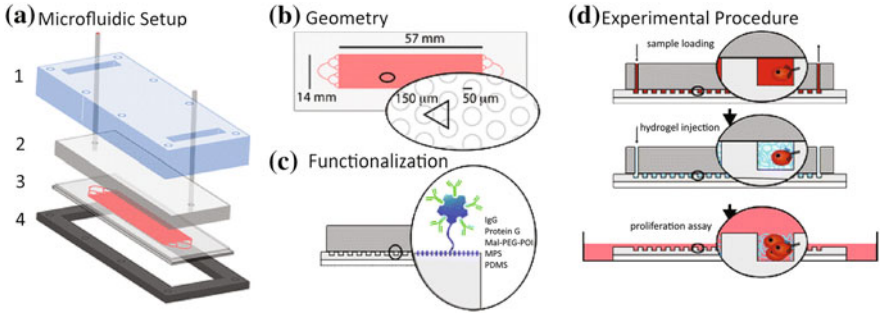

Fig. 3.4 Versatile CTC capture and culture platform. **a** Microfluidic setup. (1) Transparent PMMA cover, (2) PDMS cover with inlet tubing and outlet tubing, (3) microstructured PDMS on a glass slide, (4) metal support frame. **b** Micropillar geometry. **c** A scheme of PDMS functionalization. **d** A scheme of an experiment. In the first step, the sample is flushed through the microsystem and CTCs are captured; then, the microsystem is washed and hydrogel injected, and finally the gel-encapsulated cells are transferred to cell culture conditions. Reprinted with permission from Bichsel et al. (2012). Copyright 2012 Royal Society of Chemistry

The application of PEG hydrogels may also be useful for modeling vascular morphogenesis in the microsystem (Zanotelli et al. 2016). Human-induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) were encapsulated in PEG hydrogel integrated with MMP-degradable cross-links and CRGDS cell adhesion peptide. The cells cultured in the microdevice tended to form vascular networks according to the mechanism occurring in vivo.

Hybrid natural/synthetic hydrogels also have applications in the microfluidic devices for cellomics. Gelatin methacryloyl (GelMA) hydrogels called gelatin methacrylate is the most commonly used. GelMA hydrogels are widely used as scaffolds because of their properties resembling native ECM, the presence of mainly peptide motifs which allow for cell adhesion and exhibit MMP sensitivity. GelMA hydrogels with tunable properties are formed after light irradiation (Yue et al. 2015). Application of GelMA hydrogels may be useful in the development of a microsystem for cell alignment studies. For example, Hsieh et al. (2014) fabricated a microdevice, which allowed gradient static strains to simulate the cellular behavior in 3D hydrogel environment to be generated. The device was based on a flexible PDMS membrane on a glass substrate. The photopolymerization of GelMA hydrogel was performed in the device through a photomask with a concentric circular pattern. Thanks to that hydrogel which defined the radial direction of cell (NIH 3T3 fibroblasts), alignment was obtained.

3.2.2 Nanofibrous Scaffolds

Besides hydrogels, nanofibers find applications as scaffolds for cell cultures in the microfluidic devices as well. Nanofibrous scaffolds may be fabricated using electrospinning technology, phase separation, self-assembly, or by utilizing microfluidic devices (Lee et al. 2010; Lu et al. 2013). Nanofibers can be directly generated into fully closed microfluidic channels using a dynamic focusing method and electrospinning (Chen et al. 2016). Materials for such scaffolds include biocompatible and biodegradable polymers which exhibit suitable mechanical properties, e.g., poly(caprolactone) (PCL), copolymer poly(L-lactic acid)-co-poly (ε-caprolactone) (PLACL), poly(lactic-co-glycolic) acid (PLGA), silk fibroin

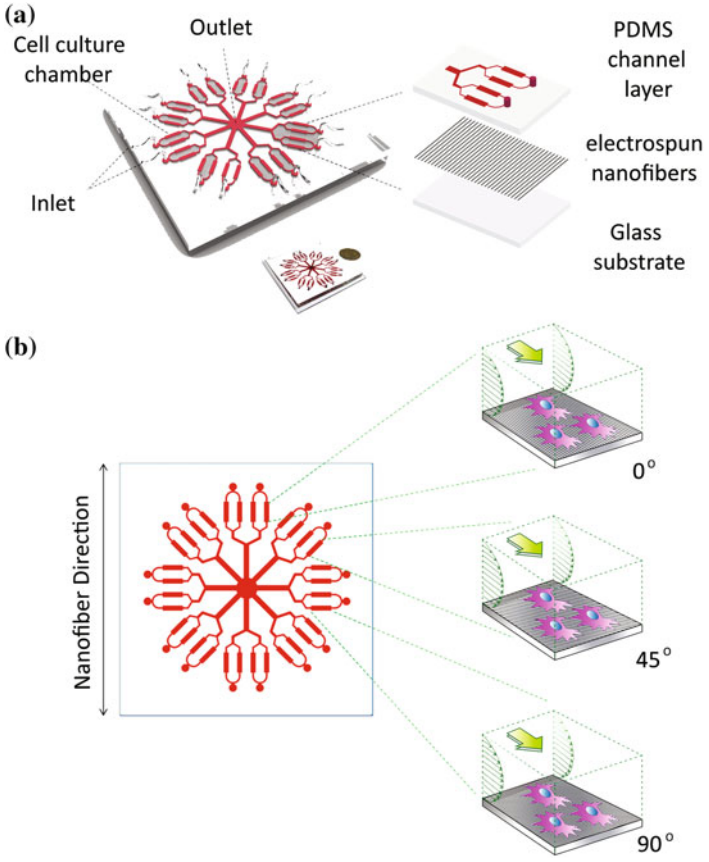

Fig. 3.5 Microfluidic-nanofiber device design. **a** Schematic diagram of the integrated microfluidic device embedded with aligned nanofibrous scaffolds used for dynamic culture. **b** The layout of microfluidic networks enables the fluid flow direction to form different angles (0°, 45°, 90°) with the aligned nanofibers simultaneously. Reprinted with permission from Zhong et al. (2013). Open Access—the Creative Commons Attribution License

(SF) (Bhaarathy et al. 2014). The nanoscaffold-based microdevices may be used to culture and differentiate various cell types. For example, Hesari et al. (2016) presented a microsystem for controlled differentiation of human-induced pluripotent stem cells (hiPSCs) into neurons. The PDMS microdevice consisted of a microchannel network, which enables medium flow and location a polymeric 3D PLGA nanoscaffolds. Nanofibrous scaffolds integrated with the microsystems most often are modified (e.g., oxygen plasma, proteins) to facilitate cell adhesion to the substrates. Because many types of nanofibrous are developed, they influence on cell proliferation and alignment is investigated. Zhong et al. (2013) presented the device composed of three layers: a glass bottom substrate, middle PLGA nanofibers, and a top PDMS layer with patterned microstructures Fig. 3.5. Effect of scaffold nanotopography and medium flow on the morphology and fibrochondrogenesis of MSC was investigated in such a microsystem.

3.3 Microfabrication of *Lab-on-a-chip* Systems

The functional requirements of the design of *LOC* systems for cellomics must take biological processes into consideration. Fabricating the microdevices has to take into account both engineered design and the material selection stages. Manufacturing on-chip features such as pumps, valves, sensors must also be considered. An increased interest has been noted in a field of micromachining technologies over the last decade. Nowadays, microelectromechanical systems (MEMS) or microoptoelectromechanical systems (MOEMS) are widely utilized in many industry segments. Since biotechnology and cellomics have entered the field of microfluidics, inventive solutions of BioMEMS, *Lab-on-a-chip,* and *Organ-on-chip* systems have been created. At present, various technologies for LOC fabrication are available (Table 3.2): etching, deposition, second cast, bonding, and maskless patterning techniques. However, photolithography is still a leading process (Kipling et al. 2015).

3.3.1 Soft Polymers

Soft polymers also known as elastic polymers are widely used for the prototyping of *Lab-on-a-chip* systems because of their simplicity and relatively low start-up costs and accessible manufacturing process. Using soft polymers is a cost-effective method for experimenting and fabricating microsystems with little experience. Additionally, integration of micropumps, microvalves, and other elements into a chip is possible because of material flexibility. Moreover, manufacturing microsystems using elastic polymers does not require an expensive laboratory, e.g., a computer numeric control (CNC) micromilling machine or a clean room (Becker 2011).

Table 3.2 Manufacture and assembly methods for materials used in LOCs for cellomics

Material	Manufacture method	Assembly method
Soft polymers	Soft lithography	Adhesive
	Replica molding	
	Rapid prototyping	Contact bonding
Rigid polymers	Hot embossing	Ultrasonic welding
	3D printing	
	Injection molding	
	Micromachining	Adhesive thermal bonding
Glass	Chemical etching	Glass fusion
	Micromachining	Anodic bonding
	Powder blasting Water jet cutting	Thermal bonding

There are multiple choices of polymers using a rapid prototyping technique; however, PDMS is the most commonly used. Since a variety of mold tools which ensure the repeatability of microdevice features are available, the mass production of soft polymer microsystems is offered. These molds can be manufactured using various materials as they are not subjected to pressure or excessive heat. Another advantage of these molds over others is the possibility of fabricating 3D and high aspect ratio features. On the other hand, manufacturing the microsystems in soft polymers is limited because of their elasticity, inherent softness, and swelling in contact with solvents. Also, curing time lowers mass production.

3.3.1.1 Soft Lithography

Soft lithography is non-photolithographic method enabling replication of the micropatterns. Using this microfabrication technique allows most structures relevant to the microfluidics (from 20 to 100 μm) to be produced without the need for a clean room. In this technique, an elastomeric structure with the embedded patterns serves as a pattern transfer agent (McDonald et al. 2000; Younan and Whitesides 1998). Soft lithography begins with the manufacturing of a PDMS replica, mold, or a master (Fig. 3.6). PDMS is a mixture of two components: a base and a curing agent in 10:1 (v/v). Only after silicon hydride groups present in a curing agent react with vinyl groups from the base, is a cross-linked elastomeric solid created. The liquid prepolymer can then be poured over the master and cured to replicate channels and features with high fidelity. Due to the low surface energy and elasticity of PDMS, it can be easily peeled from the master without being damaged (McDonald and Whitesides 2002). The master can be produced by a variety of methods including photolithography, etching in silicon, conventional machining of hard materials, or electroforming metal (Anderson et al. 2000; Becker and Gartner 2000; Duffy et al. 1998; Love et al. 2001; McDonald et al. 2002). The master can

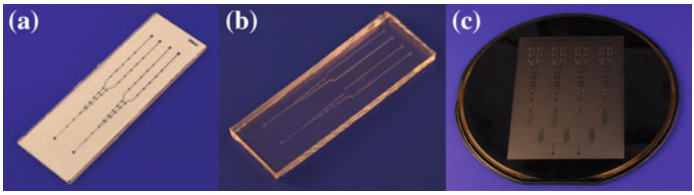

Fig. 3.6 Photograph of **a** master fabricated by so-called capillary film process, **b** PDMS replica (double casting prototyping with a thermal aging step), **c** silicon wafer

also be fabricated using thermal aging as a step in the double casting microfabrication process (Kwapiszewska et al. 2016). A non-chemically modified PDMS layer can serve as a master for double casting. Physical treatment of a surface by thermal aging is known as an extended curing process. Since low molecular weight (LMW) chains inside a PDMS are gradually cross-linked, interactions between a thermally aged master and a molded PDMS are limited. This enables such a PDMS layer to be used as a master for further PDMS casting.

3.3.1.2 Rapid Prototyping

The first step in rapid prototyping is to create a chip geometry using a computer-aided design (CAD) program. Then, a design has to be printed on a transparency using a high-resolution commercial image setter. Such a photomask enables a durable positive relief (master) of SU-8 (photocurable epoxy) photoresist on silicon wafer to be produced in contact with photolithography. The coat of SU-8 on a substrate can be made by spin coating. This technique is carried out by putting a puddle of a photoresist on a rotated substrate. The thickness of the SU-8 layer is determined by rotation speed, the acceleration, and SU-8 viscosity. Furthermore, spin coating requires a clean room. Then, the master can be used for casting PDMS microsystems. However, separating the PDMS layer from the fragile silicon wafer without fractures may be difficult. Using a hard polymer as epoxy or structural polyurethane to replicate the master may be the solution to this problem (Shaw et al. 1997).

One of the benefits of using rapid prototyping is the reduction in costs and time needed for a total design cycle, fabrication, and carrying out new experiments compared to methods using a chrome mask in the photolithographic step. Also, applying a chrome mask can be up to 100 times more expensive and time-consuming when rapid prototyping requires only hours to obtain. On the other hand, transparency resolution is > 20 μm, when using a chrome mask approximately 500 nm. The channels and other features for cellomics applications range from 50 to 100 μm and thus correspond to the capacity of rapid prototyping. For obtaining features with lower dimensions, a chrome mask needs to be used (McDonald et al. 2000).

3.3.1.3 Replica Molding

When a master is prepared, channels in PDMS can be formed by using the replica molding technique (Fig. 3.7). It involves casting a prepolymer against a master. Then, PDMS can be cured in an oven for 1 h at 60 °C prior to peeling from the master. Inlet and outlet holes for microchannels and reservoirs can be produced in a replication step by placing posts on the master or punching out the cured PDMS using a borer. The selection of technology for manufacturing masters is dictated by the production run. In the case of mass production, metal masters or these fabricated of hard materials may be used because of their durability. Although the fabrication of masters is expensive, the costs become negligible after many uses. However, in cellomics research and development where several changes are necessary to obtain a final design, metal and silicon-based molds are too expensive and time-consuming (McDonald et al. 2000). A master can also be prepared using a so-called photosensitive capillary film (Juchniewicz et al. 2009). When deposited on a solid support and exposed to UV light through a photomask, it can be developed by using cyclic washing and drying. Consequently, the microchannel network and the areas for cell culture for the replication in PDMS can be obtained.

Capillary film emulsion can also be used in the bonding-less fabrication of polymeric microsystems (Juchniewicz et al. 2009). In that case, PDMS has to be partially cross-linked. Then, the capillary film matrix is placed on the PDMS surface. After folding up the support foil of the capillary film, the emulsion remains on its surface. Once the plastic or metal tubes are placed inside the mold, the next portion of PDMS prepolymer can be poured to cover the emulsion prior to performing the cross-linking process. Using this technology, it is possible to fabricate polymeric microsystems with 2D or sandwich-like microchannel architecture without the bonding step.

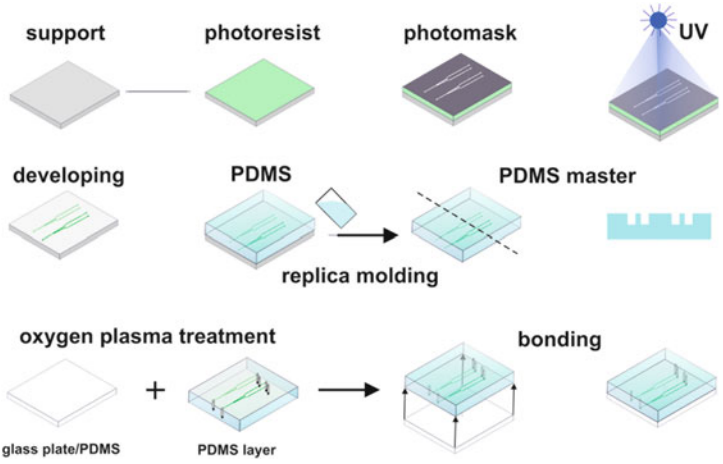

Fig. 3.7 Scheme of replica molding technology

3.3.2 Rigid Polymers

In microfluidic applications for cellomics, properties of a material such as machinability, optical properties, electroosmotic flow mobility, surface charge play a fundamental role for both fabrication and successful application. Rigid polymers are those which are characterized by high Young's modulus. They can constitute a base for creating masters or molds using micromachining techniques or may be molded with the desired features. The demand of rigid polymer-based LOCs would likely be increased with the escalation of microsystem integration in the industry or research sector, since they are the most suitable for mass production.

Injection molding stands out as the primary production method for microchips made of rigid polymers because of short manufacturing time, low price, and small amount of user input required. Other techniques are hot embossing and micromachining methods such as micromilling or microgrinding. Polymers are a diverse group of materials, and they can possess properties that can make them either desirable or entirely unsuitable for microchip microfabrication and further application. Exemplary rigid polymers used for *Lab-on-a-chip* system manufacturing are: PMMA, PC, polyimide, COC, polyethylene (PE), polyethylene terephthalate glycol, and styrene copolymers (Becker and Gartner 2008; Becker and Locascio 2002; Alrifaiy et al. 2012).

3.3.2.1 Injection Molding

Injection molding uses a variotherm process for low-cost mass production of thermoplastic polymer devices. Thermoplastic polymers suitable for injection molding are: PMMA, PC, PS, polyoxymethylene, polyvinylchloride (PVC), polypropylene (PP), polyetheretherketone (PEEK), polyamide (PA), and polyvinylidene fluoride (PVDF). This technology primarily requires the creation of a molding tool. This step is critical because each flaw or surface roughness will be transposed to every single device fabricated. The first step is closing, evacuating, and heating the two-part mold cavity (with the micromold insert) above the glass transition temperature of the polymer. Then, the heated polymer granules are transported from the hopper into the barrel and the viscous polymer is pressed into a mold. After the mold is cooled below the polymer's glass transition temperature, the polymer piece is ejected and transformed if necessary.

Although production time using injection molding is relatively short, it requires fabrication of a complex micromold insert, using specialized and expensive technology such as microelectrical discharge machining (μEDM). On the other hand, injection molding provides large features, such as reagent entry and waste holes, molded directly into a microdevice. Small features can also be fabricated because of high resolution that is possible to achieve through the machining process of the metal mold. Another benefit is limited material waste, since any shape and size of raw material granules can be manufactured. Yet, it needs to be highlighted that

plenty of parameters such as injection pressure and speed, cooling time, shot size, or polymer and mold temperature influence the exact replication of a chip using this fabrication method (Becker and Locascio 2002; Giboz et al. 2007; Mair et al. 2006).

3.3.2.2 Hot Embossing

Hot embossing as a method for plastic feature fabrication was described for the first time in the late 1990s. This fabrication method requires the production of an inverted mold with all relevant microchannels. The polymer is stamped with the mold designed from different materials and using various technologies. Primarily, wires were used for imprinting plastic microchannels. However, a silicon stamp is more common. The first stage of the hot embossing process is to design a micropattern using CAD tools. Then, the design of the features can be transferred to a photomask or high-contrast resolution transparency if the desired micropattern is greater than 20 μm. A silicon wafer is coated with masking material and then photoresist, prior to UV light source exposure through the transparency. Once the photoresist is developed, the image is transferred by etching it in hydrofluoric acid (HF) or potassium hydroxide (KOH) depending on the masking material used. The exposed silicon is subsequently etched anisotropically to create a 3D inverted image of the trapezoidal shape features. Then, the silicon stamp can be used to project microchannels in plastic materials at room or elevated temperature. Alternatively, a metal electroform may be produced using a micromachined silicon wafer as a master. In this process, two metal electroforms are created (typically using Ni). The first one is the mirror image of the master, while the second is a replica of the original silicon master. This transfers micrometer features to the robust metal substrate (Becker and Locascio 2002).

Once a mold is prepared, a microstructure can be embossed gradually by pressing on a polymeric thermoplastic substrate that is heated slightly above the glass transition temperature of the substrate. Usually, the time required for hot embossing is less than 10 min and low pressure is needed. Alternatively, the features can be imprinted at room temperature with elevated pressures, thus shortening the fabrication time by up to 2 min. After cooling, the substrate containing a glassy or semi-crystalline microstructure can be released. The advantage of hot embossing is the possibility of replicating many devices using the same mold. Mass scale fabrication using this method is more cost efficient. However, start-up costs are much higher because of the special equipment required for the process. Polymers suitable for fabrication using hot embossing are: PMMA, PC, PS, PEEK, PVC, PS, polyethylene terephthalate glycol (Pemg et al. 2009).

3.3.2.3 Three-Dimensional (3D) Printing

In recent years, advancements in 3D printing in the areas of speed and resolution have simplified the fabrication of *LOC*s to a single step. Notable advantages of 3D

printing over conventional methods include embedding of a tissue scaffold with high resolution and defined porosity into a microsystem and using a variety of biomaterials. Nowadays, living cells and growth factors can be directly printed (Gross et al. 2014; Harink et al. 2013). 3D printing offers the ability to fabricate high-precision multilayer constructions of a microvascular (of 100–300 μm) channel network with high accuracy. Minimum feature size is limited by the resolution of the printers.

3D printing begins with a digital model from the CAD software or the 3D scanners. Subsequently, a series of multiple layers by Z direction discrete from the models are obtained using slicer software. The result is that layer-by-layer printing and superposition are used for manufacturing the 3D entity (Yang et al. 2016). Moreover, 3D printing microfluidics can be assisted with a wide range of functional inks, based on high-conductance, piezo-resistive, and biocompatible soft materials, that help soft strain gauge sensors to integrate within microarchitectures (Lind et al. 2017). As a consequence, it promotes the self-assembly of physio-mimetic laminar cardiac tissues.

This technology is potentially cost-effective and rapid since many advances have been made. The most important benefit of 3D printing technology is the ability to fabricate geometries that would be very difficult or even impossible to obtain, using highly specialized equipment. For now, 3D printing is more commonly used for fabrication of radical new concepts or 'one off' production of experimental designs. Despite the great potential for the utilization of 3D printed microchips, there are some limitations of this technology. In comparison with injection molding or hot embossing, the range of materials that can be printed is restricted. This is due to thermal stability, chemical compatibility, or mechanical integrity of some polymers, which eventually limit possible applications of 3D printed microdevices (Yazdi et al. 2016).

3.3.2.4 Micromachining

Micromachining is used to fabricate polymer LOCs through two different ways. Material can be either removed directly from a stock polymer to form a desired geometry or a mold tool for injection molding or hot embossing can be produced. Micromachining techniques include microgrinding, microdrilling, and micromilling. These methods involve mechanical material removal using geometrically designated cutter edges, in which size and geometry determine the accuracy and size limit of a microchip. Microgrinding allows a workpiece with a surface of high quality and shape accuracy to be fabricated. It is possible to achieve surface roughness of about Ra = 5 nm when using fine-grained diamond grinding wheels. Moreover, ultrasounds can assist the grinding process in order to reduce the process forces (Denkena et al. 2006).

Bore manufacturing is challenging because of the reduced stiffness of microdrills. Additionally, chip transportation from the bottom of the bore can be complicated. Micromilling can be used to machine 3D microgeometries. There are

numerous microtools that can be utilized for micromilling such as double-edged end mills made of fine grain tungsten carbide or single-edged mills of monocrystalline diamond. It needs to be highlighted that to prevent the polymer from melting, and thus avoiding an excessive material build up on the tool and potential breakages, adequate cooling is necessary. Prototyping of the microsystems within micromilling can be time-consuming but cost-effective. This is important especially when numerous differing designs and configurations are needed. The micromilling step requires a CNC machine. A 3D computational model of a mold can be drawn using SolidWorks software. A CAM package such as SolidCAM converts a design into a computation code (so-called G-code) that is recognized by CNC machine software (Becker and Locascio 2002).

3.3.3 Glass

Planar glass substrates are the most commonly used to create miniaturized analytical systems for electrophoresis. In the microsystems for cellomics, glass is most often used for cell adhesion and cultivation. Fabrication methods of glass substrates using etching techniques require clean room facilities and are time-consuming. It leads to high costs of manufacturing. Nonetheless, glass has good optical properties, is efficient in dissipating heat, and is highly resistant to chemicals and mechanical stress. Glass fabrication methods, mostly including photolithography and chemical etching, are well established. Wet etching of glass results in the formation of deep microchannels with non-parallel walls. An alternative to producing an ideal microchannel is to use dry etching techniques such as deep reactive ion etching (DRIE) (Park et al. 2005).

Nevertheless, this method is expensive and requires specialized instrumentation and maintenance. Considering each of these limitations, glass is rather viewed as a special engineering material that is largely incompatible with traditional machining processes (Castano-Alvarez et al. 2008). Moreover, glass microstructures can also be fabricated using micromachining, powder blasting, laser ablation, and water jet cutting. However, these are rarely used for microfabrication of LOCs for cellomics. (Gomez et al. 2005; Schlautmann et al. 2001).

3.3.3.1 Wet Etching

Wet etching of glass is an isotropic process, which is usually performed with HF solutions. The etching rate depends on the glass composition. A low oxide content is preferable as they produce insoluble products after reacting with HF. The etch rate can be increased, by raising the temperature of the solution, annealing the glass wafer before etching, or by using ultrasonic agitation. On the other hand, diluting the HF solution would reduce the etch rate. Masking layers are the key issue in micropatterning. These include, among others, photoresist, amorphous deposited Si,

LPCVD polysilicon, Cr/Au, Ag, Mo, or Ti. However, some masking layers have drawbacks or limitations such as shallow etching (photoresist) or increased isotropy (photoresist, Ti, Ag, bulk Si, amorphous SiC). Generally, isotropic wet etching is used to achieve high etching grooves in glass (Iliescu et al. 2008).

3.3.3.2 Dry Etching

Dry etching is recommended to obtain anisotropic etching profiles. Compared to wet etching, dry etching is a rather slow process carried out at low pressure with poor selectivity relative to the mask utilization. This is due to the strong temperature gradient generated by the large amount of energy transferred to the glass substrate, during inductively coupled plasma DRIE. SF_6, C_4F_8, CF_4, or CHF_3 can be used as gas precursors in DRIE of glass. To improve etching selectivity, relatively thick masking layers such as electroplated Ni, bulk silicon, PECVD amorphous silicon are required (Iliescu et al. 2012).

3.3.4 Assembly

The aim of bonding two or more components is to create fluid channels, seal the device, and finally form a complex 3D microstructure. This process takes place on the wafer-level. Assembly techniques for miniaturized system fabrication are dependent and limited by the material used and mainly involve anodic bonding, thermal bonding, plasma-activated bonding, and the adhesive bonding. It means that assembly process has to be taken into consideration during the design step.

Most widely, the process includes sealing one plate with another containing features and inlet/outlet holes. Such a connection prevents leakages and allows manipulation of flow through the desired micropattern. Thermal bonding and adhesive bonding are most commonly used for assembly of the microstructured components that would be applied in cellomics. Plasma-activated bonding allows direct assembly of surfaces at low temperatures. The plasma removes contaminants and generates Si–OH groups, which results in surface hydrophilization with polar characteristics. It can be used for irreversibly bonding PDMS to itself or glass, silicon, silicon oxide, and SU-8. The adhesive bonding enables different materials to be assembled, particularly new combinations. It involves glues, epoxies, and other plastic agents that can be bonded by solvent evaporation or applying heat, pressure, and time. Thermal bonding forms a very strong bond of two polymers or polymer/glass surfaces by applying pressure and elevated temperature. Then, the layers are melted prior to fusion upon cooling (Becker and Locascio 2002; Leester-Schädel et al. 2016).

3.4 Summary

Functional requirements and future applications, which comprise engineered composition and material selection stages, have to be considered when designing *LOC* systems. A significant majority of the microfluidic devices are fabricated from PDMS and glass. However, other polymers (e.g., PS, PC, PMMA, COC) exhibit desirable properties and their use in microsystem fabrication is growing. Many microfabrication methods draw from conventional technologies such as wet etching and photolithography. Apart from being well established and allowing mass production manufacturing of polymeric microdevices (injection molding and hot embossing), the technologies for designing innovative and breakthrough prototypes are currently being implemented. Nowadays, the researchers focus on the simulation of in vivo conditions in the microsystems. Therefore, 3D cell culture models, which are better models of native tissues in comparison with 2D cell culture models, are formed in the microsystems. For this purpose, scaffold materials such as hydrogels (natural, synthetic, and hybrid) and nanofibers have been adapted. All these improvements are intended to broaden the applications of *LOC*s and their future integration in numerous industry segments.

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Chapter 4
Organ-on-a-chip Systems

Aleksandra Szuplewska, Michal Chudy and Zbigniew Brzozka

4.1 From Monolayer Cultures to *Organ-on-a-chip* Systems—An Introduction

Current laboratory preclinical trials for novel drugs are based on a combination of in vitro cell culturing protocols and in vivo animal models (Esch et al. 2014a; Selimovic et al. 2013). Despite the high utility of a conventional cellular monolayer (two-dimensional, 2D) model in biomedical applications, the absence of specific tissue–tissue (and organ–organ) interaction and the lack of many cell type functions preclude anticipation of biological activity (e.g., possible mechanisms of action, potential toxic effects, impact on target-free tissues) (Esch et al. 2014b; Greek and Menache 2013; Jastrzebska et al. 2015). Therefore, three-dimensional (3D) models, mainly based on hydrogel technology, are highly valued in investigations on signaling pathways (see Chap. 3). 3D models more precisely mimic the chemical and spatial complexity of living tissues in comparison to 2D models (DiMasi and Grabowski 2007; DiMasi et al. 2010; Esch et al. 2014b). Organoids, i.e., self-organizing cellular microstructures are one of the spatial models. Although self-assembling tissues represent human organ physiology and functions more realistically, one of most meaningful organoid drawbacks is a strong correlation between their behavior and specific microarchitecture (size, shape, etc.). Another example of 3D model limitation is the suppression of further genetic, biochemical, and functional (e.g., investigation on secretion, transport, or interface phenomena) analysis by the difficulties in sample preparation (Ingber 2003; Mammoto et al. 2013). Moreover, the lack of fluid flow and other stimuli such as mechanical stretching or shear fluid stress in static 3D models is the next obstacle. In addition, the critical issue for the functioning of almost every organ is the interface between

A. Szuplewska (✉) · M. Chudy · Z. Brzozka
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: aszuplewska@ch.pw.edu.pl

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neighboring tissues, which is lacking in many systems based on conventional 3D models (Huh et al. 2011; Park and Shuler 2003; Sung et al. 2013; Ye et al. 2013). Animal models, regardless of ethical questions and difficulties in the proper interpretation of the obtained results, are not representative of most human organ functions, diseases, and responses to medical treatment (Esch et al. 2014b).

An alternative solution, potentially overcoming many of the limitations listed above, is novel platforms, known as *Organ-on-a-chip* systems (Bhise et al. 2014; Bhatia and Ingber 2014; Tomecka et al. 2018; Wang et al. 2016; Zuchowska et al. 2017). The connection of bio-microelectromechanical systems (bioMEMS) (Bashir 2004), biomimetics (Bhushan 2009), and microfluidics (Whitesides 2006) more appropriately mimics the in vivo microenvironment, e.g., simulation of activity, mechanisms and physiological responses on the tissue- and organ-level, in comparison to static 2D and 3D cellular models, as well as compared to cell culturing with the use of conventional microfluidic devices. It should also be noted that *Organ-on-a-chip* systems are currently the tools, which represent the minimal functional units—answering the physiological functions and responses of organs and tissues—rather than the whole living organ. Scheme of the *Organ-on-a-chip* concept is shown in Fig. 4.1. The simplest variant of *Organ-on-a-chip* systems includes analysis of one type of tissue/organ, but *Organ-on-a-chip* technology also offers the possibility of creating more complex, multi-organ platforms known as *Body-on-a-chip* or *Human-on-a-chip* (Beebe et al. 2013; Bhatia and Ingber 2014; Esch et al. 2015; Huh et al. 2013; Moraes et al. 2012; Moyer 2011; Polini et al. 2014; Shuler and Esch 2010; Sung et al. 2014; Wang et al. 2016). Such an integration allows the research on inter-tissue and interorgan communication as well as the simulation of human metabolism which plays a key role in studies on toxic and dose-related effects of novel therapies. Moreover, *Body-on-a-chip* systems allow for the integration of human induced pluripotent stem cells (hiPSC) to create connections between the patient and specific organ, which could potentially be developed for personalized medicine (Grskovic et al. 2011). There are various organs which have been mimicked in the microsystems: e.g., lung, liver, spleen, kidney, brain, and heart. To show a wide range and potential of this research, examples of *Organ-* and *Body-on-a-chip* systems are reviewed and discussed in this chapter. Cardiac cell technologies performed in microscale are presented in the following Chaps. 7–9.

4.2 *Organ-on-a-chip* Systems: Mimicking Physiological and Pathophysiological Conditions

The great limitation of classical models, based on 2D and 3D cell cultures, was the lack of physiological accuracy and inability to mimic the microenvironment on specific organ or tissue level. Contrary, the devices based on the *Organ-on-a-chip* concept which mimics conditions in human organs have great potential in studies

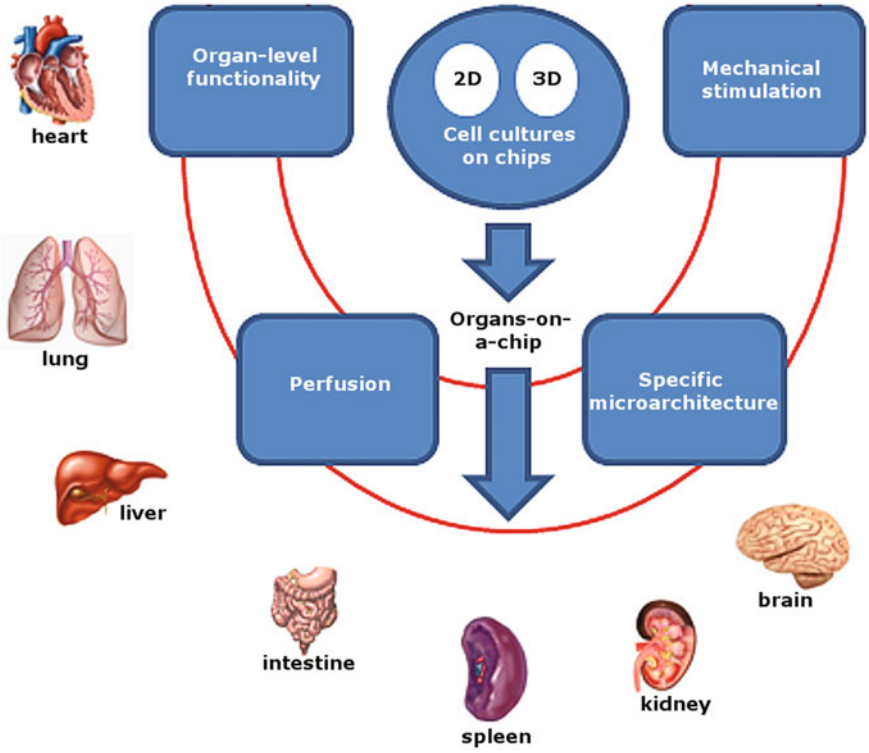

Fig. 4.1 *Organ-on-a-chip* concept

on biological phenomena by the presence of fluid flow, shear stress, and specific microarchitecture (Huh et al. 2011; Jastrzebska et al. 2016). Organs such as e.g., lung, liver, spleen, kidney, brain, and heart can be mimicked in the microsystems. It should be noted that cells coming from different organs have precisely defined properties. Therefore, to obtain the microsystems for simulation of a defined organ, specific features and parameters should also be fulfilled. Features such as dynamic fluid flow, mechanical stimulation, or material composition are taken into consideration during the fabrication of *Organ-on-a-chip* systems.

4.2.1 Mimicking of Organ Functions—The Examples

One of the key issues in the development of complex living systems is the ability to control and mimic biological 3D microarchitecture resulting from the creation of spatiotemporal, chemical microenvironments as well as the variety of used modules. The reconstruction of specific tissue functions and precise control of dynamic fluid flow has been often demonstrated (Folch and Toner 2000; Khademhosseini

et al. 2006; Mrksich et al. 1997; Whitesides et al. 2001; Whitesides 2006). Recently reported 3D models combine the reconstitution of complex tissue- and organ-level microarchitecture with mechanical as well as chemical and electrical stimulus. To achieve more accurate in vivo microarchitecture mimicking specific organs, the device based on 3D model with membrane-based multilayer compartments concept has been proposed. Such systems can be particularly useful in studies on biological barriers, as e.g., blood–brain barrier, alveolar–capillary interface in lungs, the gastrointestinal tract, the transport barrier in kidneys as well as the interface between the tumor and its external microenvironment. Some organs are simultaneously exposed to mechanical stimulation such as peristaltic motions, shear stress, or muscle contractions. Therefore, such features are also mimicked in the microsystems. Additionally, spatial arrangement of the cells (aggregates, hydrogels, nanofibers) is created inside the microsystems for more physiological accuracy of the designed systems. For example, the cerebral cortex in the brain (Alcendor et al. 2013), tumor aggregates of tumor tissue (Kim et al. 2012a; Lee et al. 2014; Mehta et al. 2012), bone marrow hematopoietic niches (Torisawa et al. 2014), or functional units of liver sinusoids (Lee et al. 2007; Schutte et al. 2011, 2009) were described as 3D models with the microenvironment regulation. To underline a wide range of the microsystem utilization for organ mimicking, we present examples of various *Organ-on-a-chip* systems in this section. Specific features, which should be fulfilled by the microsystems for simulation of specific organ, are also presented here.

4.2.1.1 Intestine-on-a-chip

A human intestine model is highly appropriable for investigation on the processes of transport, absorption, and metabolism of pharmaceutical and nutritious substances (Giacomini et al. 2010; Hodgson 2001). To mimic the specific microarchitecture, density and functionality of human intestinal villi, the microfluidic platforms for hydrogel-based spatial culture of intestinal epithelial cells are often utilized. Assembling the epithelial barrier is enabled by the use of a Transwell filter, one of the most commonly utilized conventional in vitro models of a human gut. Simultaneous reconstruction of mechanical and structural intestine properties under pathophysiological conditions along with a specific microbial flora is the key issue for biomedical applications (e.g., drug development) (Ferrec et al. 2001; Mahler et al. 2009). A porous membrane was used in a human *Gut-on-a-chip* system for investigating most meaningful intestine functions under physiological conditions, e.g., fluid flow, gut microbial flora, and cyclic mechanical strain (Huh et al. 2013; Kim et al. 2013). The designed microdevice consisted of two fluidic microchannels separated by a porous, highly flexible membrane coated with an extracellular matrix (ECM) and lined with cells derived from human intestinal epithelium (Fig. 4.2). Artificial peristaltic motions were generated on the cellular monolayer by full height vacuum chambers which caused mechanical strain (Kim et al. 2012b). The proposed model enabled observation of increase of intestinal barrier functionality.

Epithelial cell differentiation and formation of spatial villus-like aggregates were obtained thanks to the usage of low fluid flow rate (30 μL h^{-1}) and shear stress present in the living intestine (0.02 dyne cm^{-2}). The obtained results showed that the analyzed responses of the cells could potentially be enhanced by cyclic mechanical strain used in this model, in comparison to conventional cultures based on Transwell filters.

Among available in vitro human gut models, the most common solutions include the formation of polarized epithelial monolayers from established epithelial cell lines. A microsystem, which allows for forming intestinal villi and inducing Caco-2 cell spontaneous robust morphogenesis, was described by Huh et al. (2013). Analogously to normal human small intestine, basal cells continuously populate the

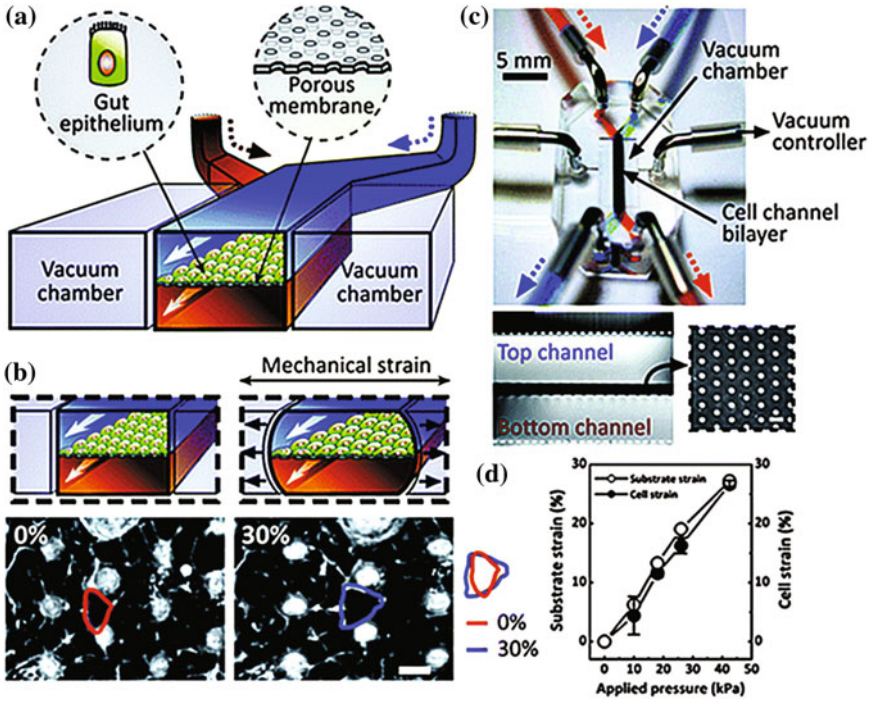

Fig. 4.2 **a** A scheme of the *Gut-on-a-chip* device with the flexible porous extracellular matrix (ECM)-coated membrane lined by gut epithelial cells crosses horizontally through the middle of the central microchannel, and full height vacuum chambers on both sides. **b** A scheme and phase contrast images of intestinal monolayers cultured within the *Gut-on-a-chip* in the absence (left) or presence (right) of mechanical strain (30%; arrow indicated direction) exerted by applying suction to the vacuum chambers. Red and blue outlines indicate the shape of a single Caco-2 cell before (red) and after (blue) mechanical strain application. **c** A photographic image of the *Gut-on-a-chip* device composed of PDMS elastomer and a cross-sectional view of the top and bottom channels (both 150 μm high) of the microsystem. **d** The graph showing dependence pressure on substrate strain. Reprinted with permission from Kim et al. (2012b). Copyright 2012 Royal Society of Chemistry

villi along the crypt–villus axis, and differentiate into four types of epithelial cells (mucussecretory, enteroendocrine, absorptive, and Paneth cell types). The intestinal surface and efficiency of absorption processes comparable to the normal human gut were obtained in the proposed model. Moreover, an enhancement of cytochrome P450 3A4 isoform-based drug metabolizing activity was observed compared with static device with Transwell membrane.

4.2.1.2 Lung-on-a-chip

Pulmonary diseases are currently one of the major causes of death worldwide. The number of these cases has been increasing in recent decades. Therefore, attentions have recently focused on some novel strategies with the aid of cell culturing models and microfluidic techniques not only for functional analysis, but also for drug screening (Doryab et al. 2016). The smallest elementary unit, distinguishable in lung tissue, is made of an endothelial and epithelial cell layer (van der Meer and van den Berg 2012). The high structural and geometric complexity, precluding the direct analysis of cells located in different regions of the lung (e.g., luminal surface coated by epithelium) as well as the mechanics of the organ and the presence of a large variety of physical stimuli, make a lung one of the most challenging organs to research under in vivo-like conditions.

Mechanical stimulation, e.g., cell exposition to fluid and continuous stress, is essential for mimicking of differences in alveoli microenvironment (Tavana et al. 2011). A strong correlation between the collapsing and reopening of alveoli causing cyclic and fluidic mechanical stresses was investigated. In acute respiratory distress syndrome, mechanical ventilation is the most commonly used form of therapy, while its use might induce further ventilator lung injury with exacerbation of acute respiratory distress syndrome (ARDS)-like symptoms (Douville et al. 2011). To mimic the cyclic stretch and fluid mechanical stress conditions, a microfluidic alveolar system was designed (Fig. 4.3). It consisted of an “alveolar chamber” and “actuation channel” which are separated by a membrane made of poly(dimethyl siloxane) (PDMS) (Douville et al. 2011).

During the process of gas exchange, air is transported between the external environments by small airways. Simultaneously, the gas exchanged in alveoli is transported via the lung barrier between blood and air (Huh et al. 2007). The alveoli gas exchange process might be impeded as a consequence of the formation of liquid plugs, a highly viscous film blocking the small airway epithelium. The observed effect might be induced by airway lumen air–liquid two-phase instabilities and accompany numerous pulmonary dysfunctions (Jacob and Gaver 2005). Respiratory crackles, i.e., a pathological sound induced by plug rupture during the process of occluded airways reopening, are one of the most widely used indicators of respiratory diseases in clinical practice (Grotberg 2001). An air–liquid two-phase microfluidic platform was proposed as the tool for studying the possible consequences of liquid plug propagation and rupture (Huh et al. 2007). The chip allow to mimic the pathophysiological conditions in respiratory system. The designed chip

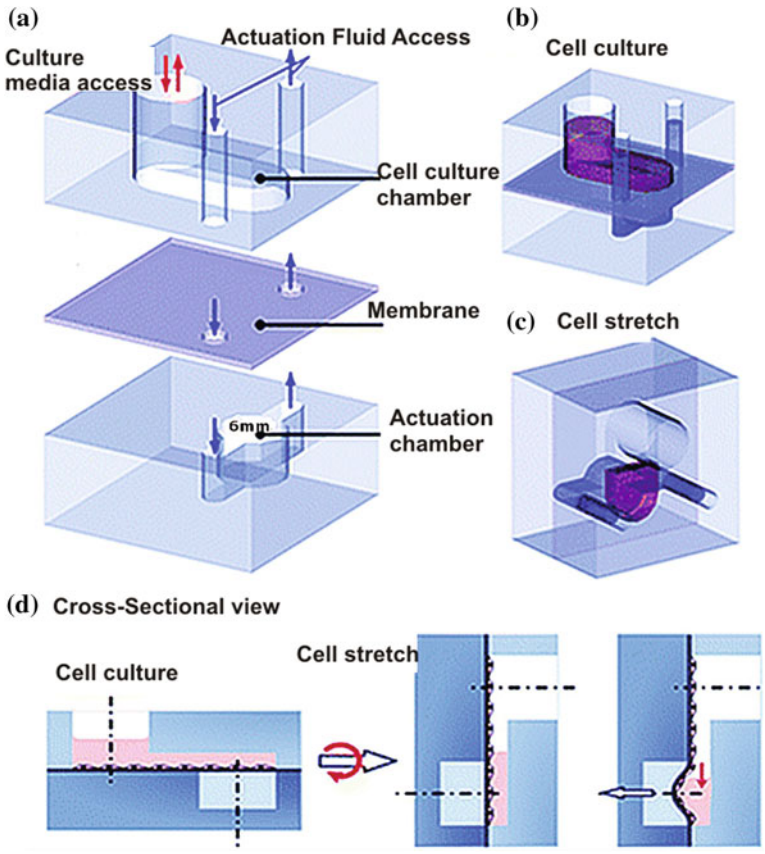

Fig. 4.3 **a** A scheme of *Alveoli-on-a-chip* system. A thin PDMS membrane separates the alveolar chamber for cell culture from the actuation channel used for deformation of the cell culture chamber. **b** The "alveolar chamber" can be filled with F-12K growth media and positioned in the horizontal orientation to allow for optimal cell adherence and growth. **c** During experiments, the "alveolar chamber" can be partially filled with fluid and positioned in the vertical configuration to establish a meniscus at the interface of fluid and air. **d** A cross-sectional view of the microsystem shows the horizontal orientation for cell culture and the vertical configuration for experimentation. Scheme of membrane deforming and cell stimulation. Reprinted with permission from Douville et al. (2011). Copyright 2010 Royal Society of Chemistry

was composed of a polymeric membrane (pore size: 400 nm), separating two chambers made of PDMS. The lower chamber was related to basal compartments of the airway epithelial layer while the upper one to the airway lumen. Working principle and design of a *Lung-on-a-chip* system presented by Stucki et al. (2015) is shown in Fig. 4.4.

The reconstruction of the main mechanical, functional, and structural characteristics of interface between alveoli and capillaries is allowed by the use of highly

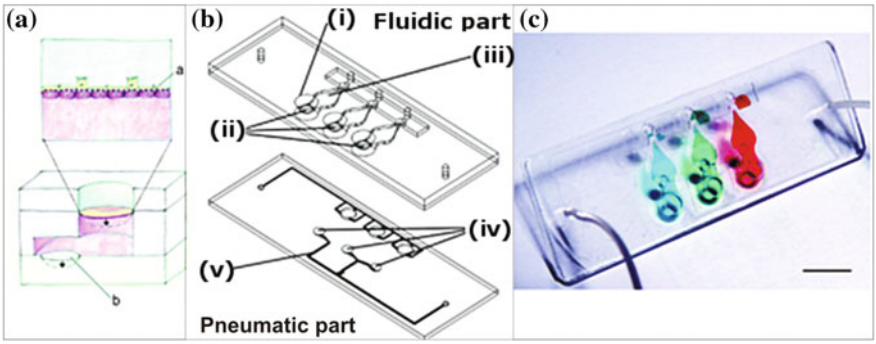

Fig. 4.4 Working principle and design of the *Lung-on-a-chip*. **a** In vitro, the 3D cyclic mechanical strain of the bioartificial alveolar membrane (a) induced by a micro-diaphragm (b) that is actuated by an electro-pneumatic setup. The bioartificial alveolar membrane consists of a thin, porous, and stretchable membrane on which epithelial and endothelial cells are cultured. **b** A scheme of *Lung-on-a-chip* system, which consists of three alveolar cell culture wells (i) and thin, porous, and flexible membranes (ii), beneath which the basolateral chambers are located (iii). The micro-diaphragms (iv) are integrated into the pneumatic part and connected to pneumatic microchannels (v). **c** Photograph of the microsystem filled with food-dye-colored solutions inside the basolateral chambers. Reprinted with permission from Stucki et al. (2015). Copyright 2015 Royal Society of Chemistry

flexible and permeable membranes. Track-etched polycarbonate (PC), as an example of a material which is quite stiff and permeable, does not accurately represent a mechanically dynamic environment. Otherwise, highly flexible, casted membranes, like those made of PDMS, act as a sealant to provide breathing mechanical movements. To create physiologically accurate alveoli, Huh et al. (2010) designed a platform based on two compartments separated by a porous, flexible membrane made of PDMS, coated with fibronectin or collagen (proteins naturally present in ECM). Mimicry of alveolar air space was made possible by introducing air into the upper compartment, where the human cells of alveolar epithelium were cultured. The human pulmonary endothelial cells were seeded on the opposite side of the intervening membrane. The structure of the chip consists of two lateral, larger microchambers for mimicry of respiratory movements, causing dynamic mechanical distortion of the alveolar–capillary interface. It was observed that the attached PDMS membrane and the adherent tissue layers stretch. Investigations on organ-level responses to cytokines inflammatory and microbial contamination were performed using the designed chip. The described system was also a useful tool for predicting potential nanotoxic effects—the obtained results showed that the cyclic mechanical strain impacts the cytotoxicity of silica nanoparticles. The reconstruction of inter-tissue borders and mechanical stimulation conditions—essential for lung functionality—makes the device reported on a suitable tool for predicting potential toxic and therapeutic effects.

4.2.1.3 Kidney-on-a-chip

One of the most meaningful issues of novel biologically active substances and alternative therapies development is potential nephrotoxic effects, often involved in a variety of processes such as active clearance, reabsorption, and local interstitial drug accumulation (Perazella 2009; Schetz et al. 2005; Tiong et al. 2014). Nephron is the minimal functional unit of kidney. Three main components such as the *glomerulus*, proximal convoluted tubule, and loop of Henle are distinguished in nephron (Weinberg et al. 2008).

The earlier reported research on the drug-related effects of tubular injuries was performed with the use of tissue slices, isolated cells, and commercially available kidney cell lines cultured on the membranes of Transwell insert dishes—porous or fabricated of polymeric materials (Baudoin et al. 2007; Gunness et al. 2010; Zhou et al. 2014). The proposed cellular kidney models based on the biology of phenotypically homogenous cells do not represent physiologically relevant phenotypic characteristics of primary renal proximal convoluted tubule cells. As a consequence of constant flow through the glomerular filtrate, the proximal tubule epithelium on both the basal and apical surfaces is stimulated under shear stress conditions. Because of the basal surface exposition to shear stress, the active transport of a number of biologically important components such as amino acids, drug metabolites, and glucose through epithelium is allowed (Jang et al. 2013). For better understanding of shear stress-related phenomena and renal functionality, a number of microfluidic platforms replacing the conventional and static Transwell culturing systems have been demonstrated (Duan et al. 2008, 2010; Jang et al. 2013). To create a basal "interstitial" space as well as an apical "luminal" channel, Jang et al. (2011) designed a microfluidic platform for culture of primary kidney epithelial cells. The microsystems consisted of one main culturing chamber separated into two adjacent channels by a porous, polyester membrane, which was coated with components present in ECM (Fig. 4.5). The formation of specific microarchitectures as well as apical fluid shear stress exposition was attained by cell culturing in the upper side of used membrane. The observed effect of attaching the epithelial cells to the cellular monolayer and enhanced by cell polarization has a significant impact on glucose reabsorption and transport of albumins. In comparison to conventional, static Transwell culturing platforms, the differences in alkaline phosphatase activity of the brush border epithelial cells were also observed. The utility for preclinical renal toxicity trials of the use of the in vivo-like kidney proximal *Tubule-on-a-chip* systems has been proven. In the microsystem, the rearrangement of the cytoskeleton, enhanced cell polarization, and reinforced cell junctions under exposition to hormonal stimuli was tested. The major role of inner medullary collecting duct (IMCD) cells in processes of water regulation and ion balance by molecular transport as the effect of hormonal stimulation in in vivo-like tubular environments was also developed.

As was mentioned before, nephron is characterized by three main components: the *glomerulus*, proximal convoluted tubule, and loop of Henle. Therefore to obtain good in vivo-like model, all nephron functionality should be mimicked. It should be

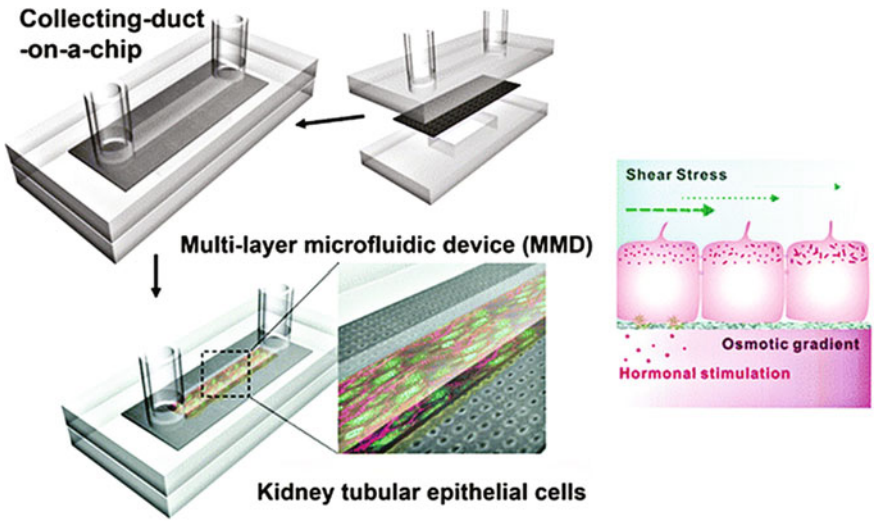

Fig. 4.5 A scheme of *Kidney-on-a-chip* system in which a flow and transport barrier functions were simulated. The microsystems consisted of one main culturing chamber separated into two adjacent channels by a porous, polyester membrane, on which kidney epithelial cells were cultured. A continuous hydrodynamic flow that produces shear stresses was also applied. Reprinted with permission from Jang et al. (2011). Copyright 2010 Royal Society of Chemistry

noted that a microfluidic system to mimic nephron features e.g., filtration of the glomerular unit with the filtration fraction was proposed by Weinberg et al. (2008). The microsystem consisted of a porous membrane, which played the role of artificial *glomerulus* and separated two microchip layers. In this part of the designed system, some components of a circulating blood sample might pass through the porous membrane into the filtrate as part of the formation of urine. Then, the filtrate flows into the proximal convoluted tubule section, where the processes of active reabsorption through the membrane take place. The reabsorption of ions and remaining water molecules takes place in the looping channels, mimicking the countercurrent mechanism of the loop of Henle. Nephron functions such as osmosis, diffusion, and active pumping are accurately reconstructed in the proposed microsystem. Thanks to that the reabsorption of remaining filtrate fluid has a final output of highly concentrated waste urine (the final concentration: 200–400 $\times$ 10^{-3} mol dm^{-3}).

4.2.1.4 Spleen-on-a-chip

The spleen is a lymphoid organ, which selectively filters infected, senescent, and damaged red blood cells as well as parasites from the *Plasmodium* family which are responsible for infections originating in the blood (Bowdler 2001). The blood surveillance function is connected with specific spleen microarchitecture, which

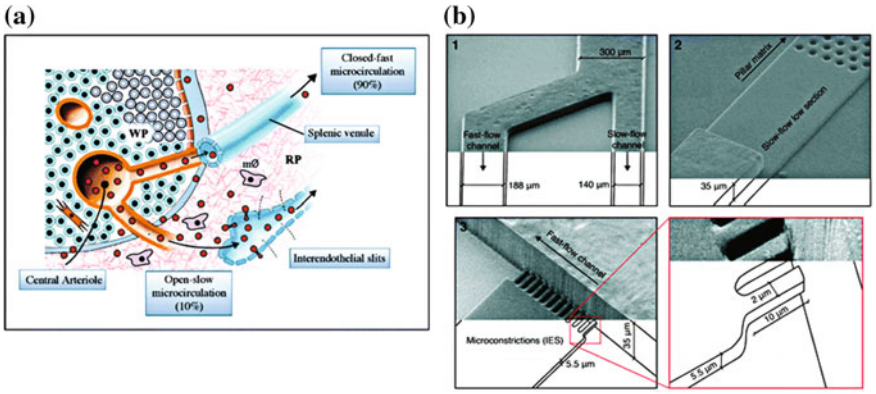

Fig. 4.6 *Spleen-on-a-chip* system: **a** diagram of the human spleen showing the closed-fast and open-slow microcirculations as well as the interendothelial slits (IES); **b** the fabricated microchannels within slow-flow channel to mimic IES. Reprinted with permission from Rigat-Brugarolas et al. (2014). Copyright 2014 Royal Society of Chemistry

consists of spleen pulp (white and red) and the marginal zone. The filtration effectiveness is due to slow blood microcirculation through the reticular meshwork of the spleen red pulp, which accompanies the increasing hematocrit. Next, specialized macrophages recognize and destroy pathologically changed red blood cells. To develop spleen in vivo-like model in the microsystem, all of the above spleen features should be taken into consideration. For example, Rigat-Brugarolas et al. (2014) have proposed a *Spleen-on-a-chip* device, which reconstructs hydrodynamic forces as well as physical and filtering properties of the minimal functional unit in spleen red pulp (Fig. 4.6).

Additionally, the proposed model gave the ability to maintain blood surveillance functions. To mimic specific microcirculation, two main microfluidic channels allowing physiological flow were designed. The pillar-based matrix, analogues to the reticular mesh, was created to mimic the hematocrit increase. The microsystem was used for investigation of new and old red blood cells, as well as cells infected by *Plasmodium vivax*. The obtained results confirmed that *Spleen-on-a-chip* system could be applied as the model for testing different types of red blood cells in both: physiologically proper and pathologically disturbed.

4.2.1.5 Brain-on-a-chip

The brain is one of the most complex and exquisite organ in the human body. Structural and functional hierarchy as well as high specialization are the specific features of the brain. Therefore, the identification of the minimal structural unit recapitulating the complexity and functions of the brain and central nervous system, even represented as a single neurotransmitter, might be potentially challenging

(Millet and Gillette 2012). Numerous processes and functions such as angiogenesis, immunological functions, or remodeling occur due to highly effective synaptic transmission through the communication of parenchyma cells within the extracellular matrix. The observed phenomena are tightly controlled by neural tissue in the central nervous system. Within the parenchyma of neural tissue, a variety of different cell types can be distinguished: primary synaptic effectors (neurons), astrocytes, microglia and oligodendrocytes (Abbott et al. 2006; Achyuta et al. 2013; Benarroch 2007).

Some brain models based on circular microfluidic coculture systems were described in the literature (Kilinc et al. 2011; Odawara et al. 2013). The structure of cerebral cortex consists of six layers with complex directional growth and multilayer connections mimic neurodevelopment. Because of that, the mimicking of specific brain microarchitecture with high accuracy requires the use of a 3D spatial model. The reconstruction of the cerebral cortex structure through control of the positions of the somata and the direction of neurite outgrowth on the basis of the orientation of collagen fibers was studied in the microsystem (Odawara et al. 2013). 3D spatial model of the brain can also be based on the cellular multilayer, obtained in alginate enriched agarose scaffolds. A novel microsystem was proposed for studying possible mechanisms of axon degeneration and death processes under control of space and specific molecular pathways (Kilinc et al. 2011). A scheme of a *Brain-on-a-chip* system is shown in Fig. 4.7. The microsystem was integrated with a porous PDMS membrane, which enabled the investigation of interaction between vascular (human brain microvascular endothelial) and neural (human neuronal and glial) cells.

Spatial arrangement of neural cells and 3D broad neural networks can be mimicked in the microsystems using neuro-spheres and spheroids networking

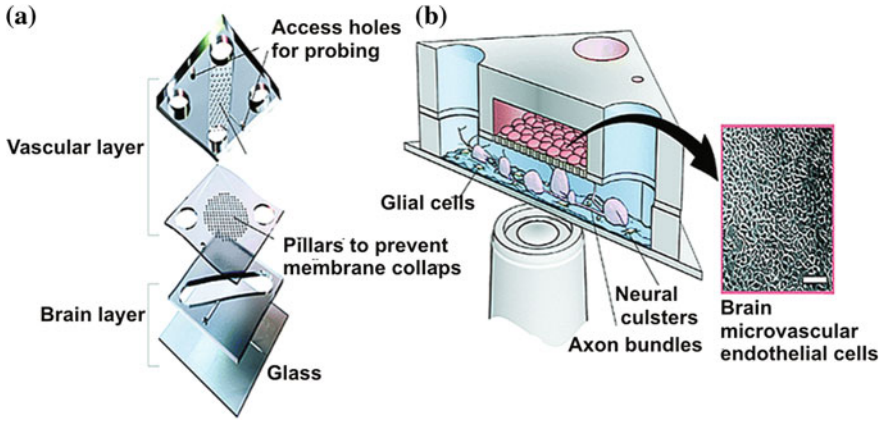

Fig. 4.7 **a** A scheme of *Brain-on-chip* system layers. **b** Cross-section of final *Brain-on-chip* platform showing culture of human neuronal and glial cells, interacting through a perforated membrane with a monolayer of human brain microvascular endothelial cells. Reprinted with permission from Kilic et al. (2016). Copyright 2016 Royal Society of Chemistry

(Jeong 2014; Kato-Negishi et al. 2013). Such models might be a tool for the analysis of morphological and functional changes of communicating neurons in order to study higher order neural networks between various brain regions. The microsystems with microgrooves can also be used for directing, isolating, and biochemical analysis of central nervous system axons.

3D organoids and multilayers also play a crucial role in the development of *Brain-on-a-chip* systems (Eiraku et al. 2008; Lancaster et al. 2013; Mariani et al. 2012). The reconstruction of various discrete regions of the hindbrain, midbrain, forebrain, as well as choroid plexus is realizable due to the formation of 3D organoids. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are used for generating three germ layers to form 3D polarized neuroepithelial structures. For example, Lancaster et al. (2013) developed bioreactor for neuroectodermal tissue generation and more complex tissue growth. The proposed system was based on a human pluripotent stem cell-derived (hPSCs-derived) 3D organoid culture, embedded in droplets of Matrigel. *Brain-on-a-chip* models, based on 3D spatial cultures and organoids from different genetic origin, have great capabilities in studies on human nervous system disease mechanisms and as a potential replacement for animals in preclinical trials.

4.2.1.6 Liver-on-a-chip

The liver is responsible for the detoxification processes, plasma protein synthesis, and glycogen storage. The liver is an organ with a highly complex structure. It contains hepatic lobules, formed of sinusoids and blood vessels lined with a permeable endothelium and surrounded by polarized hepatocytes and numerous mesenchymal cells (hepatic stellate cells (HSCs), macrophages, lymphocytes, and Küpffer cells). Hepatocytes are essential for ADMET (absorption, distribution, metabolism, excretion, and toxicity) model. These cells are closely related with liver functions. Therefore, this type of the cells is used for investigation of cytotoxicity mechanisms of novel pharmaceutical substances as well as the metabolism of xenobiotics. The examples of the microtechnologies used in studies on liver functionality and their applications are shown in Fig. 4.8.

One of the most commonly applied methods for hepatocyte activity amplification is their coculture with fibroblasts and nonparenchymal liver cells, i.e., endothelial, hepatic stellate, or Küpffer cells (Cho et al. 2010; Du et al. 2014; Kane et al. 2006; Kang et al. 2015; Khetani and Bhatia 2008). It allowed a specific intercellular communication to be investigated. For example, a miniaturized coculture platform based on 24-well plates was used for investigation fibroblasts surrounding the human hepatocytes. The hepatocytes formed well-organized colonies on micro-patterned islands coated with collagen. The importance of communication between coculture cells by canalicular transport, phase I/II metabolism, assessing gene expression profiles, secretion of products specific to liver function, as well as susceptibility to hepatotoxins was tested in the developed system (Khetani and Bhatia 2008). In comparison to models based on hepatocyte

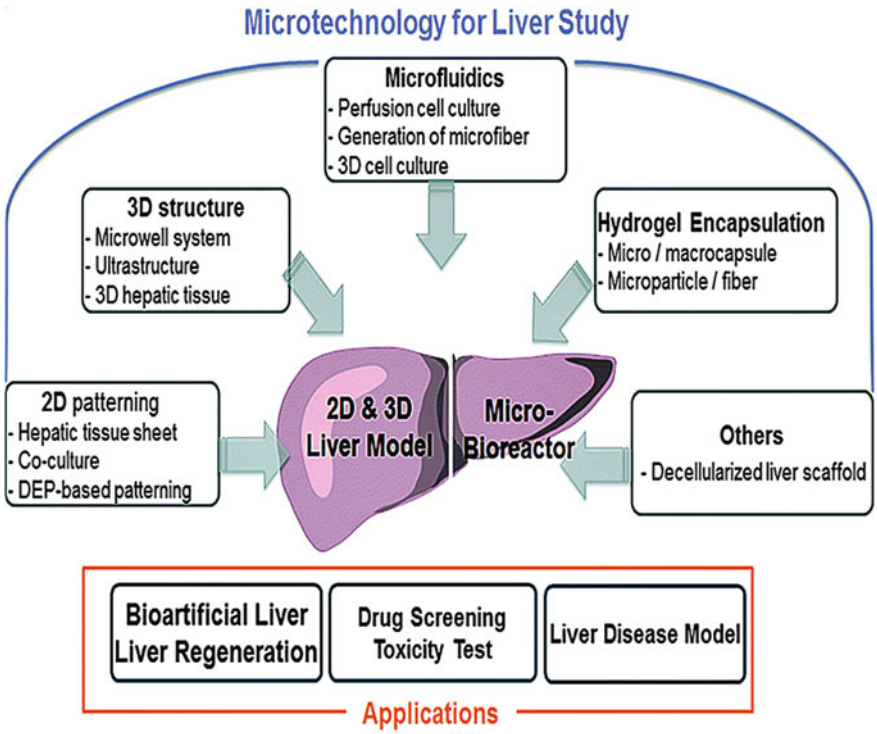

Fig. 4.8 Examples of the microtechnologies used in studies on liver functionality and their applications. Reprinted with permission from No et al. (2015). Copyright 2015 Royal Society of Chemistry

monoculture, the coculture models consisting of at least two cell types mimic hepatic function with higher efficiency. Du et al. (2014) reported encapsulation of hepatocytes with endothelial cells, differentiated from hiPSCs with the presence of recombinant protein. The cells were placed in fibers of multicomponent hydrogel and organized into 3D-patterned endothelialized liver tissue structures. The microsystem was composed of two PDMS chambers separated by a 10 μm-thick polyethylene (PE) membrane containing pores with 0.4 μm diameter (Fig. 4.9). The positive impact of stellate cell attendance on hepatocyte detoxification functions and the increased albumin secretion in a coculture with Küpffer cells were demonstrated. The relevant development in functionality of hepatocytes was determined in cocultures with cells derived from endothelium. Improved vascularization of the fiber scaffold by implanting it in a mouse model of partial hepatectomy was observed.

A hepatocyte, hepatic stellate cell, and endothelial coculture model enabled the assay of hepatic functions under static as well as perfusion conditions (Sumii et al. 2012). The flow loading also influenced hepatic function improvement (Ho et al. 2013; Prodanov et al. 2015). Cellular aggregates and spheroids were successfully

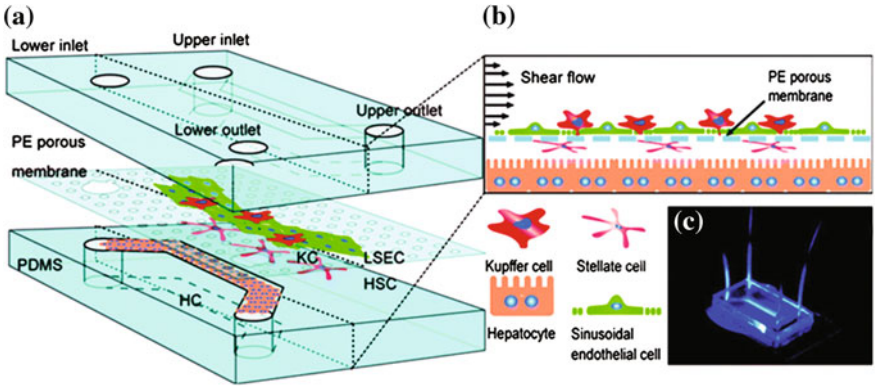

Fig. 4.9 **a** A scheme of the in vitro 3D liver sinusoid liver chip. **b** A scheme of 3D assembling. The four types of hepatic cells: liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and hepatocytes were distributed layer-by-layer in a 3D manner. **c** A photographic image of an in vitro 3D liver sinusoid liver chip. Reprinted with permission from Du et al. (2017). Copyright 2017 Royal Society of Chemistry

used for better mimicking of liver functions (Chu et al. 2009; Feng et al. 2009; Lee et al. 2013; Schutte et al. 2009, 2011). Wong et al. (2011) developed a microwell platform for quantitative and qualitative analysis of the formation processes of liver cell-based spheroids. The intercellular communication was also examined in this chip. Monoculture of primary hepatocytes as well as coculture of primary hepatocytes and hepatic stellate cells was tested in the microsystem. The investigations of cell metabolism in such aggregates showed increased cytochrome P450 enzymatic activity and albumin secretion in the heterospheres in comparison to hepatospheres. Feng et al. (2010) developed a microdevice for hepatocyte spheroid culturing based on an electrospinning technique. The described device enabled aggregates to be obtained which exhibited cell fiber structures and functionality specific for the organ. A nanofiber scaffold-based bioreactor for inducing hepatocyte spheroids to assemble with galactosylated chitosan electrospun fibers was also proposed (Chu et al. 2009; Feng et al. 2009).

The next approach to the investigation liver in a microscale is mimicking of a liver injury-on-a-chip (Zhou et al. 2015). For this purpose, the authors developed the microsystem, which consisted of two chambers for coculture hepatocytes with stellate cells and three additional microchambers integrated with miniaturized electrodes for electrochemical monitoring of liver cell response. Alcohol-related hepatotoxic effects were tested in the microsystem. The simulation of a liver sinusoid with a microfluidic artificial endothelium barrier was also developed in the microsystems. For this purpose, packing a high density of hepatocyte suspension into a microchannel is used to create liver acinus-like mass transport properties (Lee et al. 2007). Densely packed hepatocytes were fed by diffusion of nutrients across the parallel carrying microchannels. The described solution mimics the numerous mass exchange characteristics of functional liver sinusoids such as: defined tissue

and fluid transport regions, continuous nutrient exchange, and extensive intercellular contact. The microsystems are also utilized for hepatotoxicity assays (i.e., diclofenac). The novel categories of liver sinusoid models based on perfusion channels and separated chambers for cell culturing were developed with the presence endothelial-like physical barriers. This class of liver models was called an integrated microphysiological analysis platform (iMAP) (Hong and Lee 2014). The iMAP for an iPSC-derived hepatocyte model offers a lot of benefits like the possibility of obtaining enhanced drug metabolism and protein synthesis with the avoidance of polarized hepatocyte dissociation, assembling into spatial sinusoidal structures as well as facilitated organogenesis-like tissue development.

Further directions for research on modeling of fully functional artificial liver microarchitecture might be developing the space between blood sinusoids on a background of fenestrated endothelium and hepatocytes as well as basolateral villi. Domansky et al. (2010) proposed a microplatform enabled hepatocytes with stellate, Küpffer, and liver sinusoidal endothelial cells to be cultured under continuous perfusion by diaphragm micropumps integrated with a multiwell plate format. The system consisted of bioreactors separated by fluidic channels and containing scaffolds at densities comparable to normal tissue densities and specifies the functional zonation of hepatocytes. A prediction of optimal parameters for primary liver cell culturing might be established by continuously adjusting oxygen consumption and transport in the circulating culture medium. The described microplatform also allows the regulation of flow rates based on oxygen consumption and long-term steady maintenance of the oxygen gradient. Because of the highly accurate mimicking of complexity of liver sinusoid functionality and responses to numerous stimuli, this in vitro model is a promising tool for studying the liver metabolism and biological activity of novel pharmaceutical substances.

4.2.2 Cells Sources

One of the most meaningful issues during the process of designing *Organ-on-a-chip* models is the proper choice of cell sources. Animal tissues, widely used as the primary cell sources in most current experimental models, are distinguished by significantly different physiology and pathophysiology in comparison to a human's. What is more, models based on cell cultures and tissues of animal origin do not represent most human diseases and pathological conditions (e.g., neurological and autoimmunological disorders) adequately. In consequence, the investigations utilizing human cell sources with numerous inherent genetic variations are requisite (Boughton et al. 2011; van der Meer and van den Berg 2012).

There is a wide range of possible cell sources as tissues cultured under ex vivo conditions, primary cultures, stem cells, and immortalized, commercially available cells. One of the main requirements of cell sources is their ability for high reproducibility and reliability on pharmaceutical applications such as screening research and simultaneous research on a lot of novel, potentially therapeutically effective

substances (Huh et al. 2011). It is worth noting that each possible cell source has limitations. Immortalized cell lines, the most commonly established for studies on the in vitro level, are distinguished by homogenous and non-patient-specific genomes as well as by phenotype and mismatches with whole tissue. For comparison, acquisition and further culturing of the patient-specific derived from patient tissues and genetically nonhomogenous primary cells are potentially problematic (van der Meer and van den Berg 2012). The availability of ex vivo cell sources, except for tumors, is limited. Moreover, the assays based on this type of cell source must be performed within several hours due to the fact that there are rapid changes in cell function (Verpoorte et al. 2015).

Recently performed studies have brought great development in functional stem cell production technology with the use of genetic manipulation (Giobbe et al. 2015). However, the elaboration of proper protocols for stem cell differentiation under controlled conditions, with avoidance of chromosomic aberrations or epigenetic DNA changes, might still be a challenge. Stem cell technology offers a lot of potential benefits for research possibilities on neurological genetically inherited and cardiac diseases and creating novel platforms for personalized medicine (Wagner and Ho 2007).

4.3 *Human-on-a-chip*—The Integrated Multi-organ Platforms

The human physiology and the organism's functionality under pathological conditions as well as the response to the tested treatment are the results of interaction between different organs. Microfluidics is one of the most common strategies for physiological studies on the integration of cell culturing sections, each representing major organ-level functions (Blake et al. 2010; van Midwoud et al. 2011a, b). This approach allows the metabolism of therapeutically effective substances and the absorptive events as well as the response to electrical stimulation to be monitored. On the other hand, the limitation of these specimens' viability as well as the lack of normal chemical and mechanical stimulus in the described studies precludes the use of this approach in further research on possible toxic and side effects of the drugs (van Midwoud et al. 2011a). As an alternative solution, Esch et al. (2011) have proposed a *Body-on-a-chip* model. The described device was based on a system of multiple microchambers (each one contains a different type of the cells) connected by a network of microfluidic channels that permit recirculation and exchange of metabolites in a physiologically relevant manner (Fig. 4.10). The main limitations of the first variant of such approaches were the lack of optimal tissue-specific differentiation or organ-relevant microenvironments. By replacing the 2D analog cell culturing models with a more complex, spatial model based on hydrogel, it was possible to obtain more physiological accurate conditions. The human cells suspended in hydrogels (e.g., Matrigel, alginate solution) were gelled in the

microdevice. In consequence, three interconnected compartments with 3D tissue constructs (liver, bone marrow, a colon tumor) were formed. Such a solution, in contrast to the conventional cell cultures, enabled efficacy, hepatotoxicity, and hematological toxicity to be simultaneously observed as the effects of exposition to an anticancer drug (Tegafur). The results obtained were comparable to the effects observed under in vivo conditions. In the next steps, the described approach was developed by replacing the external pumps with the operation using gravity-induced flow as well as by the use of a more advanced mathematical model of the pharmacokinetic and pharmacodynamic (PK/PD) profiles of the anticancer drugs to predict the experimental data (Imura et al. 2010; Sung et al. 2010). Analogous solutions were also used as a tool for studying the biological activity of anti-breast cancer drugs in the presence of liver and intestinal models which represented human metabolism and hepatotoxicity (Imura et al. 2010).

4.4 Future Directions for Research

There are numerous challenges to overcome before *Organ-on-a-chip* systems will be widely used for biochemical applications. The greatest limitation of such systems is the problem of precisely controlling of specific microenviroment for human cell differentiation and maturation. The lack of compatibility between microdevices (owing to their small dimensions and small sample sizes) and conventional biochemical and measurement techniques hinders downstream and online analysis of biological parameters. What is more, in current laboratory practice, most of the cell

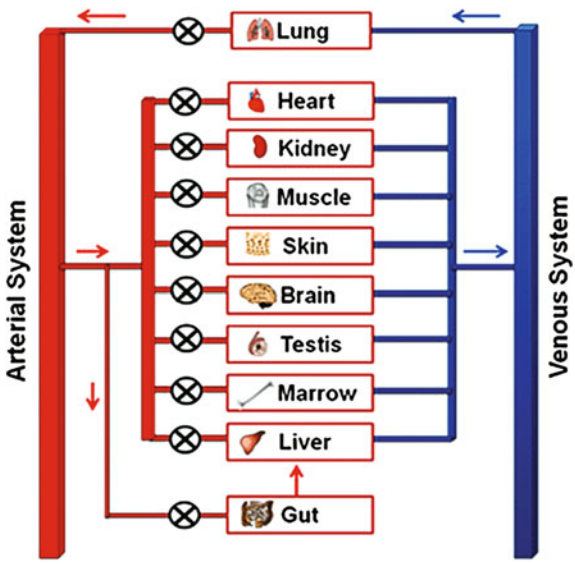

Fig. 4.10 A scheme of *Human-on-a-chip* system. Reprinted with permission from Huh et al. (2012). Copyright 2012 Royal Society of Chemistry

culture substrates are based on synthetic materials which cannot imitate the ECM adequately. One of the most common strategies is the use of chemical surface modification to minimize the effectiveness of absorption of small organic compounds (e.g., drug molecules) in widely used PDMS. Despite their limitations, *Organ-on-a-chip* systems have great potential for becoming a tool for drug or alternative therapies discovery.

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Chapter 5
Biological Bases of Cardiac Function and the Pro-regenerative Potential of Stem Cells in the Treatment of Myocardial Disorder

Karolina Anna Bednarowicz and Maciej Kurpisz

5.1 Cardiac Function and Pathophysiology of Myocardial Infarction

The heart is one of the most important organs of the organism. It pumps and distributes the blood in a closed system of vessels throughout the whole organism, nourishing and supplying oxygen to even distant tissues.

The heart consists of four cavities: the right and left atria, and two ventricles—right and left (Fig. 5.1). The atria are connected to the ventricles, while the right and left halves of the heart are completely separated from each other. The right atrium is separated from the left by the atrial septum; the ventricles are separated by the interventricular septum. Atrioventricular canals, which are surrounded by fibrous rings, to which the valves are attached, are located between the atria and ventricles. The right atrioventricular valve (tricuspid valve) is located in the right atrioventricular canal; while in the left atrioventricular canal—left atrioventricular valve, also called the mitral valve (bicuspid valve). The valves do not allow the retraction of the blood into the atria during the contraction of ventricles.

Blood to the atria is supplied from the veins, while the beginning of the arteries takes from the ventricles. From the left ventricle exits the main artery (aorta). Its branches supply the tissues with oxygenated blood and nutrients. After passing through the capillaries, blood deoxidizes and collects carbon dioxide (from the body) and then flows into the right atrium (large circulation or systemic circulation). From the right atrium, the blood enters the right ventricle and thus through the pulmonary trunk and pulmonary arteries—to the lungs. In the lungs, the blood returns carbon dioxide, receives oxygen, and is delivered to the left atrium through the pulmonary veins (small circulation or pulmonary circulation). There is a clear

K. A. Bednarowicz · M. Kurpisz (✉)
Institute of Human Genetics Polish Academy of Science,
Strzeszynska 32, Poznan 60-479, Poland
e-mail: kurpimac@man.poznan.pl

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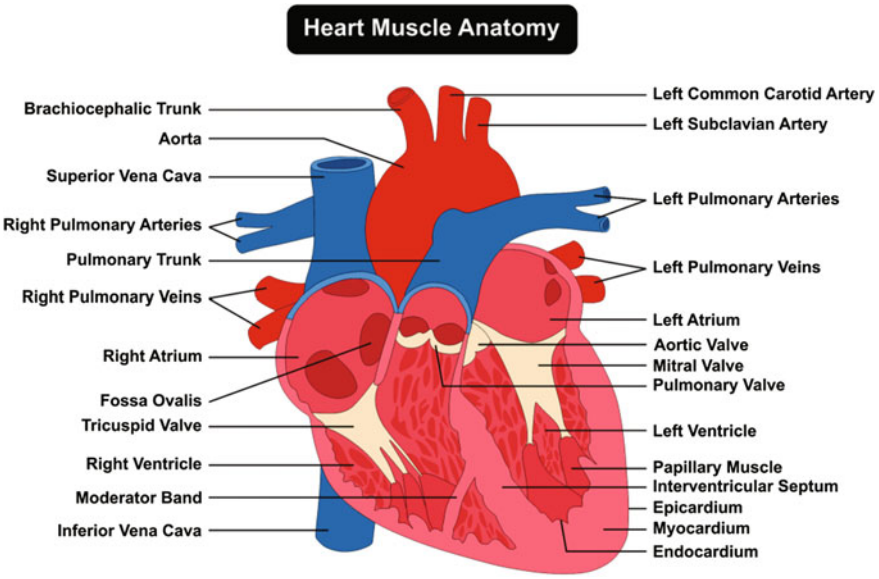


Fig. 5.1 Heart muscle anatomy. Reprinted with permission from https://www.shutterstock.com/image-illustration/human-heart-muscle-anatomy-infographic-chart-552509575

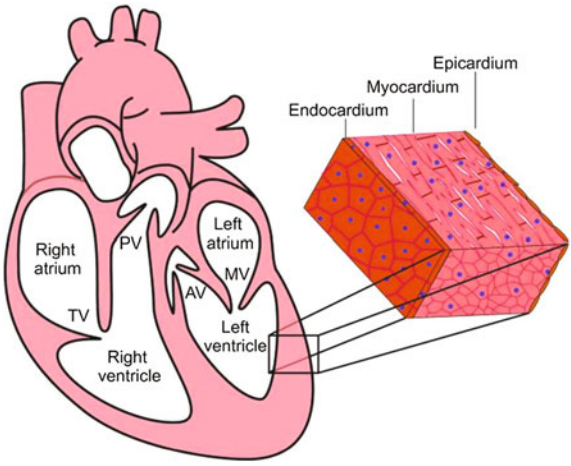


Fig. 5.2 Structure of a mammalian heart. The wall of each chamber consists of three tissue layers: endocardium, myocardium, and epicardium. *PV* pulmonary valve; *TV* tricuspid valve; *AV* atrial valve; *MV* mitral valve. Reprinted with permission from Lin et al. 2012. Copyright 2012 The Company of Biologists

division on the right half of the heart with deoxygenated blood and the left half with arterial (oxygenated) blood.

The heart wall is made up of three histological layers: the inner endocardium, middle myocardium, and outer epicardium (Fig. 5.2).

The epicardium is a thin serous membrane covering the outer surface of the myocardium along with the coronary vessels lying on its surface. At the starting point of the large vessels, the endocardium converts into outer pericardium (pericardial sac).

The endocardium is a thin, transparent membrane. It consists of a thin layer of connective tissue covered with a layer of flat epithelial cells. It covers the walls of the atria and ventricular chambers, passing directly into the membrane lining the internal surface of the vessels. The valves are made of fibrous connective tissue, covered on both sides by the endocardium.

The myocardium consists of two types of muscles. These are atrial and ventricular muscles, separated from each other by the fibrous rings surrounding the atrioventricular atrium. The myocardium is made of striated muscle tissue. It differs in its structure from skeletal muscles in that the individual cells connect the branches of neighboring cells, creating a specific muscle network with distinct histological pattern. The myocardium is made up of myogenic cells that have muscular fibrils and basic contractile elements, among others, cell filaments (Fig. 5.3). The thin filaments are formed from interconnected protein molecules—actin, and the thick filaments are built out of myosin. Thick and thin filaments are alternately arranged, with little overlap on each other. According to microscopic observations, this is visible in the form of alternating bright (actin) and dark (myosin) stripes. At half-length of the light stripe, you can see a thin, dark line (line Z). It is a membrane that divides muscle fibers into parts called sarcomers. Due to this membrane and the gap junctions, stimulating signals can be transferred from one cell to another and the cardiac muscle forms a functional syncytium. The muscle contractile phenomenon explains the "sliding" model, according to which a contraction is the result of the insertion of actin filaments between the myosin heads. This phenomenon can be observed at the level of each sarcomere, and the final effect of muscle fiber contraction is due to the sum of contractions within individual sarcomere.

Myocytes as well as the extracellular space can be distinguished in the myocardium (being the most active structural components). The space can be divided into extracellular fluid (interstitium), collagen, and fibronectin fibers. The other morphotic elements may belong to coronary vessels, i.e., endothelial cells, smooth muscle cells as well as fibroblasts underlying the myocyte layer. Myocytes represent only about 40% of the total cell population in myocardium but occupy about 75% of its volume. Extramyocyte part creates an environment, in which myocytes work. Therefore, the condition of textured extramyocytes space for cardiac function and its capacity is not less important than the myocytes themselves.

The role of the heart as a pump depends on the synchronized changes in the physical properties of the muscles. During relaxation time, the heart maintains plasticity so that blood flows into the individual cavities causing them to stretch. During stimulation this muscle generates mechanical stress and shortens, so that blood can enter the vessels, leaving the heart chambers.

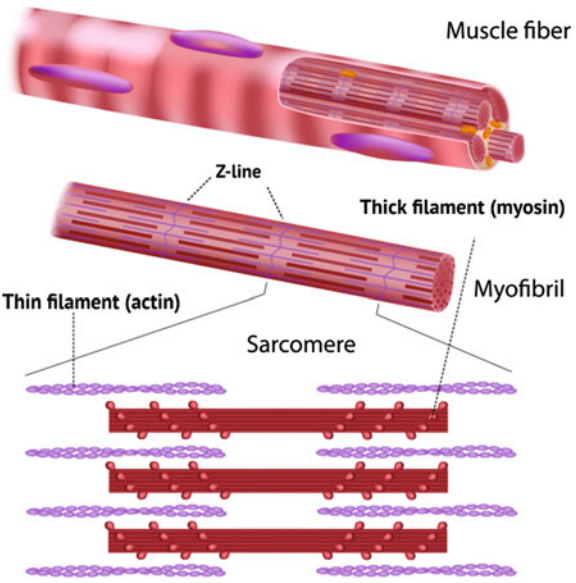


Fig. 5.3 Structure of a sarcomere. Reprinted with permission from https://www.shutterstock.com/pl/image-vector/structure-skeletal-muscle-fiber-116411524?src=5LX4lyg3Lx_7dGHE8BhYtw-1-57

The heart muscle controls its own contractions. Stimulus inducing contraction is produced in conductive heart muscle cells called Purkinje fibers (Fig. 5.4). The density as well as morphology and function of these cells is different from the other regions of the heart. They lack myofibrils and are more like smooth muscle. In these cells, there are no visible clear cross-striations. They contain more glycogen and the sarcoplasm. The cells of the conductive system stimulate themselves spontaneously and rhythmically, forming the so-called, autonomous system of the heart, in which they create contractions through depolarization. Cells of the conductive system are arranged in a very characteristic way. The sinoatrial node is the main place where synchronized stimulus arises (Keith-Flack). Stimulation propagates the first wave in the atria and then to the main chambers. In the event that the sinoatrial node is damaged, the atrioventricular one takes over as the trigger (Aschoff-Tavary). A bundle of atrioventricular (His) fibers runs from the trabecular and papillary muscles. Interruption of the connection between the atria and ventricular chambers causes on the atrioventricular block, which is manifested by the fact that the atria contract independently of the chambers.

Cardiovascular diseases (CVDs) are known to be the main cause of morbidity and mortality, particularly in highly developed countries, and are usually connected with myocardial infarction (MI). According to the World Health Organization, myocardial infarction and coronary artery disease are the reason for 29% of global mortality (Mendis et al. 2011). The predominance has been increasing constantly due to an ageing population and changes in a lifestyle.

Myocardial infarction can be a model for an acute heart ischemia. Ischemia is the result of inhibited coronary perfusion, typically because of narrowing or occlusion

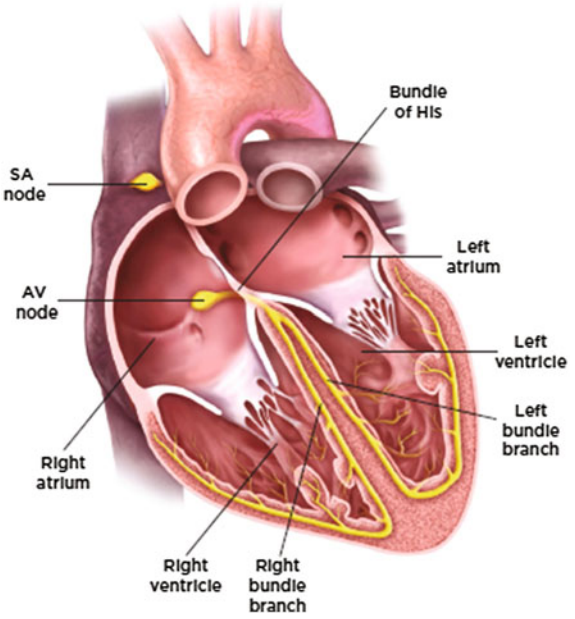


Fig. 5.4 Anatomy of the heart electrophysiological system. AV = atrioventricular; SA = sinoatrial. Reprinted with permission from Roquin 2006. Copyright 2015 Medmovie

of the coronary artery. However, human coronary collateral circulation is quite well developed. Therefore, total coronary occlusion often results in only a partial (and inhomogeneous) decrease in perfusion in the basin of the occluded artery. The accompanied decline in ATP production, proportional to the degree of perfusion limitation, increased lactic acid and cellular acidosis, decrease of myocardial contractility, and finally the death of cardiomyocytes (necrosis) may occur. The myocardial contractions may be weakened within the first few seconds of ischemia, and hypoxia is its earliest symptom. Hypoxia results in rapid acidification of heart cells, which is caused by the decomposition of ATP and the accumulation of lactic acid. Cellular acidosis is the most probable cause of early contractility disorders in ischemia, as it reduces affinity of troponin to Ca^{2+}, increases uptake, and inhibit Ca^{2+} release from the endoplasmic reticulum membrane, thus diminishing the activity of calcium channels. At a later stage of severe hypoxia (minutes, hours), loss of contractility correlates with the loss of cellular ATP and the progressive irreversible damage of cardiomyocytes. It is known that the extensive myocardial infarction reduces about 20–30% of the total heart mass (approximately 300 g), which is about 1.8–2.7 trillion cardiomyocytes.

The term "postinfarction heart remodeling" defines the structural changes in the heart following a myocardial infarction. It marks the changes at the center of the infarction zone, replacing dead myocardial tissue with connective tissue (early phase of reconstruction) as well as changes in the remaining unaffected parts of the myocardium to adapt the structures to the pathological conditions (late phase of reconstruction). The early reconstruction phase can be divided into two stages:

infarct extension associated with extension of necrosis due to the loss of cardiomyocytes in the border zone (between ischemic and healthy areas) and infarct expansion associated with passive stretching by intraventricular blood pressure and tension in the wall of the ischemic chamber. Just a few hours after the infarct, this region gets thinner, the left ventricle enlarges (becomes dilated), and its elliptical shape becomes more spherical.

Myocardial infarction is related to the interstitial fibrosis and the left ventricular (LV) dilatation, which continue to weaken cardiac efficiency and can be independent causes of morbidity and mortality after MI. LV remodeling is the consequence of overexpression of multiple factors, including angiotensin II, norepinephrine, and proinflammatory cytokines, which exert pathological effects on cardiac myocytes, non-myocyte cells, and the extracellular matrix. Current therapeutic approaches to MI (pharmacological treatment and interventional strategies) are focused on alleviation of symptoms and life extension, and they do not concentrate on the fundamental issue, which is the physical decrease (death) of cardiac viable tissue. Therefore, there is a dramatic need for new therapies that will contribute to the repair and improvement of the heart function. We shall further understand how the changes in the mechanical properties of myocardial tissue would affect the phenotype and function of cells after MI.

The most efficient therapy is the heart transplantation, but it may cause numerous problems, among others: insufficient number of donors, immunological rejection, age limitations (both of donor and recipient), complications before and after surgical intervention, and substantial medical costs. Therefore, current studies taking place around the world have been focused on stem cell-based therapies, which aim to rebuild the wasted myocardium with new functional cardiac cells. However, the results of functional stem cell therapies are limited due to multiple obstacles such as post-transplantation low cell survival, insufficient capacity of cell engraftment and their retention.

The clinical trials and experimental research attending to overpass these limitations focus on finding optimal stem cell candidates, perfect systems of cell delivery, adequate cell doses, and timing of their administration. There are two main strategies used to improve current stem cell therapy system. The first one is short term and has been focused on the pretreatment of the cells to stimulate their directed migration, retention, differentiation, and survival. The second strategy aims to ensure the optimal environment for the cells, their long-term engraftment, differentiation, and function in pathological conditions. In next section, we will summarize the latest advances on stem cell therapy and methods used to increase their efficacy for clinical application.

5.2 Stem Cells in Cardiac Regenerative Therapy; Candidates

Various types of stem cells have been included in many preclinical and clinical trials to determine their pro-regenerative ability that could be used for either direct or indirect (paracrine) effects in undertaken cell therapy. The optimal cell type should meet the postulated features: safety (no induction of immunity or tumorigenesis), pro-regenerating ability, and differentiation toward target tissues and/or organs, ease of being to be obtained with no danger of rejection and/or ethical controversy involved.

5.2.1 Embryonic Stem Cells (ESCs)

Promising candidates for cardiac pro-regenerative therapy were embryonic stem cells because of their strong ability to proliferate and differentiate into the cells from all the three germ layers: ectoderm, endoderm, and mesoderm. Nevertheless, clinical trials using these cells in humans have rarely been conducted due to bioethical issues associated with their source of origin. Other ESCs-related obstacles were possibilities for teratoma formation or immune rejection. Recently, Menasche group at Georges Pompidou European Hospital in Paris started a clinical trial (ESCORT—transplantation of human Embryonic Stem Cells-derived prOgenitors in severe heaRT failure) using pericardial flaps seeded with cardiomyocytes obtained from hESCs (Baas 2014).

5.2.2 Adult Stem Cells (ASCs)

Different populations of cells for cardiac pro-regenerative therapy have been represented by the family of adult stem cells (ASCs) including: skeletal myoblasts (SKMs), bone marrow cells (BMCs), adipose-derived stem cells (ADSCs), and endogenous cardiac stem cells (CSCs). Despite their various limitations in amount, low or variable differentiation and proliferation abilities when compared to ESCs, the efficiency and simplicity of their acquisition from the patient were the reasons that ASCs are considered to be optimal candidates for cell therapy of the heart. Moreover, the transplantation of autologous patient-derived cells eliminates ethical controversies and danger of immune rejection.

5.2.2.1 Skeletal Myoblasts (SKMs)

Mature skeletal muscle contains a reservoir of inactive and undifferentiated satellite cells, which may be transformed in a mixture of myoblasts. Myoblasts exhibit proliferative potential and can differentiate into muscle fibers regenerating damaged skeletal muscle parts. But transplanted skeletal myoblasts are mechanically and electrically detached from the host myocardium, which has been a serious obstacle for their application in cardiac pro-regenerative therapy. SKMs are one of the first cells brought into the clinical trials for heart regeneration. Small non-randomized phase I trials revealed that SKMs have a functional advantage (over the other candidates) in increasing left ventricular ejection fraction (LVEF) and improving myocardial viability. However, ventricular arrhythmias and a high loss rate of skeletal myoblasts in situ have been observed in the treated patients (Hagège et al. 2006; Siminiak et al. 2004). The first prospective randomized placebo-controlled phase II SKM trial, called MAGIC, using autologous skeletal myoblasts, showed limited or no benefits to regeneration of the postinfarction scar region (Menaschè et al. 2008). However, successive research conducted to test skeletal myoblasts as possible candidates for cardiac pro-regenerative therapy has been rather optimistic. The outcome of recent experiments demonstrated that mechanical preconditioning of transplanted skeletal myoblasts improved their interaction with recipient organ's cardiomyocytes in vivo.

5.2.2.2 Bone Marrow Cells (BMCs)

So far, autologous bone marrow cells (BMCs) have been the most commonly used for clinical therapy. The BMCs are a composition of endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs) constituting 2–4% of BMCs, mesenchymal stem cells (MSCs) < 0.1% of BMCs, and some amounts of side cell population. The induction of two major subpopulations, HSC and MSCs, were tried to provide structural elements to myocardium. The administration of MSCs into the murine myocardium demonstrated potential to differentiate into cardiomyocytes. Also, MSCs do not possess the major histocompatibility complex (MHC) antigens of class II (HLA-DR), which allow them to be applied in allogeneic transplantations. The effect of MSCs in heart regeneration is not only based on their capacity to differentiate into cardiomyocytes but is mostly related to their paracrine activities. Mesenchymal stem cells actively secrete chemokines, growth factors, and cytoprotective cytokines, which promote proliferation, differentiation, vascularization of own cardiac progenitors cells (CPC), inhibit cardiomiocyte apoptosis and myocardial fibrosis (sometimes preventing scar formation). The mechanism of MSCs is characterized by their ability to decrease the activation of NF-κB, inhibiting the expression of TNF-α and IL-6, and increasing secretion of anti-inflammatory cytokine, IL-10.

Transplantation of autologous MSCs demonstrated an increase of left ventricular ejection fraction (LVEF), decrease of the infarct size, and reversed heart remodeling

developed due to the infarction. However, based on data from meta-analysis of 49 trials performed by Nowbar and colleagues in 2014, it was only found slight myocardial recovery after BMCs transplantation (Nowbar et al. 2014). Presently, the use of BMCs in cardiac pro-regenerative therapy has shown average to modest benefits in left ventricle (LV) function. The latest research discovered that the cooperation of human MSCs with CPC's produces a better effect on the reduction of infarction size and improvement of cardiac functions than MSCs alone (Williams et al. 2013). Finally, myocardial regeneration with the appearance of new blood vessels and new cardiomyocyte populations after the BMCs transplantation has not been reported.

5.2.2.3 Adipose-Derived Stem Cells (ADSCs)

The next population of stem cells representing therapeutic potential in cardiac pro-regenerative therapy have been adipose-derived stem cells (ADSCs). The adipose tissues mostly consist of endothelial progenitor cells and adult mesenchymal stem cells, which have been shown to differentiate into a variety of cell lineages including cardiomyocytes. The application of ADSCs can effectively improve left ventricular function in preclinical animal models of myocardial infarction. For example, transplantation of ADSCs in a postinfarction rat model increased LVEF, improved angiogenesis, and reduced myocardial fibrosis (Mazo et al. 2008). Recent research has also demonstrated that human ADSCs revealed perivascular characteristics through enhanced migration in response to platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), thus leading to increased microvascular density (neoangiogenesis) (Eng et al. 2016). Additionally, ADSCs using their paracrine mechanisms can trigger native cardiac resident stem or progenitor cells (CPC) to improve heart function. Several attempts ensured that ADSCs containing a population of adult multipotent mesenchymal stem cells can improve left ventricular function but especially by the paracrine action of growth factor-mediated effects. Because the presence of cardiomyocytes within the MSC transplant seems to be rare, it is believed that MSCs control the angiogenesis mostly through paracrine pathways.

The preliminary results of clinical trials assumed that ADSCs may also improve maximal oxygen consumption and ensure stabilization of infarct size. The positive impact of ADSCs with increased cardiovascular cytoprotection and angiogenesis is believed to be associated with their multipotency and ability to secrete growth factors. Because of the potential large amount of adipose tissue in the body and the fact that ADSCs can be simply and safely obtained, this population of adult stem cells become another accessible great cell source for future cardiovascular therapy.

5.2.2.4 Cardiac Stem/Progenitor Cells (CS/PCs)

Since 2003, it has been known that the adult heart contains a population of stem/progenitor cells that can be engaged in its own regeneration process. These cells are characterized by multipotent, self-renewed, and clonogenic ability, and develop to mature cardiomyocytes, endothelial cells, and smooth muscle cells. Cardiac stem/progenitor cells were initially obtained from the adult rat heart and described as expressing the tyrosine kinase receptor c-kit and lacking any markers of hematopoietic lineage (Beltrami et al. 2003). CS/PCs have been identified in humans as well (Bearzi et al. 2007), which initiated a new direction in clinical trials. Various populations of CS/PCs have been identified and phenotyped including c-kit +, sca-1+, Is11+, cardiospheres, side cell population, epicardial, and SSEA-1+ progenitor cells. But human c-kit-positive cardiac cells are the best known and the most intensively studied CS/PC population. Some research documented the potential of CS/PCs to enhance regeneration and improvement in both LV function and structure, and heart remodeling inhibition in different animal models of post-MI cardiomyopathy (Li et al. 2011a; Linke et al. 2005; Tang et al. 2010). The results of the first clinical trial (SCIPIO) are similar to that ones obtained with the preclinical studies and assumed that intracoronary infusion of autologous CSCs provided a decrease of infarct size and an improvement of LV systolic function (Bolli et al. 2011).

Cardiosphere cardiac stem cells have also been described as a self-renewed, multipotent, and clonogenic cell subpopulation with the pro-regenerative potential to the myocardium in vivo. In the first clinical trial performed with autologous cardiosphere-derived cells (CADUCEUS), beneficial effects have been obtained (Makkar et al. 2012). The latest studies, based on this trial, but with the use of cardiosphere-derived cells from an allogenic source (ALLSTAR), resulted also in promising effects after phase I clinical trials. This led to the start of the randomized, double-blind, placebo-controlled phase 2 clinical trials to further estimate safety and efficacy of allogenic cardiosphere-derived cells in decreasing scar size in a postinfarction heart (Chakravarty et al. 2017).

The obstacle associated with CS/PCs is mainly caused by their poor retention and cell engraftment efficacy. Unfortunately, less than 1% of engrafted cells can be indentified at 4 weeks after transplantation, whereas most of the initially retained cells die due to inflammatory reaction, apoptosis, or hypoxia.

Several studies concentrated on comparing the ability of different myocardial adult stem cells types to repair damaged region. The highest rated was CSCs because of their ability to extensively produce paracrine factors, the greatest functional benefits, and the lowest dose to obtain therapeutic effects. But still, as already mentioned, the combined therapy with both CSCs and MSCs showed better results than any of these cell populations alone.

Summarizing, clinical trials focused on the postinfarction heart and based on BMSCs, CSCs, and ADSCs showed heart function improvement, also increasing the left ventricular ejection fraction and finally extending the life span of individual. However, this was mainly due to paracrine effects instead of direct differentiation

into cardiomyocytes. The new strategies, however, demonstrated another line of research when using cardiopoietic driven cell populations out of bone marrow-derived stem cells. Such manipulation could modify traditional "paracrine procedures" into more structurally oriented cells of cardiac origin (CHART-1 design) (Bartunek et al. 2016). However, conclusive documentation of such cell modification is lacking. First, genotypic and phenotypic cell characterizations were made on the grounds of MEFc nuclear/cytosol ratio, and long-term cell structural conversion has not been yet confirmed. Clinical endpoints were established at a six-month follow-up time frame, typical for "paracrine procedures" heart improvement. Further documentation is immediately needed including transplanted cell imaging to document the proof of concept.

5.2.3 Induced Pluripotent Stem Cells (IPSCs)

A pluripotent stem cell population (iPSCs) was created in 2006 by Takahashi and Yamanaka overexpressing four specific transcription factors: OCT3/4, Sox2, c-Myc, and Klf4 (so-called Yamanaka factors) in mouse fibroblasts. In this way, cells obtained revealed overexpression similar to ESC marker genes and morphology, and growth properties similar to embryonic stem cells, thus creating an opportunity to use them for regenerative medicine. Also, it is known that iPSCs differ from ESCs in respect to intensity in gene expression and DNA methylation scheme. Appeared also a concept of somatic cell reprogramming making autologous pluripotent stem cells that can be simply obtained in the laboratory, individually tailored and differentiated to specific precursors in order to eliminate rejection from the immune system of individual after aimed cellular therapy.

iPSCs may differentiate into three types of cardiomyocytes (atrial, nodal, and ventricular) with similar characteristics to "native" cardiomyocytes. No significant differences have been noticed between cardiomyocytes derived from either ESCs or iPSCs.

Most iPSCs have been obtained inducing overexpression of specific transcription factors using retroviral and lentiviral vectors (Takahashi et al. 2007), associated with the insertion of transgenes into the host cell genome. Some transcription factors, however, which generate iPSCs (c-Myc and Klf4), have been defined as oncogenes. To solve the other problems associated with induction of pluripotent stem cells, which among others is the low efficiency of iPSC output (0.001–2%), low kinetics, and no absolute safety in reprogramming, a lot of methodological corrections have been invented. For example, the reprogramming factors using Sendai viral system (SeV), established on a negative-strand RNA virus with no possibility of being stably inserted into the host cell genome, decreased the danger of random integration. Also, different methods have been expanded to fulfill the clinical application requirements through, e.g., virus-free iPSCs reprogramming (Kaji et al. 2009), piggyBac transposons (Woltjen et al. 2009), recombinant proteins (Kim et al. 2009), episomes (Yu et al. 2009), synthetic mRNAs (Warren et al. 2010),

and microRNAs (Miyoshi et al. 2011) or minicircle systems (Jia et al. 2010). Reprogramming fibroblasts to iPSCs with modified mRNA exhibited approximately 1.1% efficacy, while lentiviral transduction with miR302/367 showed 10%. Furthermore, chemical modifications have been considered as the method of choice to improve the iPSC generation (CiPSCs). Numerous small chemical molecules have been declared to replace traditional reprogramming factors in order to improve safe iPSC generation. Thus, the disadvantages of current methods could be avoided, such as the risk of tumorigenesis from random genomic integration or overexpression of harmful oncogenes.

It has been mentioned that iPSCs epigenetic characteristics, among others, histone modification, and CG methylation, may influence the pattern of the iPSC-derived cells. Since the iPSCs reprogramming from somatic cell involves global epigenetic remodeling, the efficient reprogramming process relies on chromatin modifying enzymes, for instance: inhibitors of DNA methyltransferase (e.g., 5-azacytidine) and histone deacetylase (HDAC) inhibitors (Mikkelsen et al. 2008; Huangfu et al. 2008a). For example, Valproic acid (VPA), an HDAC inhibitor, increases reprogramming efficacy by more than 100-fold. Also, VPA allows the induction of pluripotent stem cells with only Oct4 and Sox2, without using oncogene, c-Myc (Huangfu et al. 2008b).

New approaches to genetically modify human iPS cells at "safe harbor" places in the genome have been expanded, to reduce the perturbation resulting from neighboring gene expression. Safety standards made to estimate potential safe harbors are as following: a distance of at least 50 kb from the 5' end of any gene, and at least 300 kb from any cancer-related gene and microRNA, and a position outside of transcriptional units and ultraconserved regions (Papapetrou et al. 2011). Cerbini and colleagues reported observations of their latest experiments, in which they created transcription activator-like effector nucleases (TALENs) targeting the safe-harbor like gene CLYBL. It has been revealed that a target for TALEN–enhanced integrative gene-transfer, situated in intron 2 of the Citrate Lyase Beta-Like (CLYBL) gene, assured up to tenfold higher transgene expression compared to commonly used AAVS1 (Cerbini et al. 2015).

Zhang and coworkers developed a novel method of isolating a cell population with great proliferation ability and cardiovascular differentiation capacity after successful myogenic differentiation from mouse fibroblasts. They have been called, ieCPCs—induced expandable cardiovascular progenitor cells, which can differentiate into functional cardiomyocytes, endothelial cells, and smooth muscle cells. Moreover, after injection of ieCPCs into MI mouse heart the cells started to differentiate into all three subpopulations of the heart improving cardiac functions. This suggests, that ieCPCs could constitute a novel approach in pro-regenerative strategy to the heart (Zhang et al. 2016). Summary of cardiac regenerative medicine products is shown in Fig. 5.5.

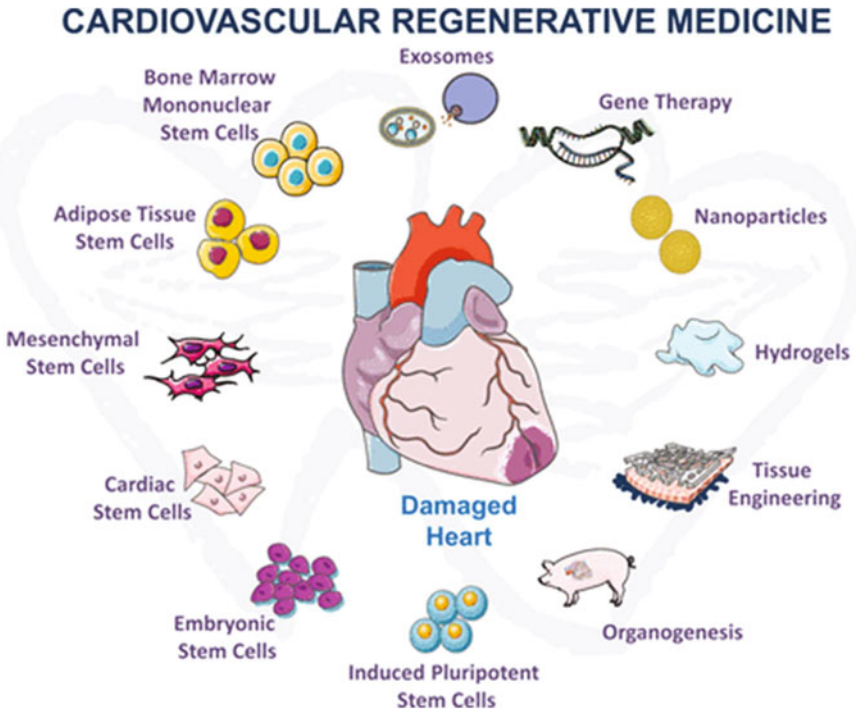


Fig. 5.5 Summary of cardiac regenerative medicine products. Reprinted with permission from Climent et al. 2016. Available from: http://circres.ahajournals.org/content/119/3/409

5.3 Novel Strategies in Cardiac Cell Therapy with Use of the Adult Stem Cells

The major issue in the transplantation of stem cells is an insufficient number of cells which may be retained in desired regions of intervention and overall low survival rate in the recipient organ. It has been proved that more than 90% of the stem cells disappear within 24 h after transplantation; this is associated with hypoxia, local ischemia, and pro-apoptotic inflammatory conditions. Furthermore, it has been documented that two hours after injection just 1.3–2.6% stem cells were retained in the myocardium and after 20 h about only 1.49% of the initial cell population was reported.

Researchers revealed that heart failure therapy based on stem cells operates within general mechanisms connected with immune surveillance, apoptosis, and angiogenesis. To address these issues, new approaches to improve the present strategies have been expanded, e.g., different methods of cells preconditioning before transplantation into infarcted myocardium.

5.3.1 Cell Preconditioning

It is known that mesenchymal stem cells cultured in hypoxic conditions exhibited increased expression of pro-angiogenic and pro-survival factors, e.g., angiopoietin-1, vascular endothelial growth factor, erythropoietin, hypoxia-inducible factor 1, Flk-1, Bcl-2, and Bcl-xL (Hu et al. 2008).

Since the time that paracrine factors were discovered to inhibit apoptosis, promote angiogenesis, and myocyte proliferation, they have been widely used to improve the therapeutic effect of stem cells transplanted into damaged myocardium. Some benefits were gained after MSCs preconditioning with cytokines and growth factors (e.g., SDF-1α), which inhibited cells apoptosis, increased their survival, engraftment, vascular density, and cooperated with SDF/CXCR4 signaling pathway improving myocardial function (Pasha et al. 2008). Recent studies reported that the preconditioning of BMSCs using the hypoxia—inducible factor 1α (HIF-1α) prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG), increased their life span and paracrine function (Liu et al. 2014), while pretreatment of adipose-derived stem cells (ADSCs) using 5-azacytidine enhanced cardiogenic differentiation (Ravichandran et al. 2013).

Similarly, preconditioning using physical factors, among others: magnetic fields, mechanical stress, and low O_2 pre-culture, has been involved in cardiac stem cell preparation. Mechanical stress inhibits the proliferation of CSCs but supports production of angiogenic factors and inflammatory cytokines (Kurazumi et al. 2011). A magnetic field was used to lead cardiac—specific differentiation into adult cardiac progenitor cells (Gaetani et al. 2009), while low O_2 conditions increased cells propagation and quality (viability) (Li et al. 2011a).

5.3.2 Genetic Stem Cell Modifications

Another strategy to improve the therapeutic ability of stem cells and enhance their chance of survival, engraftment, homing, and pro-regenerative efficiency may be a combination of the cell and gene therapies. Genetically modified stem cells have been designed to reduce the pro-apoptotic pressure after transplantation. The survival rate of MCSs has been extended after the cell modification with the anti-apoptotic gene *Bcl-2*. The results have shown the reduction of the infarct size and progress in cardiac functioning (Li et al. 2007). Improved cell survival has also been documented with MSCs transduced with heme oxygenase (*Hmox-1*), connexin43 (*Cx43*), and *Akt*, known as protein kinase B (*PKB*). Similarly, the transplantation of autologous ADSCs linked with *Hmox-1* transduction indicated improved heart function and inhibition of postinfarcted myocardium remodeling (Li and Verma 2002). Methods of genetic engineering have also been adapted to increase CPCs cell survival and proliferation. For example, overexpression of *Pim-1* kinase caused a renewal of phenotypic and functional stem cell characteristics

probably by increasing telomerase activity and extending telomere lengths (Mohsin et al. 2013). Additionally, genes responsible for the promotion of angiogenesis have also been modified, e.g., angiopoietin-1 (*Ang-1*), the vascular endothelial growth factor (VEGF), granulocyte chemotactic protein (*GCP-2*). The results obtained after MSCs transplantations in a mouse/rat model revealed significant progress in angiogenesis, cell survival index, cardiac function, and reduction of infarct size (Hua et al. 2014; Liu et al. 2012; Kim et al. 2012). Also, increased pro-angiogenic effects have been demonstrated in studies with CPCs after VEGF modification (Zhang et al. 2012) or those with high expressed Ang-1 (Zeng et al. 2012). Furthermore, human ADSCs transduced with the VEGF gene (Rehman et al. 2004) and hepatocyte growth factor (*HGF*) (Zhu et al. 2009) brought an improvement in angiogenesis and cardiac function.

Also, it has been reported that the overexpression of protein kinase G1 α (*PKG1α*) extended MSCs survival and their angiomyogenic ability in damaged myocardium. The heart function and cell survival rate were increased, but simultaneously the levels of paracrine factors and anti-apoptotic proteins (Akt, GSK3β, and Bcl-2) were raised (Wang et al. 2013).

It has been documented that genetically modified stem cells, present some problems associated with migration, hypoxic conditions, and immune response from the recipient organ. However, genetically modified MSCs by hypoxia-inducible factor-1α (*HIF-1α*) overexpression revealed an improved angiogenesis under hypoxic conditions and developed better autocrine and paracrine activities (Razban et al. 2012). Besides, intramyocardial transfection of *HIF-1α* and transplantation of genetically modified MSCs in an experimental model of rat MI showed improved angiogenesis, engraftment, cell survival, and inhibited apoptosis (Huang et al. 2014). Trying to reduce the obstacles associated with migration of the transplanted MSCs from the postinfarcted region, attempts at delivery of stromal derived factor-1α (SDF-1α), a pro-angiogenic and cardiomyocyte protective protein were initiated (Askari et al. 2003). Moreover, SDF-1α stopped hypoxia/SD-induced MSCs apoptosis through ERK1/and 2 PI3 K/Akt signaling pathways (Yin et al. 2011). It has also been shown that the transfection of mesenchymal stem cells with tumor necrosis factor receptor (*TNFR*) gene resulted in the anti-apoptotic and anti-inflammatory effects after transplantation into MI region (Bao et al. 2008). Besides, it has been documented that the enhancement of CSCs recruitment, engraftment, and reduction of infarct size was regulated through the CXCR4/PI3 K signaling pathway (Wang et al. 2012). Some research also revealed that CSCs differentiation and engraftment potential were increased simply after fibroblast growth factor (bFGF) introduction (Takehara et al. 2008).

Recently, some studies have demonstrated a new method of reprogramming mouse fibroblasts into cardiac cells using a small molecule cocktail (containing ALK4/5/6 inhibitor, GSK3 inhibitor, parnate, and forskolin) and leaving only one transcription factor—Oct4, without entering into the pluripotent state. These conditions have been very efficient and led to the creation of spontaneously beating cardiomyocytes from fibroblasts presenting heart ventricle phenotype. The introduction of small molecules reduced the dependence on genetically manipulated

transcription factors when transforming into a total pharmacological induction of cardiomyocyte development (Wang 2014).

Stem cell's ability to contract, respond, and integrate with their surrounding electrophysiological environment is critical to their reprogramming into cardiomyocytes. Vunjak-Novakovic and colleagues assumed that biomimetic electrical signals may control the internal beating properties of cardiomyocytes. They performed an electrical preconditioning of human stem cells-derived cardiomyocytes in three-dimensional (3D) culture and observed an enhancement of cardiomyocyte maturation, modification of their automaticity and increasing connexin expression, and sarcomeric pattern. Cardiomyocytes adjusted their autonomous beating rate to the frequency at which they were stimulated. This rate-adaptive action was long lasting and transferable to the adjacent cardiomyocytes. Such an electrical preconditioning brings an optimistic prospect of the implication for cell-based decrease of arrhythmia during heart regeneration (Eng et al. 2016).

5.3.3 MicroRNAs/Exosomes

It is assumed that both normal and dysfunctional cardiac mechanisms, e.g., apoptosis, myocardial fibrosis, myocyte hypertrophy, and ventricular dilation are regulated by microRNAs. The latest discovery has suggested that miR-133 and miR-1 are the main regulators of cardiac hypertrophy (Carè et al. 2007), whereas miR-499 supports differentiation of CSCs into mechanically integrated cardiomyocytes (Hosoda et al. 2011), implicating their potential therapeutic use in cardiology. Novel studies described let-7 family as the most highly upregulated family of microRNAs in cardiac maturation. Overexpression of let-7 in human embryonic stem cells-derived cardiomyocytes increased cell size, maturation of the cells, sarcomere length and contractility (Kuppusamy et al. 2015).

Another mechanism adapting the therapeutic potential of MSCs concerns the function of exosomes. They are used as vectors for miRNAs communication between cells. MicroRNA transferred via exosomes can silence compatible mRNA in target cells (Stroorvogel 2012). MSCs-derived exosomes play an important role in therapy by many anti-apoptotic miRNAs (e.g., miR-221), which initiate cell survival signaling pathway. Latest studies have simply reported that exosomal miR-221 inhibit the expression of p53 upregulated modulator of apoptosis (PUMA). Since PUMA showed an interaction with BCL-xL and p53, and activation of pro-apoptotic proteins, its inhibition by miR-221 improved CMCs survival (Yu et al. 2013). Further, last findings indicated the possibility to use the exosomes from MSCs in a few pro-regenerative attempts such as: neovascularization, anti-cardiac remodeling, and anti-inflammatory effects (Arslan et al. 2013; Salomon et al. 2013).

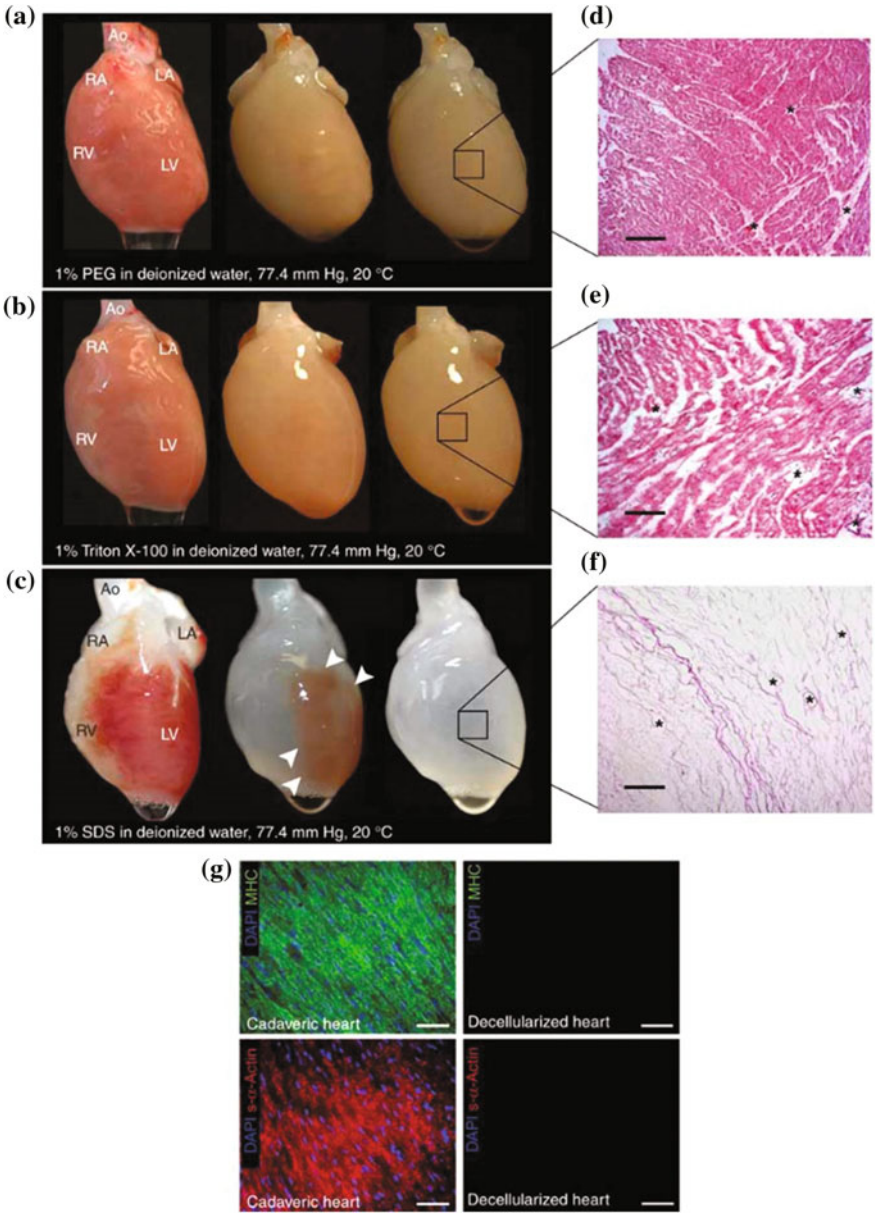
5.3.4 Drug Administration

Application of drug therapy after stem cell implantation has been tested to improve implant viability. The combined therapy with both MSCs and drugs resulted in the improvement of therapeutic effects after cell transplantation toward damaged myocardium. Statins, among others, lovastatin and rosuvastatin, are considered to have cytoprotective properties against hypoxia and serum deprivation-stimulated apoptosis via PI3 K/Akt and MEK/ERK1/2 pathways (Xu et al. 2008; Zhang et al. 2013). Also, rosuvastatin inhibited the pro-apoptotic murine proteins Bim and Bax and activated the anti-apoptotic proteins Bcl-xL, Bcl-2, and paracrine effects of MSCs (Zhang et al. 2013). Additionally, trimetazidine (TMZ) has also been described as having a protective influence on MSCs H/SD-induced apoptosis via Akt pathway as well as increasing the paracrine effect of MSCs (Gong et al. 2014). The latest research has also shown that the impact of the ASCs therapy for myocardial infarction is strengthened when the treatment is combined with cyclosporine A nanoparticles (CsA-NP) (Yin et al. 2014). Similarly, usage of 17β-estradiol (E2) increased CSCs therapeutic potential (Wang et al. 2014).

NF-κB is a transcription factor, which plays a role in the pathogenesis of heart failure activating fibrotic and inflammatory reactions (Stancovski and Baltimore 1997; Li et al. 2011b; Frangogiannis et al. 2002). The results have shown that the blockade of NF-κB action with its inhibitor decreased cell mortality and inhibited the LV remodeling. Furthermore, adapting the phosphorylation inhibitor of the IκB (the inhibitor of the NF-κB), IMD-0354 (IMD), the number of accumulated inflammatory cells in the infarcted heart regions decreased and the expression of chemokines and proinflammatory cytokines was reduced, with concomitant suppression of myocardial fibrosis (Onai et al. 2007).

5.4 Nanotechnology and Biodegradable 3D Scaffolds

A different issue in cardiac cell therapy is the stem cells injected directly into the myocardium and their migration to other distant organs. It has been reported that most of cells injected by intracoronary infusion cumulate in the border zone, not in the infarction region, whereas retention of cells within myocardium is critical for therapeutic effect. The eventual goal of tissue engineering is to replace or support infarcted regions by the transplantation of a mixture containing cells with biodegradable biomaterial scaffolds. The selection of the proper biomaterial for a scaffold that matches the conditions of the “native” microenvironment of cardiac tissue is essential. It should ensure adequate cell–material interactions and above all proper cell adhesion but not affecting the cell proliferation, differentiation, and maturation. Great progress in biomaterials production, which shows both structural


(a)
Ao
RA
LA
RV
LV
1% PEG in deionized water, 77.4 mm Hg, 20 °C
(b)
Ao
RA
LA
RV
LV
1% Triton X-100 in deionized water, 77.4 mm Hg, 20 °C
(c)
Ao
RA
LA
RV
LV
1% SDS in deionized water, 77.4 mm Hg, 20 °C
(d)
(e)
(f)
(g)
DAPI MHC
Cadaveric heart
DAPI MHC
Decellularized heart
DAPI α-cr-Actin
Cadaveric heart
DAPI α-cr-Actin
Decellularized heart

◂**Fig. 5.6** Perfusion decellularization of whole rat hearts. **a–c** Photographs of cadaveric rat hearts mounted on a Langendorff apparatus. Ao, aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Retrograde perfusion of cadaveric rat heart using PEG (**a**), Triton-X-100 (**b**) or SDS (**c**) over 12 h. The heart becomes more translucent as cellular material is washed out from the right ventricle, then the atria and finally the left ventricle. **d, e** Corresponding H&E staining of thin sections from LV of rat hearts perfused with PEG (**d**) or Triton-X-100 (**e**), showing incomplete decellularization. Hearts treated with PEG or Triton-X-100 retained nuclei and myofibers. Scale bars, 200 mum. **f** H&E staining of thin section of SDS-treated heart showing no intact cells or nuclei. Scale bar, 200 mum. All three protocols maintain large vasculature conduits (black asterisks). **g** Immunofluorescent staining of cadaveric and SDS-decellularized rat heart thin sections showing the presence or absence of DAPI-positive nuclei (purple), cardiac alpha-myosin heavy chain (green), or sarcomeric alpha-actin (red). Nuclei and contractile proteins were not detected in decellularized constructs. Scale bars, 50 mm. Reprinted with permission from Ott et al. 2008. Copyright 2008 Nature Publishing Group

and functional features like extracellular matrices, can be seen. There are two main groups of tissue constructs manufactured for cardiac therapy destination: scaffolds (based on collagen, fibrin, matrix synthetic polymers, and decellularized heart) (Fig. 5.6) and scaffold-free (cell sheets and cell aggregation technologies). There is an increasing number of evidences that nanotissue engineering is a great improvement of cellular cardiomyoplasty (Bearzi et al. 2014; Cortes-Morichetti et al. 2007; Dai et al. 2009; Maureira et al. 2012; Ott et al. 2008; Sun et al. 2014).

Different models of 3D myocardial tissues have been designed by seeding cardiomyocytes into alginate, collagen, fibrin, or synthetic polymers, e.g., poly (glycolic acid) scaffolds. The transplantation of previously invented three-dimensional myocardial tissue using cardiomyocytes and 3D porous alginate scaffolds demonstrated some positive effects. Leor and colleagues have announced nearly fully vanishing scaffold and integration into the host organ with promising recovery of heart functions, such as the decrease of LV dilatation, the improvement of LV contractility, and increased neovascularization (Leor et al. 2000).

Certain structural modifications within the extracellular myocardial matrix have been observed in the infarcted regions of myocardium. The amount of collagen type I declines from 80 to 40%. It has been defined that the collagen matrix shows some characteristics in common with cardiac tissue; therefore, it was used as a supplying vehicle inhibiting the relocation of implanted MSCs (Fig. 5.7). Much of research so far has proven that a collagen tissue patch covered with MSCs inhibits the heart remodeling process and improves myocardial function (Chachques et al. 2007; Schussler et al. 2010; Vu et al. 2012).

The studies of a rat MI model revealed that autologous mesenchymal stem cells (MSCs) placed on a collagen-1 scaffold increased perfusion and reduced an infarct size with concomitant improvement in ventricular wall thickness and promotion of angiogenesis (Maureira et al. 2012). Another collagen model helped the vascular endothelial growth factor (VEGF) distribution in genetically modified skeletal myoblasts, which improved vascularization of the damaged myocardium (Lu et al. 2001). Human embryonic stem cells and human-induced pluripotent stem

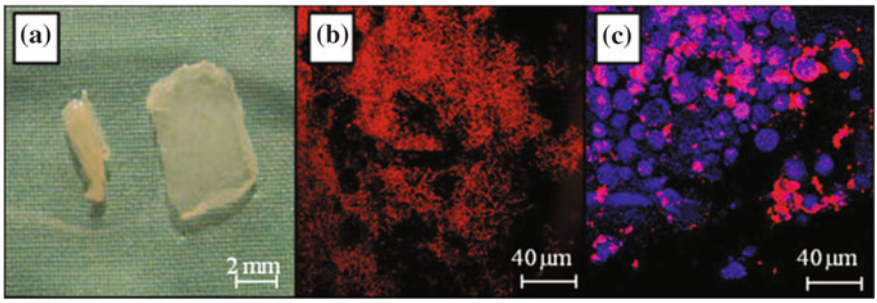


Fig. 5.7 MSC-patch characterization macroscopic view of 2 weeks in vitro cultured 3D-MSC patch (**a**), Collagen structure revealed by near-infrared and reflectance confocal microscopy (**b**), Multiphoton microscopic images of MSCs seeded in collagen patch showing positive staining for α-smooth muscle actinin (pink); the nucleus was counterstained by DAPI (blue) (**c**). Reprinted with permission from Maureira et al. 2012. Open Access—the Creative Commons Attribution License

cell-derived cardiomyocytes have also been used in a three-dimensional collagen matrix (Tulloch et al. 2011).

Fibrin patches created using both fibrinogen and thrombin with either MSCs or hESC-VCs (human embryonic stem cell-derived vascular cells) have been studied in a porcine myocardial infarction model (Liu et al. 2004; Xiong et al. 2013). Sun and colleagues also performed a test in a rat MI model, discovering that fibrin seeded with adipose-derived mesenchymal stem cells (ADMSCs) showed an improvement of left ventricle (LV) activity and reduction of LV remodeling when compared with the ADMSCs only (Sun et al. 2014).

Polyglycolic acid (PGA) (Carrier et al. 1999), epsilon-caprolactone/L-lactide (PCLA) (Matsubayashi et al. 2003; Mazo et al. 2008), poly (glycerol-sebacate) (PGS) (Marsano et al. 2010), and polyglycolic acid cloth (PGAC) (Fukuhara et al. 2005) are synthetic polymers, which have been tested in cardiac tissue engineering. The results of research with a different population of cells (BMCs, MSCs, vascular smooth muscle cells (SMCs), cardiomyocytes) conducted in various animal models have been promising for future cardiac applications. The latest results received in a mouse MI model, with both a poly (ethylene glycol)–fibrinogen (PF) scaffold and two kinds of iPSCs: MiPS (iPS cells created to secrete matrix metalloproteinase 9-MMP9) and PiPS (iPS cells made to secrete placental growth factor-PlGF), revealed an improvement in revascularization and hemodynamic parameters compared with native cells alone (Bearzi et al. 2014).

The most important benefit of three-dimensional scaffolds is its easy construction, but an emphasis has been put on the connection between the stiffness of the material and the retractile ability of the construct. It has been said that the cells stretched on scaffolds had a more mature phenotype, e.g., extended elongation, improved gap junction expression, and greater contractility. Improved survival and engraftment of implanted cells within a stretched construct have been demonstrated in a rat MI model (Mihic et al. 2014).

5.5 Whole Heart Reconstruction

Different types of putative application in the cardiac regeneration are the 3D extracellular matrix scaffolds. This model applies decellularized animal organs with a combined vascular system and extracellular matrix, e.g., three-dimensional myocardial tissue was created by reseeding cardiomyocytes into decellularized rat heart (Ott et al. 2008). The presence of structural and functional molecules in the extracellular matrix such as glycosaminoglycans, collagen, elastin, fibronectin, laminin, and vitronectin has been documented. Furthermore, it has been reported that the cardiac extracellular matrix (ECM) features, which change with time after MI, may have an influence on cardiac differentiation of implanted stem cells. The 3D extracellular matrix scaffolds as a stencil for organ reconstruction applying recellularization is believed to enhance the function and phenotype of the cells. Scaffold material obtained by decellularization is to be tested to ensure that it maintains, e.g., its integrity, bioactivity, vascular, lymphatic, and neuronal systems. Since ECM is considered to be biodegradable and does not cause an undesirable response from the host immune system, it seems to meet the requirements of the perfect biomaterial for the tissue engineering. It has been reported that the growth factors from ECM degradation, such as bFGF, VEGF, positively affect the ability for recruitment and proliferation of the cells seeded in the bioscaffold (Crapo et al. 2012; Reing et al. 2009).

The latest reports have announced that there is a chance that the 3D extracellular matrix scaffolds may support the heart muscle tissue that could recover physiological cardiac functions. Many ECM scaffold preparations (including the method of decellularization and implantation) have been examined to improve the release of growth factors and to stop the immune rejection due to cell transplantation. The outcomes of this novel research demonstrated great improvement in therapeutic effects when MSCs had been preconditioned with transforming growth factor-β (TGF-β) (Godier-Furnemont et al. 2011).

5.6 Tissue Engineering

5.6.1 Injectable Systems

The main problem of cell seeding regards the irregular distribution of cells within the scaffold. The inflexible components of the matrix isolate cardiomyocytes from each other. Therefore, a semiliquid matrix shows significant benefits over the inflexible materials, which have presented some troubles with the continuation of the myocardial structure, synchronization of the contraction, creation of vascular system, and signaling transfer. This issue has been solved by Zimmermann and colleagues, who created a combination of the cells with the soluble hydrogel of collagen type I and extracellular matrix protein (Matrigel). An electrical connection

with the host myocardium and improvement of heart function without arrhythmia has been observed (Zimmermann et al. 2002). Others who injected both stem cells and Matrigel into an infarcted heart reported improved heart geometry and function (Kofidis et al. 2005). Injectable scaffolds are considered to present some extra features, e.g., easy and minimal invasive delivery to the damaged myocardium. Matrigel is obtained from mouse sarcoma cells; therefore, the restriction of its application in clinical therapy stems from the fact that Matrigel is not tissue-specific, and it can be also associated with the high risk of tumor formation (Albini et al. 1992).

Different injectable materials studied for cardiac regenerative therapy are hydrogels based on N-isopropylacrylamide or poly (ethylene glycol) (PEG) (Rizzi et al. 2006). Some eventual obstacles of injectable materials have been observed, and they include poor mechanical support for the MI regions and the risk of blocking the blood circulation in the recipient organ.

Interesting studies have recently been reported by team of Black and colleagues. They created a hydrogel platform based on naturally derived silk fibroin containing cardiac tissue-derived extracellular matrix (cECM). These hydrogels demonstrated variable mechanical features and an adjustable rate of stiffness, which provided enhancement for cardiac fibroblast cell growth and viability throughout the in vitro culture. A cECM application increased the expression of integrins, showing an integration of cardiac fibroblasts with the cECM in the hydrogel and provoking an endogenous cell influx and cell ingrowth. These results revealed the possibility for a novel approach to more physiological constructs with a strong ability to replace healthy and injured tissue which can be also used for cardiac repair therapy (Stoppel et al. 2016).

5.6.2 Cell Sheet Engineering

Problems with scaffolds like the acute immune system response during their biodegradation and unwanted cell migration have brought about a new three-dimensional technological development. In opposition to 3D biodegradable scaffolds, a new technology, called cell sheet engineering (without scaffolds), has begun due to the invention of poly (N-isopropyl acrylamide) (PIPAAm) (Okano et al. 1993).

Shimizu et al. (2003) demonstrated that the layered cardiomyocyte sheets in vivo showed a prolonged survival rate, macroscopic pulsation, and typical construction for native heart tissue. The implantation of "layered cardiomyocyte" has been tested with cardiomyocytes from various sources (ESCs, iPSCs), and promising results were obtained (Lee et al. 2012; Stevens et al. 2009).

Sekiya and colleagues reported that cardiac cell sheets induced gene expression associated with angiogenesis; therefore, their implantation caused a neovascularization of the myocardium (Sekiya et al. 2006). Studies performed in a rat model demonstrated that the resistance and growth of transplanted myocardial cell sheets

were maintained for at least a year. Their effect on the cardiac stem cell therapy included the improvement of retractility, angiogenesis, and the reduction of fibrosis (Shimizu et al. 2006).

Improvement in LV wall thickness and a decrease of fibrosis and necrosis in the postinfarction scar region was noticed due to the cardiomyocyte sheets transplantation in a myocardial infarction in a rat model (Miyagawa et al. 2005). Also, similar results have been reported for other applied cell types: adipose-derived MSCs, mESC-derived cardiac cells, endothelial and skeletal muscle cells (Eschenhagen 2011; Masumoto et al. 2012; Miyagawa et al. 2010; Miyahara et al. 2006; Sekine et al. 2008). Furthermore, it has been described that the effect of MSCs sheets in the enhancement of the cardiac function is also associated with their paracrine activity.

Cardiomyocytes derived from pluripotent stem cells have been examined with optimistic results in both cell sheet and cell aggregation engineering. This assumes that new scaffold-free human myocardial patches may make significant progress in cardiac tissue engineering.

5.7 Concluding Remarks

Continued important elements of stem cell therapy should have been taken into consideration in order to increase the pro-regenerative impact of stem cells toward cardiac tissue, among others: the timing, cell dosage, and delivery techniques not described in this section. However, the other organs already profited from cellular therapies and medical use of stem cells shortly will become a future for biomedical technologies, overall.

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Chapter 6
Pluripotent and Mesenchymal Stem Cells—Challenging Sources for Derivation of Myoblast

Karolina Archacka, Edyta Brzoska, Maria A. Ciemerych, Areta M. Czerwinska, Iwona Grabowska, Kamil K. Kowalski and Malgorzata Zimowska

6.1 Introduction—So Many Stem Cells …

Self-renewing and regenerative capacity of mammalian tissues and organs rely on the presence of the stem cells residing within the organism. Their involvement in tissue reconstruction and/or regeneration is triggered by the signals generated as a result of the injury or disease. Under physiological conditions, endogenous stem cells are usually sufficient to sustain the renewal or regeneration processes. However, some pathological conditions cause that the resident cells fail to sufficiently secure proper tissue functioning. Such failure could be, for example, associated with certain genetic diseases, such as muscular dystrophies in case of skeletal muscles, or by tissues' injuries, as it happens during myocardial infarction. Among the treatments that could be considered to improve the tissue regeneration are those that aim to activate and/or mobilize endogenous stem cells, deliver exogenous cells, or tune the development of inflammatory response. Approaches involving the methods allowing efficient differentiation of exogenous stem cells and their delivery into malfunctioning tissue require the understanding of mechanisms governing cellular differentiation, migration, and integration within the regenerating tissue. Such a complex knowledge is prerequisite to design the proper therapeutic tactic.

Stem cells and their possible therapeutic application could be presented from various angles. Such presentation should start, however, with the definition of stem cell. Simple definition was presented by Paul Knopfler who wrote that: "scientists usually agree on what defines the stem cell. (…) First, it possesses "self-renewal," which simply means that it can divide to make more stem cells. Second, a stem cell

K. Archacka · E. Brzoska · M. A. Ciemerych (✉) · A. M. Czerwinska
I. Grabowska · K. K. Kowalski · M. Zimowska
Department of Cytology, Institute of Zoology, Faculty of Biology, University of Warsaw, Ilja Miecznikow st. 1, 02-096 Warsaw, Poland
e-mail: ciemerych@biol.uw.edu.pl

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has “potency” meaning it can differentiate into a variety of other cell types. A true stem cell has both self-renewal and potency” (Knoepfler 2013). Mammalian stem cells can remain quiescent with limited self-renewal rate or start to proliferate and differentiate, depending on the “need” to renew or regenerate the tissue they belong to. As far as self-renewal is concerned, stem cells could differ in the rate of cell divisions. Those cells that reside within actively renewing tissues, such as epithelia, are characterized by shorter intervals between cell divisions, as compared to the cells within more “quiescent” tissues and organs, for example, brain. It seems that the “speed” of self-renewal relates somehow to the potency; i.e., more “potent” stem cells, such as embryonic stem cells, are characterized by shorter cell cycles, as compared to stem cells present in adult organisms (Momčilović et al. 2011; Ciemerych et al. 2011).

The stem cell definition presented above is, however, so basic that it belittles the existence of variety of stem cells existing within the mammalian body and also of those derived by scientists and maintained outside of the living organisms. Simply by looking at stem cell potency to differentiate into a variety of other cell types, we can distinguish totipotent, pluripotent, multipotent stem cells, as well as those that are bi- or unipotent (Fig. 6.1). Totipotency characterizes exclusively embryonic cells present at the very early stages of embryo development. Thus, it is the zygote and early blastomeres that are totipotent; i.e., it can form any given tissue within the organism, including extraembryonic structures that are required for the mammalian development. As mammalian preimplantation embryo develops and forms blastocyst, which is ready to implant, cellular totipotency is being lost. Blastocyst is composed of inner cell mass which cells are pluripotent, i.e., it can form all cell types in organism, and of trophectoderm which will form extraembryonic tissues. Pluripotency is preserved also in early post-implantation embryos, in the so-called epiblast, but is gradually lost along the development. Inner cell mass and also epiblast cells are the ones which serve as a source for the cells which can only be found in the laboratory, i.e., embryonic stem cells (ESCs) (Evans 2011) and epiblast stem cells (EpiS) (Rossant 2008). Other currently known pluripotent cells, i.e., embryonic carcinoma cells (ECCs) (Robertson 1987) and induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2016), were derived from teratomas and somatic cells, respectively. The potency, or potential for differentiation, decreases along the development. Thus, in the adult organisms one did not find (at least up to now) any cells that can fulfill the stringent criteria of pluripotency, i.e., being able to form all known mammalian cells and tissues. Embryos and also adult organisms contain multipotent stem cells, as well as bi- or unipotent ones. Multipotency, i.e., the ability to form several cell types, is attributed to such cellular lineages as hematopoietic stem cells or mesenchymal stem cells. Bi- or unipotent stem cells were identified in the majority of tissues as the so-called adult stem cells, responsible for the self-renewal and regeneration of tissues and organs.

In the current chapter, we will focus at the two types of stem cells—multipotent, i.e., mesenchymal stem cells, and pluripotent stem cells, i.e., embryonic and induced pluripotent stem cells, i.e., the ones which currently attract attention due to their features and possible application in regenerative medicine. We will present the

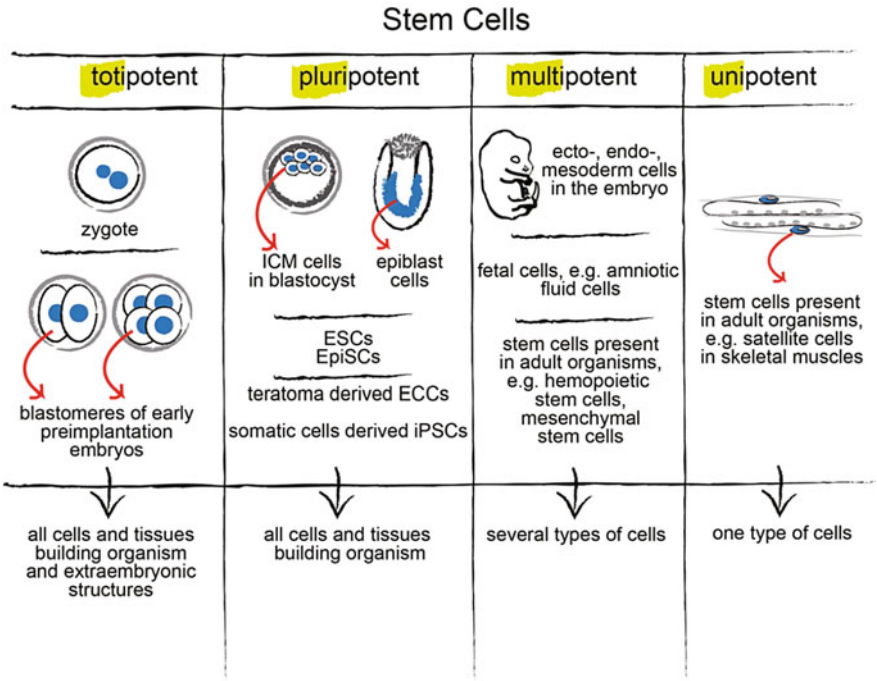

Fig. 6.1 Stem cells classification. Abbreviations used: *ICM* inner cell mass, *ESCs* embryonic stem cells, *EpiSCs* epiblast-derived stem cells, *ECCs* embryonic carcinoma cells, *iPSCs* induced pluripotent stem cells

characteristics and methods of differentiation of these cells into skeletal muscle myoblasts and cardiomyocytes. We will also give a bit of insight into current state of the clinical trials involving these cells. Unfortunately, due to the overwhelming body of evidence we were not able to cover all the issues related to the subject of pluri- and multipotent stem cells. We also regret that due to the space limitation, or our oversight, we did not mention all the relevant literature.

6.2 Becoming Skeletal or Cardiac Myoblast

The current chapter is focused at the myogenic differentiation of pluri- and multipotent stem cells, i.e., their conversion into skeletal muscle fibers (myofibers) and also cardiomyocytes. Both muscles are striated muscles, and for this reason, they share several common characteristics, e.g., mesodermal origin or mechanism of contraction. However, they also differ a lot—at the molecular, cellular, and of

course functional level. Skeletal muscles are composed of muscle fibers and satellite cells tightly connected with them. Next, these muscles are infiltrated by many other cells, such as fibroblasts, endothelial cells building blood vessels, pericytes, mesoangioblasts, and immune cells. Major cell types of heart include cardiomyocytes, cardiac conduction cells, fibroblasts, vascular smooth muscle cells, endothelial cells.

During embryonic myogenesis, skeletal muscle precursor cells originate from the dermomyotomes, i.e., part of somites that are the structures formed as a result of the segmentation of lateral mesoderm (Bryson-Richardson and Currie 2008). It has to be noted, however, that cells forming head muscles originate from prechordal and pharyngeal head mesoderm and their formation is controlled differentially from trunk and limb muscles, i.e., those one which we describe below (Shih et al. 2008). Moreover, during embryonic development myogenic precursors differentiate from the Brachyury-expressing mesoderm which will give rise to paraxial mesoderm synthesizing Pdgfrα (platelet-derived growth factor receptor α), while hematopoietic cells originate from the proximal lateral mesoderm characterized by the presence of Flk1 (fetal liver kinase 1, also known as vascular endothelial growth factor receptor 2—VEGFR2, or kinase insert domain receptor—KDR) (Kataoka et al. 1997). Thus, these markers are often used to distinguish appropriate myogenic precursors [e.g., Darabi et al. (2008), Magli et al. (2014)]. As far as muscle precursor cells present within the somites are concerned, they are characterized by the expression of Pax3 and Pax7 transcription factors, which are the master regulators controlling the expression of factors regulating myogenesis (White and Ziman 2008; Sato et al. 2010; Mayanil et al. 2001; Buckingham 2007). Among Pax3 and Pax7 direct or indirect targets are transcription factors, such as Myf-5 and MyoD [e.g., Tajbakhsh et al. (1997), McKinnell et al. (2008), Sato et al. (2010), Sassoon et al. (1989), Ott et al. (1991), Bajard et al. (2006)]. Myf-5, MyoD, myogenin, and Mrf-4 belong to the family of transcription factors called myogenic regulatory factors (MRFs) that recognize the sequences present within the promoters of genes encoding muscle-specific proteins, such as myosin heavy chains (MyHC), muscle creatine kinase (MCK), and other (Olson 1992; Olson and Klein 1994). Experimental overexpression of MRFs was shown to induce conversion of various cell types, such as neurons, fibroblasts, or ESCs, into myogenic cells [e.g., Dekel et al. (1992), Braun et al. (1989), Edmondson and Olson (1989), Weintraub et al. (1989), Lattanzi et al. (1998)]. MyoD or Myf-5 induces the cells localized within dermomyotome to create the cellular layer called myotome which will give rise to cells forming skeletal muscles (Parker et al. 2003). Muscle precursor cells present within the somites that localize at the level of forming limb buds will contribute to the formation of the limb muscles, and this is the Pax3 activity which secures their ability to propagate and migrate toward the forming limbs [e.g., Bober et al. (1994), Goulding et al. (1994), Williams and Ordahl (1994)]. Muscle precursor cells that reach the limb buds become myoblasts expressing MyoD and Myf-5 (Ott et al. 1991). At the same time, within the myotome, precursor cells differentiate into myoblasts which fuse and form multinuclear myotubes and muscle fibers. During mouse embryonic development, which usually takes 21 days, first fibers are formed

between 11th and 14th dpc (days post coitum) and then formation of the so-called secondary fibers occurs between 14th and 16th dpc. This process is controlled by "late" MRFs, i.e., myogenin and Mrf-4 (Venuti et al. 1995) and is associated with the expression of MyHC, desmin, and other muscle-specific proteins.

During the later stages of myogenesis, some of the progeny of Pax3/Pax7 expressing cells retains the expression of Pax7, localizes between muscle fiber and surrounding basal lamina (Kassar-Duchossoy et al. 2005; Relaix et al. 2005), and becomes satellite cells (Mauro 1961; Katz 1961), i.e., muscle stem cells, responsible for growth and regeneration of muscles in adult organisms. In skeletal muscles of adult organisms these cells remain quiescent until the appropriate signal, e.g., injury (Charge and Rudnicki 2004). Isolation of satellite cells from muscle also leads to their activation. Since satellite cell activation initiates molecular and cytological changes resembling those one characteristic for embryogenesis, these cells are frequently used as a model in in vitro studies of myogenic differentiation [see, e.g., our own work: Grabowska et al. (2011), Brzoska et al. (2009), Zimowska et al. (2008), Archacka et al. (2014b)].

The first sign of satellite cells activation is cell-cycle reentry and intensive proliferation [summarized in Ciemerych et al. (2011)] which is associated with the downregulation of Pax7 and activation of MRF expression; e.g., MyoD-synthesizing cells are detectable within 24 h after injury (Grounds et al. 1992; Yan et al. 2003). Thus, satellite cell activation triggers the myogenic differentiation leading to the formation of myoblasts, myotubes, and eventually functional myofibers [summarized in Ciemerych et al. (2011), Brzoska et al. (2011)] (Fig. 6.2a).

Cardiomyocytes, similarly to skeletal muscle precursor cells, originate from mesoderm. The cardiac lineages, however, consist of several subtypes of precursor cells originating within the first and the second mesodermal heart fields, FHF and SHF, respectively. Multipotent precursors give rise to cardiomyocytes that will form the heart muscle, as well as endocardium, i.e., internal layer, endocardial cushions of the valves, and epicardium forming coronary blood vessels. Some contribution to the heart formation will come from proepicardium (Spater et al. 2014). During the development, Brachyury-expressing mesodermal cells give rise to those synthesizing Flk1 and this population will form the vascular and cardiovascular progenitor cells (Huber et al. 2004; Kattman et al. 2006; Yang et al. 2008). Further differentiation decisions lead to the downregulation of Brachyury and upregulation of Eomesodermin and Bvht noncoding RNA that later on, together with Mesp1, will mediate cardiac lineage formation (Chan et al. 2013). Mesp1, except its other functions, is necessary for the cardiovascular development and initiates expression of cardiac-specific transcription factors resulting in the cascade of changes leading to the generation of cardiac mesoderm. Mesp1 acts upstream to such indispensable cardiac regulatory factors as Nkx2.5—expressed in cardiomyocyte precursor cells, Gata4—necessary to start expression of many heart-specific genes, as well as Mef2c—which controls the expression, for example, of myosin light chains (Bondue and Blanpain 2010). Thus, these genes are among the factors expressed at cardiac crescent stage (approximately 7.5 dpc) when myocardial

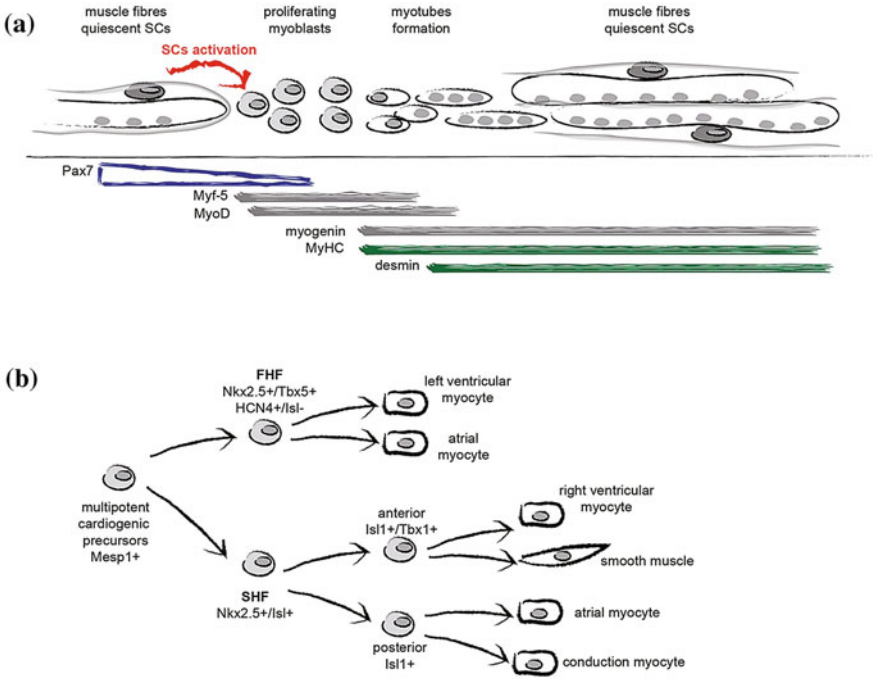

Fig. 6.2 Myogenic differentiation. **a** Myogenic differentiation of satellite cells (SCs) during skeletal muscle regeneration. SCs localized between muscle fiber sarcolemma and surrounding lamina remain quiescent. They are characterized by the expression of Pax7. Muscle injury activates SCs causing their cell-cycle reentry and differentiation into myoblasts that fuse and form multinucleated myotubes. These processes are associated with the downregulation of Pax7, induction of MRFs, i.e., Myf-5, MyoD, and myogenin expression. Next, during myotube formation and muscle fiber maturation, such structural proteins as myosins (MyHC) or desmin are expressed. Subpopulation of activated SCs retain Pax7 expression and reform SC pool in regenerated muscles [summarized in Ciemerych et al. (2011)]. **b** Specification of cardiac precursor cells. Cardiac precursors are specified within the first and the second heart field, FHF and SHF, respectively. Formation of lineages is driven by specific subset of transcription factors. FHF cells differentiate into left ventricular cardiomyocytes and atrial cardiomyocytes. SHF cells form anterior and posterior precursors that further differentiate: anterior precursors form smooth muscle cells and right ventricle cardiomyocytes, and posterior ones form atrial cardiomyocytes and conduction cardiomyocytes. Each type of these cells forms subpopulation characterized by the distinct subset of molecular markers [summarized in Spater et al. (2014), Paige et al. (2015)]

differentiation is initiated (Fig. 6.2b). Moreover, Mesp1 promotes expression of genes involved in the migration of precursor cells committed to become cardiomyoblasts. The fate of multipotent precursors initially present within the cardiac crescent greatly depends on their origin, i.e., if they come from first or the second heart filed. FHF progenitor cells express Nkx2.5, Tbx5, and Hcn4. They will give rise to left ventricular cardiomyocytes, atrial cardiomyocytes, cardiac conducting cells, atrioventricular conducting cells, and other cell types. SHF progenitors

expressing Flk1, Nkx2.5, and Isl1 will differentiate into such cells as right ventricular cardiomyocytes, atrial cardiomyocytes, cardiac conducting cells, vascular smooth muscle cells, or endothelial cells. Proepicardial cells, which constitute a separate complex subpopulation of progenitors, form ventricular cardiomyocytes, fibroblasts, endothelial cells, vascular smooth muscle cells (Spater et al. 2014; Buckingham et al. 2005). Formation of fully functional heart also, as in case of skeletal muscle fibers, involves the participation of neurons. Each subpopulation of cardiomyocytes proliferates and then undergoes final differentiation characterized, for example, by the expression of proteins typical for striated muscles, such as myosin heavy and light chains, and also those one specific for heart muscle, such as atrial natriuretic factor (Anf). The detailed description of the complex interactions occurring at the molecular and cellular levels and resulting in the formation of functional heart could be found elsewhere (Buckingham et al. 2005; Paige et al. 2015; Spater et al. 2014; Leri et al. 2015).

6.3 Stem Cell Niche

Stem cell niches, which are defined as a specialized local tissue microenvironment capable of housing, regulating, and maintaining of stem cells, are also considered as "factors" indispensable for the proper stem cell function (Morrison and Spradling 2008). Since the niche is essential for the proper self-renewal of stem cells and their effective proliferation and differentiation, recreating such environment should be also considered in future applications of stem cells in therapies. For this reason, it is important to understand the effect of the niche components on maintaining homeostasis of stem cells. In mammals, stem cell niches have been studied in many tissues, such as in bone marrow, intestinal epithelium, skin epidermis, hair follicles, testis, and many others. The structure of the niche differs depending on the specific tissue. Generally, two basic types of niche have been recognized—"stromal cell" and "epithelial" niches. The stromal cell niche consists of adjacent stromal cells that regulate the population of stem cells and "hold" them in a specific location. In this model, the stem cells have direct contact with the niche cells and are anchored by cadherin-mediated cell–cell adhesion between the stem cells and the niche cells. In the epithelial niches, the stem cells are associated directly with the extracellular matrix (ECM) and basement membrane. They are anchored to the niche by forming integrin-mediated cell-to-ECM adhesion (Xi 2009). Thus, ECM is an essential component of the niche providing much more than mechanical support. It forms complex three-dimensional scaffold composed of variety of molecules, such as structural proteins, e.g., collagens, elastin, laminin, fibronectin, proteoglycans, and glycosaminoglycans. The structure and functioning of the niche strongly depends on the proportions of these components, although their specific contribution depends on the tissue; e.g., hematopoietic niches contain tenascin-C and osteopontin, whereas collagen IV and β1 integrin are found in epithelial/epidermal stem cell niches (Votteler et al. 2010).

Skeletal muscle-specific stem cell niche, i.e., satellite cells, exists at the periphery of muscle fibers, between the sarcolemma and basal lamina. In skeletal muscle, ECM components are mainly secreted by interstitial fibroblasts but can be also synthesized by myoblasts (Kovanen 2002). Skeletal muscle ECM contains proteoglycans such as perlecan, decorin, and biglycan (Thomas et al. 2015). Among other primary components of this niche are collagen type IV, laminin-2 (formed by α2, β1, and γ1 chains), and nidogen (Thomas et al. 2015). The satellite cell niche remains also influenced by the various factors released by endothelial cells building blood vessels, as well as other types of cells residing within the muscle (Yin et al. 2013). The interactions between satellite cells and their niche specifically regulate the behavior of these cells, and even small modifications can have profound effects on their localization, quiescence, self-renewal, proliferation, and differentiation. Skeletal muscle ECM not only forms a mesh but also sequesters a wide range of growth factors, such as hepatic growth factor (HGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and insulin-like growth factor isoforms (IGF-I, IGF-II) [summarized in (Brzoska et al. 2011)]. Next, among the stem cell niche components are also the factors secreted by stem cells and other cells present within the niche, such as Wnt and Notch, which are responsible for activating specific cell signaling pathways (Brzoska et al. 2011; Ciemerych et al. 2011; Yin et al. 2013; Otto et al. 2008; Mourikis et al. 2012; Conboy and Rando 2002). Thus, it provides a local reservoir of factors necessary for coordination of satellite cell activation, proliferation, and differentiation upon muscle injury.

Structural proteins of ECM are directly attached to the cell cytoskeleton by the adhesion proteins—integrins and cadherins. Both of them play a role also in signal transduction being responsible not only for the adhesion but also for variety of other phenomena, such as stem cell self-renewal, proliferation, and differentiation. Satellite cells, which reside between two opposing microenvironments, i.e., the basal lamina and the myofiber sarcolemma, are linked to the myofiber by M-cadherin, whereas integrins α7 and β1 are involved in their binding to laminin on the basal membrane side (Dumont et al. 2015). Interestingly, integrin expression varies between quiescent and activated satellite cells. Activated cells express integrin β3, which likely complexes with integrin αv to bind fibronectin, osteopontin, or collagens (Liu et al. 2011), as well as integrin α5 which complexes with β1 and binds fibronectin (Blaschuk and Holland 1994).

As far as cardiac muscle is concerned, our knowledge on the cardiac stem cells and niches specific for them is still less satisfactory as compared to skeletal muscles. Cardiac stem cell niche seems to be settled by populations of stem/progenitor cells that manifest the potential to generate cardiomyocytes (Beltrami et al. 2003). Except these cells, cardiac niches also contain fibroblasts that act as niche-supporting cells (Leri et al. 2014). The precise localization of niches is, however, difficult. Several reports indicate that cardiac stem cells are localized in both myocardium and epicardium (Martin-Puig et al. 2008; Wu et al. 2008). However, their involvement in the renewal of cardiac muscle during normal aging and disease is limited (Kajstura et al. 2012; Laflamme and Murry 2011). Within the myocardial niches, cardiac stem cells and progenitor cells are typically clustered

together. Their number was shown to be higher in the atrial and apical myocardium than in the base–mid-region (Sanada et al. 2014; Urbanek et al. 2006). Resident cardiac progenitors have been also identified in the epicardium of the developing and adult mouse heart (Chong et al. 2011). The specific cell–cell or cell–ECM interactions, acting as a stem cell niche, in adult epicardium, are not precisely defined. Importantly, the niche supports multipotent stem cells, which have the capacity to differentiate into fibroblasts, vascular endothelial cells, smooth muscle cells, and cardiomyocytes, having the potential to contribute to cardiac homeostasis and repair (Zhou et al. 2008).

Taking together, proper and effective proliferation, differentiation, and self-renewal of stem cell populations depend on stem cell niche. This local environment largely controls the maintenance and the fate of cells in the niche. Moreover, some anomalies, e.g., in stem cells homeostasis, may be attributable to the niche dysfunction. Thus, any therapeutic strategy aiming at the transplantation of stem cells into skeletal or cardiac muscle needs to take into account the "niche" issue in order to support the reconstruction of functional muscle tissue.

6.4 Mesenchymal Stem Cells—What Are They and Where Do They Come from?

Mesenchymal stem cells (MSCs) are heterogeneous population of postnatal cells that are present in adult organisms. The term "mesenchymal stem cells" is pretty widely used to describe the populations of cells which differ in their localization and properties. Moreover, MSC term has also alternative meanings, such as multipotent stromal cells or mesenchymal stromal cells (Dominici et al. 2006). As a result, many controversies, for different reasons, arise around "MSCs." First, MSCs are described as the multipotent, self-renewing "skeleton-related" stem cells, i.e., present within the mammalian bone marrow stroma. According to this concept, bone marrow stromal cells play a role in the growth and turnover of the bone, form hematopoietic microenvironment, and also are able to initiate and complete bone organogenesis in vivo (Bianco et al. 2013; Bianco 2014, 2015). Second, MSCs are often described as ubiquitous cells, present within the connective tissue of different organs, and able to adhere to the plastic during in vitro culture and also to differentiate into chondrocytes, adipocytes, and osteocytes (Dominici et al. 2006). Thus, each time one confronts the term MSCs, one has to ask the question about the characteristic of these cells.

The stringent and rigorous assays allowing to define stem cells base on the following clues: stemness probed during in vivo serial transplantations, self-renewal, i.e., ability to generate identical population of stem cellsin vivo and clonogenity in vitro, i.e., ability to proliferate and give rise to cell colonies, long-term expansion in vitro without phenotypic change, and finally multipotency probed by in vivo differentiation assay (e.g., heterotopic transplantation) (Bianco

et al. 2008, 2013). However, difficulties connected with in vitro culture and analysis of stem cells, in general, and mesenchymal stem cells, in particular, not always allow simple identification of a cell as a stem cell (Bianco et al. 2013).

The story of MSCs started in the 1960s when Friedenstein found skeletal stem/progenitor cells in the bone marrow stroma (Friedenstein et al. 1966). These bone marrow stromal cells (BMSCs) enclosed in a diffusion chamber and heterotopically transplanted subcutaneously produced bone and bone marrow (Friedenstein et al. 1987). The BMSCs grafted without chamber, i.e., in open transplants, formed bone that was colonized by host hematopoietic cells (Tavassoli and Crosby 1968). Thus, the naïve osteogenic potential of BMSCs and their ability to form hematopoietic microenvironment and hematopoietic stem cell (HSC) niche have been showed unquestionably (Friedenstein et al. 1968; Friedenstein and Kuralesova 1971). Further studies documented that the population of adherent BMSCs contains cells that are able to grow in vitro when cultured at clonal density and to form so-called colony-forming units-fibroblastic (CFU-F). These clonogenic cells, i.e., cells able to proliferate and give rise to cell colonies, were able to generate bone after their heterotopic transplantation in vivo (Friedenstein 1980; Friedenstein et al. 1987). Importantly, even single bone marrow-derived cell was able to give rise to bone, cartilage, and adipocytes, i.e., other types of connective tissue, after heterotopic transplantation (Friedenstein et al. 1987; Owen and Friedenstein 1988). Therefore, it was indisputably shown that cells residing within the bone marrow stroma, i.e., BMSCs, are multipotent progenitor cells.

Further characterization allowed more precise characteristic of BMSCs. CD146 appeared to be very useful marker to select and enrich human bone marrow-derived cells and to distinguish BMSCs from other osteogenic (trabecular bone cells, periosteal cells, endosteal cells), nonosteogenic (fibroblasts, endothelial cells, adipocytes, chondrocytes), or hematopoietic cells (Sacchetti et al. 2007). If no selection strategy was used, i.e., cells were not selected on the basis of CD146 presence, osteoblasts, fibroblasts, endothelial cells, adipocytes, and smooth muscle myoblasts were present in primary culture of cells isolated from the bone marrow (Sacchetti et al. 2007). Thus, such non-BMSCs could be excluded from the cell culture by cell sorting, serial passaging, or culture in clonal density. Combination of these methods, i.e., the isolation of cells based on surface phenotype and their culture at clonal density, allows to obtain highly purified population of BMSCs (Bianco et al. 2013). Among other markers used to characterize and isolate human BMSCs, in addition to CD146, are: STRO-1, CD105, alkaline phosphatase (ALP), CD49a, and CD271. Mouse BMSCs are characterized by the expression of nestin, CD105, VCAM-1, CD90, and other markers (Bianco et al. 2013; Boxall and Jones 2012; Lv et al. 2014).

Fraction of human BMSCs expressing CD146 (CD146+) was shown to be perivascularly located, and thus, it seems to be corresponded to adventitial reticular cells, i.e., ARCs (Sacchetti et al. 2007). These cells present in mouse bone marrow were described as expressing nestin and corresponding to CXCL12 abundant reticular cells, i.e., CARs (Mendez-Ferrer et al. 2010). Subpopulation of human CD146+ BMSCs was able to differentiate into bone and form hematopoietic

microenvironment, i.e., bone marrow, after their subcutaneous transplantation into immunocompromised mice (Sacchetti et al. 2007, 2016). Importantly, neither muscle nor skin fibroblasts were able to generate bone or bone marrow under the same conditions. Human CD146+ BMSCs was also shown to support organization of endothelial cells into functional blood vessels, both in vitro and in vivo, and such feature was also not characteristic for fibroblasts (Sacchetti et al. 2007, 2016). These cells were also able to differentiate into cartilage (Sacchetti et al. 2016; Serafini et al. 2014). Thus, the multipotency of human CD146+ BMSCs was proved. Moreover, Sacchetti and co-workers showed that human cells isolated from heterotopic ossicles formed by subcutaneously transplanted CD146+ BMSCs cultured at clonal density were still able to generate colonies (Sacchetti et al. 2007) and formed another heterotopic ossicles after serial transplantation (Serafini et al. 2014). Thus, CD146+ BMSCs fulfill the criteria of stem cells; i.e., they are able to self-renew and differentiate into several cell types.

BMSCs were also described using markers other than mentioned above. Ghazanfari et al. (2016) described these cells as the ones expressing high levels of CD271 but low levels or none of CD140. Other researchers used nestin as a suitable marker (Mendez-Ferrer et al. 2010). Mouse nestin+ BMSCs, able to self-renew and expand after serial transplantation, manifested adipo-, osteo-, as well as chondrogenic potential and were shown to be required for hematopoietic stem cell maintenance in the bone marrow (Mendez-Ferrer et al. 2010). Thus, the self-renewal, clonal, and multipotential capacity of human and mice BMSCs, identified by markers indicated above, was proved (Mendez-Ferrer et al. 2010; Sacchetti et al. 2007; Serafini et al. 2014; Ghazanfari et al. 2016). Therefore, we can conclude that BMSCs are perivascular multipotent stem cells that correspond to mesenchymal stem cells. However, the question if BMSCs are able to differentiate in the cells of tissues other than connective tissue, i.e., other than of mesodermal origin, remains open.

Caplan and co-workers used the term mesenchymal stem cells to describe stromal stem cells present in connective tissues such as bone, cartilage, and adipose tissue (Caplan 1991; Caplan and Correa 2011). This term was then extended to include cells isolated from other connective tissues, such as tendon, and other of mesodermal origin, i.e., skeletal and smooth muscles. In vitro MSCs were characterized as the adherent ones, growing in culture without addition of serum, expressing specific markers (CD105, CD90, CD73), lacking hematopoietic and endothelial markers, as well as being able to differentiate into adipocytes, chondrocytes, and osteocytes (Dominici et al. 2006). MSCs fulfilling this definition were isolated as described above from bone marrow, but also from dental pulp, brain, umbilical cord, umbilical cord blood, amniotic fluid, adipose tissue, and liver (Erices et al. 2000; Shi and Gronthos 2003; Lee et al. 2004; Kang et al. 2010; Zebardast et al. 2010; Cai et al. 2011; La Rocca et al. 2009; Iacono et al. 2012). However, it is still questionable if non-bone marrow MSCs are progenitor or stem cells and whether they are unipotent, tissue-specific cells, or truly multipotent ones. Next, it is still not clear which cell could be acknowledged as mesenchymal stem type of cells. Moreover, the population of MSCs isolated from various sources was

usually very heterogonous. Observed heterogeneity could be a result of the composition of the population of isolated cells or be generated by the in vitro culture conditions (Bianco 2014). Thus, usually only a part of in vitro cultured MSCs are truly multipotent and self-renewing stem cells. For example, approximately 50% of CD146+ BMSCs met stringent criteria of being the stem cell (Sacchetti et al. 2007).

Among the tissues that contain cells of MSC characteristics is adipose tissue (Huang et al. 2013). Similar to BMSCs and other MSCs tested, also adipose-derived mesenchymal stem cells (ADSCs) manifest adipogenic, chondrogenic, and osteogenic potential in vitro (Zuk et al. 2002; Peng et al. 2008; Guilak et al. 2006). However, human ADSCs did not form bone, cartilage and marrow when transplanted subcutaneously into immunodeficient mouse (Reinisch et al. 2015). Thus, in case of ADSCs, the term MSCs should be used carefully and rigorous characterization should be performed, especially if these cells are planned to be used in translational studies aiming to design the cell-based therapy.

Among the cells which were included to MSCs are also perivascular pericytes located in the close vicinity of endothelial cells forming the microvasculature and present in nearly all vertebrate tissues [e.g., Caplan (2008), Crisan et al. (2008), da Silva Meirelles et al. (2008)]. Thus, some authors suggest that non-bone marrow MSCs could be considered as perivascular pericytes (Caplan 2008). Pericytes play an important role in the vessel stabilization, regulation of vascular tone, and immunologic defense (Diaz-Flores et al. 2009). They were shown to be of mesodermal origin but also suggested to be formed from the neural crest cells, i.e., to be of neuroectodermal origin (Diaz-Flores et al. 2009). Interestingly, potential of pericytes is defined by their origin. For example, pericytes isolated from skeletal muscles manifested myogenic and those ones isolated from adipose tissue—adipogenic potential (Dellavalle et al. 2007, 2011; Tang et al. 2008).

As it was shown for pericytes, MSCs present in adult organisms seem to differ in their characteristics and potential depending on their developmental origin (Sacchetti et al. 2016; Reinisch et al. 2015). Thus, human BMSCs and also non-bone marrow MSCs vary in their transcriptional profile and differentiation potential (Sacchetti et al. 2016). Cord blood MSCs were shown to be characterized by overrepresentation of genes related to proliferation and cell-cycle regulation, while muscle MSCs overexpressed myogenic cell-specific genes, such as *PAX7*. Analysis of naïve potential of MSCs using heterotopic transplantation showed that under such conditions, BMSCs formed bone, bone marrow stroma, and also adipocytes, cord blood MSCs formed bone and cartilage, periosteum MSCs formed bone, but muscle MSCs did not reveal spontaneous osteogenic potential. The bone marrow, cord blood, and muscle MSCs were able to form nascent blood vessels in co-cultures with human umbilical vein endothelial cells (HUVECs) in vitro (Sacchetti et al. 2016). The muscle-derived MSCs presented high myogenic potential and were able to self-renew. Bone marrow, cord blood, and periosteum MSCs also fused and formed myotubes but only in the presence of C2C12 myoblasts (Fig. 6.3). These results strongly suggested that the source of MSCs impacted their potential and that MSCs from sources other than muscles were not inherently myogenic. However, there are many other studies showing that using

different protocols, myogenic differentiation of MSCs from different sources could be induced. Study of Sacchetti and co-workers, however, bring us information showing that MSCs are tissue-specific stem/progenitor cells, most possibly of different developmental origin (Sacchetti et al. 2016).

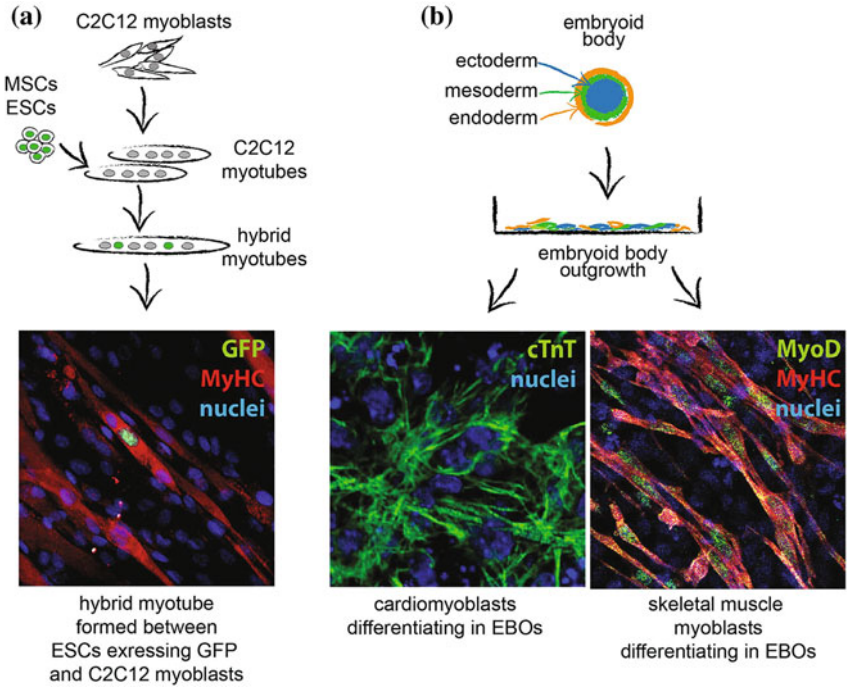

Fig. 6.3 In vitro differentiation of stem cells. **a** Myogenic potential of stem cells, such as MSCs, ESCs, or iPSCs, could be tested by their co-culture with myoblasts. First, myoblasts, such as C2C12, are cultured in vitro until they start to form myotubes. Next, tested stem cells are seeded into them. Presence of the hybrid myotubes formed by fusion of C2C12 myoblasts and stem cells indicates the myogenic potential of the latter ones. Image shows myotube formed by C2C12 myoblasts and ESCs expressing histone H2A fused with GFP (Green Fluorescence Protein, green). One of the nuclei within the myotube is of ESC origin. Immunolocalization with specific antibodies allows to visualize myosin heavy chains (MyHC, red). Nuclei are stained with DNA incorporating fluorescent dye (blue). **b** Embryoid bodies formed by ESCs or iPSCs are contain three cellular layers. Outer layer is composed with endodermal cells (orange), inner layer with ectodermal cells (blue), mesoderm is positioned in between (green). In embryoid body outgrowths (EBOs), this organization is lost. Cardiomyocytes present within the EBOs are visualized by immunodetection of cardiac troponin T (cTnT, green, left image). Skeletal muscle myoblasts and myotubes are visualized by immunodetection of MyHC (red, right image) and MyoD (green, right image). Nuclei are stained with DNA incorporating fluorescent dye (blue). Images taken using Zeiss scanning confocal microscope equipped with LSM software

6.5 Myogenic Differentiation of Mesenchymal Stem Cells

Many lines of evidence document that MSC differentiation into myoblasts and cardiomyocytes can be induced. However, proving the myogenic potential of these stem cells is not an easy task. Generation of skeletal muscle myofibers involves cell fusion mediated by the precise interaction of many adhesion proteins. Derivation of functional cardiomyocytes also requires formation of complex cell–cell connections. Next, in case of skeletal muscle fibers, it is difficult to judge whether the myogenic differentiation was initiated before the fusion or resulted from the fusion of stem cells with other already differentiated myoblasts or myotubes. For this reason, some authors accept only such in vitro assays during which stem cells are induced to differentiate in the absence of exogenous myoblasts, what allows to uncover myogenic potential of tested cells (Bianco 2014). Thus, it is important to distinguish the reprogramming (change of potential) from the differentiation (expression of potential) in the case of stem cell myogenic differentiation. However, in our opinion, cells that are able to fuse in vivo with myoblasts, form new myofibers during muscle regeneration, and to reconstruct the population of satellite cells could be considered as cells characterized by the myogenic potential.

As we described above, MSCs could be routinely induced to differentiate into osteogenic, chondrogenic, and adipogenic cells. However, derivation of functional skeletal muscle myoblasts is still considered as challenging (Table 6.1). First evidence showing that regeneration can be improved by the transplantation of BMSCs into injured skeletal muscle was published in 1983. Whole population of allogenic BMSCs was injected directly into regenerating muscle of baboon. As a result, improvement in muscle regeneration was observed giving the hope that BMSCs could be used in clinics. However, at the time the experiment was conducted the technical limitations did not allow to prove that BMSCs directly participated in the skeletal muscle regeneration (Meyer and Yarom 1983). One of the first evidences showing that BMSCs can differentiate into myotube-like structures resulted from the in vitro experiments during which rat BMSCs were exposed to DNA demethylating drug—5-azacytidine (Wakitani et al. 1995). In 1998, Ferrari and co-workers confirmed that BMSCs can be incorporated into the regenerating mouse muscle and form new muscle fibers as well as migrate from the bone marrow niche to the site of the injury (Ferrari et al. 1998). This study documented the capacity of BMSCs to incorporate into skeletal muscle, but it was not verified whether muscle fibers formed with the participation of these cells were functional. To address this issue, BMSCs isolated from wild-type mice were transplanted to irradiated mdx mice, i.e., animals lacking functional dystrophin gene (Sicinski et al. 1989). Such transplantation showed that BMSCs participated in formation of muscle fibers leading to the resurrection of dystrophin expression (Gussoni et al. 1999). Transplantation of BMSCs into irradiated newborn mice gave even better results. BMSCs participated in the skeletal muscle regeneration, colonized satellite cell niche, and also took part in the subsequently induced round of muscle regeneration. Thus, it was shown that transplanted cells differentiated into functional satellite

cells (Fukada et al. 2002). However, the proportion of muscle fibers formed with the participation of donor BMSCs never exceeded few percent of total fibers within the reconstructed muscle. Other studies documented that BMSCs are able to participate in the regeneration of mouse skeletal muscle and form satellite cells; however, the efficiency of the procedure was very low, i.e., never exceeded 3.5% of total fibers formed (LaBarge and Blau 2002). Apparently, the frequency of BMSC incorporation into regenerating muscle depended on muscle type and varied from 0.0022% in case of tongue to 5.23% in case of *Panniculus carnosus* muscle (Brazelton et al. 2003). Thus, even the maximum efficiency of the formation of the muscle fibers by BMSCs was not sufficient to support the application of these cells to truly improve muscle functions (Ferrari et al. 1998; LaBarge and Blau 2002). Obtained results, however, certainly evidenced that BMSCs are characterized by the ability to participate in the formation of muscle fibers and that the future projects should focus at the designing of the methods allowing to improve their myogenic potential. BMSCs are also considered as a source of cells to ameliorate the consequences of cardiac failure and to improve myocardiac regeneration. Among the first projects involving BMSCs were those showing that these cells were able to participate in cardiac regeneration (Orlic et al. 2001a, b, 2001c) and that approximately 68% of infracted portion of heart muscle was occupied by cells originating from injected BMSCs (Orlic et al. 2001b).

MSCs other than BMSCs were also explored in the studies on skeletal muscle and cardiac regeneration. Among such cells were adipose tissue-derived mesenchymal stem cells (Zhang et al. 2015), synovial membrane MSCs (De Bari et al. 2003), or MSCs isolated from umbilical cord blood or connective tissue (Brzoska et al. 2006; Grabowska et al. 2012b; Pereira et al. 2014). However, as mentioned above, MSCs from various sources possess different abilities to undergo myogenesis (Sacchetti et al. 2016).

Among the approaches allowing to increase myogenic differentiation of MSCs are the simple ones, such as their co-culture with myogenic cells and more "harsh" such as the manipulations of signaling pathways involved in the regulation of myogenesis as well as overexpression of factors such as MRFs or adhesion molecules necessary to induce myoblast differentiation and fusion [summarized in (Grabowska et al. 2012a)]. The "simplest" approach to test myogenic potential of stem cells is to co-culture them with myogenic cells, such as myoblasts or myotubes. Under such conditions, differentiation can be stimulated by both direct cell–cell contact and/or molecules secreted by myogenic cells. However, conditions of such experiments are never precisely controlled and usually impossible to routinely reproduce. Nevertheless, using such an assay ability to participate in the myotube formation was shown for human BMSCs (Shi et al. 2004) and human umbilical

Table 6.1 Myogenic differentiation of BM cells and BMSCs in vitro and in vivo—selected examples

Type of cells	In vitro differentiation	In vivo myofiber formation and satellite cell niche colonization	References
Whole population of rat BM cells	NT	Autogenous transplantation of BM cells into rat muscle damaged by cutting (*Rectus femoris)* resulting in improved muscle regeneration and decreased fibrosis	Meyer and Yarom (1983)
Rat BMSCs	Myogenic differentiation induced by DNA demethylating drug—5-azacytidine. Multinucleated myotubes were observed	NT	Wakitani et al. (1995)
Whole population of BM cells isolated from transgenic mice expressing β-galactosidase under the promoter of muscle-specific myosin light chain 3F (MLCF-*nlacZ*)	NT	Transplantation of MLCF-*nlacZ* BM cells directly into chemically injured mouse *tibialis anterior* muscle of gamma ray irradiated mouse. Five weeks after transplantation muscle were injured and analyzed 2–3 weeks later. β-gal$^+$ nuclei were observed within the muscle fibers	Ferrari et al. (1998)
Whole population of BM cells	NT	Transplantation of BM cells via tail vein into irradiated mdx mice. Twelve weeks after transplantation approximately 10% of muscle fibers expressed dystrophin	Gussoni et al. (1999)
Whole population of BM cells isolated from transgenic mice expressing GFP	NT	Busulfan-treated control or mdx neonatal mice were injected intrahepatically with GFP$^+$ BM cells. *Tibialis anterior* muscle of 4-week-old mice was injured with cardiotoxin. 13 weeks after muscle	Fukada et al. (2002)

(continued)

Table 6.1 (continued)

Type of cells	In vitro differentiation	In vivo myofiber formation and satellite cell niche colonization	References
		damage approximately 0.89—2.63% of GFP^+ fibers were observed within the muscle. GFP was detectable in mononuclear cells residing beneath the basal lamina of muscle fiber, i.e., in the satellite cell niche	
Whole population of BM cells isolated from transgenic mice expressing GFP	NT	Transplantation of GFP^+ BM cells via tail vein into irradiated mouse. After 2–6 months, GFP^+ cells were present in satellite cell niche. Next, GFP^+ "satellite" cells were isolated from chimeric mice and injected into regenerating *tibialis anterior* muscle. GFP^+ fibers were observed 7 days after transplantation	LaBarge and Blau (2002)
Whole population of BM cells isolated from transgenic mice expressing GFP	NT	Transplantation of GFP^+ BM cells via tail vein into irradiated mouse. Muscle injury was not induced. Sixteen months after transplantation, different proportions of GFP^+ muscle fibers were detectable in various muscles; e.g., 5% of GFP^+ BM-cell-derived fibers were observed in p*anniculus carnosus muscle*	Brazelton et al. (2003)
BMSCs isolated from human bone marrow and transfected with lentiviral vectors expressing the enhanced GFP	In vitro culture of BMSCs using trans-well system in the presence of C2C12 (to avoid direct cell–cell contact) did not result in the myogenic differentiation. Co-culture of BMSCs with C2C12 myoblast in the medium containing	Transplantation of GFP^+ BMSCs into the cardiotoxin injured *tibialis anterior* muscle of NOD/SCID mice resulted in the formation of 0.023% of GFP^+ muscle fibers	Shi et al. (2004)

(continued)

Table 6.1 (continued)

Type of cells	In vitro differentiation	In vivo myofiber formation and satellite cell niche colonization	References
	insulin and transferrin resulted in hybrid myotubes expressing human muscle-specific genes. The proportion of hybrid myotubes was 0.038%		
Whole population of BM cells isolated from transgenic mice expressing GFP	NT	Transplantation of GFP^+ BM cells by retro-orbital injection into irradiated mouse. Muscle injury was not induced. Abundant number of GFP^+ mononuclear cells was found in the interstitial connective tissue and satellite cell niche. The proportion of GFP^+ cells found in satellite cell niche was 4.1% after 1 month, 9.4% after 3 months, and 14.4% after 6 months post-transplantation. 1.3% of GFP^+ muscle fibers was detected in *tibialis anterior* after 6 months post-transplantation	Dreyfus et al. (2004)
Whole population of BM cells isolated from transgenic mice expressing GFP	NT	GFP^+ BM cells were transplanted by direct injection into the liver of newborn mice or by retro-orbital injection into irradiated adult mouse. GFP^+ muscle fibers were not detected in uninjured *triceps surae* muscles. GFP^+ muscle fibers were detected in mechanically or cardiotoxin injured *tibialis anterior* and *triceps surae* muscles. The proportion of GFP^+ muscle fibers was 0.4%. Repeated muscle injury increases the number of GFP^+ muscle fibers	Sherwood et al. (2004)

(continued)

Table 6.1 (continued)

Type of cells	In vitro differentiation	In vivo myofiber formation and satellite cell niche colonization	References
Whole population of BM cells isolated from transgenic mice expressing GFP	NT	Transplantation of GFP^+ BM cells into irradiated mouse. Then parabiotic pairs were created between GFP expressing mouse and wild-type mouse or between wild type and mouse with transplanted GFP^+ BM cells. Three weeks after parabiotic joining the muscles of wild-type mice were injured with notexin. Two or four weeks after the injury GFP^+ muscle fibers were observed in regenerated *tibialis anterior* muscle. The proportion of GFP^+ muscle fibers was 10–17%. GFP^+ BM cells contribution to myofibers resulted in increase in frequency in response to physiological cues (hypertrophy)	Palermo et al. (2005)
Human and rat BMSCs	BMSCs culture in the presence of bFGF, forskolin, PDGF, and neuregulin followed by the overexpression of NICD and further culture in medium containing either 2% HS or in ITS serum-free medium. Such "induced" BMSCs formed myotubes in the response to HS (fusion index 24%) or ITS serum-free medium (fusion index 12%). Differentiated BMSCs expressed Pax7, Myod1, and myogenin	"Induced" human BMSCs were transfected with GFP and transplanted by local injection into muscle or by intravenous injection into immunosuppressed rats which gastrocnemius muscles were damaged with cardiotoxin. Within two weeks after transplantation, GFP^+ BMSCs took part in the formation of muscle fibers which expressed human dystrophin—the proportion of GFP^+ muscle fibers was 16.5%. BMSC-derived cells were also present in the satellite cells niche and were able to participate in the next rounds of regeneration	Dezawa et al. (2005)

(continued)

Table 6.1 (continued)

Type of cells	In vitro differentiation	In vivo myofiber formation and satellite cell niche colonization	References
Rat BMSCs (adherent fraction of BM cells)	BMSCs transfected with a constitutively active β-catenin and "induced" by culture in the presence of 2% HS. After 3–5 days few multinucleated cells were observed. After 5 days the fusion index was 8.65% and after 15 days 27.1%. Such cells expressed Pax7, Myod1, Myf5, myogenin, MyHC, and desmin	NT	Shang et al. (2007)
Human and mouse BMSCs	Pax3 overexpression in mouse BMSCs cultured in MSC expansion medium resulted in Myod1, Myf5, and myogenin, but not MyHC and MCK expression Pax3 overexpressing mouse BMSCs, cultured in FBS and/or HS containing medium, fused and expressed MyHC and MCK. Human BMSCs overexpressing Pax3 induced Myod1, Myf5, myogenin, and MyHC expression when cultured in medium containing either 10% FBS, bFGF, neuregulin, PDGF-AA and forskolin, or 3.2% HS or 10% FBS and 5-azacitidine, or 10% FBS and PDGF-BB/EGF, or in medium without growth factors	Human BMSCs overexpressing Pax3 were transplanted into cardiotoxin injured *tibialis anterior* muscle of mdx-nude mice. Four weeks later myofibers which expressed human dystrophin (11% of total muscle fibers) were detected. Transplantation did not result in the functional improvement of muscle function	Gang et al. (2008, 2009)

(continued)

Table 6.1 (continued)

Type of cells	In vitro differentiation	In vivo myofiber formation and satellite cell niche colonization	References
Human $CD146^+$ BMSCs	$CD146^+$ BMSCs expanded on plastic with 20% FBS, and then replated on Matrigel with 2% horse serum did not fuse and did not form myotubes. $CD146^+$ BMSCs co-cultured with C2C12 rarely fuse and formed hybrid myotubes	$CD146^+$ BMSCs were transplanted into cardiotoxin injured *tibialis anterior* muscle of SCID mice. Four weeks later, they failed to contribute to form myofibers or satellite cells in the regenerating muscle. Heterotopically transplanted $CD146^+$ BMSCs did not form muscle tissue	Sacchetti et al. (2016)

BM bone marrow; *BMSCs* bone marrow mesenchymal stem cells (adherent fraction of BM cells); *bFGF* basic fibroblast growth factor; *DMEM* Dulbecco's modified Eagle's medium; *FBS* fetal bovine serum; *G-CSF* granulocyte colony-stimulating factor; *GFP* green fluorescent protein; *HS* horse serum; *ITS* insulin–transferrin–selenite; *mdx* X-chromosome-linked muscular dystrophy; *NICD* Notch1 intracellular domain; *SCID* nonobese diabetic–severe combined immunodeficient; *MCK* muscle creatine kinase; *MyHC* myosin heavy chains; *PDGF* platelet-derived growth factor; *NT* not tested

cord MSCs (Grabowska et al. 2012b). Human cardiomyocytes induced the expression of cardiac-specific mRNAs, such as cardiac troponin T (cTNT) or β-actin in human BMSCs (Rangappa et al. 2003) or desmin, cTNT, and α-actin in rat BMSCs (Wang et al. 2006). Unfortunately, such treatment led only to upregulation of the cardiac markers but did not result in derivation of fully differentiated and functional cardiomyocytes (Rose et al. 2008).

Manipulation of Wnt signaling pathways that play crucial role in the variety of developmental processes, including myogenesis and skeletal muscle regeneration (Polesskaya et al. 2003; Munsterberg et al. 1995), may also lead to the induction of myogenic differentiation. Activation of canonical Wnt pathway prevents the degradation of β-catenin allowing it to act as a transcriptional activator. Thus, constitutively active β-catenin was shown to induce myogenic differentiation of rat BMSCs at the same time preventing their differentiation in the adipose cells (Shang et al. 2007). Another signaling pathway involved in the myogenic differentiation relies on Notch and is crucial for the establishment of cell fate and Pax7

upregulation (Wen et al. 2012). Administration of NICD (Notch1 intracellular domain) allows to activate Notch pathway in BMSCs and to induce them to express MyoD and also MyHC (Dezawa et al. 2005). Furthermore, cells overexpressing NICD were able to participate in the skeletal muscle regeneration; on average 16.5% of myofibers present in the regenerating mouse muscle was generated with the participation of transplanted BMSCs (Dezawa et al. 2005). This was truly impressive result. However, until now it is the only example of efficient differentiation of MSCs into myoblasts. Also in case of cardiac differentiation, excess of one of the Wnts, namely Wnt11, increased the differentiation of BMSCs into cardiomyocytes. As a result, these cells started to express cardiac markers, such as GATA-4 or BNP (B-type Natriuretic Peptide), and formed significantly more cardiomyocytes in vitro (He et al. 2011).

Forced expression of genes encoding crucial regulators of embryonic myogenesis leads to the in vitro differentiation of MSCs. For example, overexpression of *PAX3* in human BMSCs induced expression of MYF5, MYOGENIN, MRF-4. Unfortunately, such manipulation did not increase the ability of BMSCs to participate in the formation of new muscle fibers within the regenerating skeletal muscle and also did not correlate with the functional improvement of muscle function (Gang et al. 2008, 2009). MyoD was another factor which overexpression enhanced myogenic differentiation. Its introduction to MSCs isolated from adipose tissue induced myogenic differentiation and resulted in derivation of cells that were able to participate in the formation of new muscle fibers (Goudenege et al. 2009).

Approaches presented above are not the only ones applied in the studies focusing at the induction of myogenic differentiation of MSCs. Among other approaches one can list the manipulations of epigenome, modifications of stem cell niche or methods improving homing of transplanted stem cells, such as Sdf-1 which action we extensively explore in our studies [e.g., Kowalski et al. (2016a, b)]. Hopefully, they can lead us in the future to the application of MSCs in the therapies of injured or malfunctioning cardiac and skeletal muscles.

6.6 Embryonic Stem Cells and Induced Pluripotent Stem Cells

Almost 40 years ago, independently, Evans and Kaufman (1981) and Martin (1981) derived first lines of mouse embryonic stem cells. Seventeen years later, first human embryonic stem cells were derived (Thomson et al. 1998). In both cases, i.e., mouse and human, these cells were derived from preimplantation embryos, i.e., blastocysts, what rose almost immediate ethically driven discussion. Currently, at least in some countries, human ESCs can be derived and studied in vitro providing unique opportunity to analyze mechanisms driving human development. Since these cells originate from the embryo, they resemble embryonic cells. Thus, they present the ability to differentiate into any given tissue

in vivo and into numerous cell types in vitro. Since their "discovery," they were extensively tested what allowed to characterize molecular basis of their self-renewal, pluripotency, and differentiation.

Derivation of pluripotent ESCs, which can differentiate into any type of cell building the mammalian body, not only allowed to understand many aspects of cellular differentiation, but also gave hope to develop therapies aiming to replace damaged tissues and organs. It also rose, as mentioned above, ethical concerns resulting from the fact that the commonly used ESC derivation method required the damage of the preimplantation embryos. These ethical issues over human ESCs became a history in 2006. That year Takahashi and Yamanaka described in vitro reprogramming of mouse fibroblasts to stem cells and presented induced pluripotent stem cells—iPSCs (Takahashi and Yamanaka 2006). Derivation of iPSCs was achieved by viral vector-driven expression of transcription factors which were previously shown to govern cell pluripotency of early embryonic cells and ESCs, namely Oct4, Sox2, Klf4. The fourth factor used, c-Myc, was the oncogene controlling cellular proliferation. In 2007 Yamanaka's (Takahashi et al. 2007a, b) and Thomson's groups derived human iPSCs (Yu et al. 2007). At the first glance, ESCs and iPSCs look almost the same; however, past and ongoing detailed analyses led to the new questions about many aspects of pluripotency of these two types of cells. Nevertheless, iPSCs, since they can be derived from the patient's cells, are currently considered as a great hope of regenerative medicine.

Standard procedure of derivation and culture of ESCs requires the "support" of feeder layer cells, usually provided by mouse embryonic fibroblasts (MEFs) which proliferation is inhibited either by gamma irradiation or by DNA damage induced by mitomycin C (Hogan et al. 1994). It soon became apparent that the feeder layer can be combined or even replaced by supplementing the culture medium with leukemia inhibitory factor (LIF), i.e., a cytokine of the interleukin-6 (IL-6) family responsible for the induction of a signaling pathway leading to the activation of STAT3 (Signal transducer and activator of transcription 3) (Smith et al. 1988; Williams et al. 1988). STAT3 activates expression of Klf4 and Tbx3 which control the expression of *Nanog*, coding one of the most important "guards" of pluripotency, which in both mouse and human cells controls the expression of genes involved in maintaining the ability to self-renew and preventing the differentiation of ESCs (Boeuf et al. 1997; Mitsui et al. 2003; Chambers et al. 2007). Among the factors involved in securing the ESC and also iPSC pluripotency are also Oct4 (encoded by *Pou5f1* gene) and Sox2 (Pera and Tam 2010). In mouse and also human pluripotent cells, these proteins bind to the promoters of genes encoding the factors involved in the regulation of transcription, signal transduction, and epigenetic modifications of chromatin (Pera and Tam 2010). Analysis conducted at the genomic scale using microarrays and chromatin immunoprecipitation indicated broad range of Oct4 targets (Boyer et al. 2005). It binds to the promoters of 623 protein-coding genes and 5 genes encoding miRNA, i.e., 3% of all genes expressed in human ESCs. Approximately 50% of promoters which are targeted by Oct4 also bind Sox2. Next, 90% of promoters that are "chosen" by both Oct4 and Sox2 are also bound by Nanog (Boyer et al. 2005). In addition, Sox2 and Nanog together

regulate the activity of genes, which are not regulated by Oct4. Besides Oct4 and Nanog "pool" of markers of undifferentiated mouse ESCs include a surface protein SSEA-1 [Stage-specific Embryonic Antigen-1; (Solter and Knowles 1978)], alkaline phosphatase [AP; (Wobus et al. 1984)], and active telomerase (Armstrong et al. 2000). Not all of these factors are characteristic for human ESCs. For example, human cells miss SSEA-1 but express SSEA-3 and SSEA-4. Interestingly, they are not sensitive to LIF (Daheron et al. 2004; Humphrey et al. 2004). Instead, their in vitro self-renewal and pluripotency are supported by the FGF2 and Activin/Nodal signaling which induce *NANOG* expression [summarized in (Suwinska and Ciemerych 2011)].

iPSCs are the "products" of adult somatic cell reprogramming that led to the global changes in gene expression allowing regaining the pluripotency and ability to self-renew. The original "cocktail" used for reprogramming of mouse cells included Oct4, Sox2, Klf4, and c-Myc (so-called OSKM) encoded by retroviral vectors (Takahashi and Yamanaka 2006, 2016), while in case of human cells—Oct4, Sox2, Nanog, and Lin28 (Yu et al. 2007). Many other combinations of "pluripotency-inducing" genes were then tested. Reprogramming efficiency was and still is very low and depends on the expression of OSKM transcripts sustained for about 10 days. After this time, endogenous expression of Oct4, Sox2, and telomerase is induced and also epigenetic changes allowing decondensation of X chromosome, in case of female cells, are observed (Stadtfeld et al. 2008). Since the derivation of first iPSC lines, the available methods of reprogramming were perfected and new ones were developed—aiming at the elimination of genome integrating vectors, such as retroviruses. Thus, iPSCs could be currently generated using non-integrating vectors such as adenoviruses or plasmids, "removable" vectors, mRNAs, microRNAs, and proteins [for summary see, e.g., Adlakha and Seth (2016)]. In addition to the experiments demonstrating that the iPSCs can be obtained from fibroblasts, many lines of evidence documented that any somatic cell tested could be "reprogrammable." However, iPSCs derived from cells of different origin (e.g., fibroblasts, myoblasts) were characterized by specific "memory", possibly caused by epigenetic modifications, that could be erased by repeated passages (Polo et al. 2010; Quattrocelli et al. 2011; Bar-Nur et al. 2011; Kim et al. 2010). As a result, some iPSCs might be difficult to differentiate to lineages other that those of their origin. However, such memory was not always observed. For example, iPSCs derived from skeletal muscle-specific stem cells, i.e., satellite cells, did not manifest the tendency to preferably differentiate into muscle tissue (Tan et al. 2011). Similarly, various lines of ESCs and iPSCs differed in the gene expression patterns and, therefore, differentiation ability. Surprisingly, this phenomenon depends also on the laboratory of the origin of ESC or iPSC lines (Newman and Cooper 2010) what reveals the urgent need to carefully standardize the procedures of pluripotent stem cell derivation and culture.

Pluripotency of ESCs and iPSCs was proven in in vivo tests, i.e., by generation of chimeric animals or teratomas, and also in vitro using defined culture conditions, or using molecular analyses [see, e.g., Grabowska et al. (2012a), Singh et al. (2016)]. This most prized ability of pluripotent stem cells is, however, also their

curse. Since they are prone to differentiate into variety of tissues, in case of some cell types even without any specific stimulation, they have the ability to form teratomas, which are a nonmalignant tumors composed of variety of tissues of ecto-, endo-, and mesodermal origin. Thus, the idea of pluripotent stem cell application in clinics has its limitation. Regardless of the method used to derive ESCs or iPSCs, these cells need to be properly tested; i.e., their ability to differentiate into cells characteristic for three germ layers has to be documented. On the other hand, perfect methods of their differentiation into required cell types need to be designed to avoid uncontrolled differentiation. Only then ESCs and iPSCs can be considered as a safe "material" for cell therapies.

6.7 Myogenic Differentiation of Pluripotent Stem Cells

ESC and iPSC characteristics make them an attractive source of cells to use in therapies. Their myogenic potential was documented using in vivo tests, including most stringent ones as the generation of chimeric mice and teratomas, as well as in vitro tests such as the ability to form hybrid myotubes with C2C12 myoblasts or to differentiate into myoblasts when cultured in colonies or within the embryoid bodies (see below) (Archacka et al. 2014a; Brzoska et al. 2015; Helinska et al. 2017; Grabowska et al. 2012a; Czerwinska et al. 2016a, b) (Fig. 6.3).

Pluripotent stem cell potential, however, does not guarantee their immediate application in clinics. Transplantation of undifferentiated cells is ineffective and dangerous, bringing the risk of teratoma formation. Thus, effective and reproducible methods to induce derivation of cells of interest are necessary. Many attempts have been made to propose an approach that would be good enough to induce differentiation into such cells as skeletal myoblasts or cardiomyocytes. For many years, the most effective methods were based on the genetic modifications, i.e., induction of expression of genes known to be involved in myogenesis or cardiogenesis. For example, overexpression of MyoD (Dekel et al. 1992; Shani et al. 1992), Pax3, or Pax7 (Darabi et al. 2008, 2011) in case of skeletal myoblasts or Gata factors (Turbendian et al. 2013) in case of cardiomyocytes was sufficient to induce directed differentiation of mouse ESCs. Recently, Bai and co-workers showed a way to force endogenous gene expression that could be an alternative to genome modifications. Instead of using gene-coding vectors, e.g., viruses which could be potentially dangerous due to their ability to integrate into DNA, differentiation was directed using bacterial type III secretion system. Using such method, Gata-4, Mef-2c, and Tbx-5 proteins were introduced in differentiating cells. Such treatment resulted in the formation of cardiomyocytes with 51% efficiency (Bai et al. 2015).

Except for induction of gene expression, also adequate environment, i.e., culture conditions or medium composition, was shown to be important modulator of differentiation. Importantly, spatiotemporal interactions between differentiating ESCs or iPSCs were shown to support the differentiation. One of the methods to induce differentiation relies on the formation of the so-called embryoid bodies (EBs) that

are tridimensional structures in that cellular interactions mimic those ones occurring during embryonic development (Fig. 6.3). As a result, endo-, ecto-, and mesoderm are formed in EBs, facilitating further differentiation into more specialized cells. Thus, during differentiation of pluripotent cells within EBs, many types of cells are formed. For example, cells expressing Pax7 or myogenin can be identified in EBs cultured for 18 days (Karbanova and Mokry 2002). Next, contracting groups of cardiomyocytes were reported to form spontaneously in EBs formed from mouse ESCs and cultured only for 8 days (Doetschman et al. 1985). Also, human pluripotent cells spontaneously differentiating in EBs were proven to be able to become cardiomyocytes (Itskovitz-Eldor et al. 2000).

Spontaneous, i.e., "non-directed" by the gene overexpression or use of specific culture conditions, differentiation of pluripotent stem cells in EBs is highly inefficient. Myoblasts and cardiomyocytes are generated not in all of EBs and consist of just a fraction of differentiating cells. For this reason, it is hardly possible to predict and estimate the proportion of cells of interest that could be derived using such simple protocols. Since EBs are composed of variety of cell types, it creates the need to select chosen ones. This is crucial if cells derived in EBs would be designated for therapeutic purposes. Injection of any non-differentiated cell may result in the formation of teratoma. In case of cardiomyocytes, their separation from other cell-types may be a bit facilitated due to their contracting activity. Beating areas may be "simply" microdissected from EBs' outgrowths. Such approach was used in an elegant study in that dissected "tissue" fragments were used to record electrophysiological properties of cardiomyocytes obtained via differentiation of human iPSCs (Zwi et al. 2009). Surprisingly, however, cardiomyocytes comprised only 3.4% of the cells present in the culture of dissected fragments (Klug et al. 1996). More specific technique to select cells of interest from in vitro cultured EBs is sorting them by FACS using specific antibodies against appropriate cell type-specific markers. Precursors of skeletal muscle myoblasts can be identified and selected based on either the presence of Pdgfrα, which characterizes paraxial mesoderm, and lack of Flk-1 which is specific for lateral mesoderm (Darabi et al. 2008), or simultaneous expression of CXCR-4 and c-Met (Borchin et al. 2013), or presence of yet unknown satellite cell surface antigen detectable by SM/C-2.6 antibody (Chang et al. 2009; Mizuno et al. 2010). Cardiomyocytes could be isolated based on the presence either of SIRPA, i.e., signal regulatory protein alpha (Dubois et al. 2011), or VCAM-1 (Uosaki et al. 2011). Interestingly, cardiomyocytes stain intensively with fluorescent dye that labels mitochondria. Thus, this dye used to selectively enrich cardiomyocytes derived in EBs by FACS allowed to obtain >99% pure population (Hattori et al. 2010).

During differentiation of ESCs and iPSCs, cardiomyocytes originate from the cells expressing Flk-1 which is characteristic for cardiovascular progenitors. It should be noted, though, that expression of Flk-1 is characteristic for cardiovascular progenitors only at a specific time interval. At the beginning of EBs' culture, cells expressing Brachyury and Flk-1 are primed for differentiation into hemangioblast cells, while Brachyury+/Flk-1- phenotype is characteristic for mesodermal cells that can be further differentiated into cardiomyocytes (Kouskoff et al. 2005).

Re-aggregation of those cells and their further culture result in the induction of Flk-1 synthesis in a subpopulation of those cells. Only separation of Flk-1+ cells at this stage and their further culture led to the formation of colonies out of which 25–90% contained contracting cardiomyocytes (Kattman et al. 2006).

Except for sorting, also other techniques allowing to enrich the cell culture in cardiomyocytes were proposed over years. For example, taking advantage of metabolic properties of cardiomyocytes, Tohoyama and co-workers proposed a protocol in that human or mouse ESCs differentiating in EBs for 20–30 days were shifted to the glucose-free medium supplemented with lactate (Tohyama et al. 2013). Such approach led to the elimination of cells other than cardiomyocytes. Cardiomyocyte selection can also be obtained via genetic manipulation of ESCs, i.e., linking expression of cardiac-specific gene, such as Ncx1, with sequence encoding enzymes providing the resistance to antibiotics (Fijnvandraat et al. 2003). For example, generation of ESCs carrying gene-encoding aminoglycoside phosphotransferase that is expressed together with α-cardiac MyHC ensures resistance to geneticin only in cardiomyocytes. Thus, using antibiotic selection, it is possible to select differentiated cells obtaining >99% purity (Klug et al. 1996). Enrichment in cardiomyocytes could be also achieved by centrifugation of differentiating ESCs in Percoll gradient. However, the efficiency of this procedure was not satisfactory and under various conditions allowed to derive the population of cells containing between 17 and 75% of cardiomyocytes (Laflamme et al. 2005; Xu et al. 2002).

To increase the number of derived cells of interest, various modifications of EB culture conditions were proposed. The substantial improvement in EB-based pluripotent stem cell differentiation was made by supplementation of the culture medium with the factors active during embryonic development and controlling formation of given cell line or tissue. Thus, such approach aimed at mimicking temporal changes associated with morpho- and organogenesis occurring in developing embryo. Differentiation of mouse ESCs into skeletal muscle myoblasts in EBs was shown to be enhanced by temporal addition to the culture medium of ascorbic acid and activin A (Tian et al. 2008), or retinoic acid (RA) (Wobus et al. 1994), or RA and DMSO (Kennedy et al. 2009; Ryan et al. 2012), or supplement composed of insulin, transferrin, and selenium (ITS), as well as dexamethasone and epidermal growth factor (EGF), or only horse serum (HS) (Zheng et al. 2006). Induction of pluripotent stem cell differentiation using those factors was, however, not as specific as expected. For example, RA induced both myogenic and cardiac differentiation, though efficiency of each of the processes varied depending on RA concentration and time of treatment. RA applied in lower concentration stimulated neuro- and myogenesis, while RA used in higher concentration favored cardiomyogenesis (Wobus et al. 1994). Cardiac differentiation may be also enhanced by the addition of Bmp-4 to the medium in that EBs were cultured (Takei et al. 2009). Interestingly, cardiomyocyte derivation was more effective after supplementation of culture medium with Wnt-3a instead of Bmp-4 (Tran et al. 2009). Wnt signaling pathway can be also activated by CHIR99021, an inhibitor of Gsk-3, which is negative regulator of β-catenin (Lian et al. 2012). Cardiomyocyte derivation using EBs was also facilitated by their culture on laminin-coated

microcarriers in serum-free medium supplemented with p38 MAP kinase inhibitor (Ting et al. 2012).

Another system allowing the induction of pluripotent stem cell differentiation relies at the signals released by other cells. Such strategy was used in experiments in that EBs, initially grown in suspension, were co-cultured with myoblasts. Next, those ESCs-derived cells were transplanted into skeletal muscle in which they participated in the formation of new myofibers. Thus, interactions with myoblasts induced myogenic differentiation of mouse ESCs (Bhagavati and Xu 2005). On the other hand, cardiac differentiation was enhanced when human ESCs were cultured using mitotically inactive mouse visceral endoderm-like cells, END-2, as a feeder layer (Mummery et al. 2003). Interestingly, the efficiency of cardiomyocyte differentiation was increased when co-culture was maintained in a serum-free medium (Passier et al. 2005).

Differentiation of pluripotent cells within EBs is poorly controllable, so the influence of factors known to push differentiation toward selected cell types was also tested in monolayer cultures. An interesting protocol, so-called matrix "sandwich" bases on the monolayer culture that "evolves" into tridimensional system (Zhang et al. 2012). At first, cells are allowed to grow on Matrigel-coated surface, and before they reach the confluence, they are covered with another layer of Matrigel. Formation of such a Matrigel "sandwich" enabled multilayer culture of ESCs and facilitated differentiation, somehow mimicking embryonic environment, as it happens in case of embryoid bodies.

As described above, differentiation of pluripotent stem cells in EBs can be directed by the addition of factors known to play roles during embryogenesis. It was tempting to try to mimic embryonic environment also in monolayer culture by using a combination of factors allowing sequential activation and/or inhibition of signaling pathways crucial during embryogenesis. It turned out that efficient derivation of mesodermal cells from pluripotent stem cells could be induced by inhibiting Gsk-3 using CHIR99021 (Borchin et al. 2013; Shelton et al. 2014). Further improvement of this protocol led to the derivation of myogenic cells with 90% efficiency (Shelton et al. 2014). Only recently very elegant study, starting with transcriptome analysis of mesodermal cells in developing mouse embryos, was published (Chal et al. 2015). This initial analysis showed which factors regulate segmentation of presomitic mesoderm. Based on this knowledge, Chal and co-workers proposed a protocol involving sequential and combined use of factors necessary during embryonic development and inhibition of selected signaling pathways. As a result, induction of pluripotent stem cells to differentiate first into mesoderm and then into myogenic precursors and myoblasts, able to form myotubes and participate in regeneration of skeletal muscle, was possible (Chal et al. 2015, 2016). Similar approaches were applied to derive cardiomyocytes. Human ESCs culture in the medium supplemented with activin A followed by BMP-4 resulted in the derivation of more than 30% of cardiomyocytes that could positively influence cardiac structure and contractile function after transplantation into rat myocardium after infarction (Laflamme et al. 2007). The efficiency of cardiac differentiation was further increased by Percoll gradient centrifugation (Laflamme

et al. 2007). Yang and co-workers proposed the protocol of human ESC differentiation in that combined and time-controlled use of such factors as BMP-4, bFGF, activin A, VEGF, and DKK-1 resulted in the cell culture contained 40% of cells expressing cTNT (Yang et al. 2008). Alternatively, as in case of the method recapitulating skeletal myogenesis in vitro (Borchin et al. 2013; Shelton et al. 2014; Chal et al. 2015), cardiac differentiation of human iPSCs may be induced by the use of Gsk inhibitor—CHIR99021 (Lian et al. 2012). Interestingly, the effectiveness of such treatment was dramatically enhanced if initial induction of Wnt signaling using Gsk-3 inhibition was then followed by inhibition of Wnt signaling (Lian et al. 2012).

Comparison of 3 methods of cardiogenesis induction in monolayer culture of human iPSCs was presented by Burridge et al. (2014). Selection of the best medium and further optimization of culture conditions led to the formulation of CDM3 medium, consisting of RPMI-1640 medium containing recombinant human albumin and ascorbic acid. The use of this protocol resulted in the derivation of 80–95% of cells expressing cTNT and contracting (Burridge et al. 2014). Additionally, Burridge and co-workers made a comparison of various matrices on which cells were grown during differentiation. It turned out that all substrates tested, i.e., vitronectin peptide, E-cadherin, laminin, fibronectin, supported the differentiation of human iPSCs in CDM3 medium and only laminin resulted in higher growth rate (Burridge et al. 2014).

Summarizing, the currently available protocols enable the efficient derivation of both skeletal myoblasts and cardiomyocytes. The next step should be verification if cells obtained using those methods are truly functional in vivo.

6.8 Mesenchymal and Pluripotent Stem Cells—From Bench to Bedside

Since the derivation of the first ESC lines, pluripotent cells have attracted a lot of attention as a potential universal source of cells for transplantation aiming to repair or replace dysfunctional cells, tissues, and organs. Generation of iPSCs raised both scientific and public expectations about progress in the regenerative medicine since —as mentioned before—these cells have all unique potential for differentiation and lack the ethical controversies of ESCs. However, first successful pluripotent stem cell-based therapy was conducted using human ESC but not iPSC derivatives (Schwartz et al. 2012, 2015). Although, over the years, several types of functional cells were generated from differentiating pluripotent stem cells, some of eye diseases were the first successfully treated. Schwartz and co-workers transplanted retinal pigment epithelium cells (RPECs) generated from human ESCs to treat patients suffering from macular degeneration, for which no other therapeutic options had been available. Macular degeneration results from the progressive deterioration of light-sensing photoreceptors in the eye and remains one of the main

causes of blindness. Transplantation of RPECs to central visual area of the retina led to the improvement of the vision acuity in 10 out of 18 treated eyes (Schwartz et al. 2012, 2015). Importantly, no adverse effects, such as inflammation, abnormal proliferation, or retinal detachment, have been observed. It has been noticed, however, that the coverage of the macula by transplanted cells was patchy. For this reason, currently, ESCs-derived RPECs are inserted under photoreceptor layer on scaffolds to cover the entire macula (Binder 2011). Also, RPECs derived from human iPSCs were used in cell therapy of macular degeneration (Kamao et al. 2014). Despite that currently, i.e., in 2016, clinical trial using iPSC-derived RPECs is on hold, it shows that iPSC-based therapies will most probably reach clinical application in a very short time. However, many challenges and obstacles on the way to clinical application of pluripotent stem cells still exist, including safety, quality, and purity of obtained cell populations.

As mentioned above, during the last few years, a significant improvement of safety and efficiency of iPSC derivation has been made (Sayed et al. 2016). Also, several stepwise differentiation protocols have been developed to recapitulate the developmental pathways leading to generation of functional cells of selected types such as skeletal muscle myoblasts or β islet cell progenitors [see, e.g., Schulz (2015), Swierczek et al. (2015)]. Derivation of specialized and functional cells is of great importance as there are still many human conditions that have limited treatment options. Although only two stem cell-based therapies, i.e., bone marrow transplants for blood diseases and cancer, and *Holoclar* therapy for limbal stem cell deficiency caused by corneal burns (Abbott 2015), have been already approved for clinical use in humans, there are many trials testing the therapeutic potential of stem cells in different medical conditions. Among the major clinical targets there are cardiovascular conditions and blood diseases, diabetes, neurology, opthalmology, and gastroenterology, as well as skeletomuscular conditions and cancer. Apart from RPECs among the most advanced pluripotent stem cells-derived products in clinical development, there are: human ESC-derived pancreatic precursor cells for type 1 diabetes (*Viacyte* company), oligodendrocyte progenitors for spinal cord injuries, used in the world's first clinical trial of human ESC-based therapy initiated in 2009 (*Geron* company, *Asterias Biotherapeutic*), as well as human ESC-derived cardiac progenitors for severe heart failure, used in the clinical trial initiated in Hopitaux de Paris in France (Ilic et al. 2015). Derivation of functional cardiac progenitors is of special social importance as heart failure still stays one of the major death causes all over the world.

ESCs and iPSCs are extensively used to study the mechanisms of myogenic differentiation and to design the methods allowing their future use in clinics. However, until now no clinical trials using these cells in skeletal muscle therapy have been reported. In 2016, an important study revealing the results of five-year follow-up after transplantation of stem cells into dogs suffering from Duchenne muscular dystrophy (DMD) was published. DMD is X-chromosome inherited, genetic muscular dystrophy leading to progressive degeneration of skeletal muscle. Improvement in the function of dystrophic dogs' muscles was noticeably ameliorated after the transplantation of stem cells expressing CD133. Although

transplanted cells were isolated from muscles and not derived from differentiating pluripotent stem cells, this study is an important step toward the skeletal muscle cell therapy using stem cells (Sitzia et al. 2016).

In contrast to skeletal muscles which are able to regenerate after the injury, the endogenous regenerative capacity of the heart is very low. Therefore, serious cardiac muscle damages resulting from heart infarctions are not repaired efficiently what results in the impaired functioning of the heart. As a solution, the therapy aimed at replacing or promoting cardiomyocytes recovery is being considered. There have been numerous preclinical research focused on the assessment of different stem cell utilities in such therapies for cardiac conditions (Sanganalmath and Bolli 2013). Among the first cells tested in clinical trials for heart ischemia were myoblasts derived from satellite cells. However, instead of any significant clinical benefits occurring after transplantation of these cells, arrhythmia was observed (Duckers et al. 2011; Povsic et al. 2011). In many other clinical trials (including >2000 patients in total), unfractionated bone marrow mononuclear cells (BMMNCs) were used but with different outcomes. Although in most of the trials reduced infarct scar in ischemic heart was described, discrepancies in left ventricular ejection fraction (LVEF) appeared. In BOOST trial, the increase in LVEF occurred after 6 but not 18 months after the treatment, indicating that sustained improvement in LVEF can be a challenge in the therapy for ischemic heart failure (Young and Schafer 2015). Discrepancies in obtained results can be partially explained by the differences in cell doses (ranging from 10^5 to 10^9 cells) as well as distinct delivery routes, i.e., direct intracoronary injection, transendocardial injection, and transepicardial injection used in the clinical trials (Sanganalmath and Bolli 2013). Similar to skeletal muscle myoblast transplantation, arrhythmias have also been observed in the studies in which pluripotent stem cell derivatives were used (Chong et al. 2014). Despite different concerns and disappointments over heart failure cell therapies, several clinical trials using pluripotent stem cell derivatives have been already initiated. Among them is the clinical trial started in 2014 in France and based on the transplantation of the cardiac progenitors to the patients with low LVEF and akinesia resulting from myocardial infarction. Initial results indicated that no adverse effects were observed while increase in LVEF as well as heart revascularization occurred (Menasche et al. 2015). Pluripotent stem cells and their derivatives have been also used in modeling of different cardiac diseases as well as drug screening (Sayed et al. 2016). However, it should be remembered that cardiomyocytes derived from iPSCs are smaller and their sarcomeric organization is affected with decreased force generation, so they resemble rather fetal than mature cells building adult heart (Karakikes et al. 2015).

Although—due to extraordinary potential for differentiation—clinical trials are now evolving into the use of pluripotent stem cells and their derivatives, there are numerous studies testing different types of stem cells with a prevalence of MSCs. The first MSC product—Prochymal—was registered in Canada and New Zealand in 2012 as a treatment for graft versus host disease in children who do not respond

to steroids (Kurtzberg et al. 2014). The field of clinical trials concerning application of MSCs is growing year by year due to numerous advantages of MSCs described before, *inter alia* access to different sources of MSCs, available methods of their expansion in vitro, and few if any adverse effects documented in thousands of patients treated with MSCs, so far. Another advantage of MSCs is that—in contrast to pluripotent stem cells—these cells do not form teratomas. Among challenges on MSC translation into clinic are difficulties in rigorous characterization of these cells which may vary depending on the source affecting future therapeutic effect. More efforts is needed to define MSCs as none of the currently known and used MSC markers are unique for these cells (Prockop 2017). Many studies indicate that the beneficial effect observed after transplantation of MSCs is not related to their engraftment and differentiation into cells of particular tissues but to the secretion of paracrine factors. Nevertheless, in many cases, the exact mechanism of MSC action is not clear. Despite these challenges, MSCs are currently used in over 60% of clinical trials for a range of conditions with some already demonstrated clinical benefits including anti-fibrosis, anti-apoptotic, and neuroprotective effects. Numerous studies revealed that MSCs can efficiently suppress excessive inflammation that underlies many diseases, for example, osteoarthritis or Crohn's disease (Orozco et al. 2013; Dalal et al. 2012). Beneficial effects of MSC transplantation have also been observed in cardiac conditions, particularly in myocardial infarct in which secretion of activin A, epiregulin, endothelin, VEGF, and other factors by MSCs was shown to improve cardiac function (Williams and Hare 2011). Also, MSC-conditioned medium has been shown to reduce infarct scar and improve heart remodeling after myocardial infarct (Timmers et al. 2011). However, in these studies, detailed mechanism of MSC action remains unknown. Better insight into MSC influence on heart infarct was described by Lee and co-workers who noticed that although most of the MSCs injected intravenously became trapped in the lungs, these cells still acted at a distance reducing injury of the heart. The beneficial effect of MSCs relied on secretion of the product of *TSG-6,* i.e., TNF-stimulated gene 6, which reduced inflammatory response in damaged heart, leading to the reduced myocardial infarct scar size and improved function of the left ventricle (Lee et al. 2009). In another study, i.e., C-Cure trial (Cardiopoietic stem Cell therapy in heart failURE), endomyocardial injection of MSCs was preceded by their incubation with the cocktail inducing cardiogenic stem cell differentiation. Again, certain, but not dramatic, improvement was observed (Bartunek et al. 2013a, b). Despite some promising initial results, however, no advanced MSC products are currently available in the therapy for heart as well as skeletal muscle diseases. Thus, similar to the studies on pluripotent stem cells and their derivatives, that ones are also still on the very early stage. It is currently clear that in both cases, the road from bench to bedside is still long.

As a scientific community, we do have an access to the great knowledge about sources of stem cells, methods of their isolation, culture, and differentiation. However, we still face numerous issues that must be addressed before the next great step in global stem cell medicine will be possible. Among them are: development of worldwide cell banks ensuring cell quality and identity, rigorous characterization of

cells used in studies, generation of mature and functional cells for transplantation with scale-up approach, understanding molecular and cellular stem cell influence on tissue repair, careful interpretation of clinical trial data as well as scrupulousness in addressing safety issues including adverse effect description. Last but not at least, many countries still lack any regulatory guidelines. However, despite the challenges on the translation of discovery into clinical product, several cellular products were approved in the last few years, including Carticel and Epicel (Heathman et al. 2015). Therefore, although currently few clinical trials based on pluripotent stem cells and mesenchymal stem cells have demonstrated sufficient clinical benefits, it does not annul the expectations that stem cell-based therapies will bring significant relief to patients suffering a wide range of conditions, including skeletal muscle and heart disorders.

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Chapter 7
Microfluidic Systems for Cardiac Cell Culture—Characterization

Elzbieta Jastrzebska and Zbigniew Brzozka

7.1 Research Issue—Heart Diseases

Biomedical studies play a significant role in both science and daily life. Deep study in this research field, development of medical products and fabrication of medical devices, repeatedly contribute to save human lives. To diagnose and cure a particular illness, it is important to have a specific method, which enables scientists to detect and defeat a given ailment. However, the available methods for diagnosis and treatment of diseases are often not enough to recognize and diagnose all mechanisms responsible for an illness (Fryburg et al. 2014). Additionally, selecting the best treatment in clinical cases requires earlier investigation of the disease mechanisms. In vivo or in vivo-like assays are the best ways to test new drugs and treatment methods (Salyers 2009; Zhang et al. 2012). The existing in vitro testing models have many disadvantages (Katt et al. 2016). Consequently, it is necessary to conduct research for the development of new testing models, tools and methods, which could improve disease treatments. The developed solutions could be used by doctors not only to test new drugs but also to optimize treatment parameters. The cardiovascular system is one of such a research area, in which such solutions are needed.

Statistical analysis shows that cardiovascular diseases (CVDs), next to cancers, are the most common cause of death all over the world (World Health Organization 2014). CVDs increase the loss of cardiomyocytes (CMs), which simultaneously deprive cells of their ability to regenerate (Laflamme and Murry 2011). Treatment of heart diseases may include the usage of: various medicines, medical and surgical procedures as well as cardiomyocyte (CM) regeneration (Mampuya 2012; Sheng et al. 2013). The goals of the above methods are, among others, to relieve symptoms and reduce risk factors, which can cause heart attack as well as prevent

E. Jastrzebska (✉) · Z. Brzozka
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: ejastrzebska@ch.pw.edu.pl

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CVD complications (Hobbs 2004). Lifestyle changes (healthy diet, no smoking, physical activity) are also crucial in treating and preventing CVDs. There are various kinds of drugs with different mechanism actions, which are used in cardiac therapy (Lundin et al. 2016). The common cardiac medications are divided into such groups as: anticoagulants, beta blockers, calcium channel blockers, digitalis preparations, cholesterol-lowering medications, angiotensin-converting enzyme (ACE) inhibitors, diuretics, and vasodilators. The effects of these drugs include, among others: the regulation of blood clotting (coagulating), decreasing blood pressure, decreasing low-density lipoprotein (LDL) cholesterol, reducing swelling from excess buildup of fluid in the body, and mitigation of chest pain (angina) (Lundin et al. 2016). The nonsurgical procedure—percutaneous coronary intervention (PCI, angioplasty) that opens blocked or narrowed coronary arteries is also used for the treatment (Hoyt et al. 2013). Another method is coronary artery bypass grafting (CABG). CABG involves removing arteries or veins from other areas of the body and using them to bypass narrowed or blocked coronary arteries (Iqbal et al. 2013). There are also advanced methods for treating heart failure such as heart transplants, artificial hearts, and mechanical devices supporting left ventricular function (Kozar-Kaminska 2012). However, these treatments are not widely available, and their usage is limited to a small group of patients. Because heart failure is becoming increasingly common, great emphasis is placed on the development of new methods, which improve and restore heart functions. Regenerative medicine has been playing an important role in cardiology. At the end of the previous century, it was stipulated that with the use of acquired knowledge and experience in tissue engineering, it should be possible to grow a fully functioning heart. However, engineered heart tissue (EHT) is still being developed. Stem cells (SCs) are more commonly used as an alternative therapy for heart diseases (Beeres et al. 2007; Zhang et al. 2015). SCs have the ability to regenerate and differentiate into other types of cells. Appropriate growth factors and external stimuli (electrical, mechanical, optical, or magnetic pulses) can differentiate SCs to the cardiac cells (Batalov and Feinberg 2015; Nadal-Ginard et al. 2014). Therefore, SCs constitute the material filling the destroyed CMs, which lack the ability to regenerate (Cambria et al. 2016). Additionally, scaffolds and multilayer cell cultures are used to improve in vivo conditions. EHT provide an in vitro model reproducing heart tissue, in which therapies for patients with CVDs can be investigated (Doppler et al. 2013; Schaaf et al. 2011; Sondergaard et al. 2012). Research conducted so far has shown that heart regeneration is a long and complex process. It could result from the fact that conventional in vitro methods have some obstacles restricting their application (Bernstein and Srivastava 2012; Lovell and Mathur 2010). Despite long and advanced research based on EHT development and improvement in regenerative medicine there are many aspects, which should be solved and improved. First of all, a large amount of SCs is required. The creation of a heart model with an appropriate system of vascularisation (angiogenesis) responsible for supplying nutritive substances is also difficult. Therefore, scientists have developed new methods for cardiac cell analysis and propose new in vivo-like models to understand processes present in a fully functioning heart.

7.2 *Lab-on-a-chip* Systems—A New Approach for Heart Investigation

Elaboration of new therapeutic methods for heart diseases is a great challenge for scientists and doctors dealing with this type of ailment. Therefore, to avoid problems which appear during research conducted in conventional laboratories, *Lab-on-a-chip* systems were developed (Mehling and Tay 2014; Ziolkowska et al. 2011). The aim of the fabrication of such systems is to develop an in vivo-like cardiac model, in which the investigation of cardiac cell processes, as well as the elaboration of new therapies for heart failure will be possible. In such microsystems, fully functioning and vascularized heart tissue could be mimicked (Jastrzebska et al. 2016; Lee et al. 2015). These models, called *Heart-on-a-chip* systems, are specific types of *Organ-on-a-chip* systems. The usage of the microsystems for cardiac cell engineering has many advantages. First of all, miniaturization allows for a reduction in volumes of reagents used for experiments. The microsystems allow also to shorten the time of assays and to automate the whole process of biological sample studies (Halldorsson et al. 2015; Sackmann et al. 2014; Tehranirokh et al. 2013). Additionally, it is possible to control precisely the spatio-temporal phenomena present in a microsystem. This is particularly important when difficult-to-isolate or expensive cells (e.g., primary and stem cells) are examined (Visone et al. 2016). However, the most important advantage of *Heart-on-a-chip* systems is the possibility to mimic in vivo conditions better than in conventional (two-dimensional, 2D) culture methods (Jastrzebska et al. 2016; Lee et al. 2015). A network of microstructures can be designed in such a way that the microenvironment created in Lab-on-a-chip systems is similar to natural cell growth. Additionally, microstructure dimensions are similar to the dimensions of the cells (Bhatia and Ingber 2014; Mehling and Tay 2014). The in vivo cardiovascular system is characterized by blood circulation, which allows necessary nutrients, oxygen, and hormones taken from components of the metabolism to be supplied to the cells (Pittman 2011). Because the microsystems provide dynamic conditions, culture models obtained in microscale brings even more to in vivo. The flow environment is an important property especially for cardiac cell cultures (Kobuszewska et al. 2017). The next feature is that the cells in tissue are surrounded by an extracellular matrix (ECM), the mixture secreted by the cells and filling the space between them (Alberts et al. 2002). ECM plays a significant role in cell behavior. It regulates intercellular communication and a dosage of various cellular growth factors absorbed in this matrix. Moreover, ECM maintains a spatial (three-dimensional, 3D) arrangement of the cells. Scaffolds, hydrogels, and multicellular spheroids are used to culture cells in such conditions (Bray et al. 2015; Nath and Devi 2016; Tomecka et al. 2018; Yan et al. 2015). However, conventional techniques (macroscale) have still not been optimized.

Cell culture methods used so far in conventional biological laboratories (e.g., using 96-well plates) do not ensure the conditions described above (Yamada and Cukierman 2007; Ziolkowska et al. 2011). A scheme of cell environments provided

in vivo and in vitro monolayer cultures is shown in Fig. 7.1. Monolayer cell culture does not correspond well to natural tissue conditions. However, the usage of the microsystems for spatial heart culture can allow to mimic in vivo environment and to control culture conditions precisely.

The systems based on *Heart-on-a-chip* have two main approaches (i) the creation of a beating heart culture model, which mimics heart tissue and (ii) creation of a whole vascular system, which mimics blood flow in vessels (Fig. 7.2) (Lee et al. 2015; Ribas et al. 2016; Simmons et al. 2012). The development of a beating heart model allows to investigate cardiomyocyte contraction under various conditions (static and dynamic). *Heart-on-a-chip* systems can also be utilized for investigation of an external stimuli influence on cardiomyocyte growth as well as stem cell cardiogenesis. Parameters such as oxygen concentration, pH value, hydrodynamic

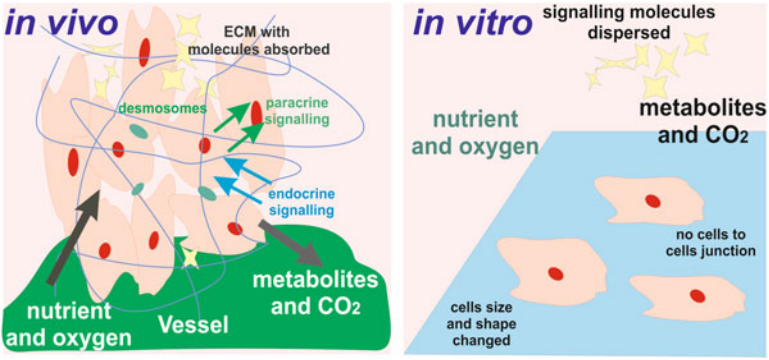

Fig. 7.1 A scheme of microenvironments provided in vivo and in vitro two-dimensional (2D) culture

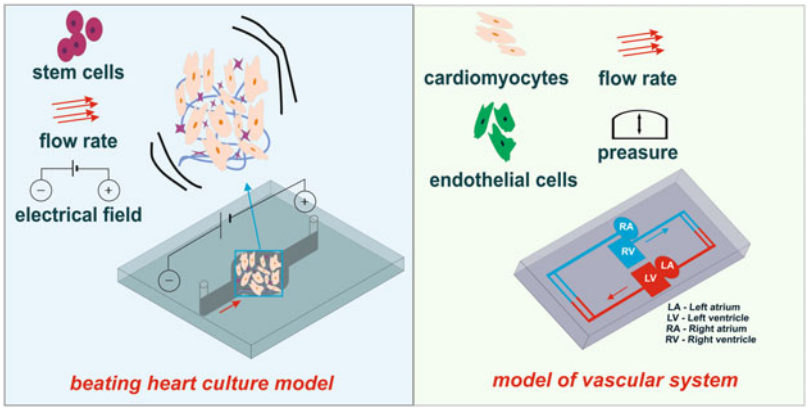

Fig. 7.2 Types of *Heart-on-a-chip* systems: model of beating heart culture, which mimic heart tissue (left) and model of a whole vascular system, which mimic blood flow in vessels (right)

stress, uptake of intracellular calcium ions, and lactic oxidase level were monitored in cardiac cultures maintained in microscale. Additionally, cytotoxicity of new compounds with cardiac therapeutic activity can be investigated. The microsystems mimicking either heart tissue or the whole vascular system can be good models for the understanding of heart phenomena as well as to verify drug usefulness in heart disease treatment (Jastrzebska et al. 2016; Lee et al. 2015; Simmons et al. 2012; Zhang et al. 2016a).

7.3 *Heart-on-a-chip* Systems—What Is Specific?

Organ-on-a-chip systems are in vivo-like models, which mimic functioning organs as well as which are used for investigation of both tissue disorders and drug cytotoxicity (An et al. 2015; Young 2013). Each in vitro organ model has parameters which are specific for that tissue. Therefore, to create a fully working heart model in microscale, it is necessary to know the properties of the specific tissue. Native myocardium is characterized by both parallel cardiac muscle fiber and complex electrochemical dynamics. Therefore, these facts are considered during the development of *Heart-on-a-chip* systems (Ribas et al. 2016). Electrochemical signals present in heart tissue are important because they regulate heart function and demonstrate spontaneous beating of CMs. A laminar flow and a pulsatile flow in the vasculature system are the next essential feature. The electrical field, stretching, the usage of hydrogels, or nanofibers are the key signaling parameters used to mimic native myocardium. Additionally, heart cell culture in the microsystems is often used to simulate heart diseases and investigate heart regeneration using stem cells (Fig. 7.3) (Ghafar-Zadeh et al. 2011; Visone et al. 2016).

7.3.1 Vasculature and Dynamic Conditions

The microenvironment occurring in the vasculature system, provided by blood flow, is characterized by dynamic conditions. Mimicking these conditions in vitro is important to study the influence of shear flow and shear stress on cell proliferation, morphology, and viability (Cheng et al. 2003; Dahl et al. 2010; Li et al. 2005; World et al. 2006). However, the imitation of a vascular environment is difficult. It results from the fact that components of such a system (arteries, veins, venules, and capillaries) have varying compositions. Because of the dynamic microenvironment presented in a body, the microfluidic systems are excellent tools for mimicking these conditions (Wong et al. 2012). This results from the fact that laminar flow is provided in the microsystems (Young and Simmons 2009). A low Reynolds number and shear stress profile in the microsystems are similar to these, which are presented in vivo. Another feature of microscale, which helps in developing

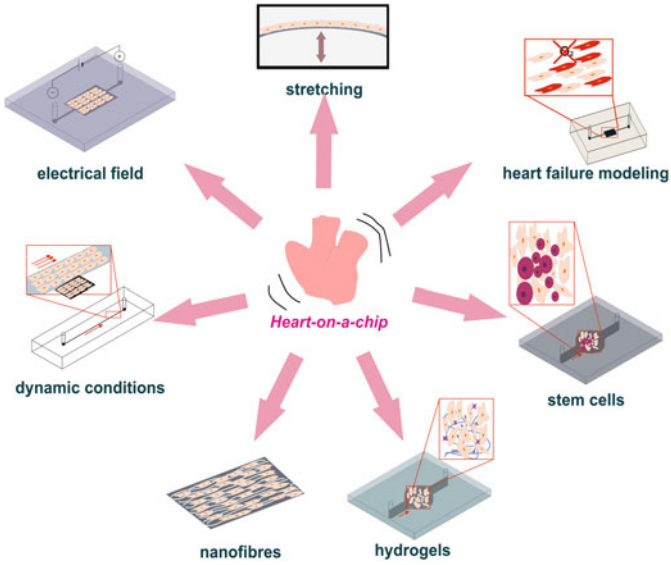

Fig. 7.3 Key signaling parameters used to mimic native myocardium in *Heart-on-a-chip* systems

Heart-on-a-chip systems is the fact that microtechnology allows various flow profiles to be precisely designed. Thanks to this, a network of microstructures can be designed in such a way that in various areas of a microsystem different cell types can be cultured and simultaneously different shear stress values can be generated (Rossi et al. 2009). Endothelial cells cushion the inner heart chamber and blood vessels, therefore these cells are most often used to mimic the vasculature system in microscale (Hasenberg et al. 2015; Morgan et al. 2013). So far several types of the microsystems have been developed to mimic vessel connections and angiogenesis formation. Additionally, the microsystems for chemotaxis study and vascular disease modeling have been fabricated (Ribas et al. 2016).

Perfusion is also an important factor in fabricating a model of a beating heart culture. Due to the fact, that the heart is still simulated by a flow rate, assays based on the comparison of static and dynamic conditions are performed in the microsystems. These tests are able to check how a flow rate influences on proliferation and contraction of CMs. Additionally, perfusion can enhance parallel CM arrangement, which is specific for native myocardium (Kobuszewska et al. 2017; Xiao et al. 2014).

7.3.2 Materials Versus Cell Alignment

Mimicking of cardiac tissue microenvironment is also related to the selection of construction materials of *Heart-on-a-chip* systems. To fabricate such systems, poly

(dimethyl siloxane) (PDMS) and glass are often used (Halldorsson et al. 2015; Ren et al. 2013). It results from the beneficial properties of these materials, which have been described in Chap. 3. To obtain a suitable microstructure/surface for cardiac cell culture, PDMS and glass are additionally modified. Native myocardial tissue is characterized by a parallel arrangement of the cells. Lack of such cell orientation is the main distinction between in vivo and in vitro cultures. Therefore, parallel cardiomyocyte alignment should be simulated in 2D and 3D in vitro models performed in microscale. Microcontact printing is a method used for obtaining ECM proteins on PDMS or glass plates and parallel CM alignment (Guillemette et al. 2010). Proteins such as collagen, laminin, and fibronectin have been used for this purpose. A fabrication of microgrooves in the culture surface has also been used to CM align (Motlhagh et al. 2003; Yang and Ma 2012). Scaffolds and hydrogels are other methods used to create a native myocardium environment. This can be achieved by the usage of regular nanofibers. Nanofibers have many advantages, which are useful for cell cultures: they have a high surface-to-volume (SAV) ratio and high porosity. Moreover, their structure and nanofiber organization influences the parallel orientation of CMs (Ashammakhi et al. 2012; Carletti et al. 2011). CM arrangement has been tested on nanofibers made of materials such as: poly(L-lactide-co-ε-caprolactone) [P(LLA-CL)] copolymer, poly(lactide-co-glycolide) (PLGA), poly(ε-caprolactone) (PCL), poly(hydroxybutyrate) (PHB), chitosan-polycaprolactone, polymethylglutarimide (PMGI) (Tomecka et al. 2017). The above mentioned materials can be placed on PDMS or glass surfaces as well as inside of the microsystems. Other method used for the creation of a spatial cardiac cell arrangement in the microsystems is the use of hydrogels. They allow to obtain uniform distribution of nutrients in 3D culture (Annabi et al. 2013). Hydrogels which are gelled under the influence of various external factors (ultraviolet irradiation, temperature, chemical factors) were used for the 3D culture in the microsystems (Hoffman 2012; Zhang et al. 2016b; Zuppinger 2016).

7.3.3 Stretching

Cell stretch, is the next feature, simulated in the microfluidic devices (Simmons et al. 2012). Regulating/moving of a surface, on which the cells are cultured, is used to stretch the cells. This is obtained by changing the pressure. Longitudinal, in-plane and out-of-plane mechanical simulations are used for this purpose. However, pressure microchannels are most often used to stretch a thin membrane made from elastic material (Huh et al. 2010; Moraes et al. 2010). This allows to mimic native conditions of heart cells.

7.3.4 Electrical Field

Electrical depolarization begins in Purkinje fibers and next propagates between the cardiomyocytes. This phenomenon is responsible for heart beat. Electrical stimulation is one method, which can be applied to cardiomyocyte depolarization and stimulation for contraction (Maidhof et al. 2012). Different types of electrical stimulation can be used for tissue engineering. It can be monophasic or biphasic stimuli, in a form of sinusoidal or square waves. Electrical signals can be delivered in pulses or continuously (Balint et al. 2012). The placement of an anode and cathode in the culture medium is the simplest method of inducing cell depolarization (Ribas et al. 2016; Serena et al. 2009). This allows to obtain a uniform electrical field across the cell culture. Either rods or microelectrodes are used in microscale. The integration of the microfluidic devices with planar electrodes and multi-unit electrode arrays (MEA) is the next method, which allows cell stimulation (Ma et al. 2012). In this case, the stimulation can be performed between two or more electrodes on which cells are cultured. Electrodes made of various types of materials are utilized for cell stimulation: stainless steel, carbon, platinum, gold, indium tin oxide (ITO). The integration of the microsystems with the electrodes allow to obtain controllable conditions, which influences cell contraction, alignment as well as differentiation.

7.3.5 Heart Failure Modeling

Lab-on-a-chip systems are used most often to analyze cell proliferation, migration, and interactions. Cytotoxicity assays of new drugs administered into the cells in a form of either solution or nanoparticles are also performed in the microsystems. Additionally, the mimicking of different organs is performed in microscale (Bhise et al. 2014). However, disease modeling is most often performed in *Heart-on-a-chip* systems. Arythmia, ischemia, and myocardium infarction were simulated and tested in microscale (Ren et al. 2013; Ribas et al. 2016). Ischemia was simulated by limiting the oxygen level or by the usage of a specific oxygen consumption blocking reagent: cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The microfluidic systems provide many possibilities to investigate the repair of "damaged" cardiac cells. Additionally, processes responsible for heart diseases can be studied in detail using in vivo-like models. Modeling of heart failure could be helpful in better understanding drug development and heart regeneration. The utilization of electrical fields, mechanical stimulation, SCs, or myocytes could be used to test cardiac cell regeneration in microscale.

7.3.6 *Co-culture with Stem Cells*

The microsystems used especially for heart cell cultures have also been used for co-culture. Because of the high importance of SC usage in regenerative medicine, these cells are most often used in co-culture with cardiac cells. They are utilized as a potential method to regenerate CMs (Garbern and Lee 2013). SCs can be differentiated into cardiac cells using different biochemical, mechanical, and electrical methods. Although, differentiation to various types of the cells is described in the literature there still are not too many reports about SC differentiation into cardiac cells (Ghafar-Zadeh et al. 2011; Jastrzebska et al. 2016). Most often such differentiation is started in macroscale, using conventional cultures and later differentiated cells are introduced and cultured in the microsystems (Mathur et al. 2015). The potential interaction between the cells as well as repair properties has been investigated using SCs (He et al. 2014).

7.4 Summary

Lab-on-a-chip systems for mimicking and studying heart cells are more often presented in the literature. Microtechnology allows in vivo conditions well to be mimicked. Therefore, the microsystems are increasingly used to simulate the vascular system, to culture cardiomyocytes (CMs) and to test their action after stimulation with various external factors. The microsystems for heart cell cultures have properties, which are the same as in another microsystems used especially for cell engineering, e.g., similar microstructure dimensions to cell dimensions, a laminar flow, high surface-to-volume (SAV) ratio, and effective culture volume (ECV). Besides that *Heart-on-a-chip* systems have additional features, which are specific for heart cells. Developing *Heart-on-a-chip* systems brings many challenges. It results from the fact that dynamic conditions, stretching, and electrical stimulation should be obtained in such microsystems. In the literature, various approaches and solutions have been presented, e.g., utilization of hydrogels and nanofibers, simulation of heart diseases. The microsystems presented so far have also a good potential to test the heart's function and regeneration with SCs. However, there are many aspects, which should still be investigated and improved in microscale, e.g., long-term CM culture, SC differentiation.

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Chapter 8
Heart-on-a-chip Systems

Magdalena Bulka and Elzbieta Jastrzebska

8.1 Cardiac Cell Cultures in *Lab-on-a-chip* Systems

Heart-on-a-chip systems are developed to create culture models, which are able to mimic functional heart tissue (Selimović et al. 2013). Such cellular models could lead to a better understanding of mechanisms responsible for heart diseases. In vivo-like cardiac cultures obtained in *Lab-on-a-chip* systems can be utilized for (1) the investigation of cardiac cell physiology under conditions close to in vivo, (2) the evaluation of the cardiotoxicity of drugs used for CVD therapy, (3) the investigation of side effects generated by different types of drugs, and (4) the study of mechanisms responsible for heart regeneration (Jastrzebska et al. 2016; Ribas et al. 2016; Zhang et al. 2016a, b).

Two-dimensional (2D) cell cultures (monolayers) are the most common in vitro models of the heart and cardiovascular system. However, they do not mimic the conditions present in a living organism accurately. Conventional cardiac cell cultures are based on a monolayer culture, where the cardiac cells are cultured on a flat surface of the culture flask. In this case, the culture is carried out under static conditions, where a culture medium is exchanged periodically (Ralphe and de Lange 2013). Additionally, the arrangement of the cells to each other is usually random in a conventional monolayer culture. The cells are arranged parallelly to each other in living organisms (Bhaarathy et al. 2014; LeGrice et al. 1995). It should be noted that such cell arrangement is essential in a cell model. The orientation of myocardial cells is determined by many factors. Mechanical factors play the most important role. In the native environment, heart tissue is constantly in contact with body fluids (blood, lymph) (Kujala et al. 2016). The continuous stimulation of heart cells by blood flow and physical forces in vivo (i.e., shear

M. Bulka · E. Jastrzebska (✉)
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: ejastrzebska@ch.pw.edu.pl

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stress) is crucial for the determination of cardiac cell structure, phenotype, and alignment to the other cells. Therefore, the dynamic conditions should be provided to in vitro cardiac cell cultures. The *Lab-on-a-chip* systems are suitable tools, in which biochemical, mechanical, and physical factors of heart tissue can be mimicked (Chen et al. 2017; Giaseddin et al. 2017; Simmson et al. 2012). Key signaling parameters used to mimic native myocardium in the microsystems are described in detail in Chap. 7.

Heart and cardiovascular systems are composed of different cell types: e.g., cardiomyocytes (CMs), fibroblasts, endothelial cells, smooth muscle cells, and pericytes (see Chap. 5 and Fig. 8.1). CMs are myocardial cells, which are responsible for heart contractions. These cells have contractile proteins such as sarcomeres and myofibrils. Fibroblasts are responsible for producing an extracellular matrix (ECM) and the arrangement of other heart cells (Ugolini et al. 2016). Endothelial cells build heart atriums and produce atrial natriuretic peptide (ANP), which prevents heart failure (Maksimov et al. 2015). Pericytes build small blood vessels. Their function is very important, because they strengthen the blood vessels. Pericytes can also differentiate into other cells (fibroblasts or smooth muscle cells) depending on tissue requirements (Hall et al. 2014).

Cardiac and vascular cells are characterized as having a high dependency on the external environment (Conant et al. 2017). They exhibit morphological and physiological changes as a result of mechanical, biological, chemical, and electrical stimulations (Dahl et al. 2010; Simmson et al. 2012). CMs are the most commonly used for in vitro studies. Cardiomyoblasts (precursor of CMs) are the next cell type often utilized for in vitro experiments. Cardiomyoblasts are mesodermal cells, which can differentiate into transverse striated heart muscle cells (Simmson et al. 2012). The cells, utilized for in vitro assays, can be mainly derived from human, mouse, or rat. However, it should be noted that there are significant differences

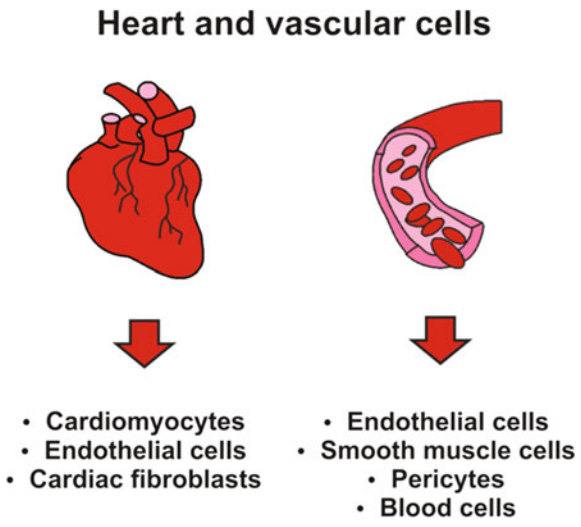

Fig. 8.1 Types of cells, which build in vivo cardiovascular system

between human and animal cardiac cells. Rat CMs have a higher percentage of protein chains, which build filaments fibers (e.g., α-myosin) and a fivefold higher resting heart rate than human CMs. A calcium transport through the rat cell membrane is also carried out by other mechanism than in human cells. Mouse CMs differ from human heart cells even more than rat CMs. The resting heart rate of mouse CMs is tenfold higher than in human cells. Moreover, the way of potassium repolarization in mouse CMs is different than in human cardiac cells. This can influence the permeability of chemical compounds (e.g., drugs) through cell membranes. Finally, different drug cytotoxicity could be obtained depending on cell origin (Simmson et al. 2012). For this reason, stem cell-derived cardiomyocytes (SC-CMs) have been used for in vitro studies in recent years (Au et al. 2009). Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSC) are predominantly differentiated in CMs using conventional methods (macroscale). Moreover, SC differentiation into CMs using different types of stimulation (biochemical, mechanical, and electrical) can be performed in microscale. These methods are described in detail in Chap. 9.

The *Lab-on-a-chip* systems used to create both a beating heart culture model and a whole vascular system have been presented in the literature in the last few years. Two-dimensional (2D) and three-dimensional (3D) cell cultures are obtained in the developed microsystems (Cheng et al. 2006; Ges et al. 2008; Horiguchi et al. 2009; Yue et al. 2014). A scheme of culture types, which can be obtained in *Heart-on-a-chip* systems, is shown in Fig. 8.2.

Poly(dimethyl siloxane) (PDMS) and glass are most commonly used for fabrication of *Heart-on-a-chip* systems. It results from the properties of these materials (see Chap. 3). It should be noted that PDMS is highly hydrophobic. 2D culture of CMs on PDMS surface is limited, because its hydrophobic properties inhibit cell attachment. In turn, a hydrophobic surface of PDMS enhances 3D cultures

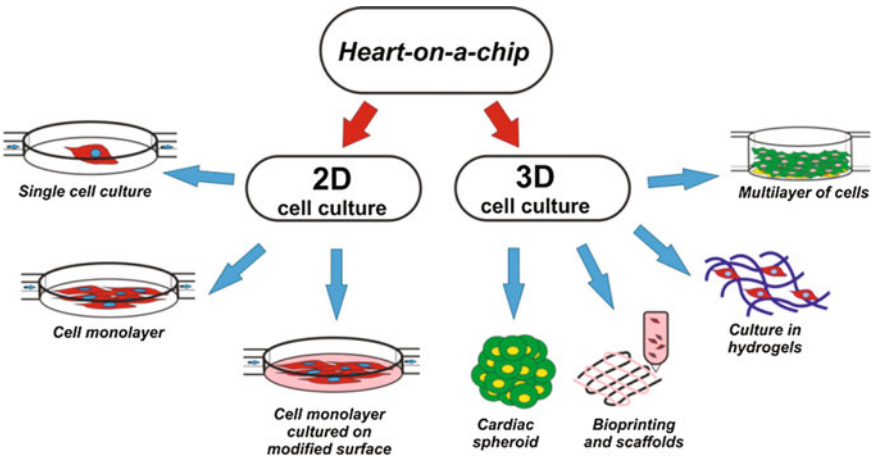

Fig. 8.2 Types of cardiac and vascular cell cultures performed in *Heart-on-a-chip* systems

(spheroid formation). There are many physical and chemical methods, which can be used to increase PDMS hydrophilicity (Zuchowska et al. 2016). PDMS surfaces utilized for CM cultures are often modified by coating them with proteins such as collagen type I, fibronectin, laminin, or gelatin (Ugolini et al. 2016). The addition of proteins to the culture environment mimics a natural extracellular matrix (ECM). Moreover, proteins have an impact on the spatial organization of the CMs, their shape, contractility, and transport of calcium ions (Boudou et al. 2012). The culture surface can also be modified by changing the surface topography. For this purpose, the microgrooves with controlled depth and width are formed. Additionally, nano- and microfibers are used to change properties of the surface for CM cultures (Simmson et al. 2012; Tomecka et al. 2017). Modifications of culture surfaces are mainly carried out to stimulate anisotropic orientation of cardiac cells. Because of this, the cells can be parallelly arranged as in the native environment.

As was mentioned in the previous chapters, there are significant differences between 2D and 3D cultures. Both of them have important benefits and disadvantages to use. 3D cultures are similar to the in vivo microenvironment, whereas 2D cell models enable fast and precisely controlled analysis. A single-cell culture is considered to be a 2D culture. There are not physical and biochemical intercellular interactions in such a culture model. Single-cell culture is free of tissue-specific intercellular connections, intercellular spaces and gaps with natural ECM. The natural structure and morphology of the cytoskeleton are also not preserved in a single cell culture. However, the simplicity of such cultures allows for quick analysis of cell functions. It is possible to measure the contraction of a single cell. Kaneko et al. (2007) presented an interesting solution for single-cell analysis (Fig. 8.3a). The microplatform was composed of a glass plate with three layers: chromium, collagen type I, and agarose. Single cardiomyocytes isolated from 13- to 14-day-old mouse embryos were trapped into each microchamber fabricated in the agarose layer. Next, a CM contraction after introduction of haloperidol was measured using a microscope and a video image recording method. The results indicated that the direct single-cell-based measurement enabled precise and quantitative control of cytotoxic effects of the tested drug. The device for trapping a single cardiac myocyte and the measurement of pH in the extracellular environment was also presented in the literature (Ges et al. 2005, 2008). To trap a single cell, the microsystem was equipped with an integrated system of microchannels and microvalves. Each trapped cell was cultured in the microsystem for 1–2 h. Next, the influence of chemical compounds on cell physiology was studied based on the formation of metabolites (pH changes). The microsystem was made of a glass plate covered with a thin layer of two pH-sensitive iridium oxide (IrOx) electrodes and PDMS layer containing microstructures (Fig. 8.3b). Wild-type (WT) single CMs isolated from a mouse were tested in the microsystem. The proposed microsystem can be applied to study ischemia, reperfusion injury, or disorders of other biological systems.

2D monolayer culture is the next model often used to investigate CMs in microscale. Tanaka et al. (2007) have started research based on 2D cardiac cell culture in the microsystems. A microplatform (bio-microactuator) was made of

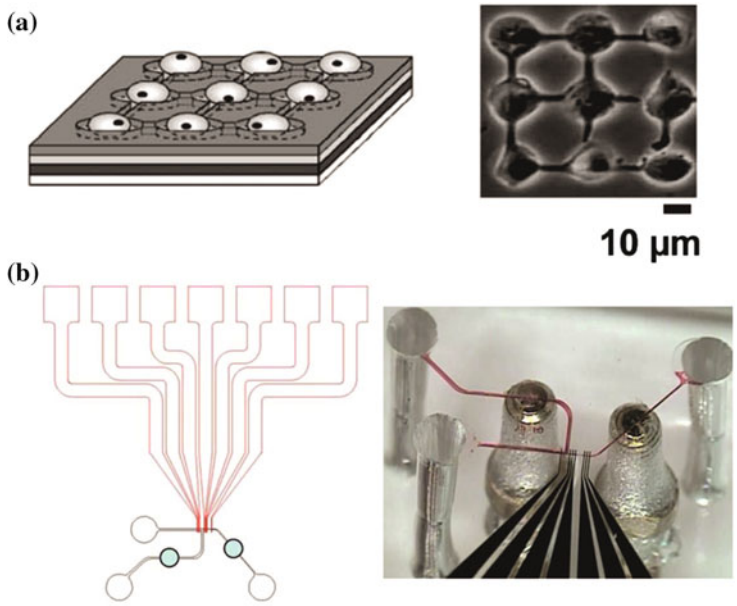

Fig. 8.3 Geometries of the microsystems for single cardiac cell analysis. **a** The microsystem for the single-cell-based cardiomyocyte culture with nine single microchambers (left) and microscopic image of the nine growing cardiomyocytes (right). Reprinted with permission from Kaneko et al. (2007). Copyright 2007 Royal Society of Chemistry. **b** A scheme of platinum microelectrode array and image of microchannel network in the microsystem. Reprinted with permission from Ges et al. (2008). Copyright 2007 Springer

PDMS using a replica molding technique. The microplatform consisted of a series of specific micropillars (10 µm high) designed for CM placement (Fig. 8.4a). Additionally, the PDMS surface was modified by fibronectin to enhance cell attachment to the micropillars. Primary neonatal rat CMs were cultured in the designed microplatform for 3 days. After that time, heart cell attachment to the micropillars and cell spontaneous beating was noticed. Cell contractions caused micropillar dilatation/deflection (1.4 Hz, ~3 µm displacement). The results showed that continuous cell-to-cell contacts and interactions indicated spontaneous and regular cardiac contraction without the use of any additional biochemical agents. The proposed bio-microactuator is a simplified type of an actuator, in which chemical energy generates mechanical energy (associated with cardiac muscle contractions).

To improve culture and beating of cardiomyocytes in the microsystems, the cells are exposed to additional factors. For example, methacrylated tropoelastin (MeTro) and methacrylated gelatin (GelMA) hydrogels were used for 2D CM cultures (Annabi et al. 2013). The cellular attachment, alignment, and beating of neonatal rat CMs cultured on the modified PDMS layers were compared. The obtained results indicated that PDMS coated with MeTro hydrogel had a high impact on cardiac cell

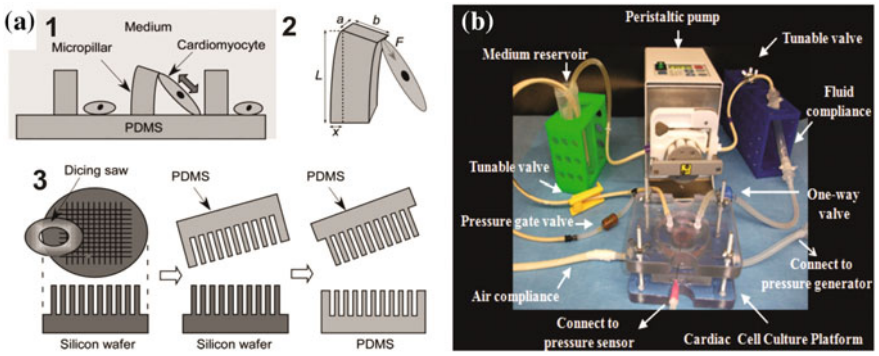

Fig. 8.4 **a** 1 PDMS micropillars in the microsystem for monolayer cardiomyocytes culture, 2 Scheme of cardiomyocyte adhesion to the micropillar. 3 A scheme of the micropillar fabrication. Reprinted with permission from Tanaka et al. (2007). Copyright 2006 Royal Society of Chemistry. **b** An experimental setup of 2D cardiac cell culture platform. Reprinted with permission from Nguyen et al. (2015). Open access

proliferation. Moreover, this method of surface modification could be useful for the culture of other cell types derived from the cardiovascular system (e.g., blood vessel cells).

The cells in heart tissue are exposed to dynamic flow conditions and stretching. Therefore, it is important to mimic these features in the *Heart-on-a-chip* systems. The microsystems, in which perfusion conditions are simulated, were often presented in the literature (Chen et al. 2017; Kobuszewska et al. 2017; Kujala et al. 2016; Nguyen et al. 2015). It results from the fact that flow conditions play a critical role in the early development and functional maturation of CMs. Moreover, the flow affects cell organization, intercellular interactions, and the transmission of chemical and physical signals (e.g., responsible for contraction) (Mannhardt et al. 2017). For example, Kobuszewska et al. (2017) studied how the geometry of a microsystem and microenvironmental conditions (static and perfusion) influence the proliferation, morphology, and alignment of rat cardiomyoblasts—H9C2 cells. Three different microsystems with a circular chamber, a longitudinal channel, and three parallel microchannels separated by two rows of micropillars were used in the experiments. It was found that perfusion conditions enhanced cell proliferation and induced parallel arrangement of the cells more than static conditions. Additionally, it was observed that the parallel orientation and elongation of the cells are dependent on microchamber geometry.

Stimulation of the cells using two different external agents enhanced CM proliferation. The usage perfusion conditions and mechanical stimulation resulted in increasing of cardiac gene expression (e.g., α-actin sarcomere, cardiac troponin T) and protein synthesis for calcium transport. For example, Nguyen et al. (2015) presented a fully automated platform for 2D cell culture of the embryonic ventricular chick CMs (Fig. 8.4b). The cells were cultured under perfusion conditions and cyclic mechanical stimulation. The obtained results showed that mechanical

stimulation of embryonic CMs is crucial to enhance cell proliferation and to create calcium transporting proteins, which are necessary in the process of cell contraction. It was investigated that mechanical conditions can be essential for the development of functional cardiac fragments (implants), which could replace damaged parts of the heart.

A muscular thin film (MTF) platform consisted of an anisotropic fragment of heart muscle cell tissue is the next interesting solution proposed as a functional cellular model (Grosberg et al. 2011). Such a 2D culture can compete with complex and advanced 3D models. Deformable elastic and flexible thin films made of PDMS, which are the component of MTF, were utilized to culture the neonatal rat ventricular myocytes. To enhance cell organization into an anisotropic form, thin PDMS layers were modified with ultraviolet (UV) light and fibronectin. The modification of culture surfaces can significantly increase cell viability and proliferation, and it can influence parallel cell orientation to each other. The CMs cultured on the designed microplatform were additionally electrical stimulated (square wave pulse, 5–20 V, 2 Hz, 10 ms duration). Thanks to the measurement of the curvature radius of MTF, the cell response to the external stimuli and cell contraction could be established.

Cocultures and multilayers are more advanced models of cardiac cell cultures, which mimic in vivo conditions better than monolayer cultures (Akins et al. 1999; Cheah et al. 2010; Horiguchi et al. 2009). CM cocultures with other types of heart cells allow enrichment of ECM with additional proteins and improvement of a 2D cell model (Gupta and Grande-Allen 2006; Liu et al. 2017). Cell coculture stimulates cell proliferation and increases the efficiency of cell signaling. Endothelial cells and cardiac fibroblasts are the cells, which are capable of producing complex ECM (Garzoni et al. 2009; Hussain et al. 2013; MacKenna et al. 2000; Saini et al. 2015). Additionally, because stem cells (SCs) play a very important role in regenerative medicine, these cells are also used in coculture with cardiac cells. They are utilized as a potential method to regenerate CMs (Garbern and Lee 2013; Ou et al. 2016). However, three-dimensional (3D) heart cultures are the most advanced cellular models used for heart research at the laboratory level. Spatial culture using heart tissue fragment (biopsy) can be performed in a microfluidic device (Cheah et al. 2010). The example of such a microsystem is shown in Fig. 8.5a. The microsystem consisted of a single flow chamber with a diameter of 7 mm. Right ventricular tissue from rat and right atrial tissue biopsies from patient were tested in the presented microdevice. The samples were placed in the microchamber, which was equipped with platinum electrodes for electrical stimulation. The presented microfluidic device was successfully used for real-time electrochemical monitoring of reactive oxygen species (ROS) release from a fragment of heart tissue. Additionally, cell damage was determined by measuring lactate dehydrogenase (LDH).

The scientists have also developed other methods for 3D cardiac cell cultures. 3D structures of CMs can be obtained by electrospinning, which uses the aligned biopolymer fibers as spatial scaffolds. Arrangement of CMs has been tested on nanofibers made of materials such as: poly(l-lactid-co-ε-caprolactone) [P(LLA-CL)]

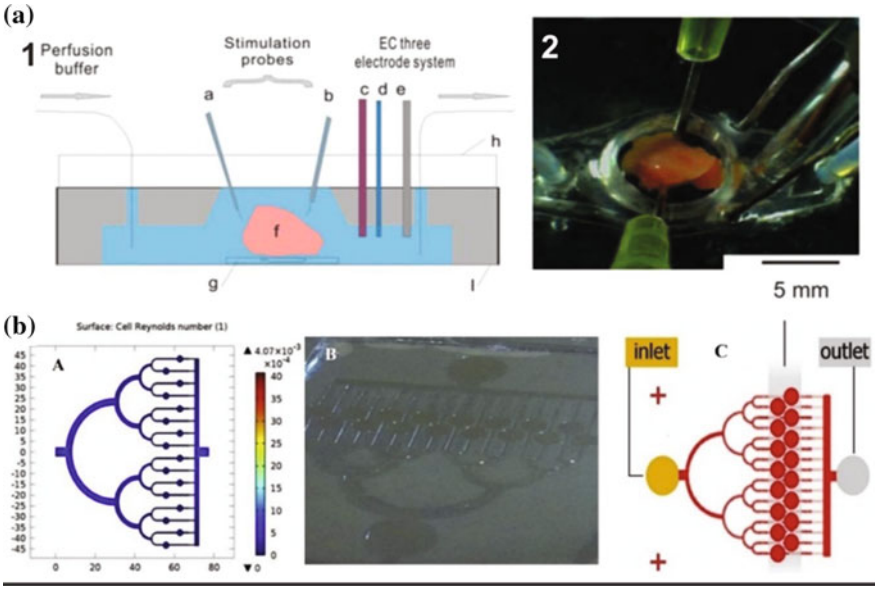

Fig. 8.5 **a** The microsystem for 3D tissue fragment analysis (a, b—stimulation electrodes, c, d, e—working, reference, and counted electrodes, f—holder, g—layer of PDMS, h—petri dish lid, i—petri dish). Reprinted with permission from Cheah et al. (2010). Copyright 2010 Royal Society of Chemistry. **b** The geometry, mold, and pattern of microsystem for cardiac cell culture in a hydrogel. Reprinted with permission from Ghiaseddin et al. (2017). Copyright 2017 Elsevier

copolymer, poly(lactide-co-glycolide) (PLGA), poly(ε-caprolactone) (PCL), poly (hydroxybutyrate) (PHB), chitosan–polycaprolactone, polymethylglutarimide (PMGI) (Mannhardt et al. 2017; Rogozhnikov et al. 2016; Tomecka et al. 2017; Visone et al. 2016). Multilayers without the use of scaffolds can also be used to obtain 3D cell cultures. For this purpose, thermo-sensitive polymers (e.g., poly (N-isopropylacrylamide) can be applied. They are disintegrated at a cell safe temperature, at the last stage of cell culture (Kikuchi and Okano 2005; Shimizu et al. 2002, 2003). A laser microablation is also used to create 3D structure in biodegradable polymers [e.g., poly(glycerol sebacate)], characterized by high porosity and elasticity (Simmons et al. 2012). Hydrogels, which can be gelled under the influence of various external factors (ultraviolet irradiation, temperature, chemical factors), are also utilized for creation of CM spatial arrangement. For example, 3D cultures with controlled size and architecture can be performed using, e.g., fibrin-based or collagen-based hydrogel matrix generated by soft lithography technique (Ghiassedin et al. 2017; Visone et al. 2016; Zhang et al. 2016c). A micro-bioreactor with a network of microchannels, in which cardiac cells with chitosan hydrogel were successfully cultured, is shown in Fig. 8.5b. A high density of mice cardiac progenitor cells (CPC) were loaded into the microchambers and cultured for 10 days. Based on this method, spatial forms of cardiac tissue were created. Marsano et al. (2016) as one of the first examined how the simultaneous

mechanical and biochemical stimulations affected the 3D culture of heart cells. A fibrin gel matrix was used to create 3D culture in a microsystem consisted of two PDMS layers with micropillars and a PDMS membrane. The micropillars in a top layer were used to create spatial models from both neonatal rat and human-induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM). A bottom layer with the micropillars and a PDMS membrane was used to induce homogeneous cyclic strains of 3D cell constructs. It was noted that the cyclic strain enhanced cardiac differentiation. High expression of cardiac markers such as cardiac troponin I and sarcomere α-actin was measured. Mechanical stimulation also influenced higher spontaneous cell beating. The proposed *Heart-on-a-chip* device was also used for evaluation of drug cytotoxicity.

The main challenge for the scientists is to develop an in vitro culture model, which has the ability to contract spontaneously. Additionally, heart cell models integrated with a microvessel network should be elaborated to mimic native heart tissue more. Such a network is responsible for proper distribution of oxygen and nutrients as well as removal of waste products (Gao et al. 2015). Bioprinting is a new technique used for tissue engineering. Compared with other tissue engineering approaches (e.g., the usage of scaffolds or hydrogels), bioprinting is an attractive method thanks to which fabrication of complex tissues also integrated with a vascular network is possible (Kolesky et al. 2016). A microsystem based on bioprinting can be a new approach in regenerative medicine, drug screening, and modeling of CVDs (Jia et al. 2016; Murphy and Antala 2014; Ozbolat 2015). There are a few bioprinting methods: bioprinting of cell-laden hydrogel 3D structures (Dhariwala et al. 2004), cell sheet lamination (Haraguchi et al. 2012), inkjet bioprinting (Boland et al. 2006), laser-assisted bioprinting (Yan et al. 2013), and extrusion-based bioprinting (Beyer et al. 2013). The materials used for bioprinting have specific properties. Functional inks based on piezo-resistive, high-conductance, and biocompatible soft materials (e.g., alginiane, gelatin methacryloyl) are most often used in this technology. Lind et al. (2016) presented an interesting microphysiological device, which was made using 3D bioprinting. To fabricate the microdevice, six inks with different properties were utilized. The sterilized and fibronectin-modified microsystem was used to culture neonatal rat ventricular myocytes (NRVMs) and human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs). The cells were exposed to the isoproterenol and verapamil as well as mechanical strain. The fabricated microsystem enabled the electrical measurement of cell functions and microscopic observation of the immunostained cells (i.e. sarcomere α-actin).

Zhang et al. (2016b) developed a microfluidic bioreactor with the network of blood vessels. Two PDMS layers created a main chamber with bioprinted scaffolds. The main chamber was additionally equipped with four micropillars, which were used to avoid scaffold movement during the flow of a culture medium. Alginate, gelatin methacryloyl (GelMA), photoinitiator Irgacure 2959 were the components of the bioink used for the bioprinting. Human umbilical vein endothelial cells (HUVECs) mixed with the bioink were bioprinted in the scaffolds. Next, CMs (neonatal rate CMs or hiPSC-CMs) were seeded on the created scaffolds. Finally,

cell contraction and high expression of cardiac markers (e.g. sarcomere α-actin, connexin-43) were observed. The bioprinting procedure scheme is shown in Fig. 8.6a. It was noticed that HUVECs migrated toward the peripheries of the microfibers and formed a layer of a confluent endotheliumin. Coculture with cardiac cells created a complex and technologically advanced 3D heart model. The developed microsystem based on bioprinting scaffolds can also be successfully used for screening the efficacy and toxicity of cardiovascular drugs.

Multicellular spheroids are the next known 3D models of cell culture. Spheroids exhibit a number of characteristic in vivo features such as: spatial physical and biochemical intercellular interactions, morphology of the cytoskeleton similar to in vivo morphology, the presence of ECM as well as nutrient and oxygen gradients (Hamilton 1988). Although spheroids are most often considered as tumor model, they are also used to spatial cardiac cell culture (Lee et al. 2013; Oliveira et al. 2013; Ota et al. 2010). Garzoni et al. (2009) presented a 3D spheroid coculture of murine embryos with endothelial cells or bone marrow-derived mesenchymal stroma cells (Fig. 8.6b). New approach for cardiac spheroid formation was also

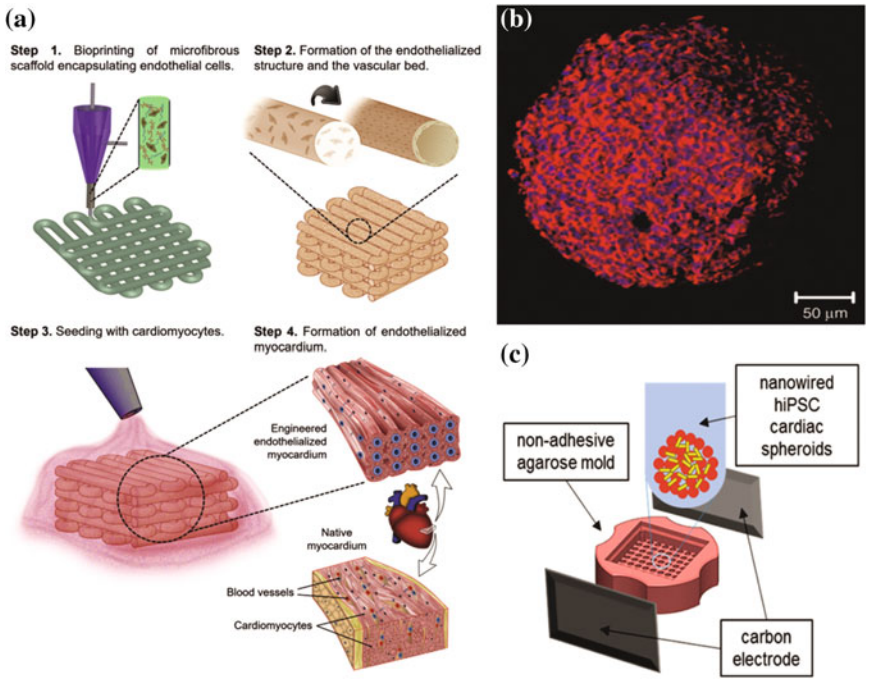

Fig. 8.6 **a** A scheme of a bioprinting procedure utilized to obtain 3D cardiac cell model. Reprinted with permission from Zhang et al. (2016b). Copyright 2016 Elsevier. **b** A 5-day-old cardiac spheroid stained with fluorescent dyes (DAPI and Evan's blue). Reprinted with permission from Garzoni et al. (2009). Copyright 2009 Elsevier. **c** The microsystem for cardiac spheroid culture with nanowires. Reprinted with permission from Richards et al. (2016). Copyright 2016 American Chemical Society

proposed by Richards et al. (2016). They developed a microsystem for spheroid culture of hiPSC-CMs with silicon nanowires (Fig. 8.6c). Electrical stimulation was used to form the intercellular connections and the spontaneous contraction of CMs. The formed cardiac spheroids were used as a 3D model for in vitro investigation of heart muscle contraction.

8.2 Toxicity Assays

The pharmaceutical industry spends a lot of money on implementing new drugs in the market. In addition, it is a long-term process (about 10–15 years). Nonetheless, many of the implemented drugs are removed from the market due to their side effects, which are very often associated with cardiotoxicity. Some of the nonsteroidal anti-inflammatory drugs (NSAID) (e.g., rofecoxib, cisapride, tegaserod) belong to this group of compounds (Mordwinkin et al. 2013). Preclinical drug development can be limited by high cardiotoxicity of compounds developed to treat CVDs and other diseases. Detecting the potential side effects of drug action can reduce the cost of drug production and implementation. Therefore, in vitro cardiac cell models have two main purposes: (1) evaluating cardiotoxicity of new and approved drugs used for the treatment of various diseases, (2) evaluating therapies and drugs used to treat CVDs (Fermini and Fossa 2003). Toxicological and pharmacological tests performed on cardiac models play an important role in the development of personalized medicine (Zhang et al. 2016d). In vitro studies are conducted to investigate both the degree of cardiotoxicity and the preliminary assessment of the drug dose, which is safe for the heart. There are many classes of drugs, including non-cardiovascular ones, that cause significant side effects to heart tissue (e.g., anthracyclines and other cytostatic antitumor drugs, some antipsychotic drugs, and NSAIDs). Anthracyclines (e.g., doxorubicin, daunorubicin, epirubicin) as well as mitoxantrone, dasatinib, imatinib, and trastuzumab are anticancer drugs, which exhibit high cardiotoxicity (Kim et al. 2011; Zhang et al. 2017). Antipsychotics (e.g., clozapine, droperidol, thioridazine) can also disturb heart functions. They can cause heart inflammation, delay of cellular depolarization or tachycardia (Nozaki et al. 2014). Based on in vivo and in vitro studies, it is also known that analgesic drugs (e.g., naproxen, diclofenac, celecoxib, ketoprofen, ibuprofen) may also increase the risk of CVDs (Force and Kolaja 2011).

So far, cardiotoxicity is most often studied based on conventional, macroscale tests (Esch et al. 2014). To test the effect of chemical compounds and drugs, the viability of the cells is evaluated. Additionally, expression of cardiac markers is analyzed. Because CMs have a specific feature (a spontaneous contraction), the influence of compounds on cell beating is also investigated. Parameters such as the level of calcium ions and ROS generation are also monitored in CMs after drug exposure (Cheah et al. 2010; Saric et al. 2016). An important goal of cellular engineering is the development of in vivo-like heart culture models, in which cardiac cell functions as well as cardiotoxicity of various drugs will be possible to

measure (Chan et al. 2013). There are several reports on *Heart-on-a-chip systems,* which have been reported to be promising tools for drug testing (Boudou et al. 2012; Kaneko et al. 2007; Sidorov et al. 2017) (see Table 8.1). Drugs used for CVD therapy, e.g., isoproterenol (nonselective β-adrenergic agonist used to treat bradycardia) and verapamil (calcium channel blocker with antiarrhythmic properties) have often been investigated in *Heart-on-a-chip systems* (Agarwal et al. 2013; Marsano et al. 2016; Tomecka et al. 2018). Agarwal et al. (2013) presented a microfluidic system based on muscular thin films (MTFs) as a new approach for cardiotoxicity measurements (Fig. 8.7a). The microsystem was integrated with a transparent polycarbonate layer for recording MTF deformation, aluminum heating elements, and platinum electrodes for electrical stimulation. Neonatal rat ventricular myocytes were cultured on a thin flexible PDMS film, which was microprinted with fibronectin. Cardiac contraction was investigated after isoproterenol exposure (1 nM–100 μM). It was observed that cell contraction is dependent on drug dosage and concentration. The proposed microplatform is a promising tool possible to use as a commercial platform for cardiotoxicity analysis of various drugs. Cytotoxicity assays performed on spatial cultures could have a high impact on development of heart research. Such approach has been presented by Marsano et al. (2016). They investigated cardiotoxicity of isoprotenerol on human cardiac microtissues (3D culture) in a *Heart-on-a-chip* system. Mechanical and electrical stimulations were used to mimic in vivo-like conditions during the experiments. An increase in the cardiac cell contraction rate was observed after cell exposure to isoproterenol (10^{-12}–10^{-6} M) and electrical stimulation (square pulse, a duration of 4 ms, a frequency of 1 Hz). A microscopic observation is most often used to measure cell viability in the microsystems. Therefore, electrical impedance spectroscopy was proposed as the noninvasive, real-time, and quantitative method to study cardiotoxicity in a microdevice (Zhang et al. 2016a). The device consisted of polystyrene chambers and nonconductive substrate with gold electrodes (Fig. 8.7b). Verapamil (in the range of 0–2 μM) as a drug used for CVD treatment and doxorubicin (in the range of 0–15 μM) as an anticancer drug were selected for the experiments. The viability and contractions of CMs isolated from neonatal rats after exposure to the tested compounds were studied. The results showed that impedance spectroscopy can be successfully used for real-time determination of drug cardiotoxicity in the microsystems.

As it was mentioned above, there are drugs, which cause significant side effects to heart tissue. Based on this, the *Heart-on-a-chip* systems applied for cardiotoxicity analysis of antipsychotic and anticancer drugs are more and more developed (Grosberg et al. 2011; Hansen et al. 2010; Kaneko et al. 2007). For example, Grosberg et al. (2011) used a static culture of neonatal rat ventricular myocytes to study the cardiotoxicity of epinephrine—a catecholamine neurotransmitter with properties that stimulate myocardial contractility. Kaneko et al. (2007) presented a microsystem for investigation of the cardiotoxicity of haloperidol (antipsychotic drug) on a single cardiac cell culture. It should be noted that a new microplatform based on engineered heart tissue (EHT) was also developed to test drug

Table 8.1 Toxicity assays based on *Heart-on-a-chip* systems

Type of cells	Type of culture	Culture conditions	Investigated drug	Drug properties	Assay(s)	References
Neonatal rat ventricular myocytes	Multicellular cell culture (3D)	Perfusion	Isoproterenol (1 nM–100 μM)	Nonselective beta-adrenergic agonist, used for treatment of heart disease (e.g., heart attack, congestive heart failure) Possible side effects: • tachycardia, • palpitations, • ventricular arrhythmias, • tachyarrhythmias	Optical analysis of contraction (conductivity measurements) and immunostaining	Agarwal et al. (2013)
Neonatal rat heart cells	Multicellular cell culture (3D)	Static	Chromanol 293 B (1–100 μM) Doxorubicin (0.1–1 μM)	Antiarrhythmic drug, blocker of the slow delayed rectifier K^+ current via potassium channels. Anticancer drug, anthracyclines antibiotic with cytostatic effect	Optical analysis of contraction and relaxation time	Hansen et al. (2010)
Neonatal rat ventricular myocytes	Multicellular cell culture (3D)	Static	Epinephrine (10^{-12}–10^{-4} M)	Sympathomimetic amina, hormone and neurotransmitter, drug for stimulating cardiac contraction. Used to treat anaphylaxis and cardiogenic shock	Measurement of cell contraction, and quantification of action potential propagation, immunostaining	Grosberg et al. (2011)
Embryonic mouse cardiomyoblast	Single cell	Static	Haloperidol (1 μM)	Antipsychotic drug Possible side effects: • ventricular arrhythmias • abnormal beating state of cardiac tissue	Measurement of beating rhythm with a video image recording method	Kaneko et al. (2007)

(continued)

Table 8.1 (continued)

Type of cells	Type of culture	Culture conditions	Investigated drug	Drug properties	Assay(s)	References
Neonatal rat cardiomyocytes	Multicellular cell culture (2D and 3D)	Perfusion	Isoprenaline (Isoproterenol) (10^{-12}–10^{-6} M)	Adrenoreceptors (β_1 and β_2) agonist, drug used for the treatment of bradycardia (slow heart rate), heart block, and rarely for asthma	Viability tests: fluorescent staining with calcein AM and ethidium homodimer-1. microscopic observation, measurement of beating rhythm with a video image recording method, immunostaining	Marsano et al. (2016)
Rabbit ventricular myocytes	Multicellular cell culture (3D)	Perfusion	FCCP	Protonophore (H^+ ionophore) and uncoupler of oxidative phosphorylation in mitochondria, inhibit a background K^+ current and induce a small inward current and induce a rise of intracellular Na^+, stimulates Mg^{2+}-ATPase activity; used to simulate heart hypoxia	Monitoring myocyte shortening and intracellular Ca^{2+} transients (using fluo-3 fluorescence)	Klauke et al. (2003)
Rat heart myocardium	Multicellular cell culture (3D)	Perfusion	FCCP	As above	Analysis of mitochondrial membrane potential and caspase-3 activity, fluorescent staining and microscopic observation	Ren et al. (2013)

2D two-dimensional, *3D* three-dimensional, *FCCP* cyanide-p-trifluoromethoxyphenylhydrazone

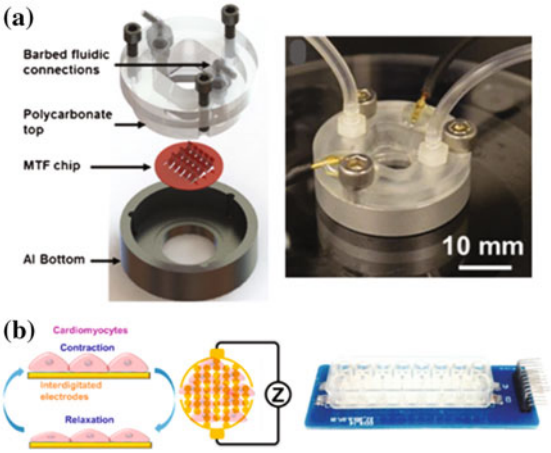

Fig. 8.7 **a** A scheme and photograph of the MTF (muscular thin films)-based microsystem. Reprinted with permission from Agarwal et al. (2013). Copyright 2013 Royal Society of Chemistry. **b** A scheme and a photograph of *Heart-on-a-chip* system with integrated electrodes for verapamil testing using impedance detection method. Reprinted with permission from Zhang et al. (2016a). Open access

cardiotoxicity (Hansen et al. 2010). The microplatform was used to study compounds such as: chromanol 293B (potassium channel blocker), quinidine and erythromycin (both used to treat arrhythmia), and doxorubicin (an anticancer drug). To create EHT-like culture model, neonatal rat heart cells were mixed with fibrinogen and Matrigel with thrombin, and they were cultured in the microdevice. Cell contraction was observed 8–10 days after cell seeding. Such cultures were exposed to the above listed drugs. Cell response (e.g., contraction) to the drugs was monitored using a video-optical technique. The authors confirmed that chromanol, quinidine, and erythromycin (depending on the concentration) increased the relaxation time, and doxorubicin decreased the contraction force. The developed microplatform can be useful for clinical pharmacology studies.

The Heart-on-a-chip systems can be used not only for analysis of drug cardiotoxicity, but also for mimicking CVDs. Heart diseases such as hypoxia, arrhythmia, ischemia, or myocardial infarction have been analyzed in the *Heart-on-a-chip* systems (Chen et al. 2017; Grosberg et al. 2011; Klauke et al. 2003; Ren et al. 2013; Sidorov et al. 2017). Ren et al. (2013) presented a PDMS microsystem for the dynamic study of hypoxia-induced myocardial injury in a controlled microenvironment. The microsystem consisted of three microchannels: central one used for culture and investigation of rat myocardium cells (H9C2) and two side microchannels for introduction of a culture medium and tested solutions (Fig. 8.8a). Hypoxia-induced myocardial injury was simulated using FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) (50 μM for 2 h). To study the hypoxic injury dynamics of H9C2 cells, the mitochondrial membrane potential and caspase-3 activity of the cells were analyzed. The usage of FCCP caused disintegration of the cytoskeleton and loss of mitochondrial membrane potential of the myocardium cells. It was noticed that the developed microsystem can be successfully used to mimic physiological and pathological conditions in heart tissue, and it has a high potential to study heart regeneration. The second microsystem, which can have also high impact on heart-based research, is a microsystem mimicking the

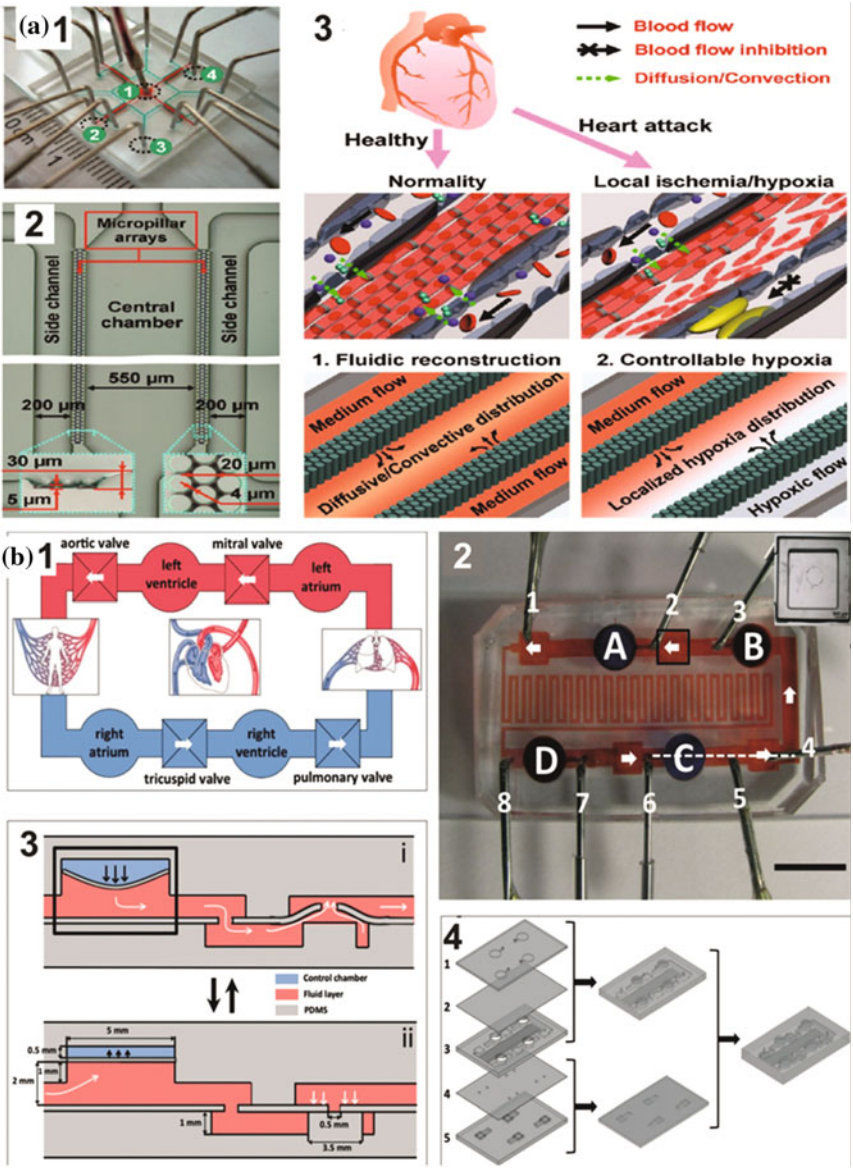

Fig. 8.8 **a 1**, **2** Geometry and photograph of the designed microsystem. **3** A scheme of processes performed in the microsystem for studying controllable myocardial hypoxia. Reprinted with permission from Ren et al. (2013). Copyright 2013 American Chemical Society. **b 1** Scheme of the microfluidic circulatory system. **2** A photograph of the fabricated microsystem. **3** A scheme of pumps and valves system. **4** The geometry of the microsystem and layers forming the microsystem. Reprinted with permission from Chen et al. (2017). Copyright 2017 Royal Society of Chemistry

function of a whole cardiovascular system (Chen et al. 2017). The designed microsystem was integrated with a cardiac-like, on-chip pumping system. It consisted of four pumps and valves, which imitated heart atriums, ventricles, and valves. It generated a one-way, circular flow of a culture medium (Fig. 8.8b). HUVECs were cultured in the microsystem, and their response to mechanical forces generated inside the microchannels was investigated. The fabricated microsystem can also be used as a microtool for modeling vascular diseases such as: bradycardia (a lower heart beat rate compared to healthy tissue) and hypotension (lower peak pressure compared to healthy tissue). The methods based on the pressure changing inside the microchannels were used to mimic these diseases in the microsystem.

8.3 Electrical Field

CMs in the native heart tissue form a complex network of neighboring and contacting cells. The pacemaker cells, which are 1% of cardiac cells, regulate the transmission of electrical pulses to the other cells of the heart. These cells are responsible for the generation of electrical pulses or action potentials that maintain electrical connectivity across the tissue (Dorn et al. 2015). Because electrical pulses are presented in the native heart tissue, such conditions should also be mimicked in vitro. For this purpose, an external electrical field is utilized. Electrical stimulation influences the rate, duration, and number of CM action potentials. It induces CM contraction and increases the number of spontaneously beating cells. Moreover, the electrical field affects the transport of calcium ions between the cells. Electrical stimulation of CMs was studied in both macro- and microscale (Barash et al. 2010; Maidhof et al. 2012; Zhang et al. 2013a; Vacek et al. 2011). Studies have shown that electrical stimulation influences CMs at the molecular level. It has influence on the formation of gaps and intercellular connections and the increase of the expression of cardiac markers. An electrical field was used to stimulate cell migration and orientation (parallel to each other) (Mannhardt et al. 2017; Shin et al. 2016).

Parameters such as electrical field value, signal type, frequency, pulse duration, and exposure time have to be optimized during electrical stimulation (Tandon et al. 2010, 2011). Biphasic square pulses, in the range of 1–20 V cm^{-1}, at a frequency of 1–10 Hz and duration impulse between 1 and 4 ms are parameters most often used for CM stimulation. There are two main methods utilized for generation of electrical field in the *Heart-on-a-chip* systems. The first is based on the placement an anode and a cathode in a culture medium. Thanks to this, a uniform electrical field is obtained (Ribas et al. 2016; Serena et al. 2009). Although this method can be easily integrated with the microsystems, it has a few disadvantages. For instance, a single cell is not well characterized during the stimulation and it is possible to generate a pH gradient in the culture microenvironment. The integration of the microsystems with planar electrodes and multi-unit electrode arrays (MEAs) is the next method used to generate electrical field (Ma et al. 2012; Natarajan et al. 2011;

Simmons et al. 2012; Yu et al. 2012). The example of MEA used for CM stimulation is shown in Fig. 8.9a (Natarajan et al. 2011). MEA allows electrical field to be precisely regulated. Thanks to the utilization of such microarrays, it is possible to stimulate a whole cell population as well as a single cell. MEAs can be used to generate a high value electrical field (0.1–10 V) on a small working surface (instead of using high absolute voltages). An important benefit of MEAs is that they allow both stimulation of the CMs and real-time recording of CMs exposure to various external factors (Werdich et al. 2004).

Electrodes made of various types of materials are utilized for cell stimulation in *Heart-on-a-chip* systems: e.g., stainless steel, carbon platinum, gold, indium tin oxide (ITO) (Table 8.2) (Jastrzebska et al. 2016; Serena et al. 2009; Tandon et al. 2010; Wei et al. 2011). Gold or platinum wires are most often integrated with the microsystems (Chen et al. 2009; Klauke et al. 2003). Stretchable and flexible electrodes made of a thin layer of gold are also used (Rogers et al. 2010). Spiral electrodes made of carbon nanotubes (Khang et al. 2008) and silver nanoparticles (Ahn et al. 2009) have also been reported as new types of electrodes integrated with the microsystems.

Different types of cell cultures (single-cell, monolayer, and 3D cultures) were stimulated with an electric field in the microsystems. The type of culture model has

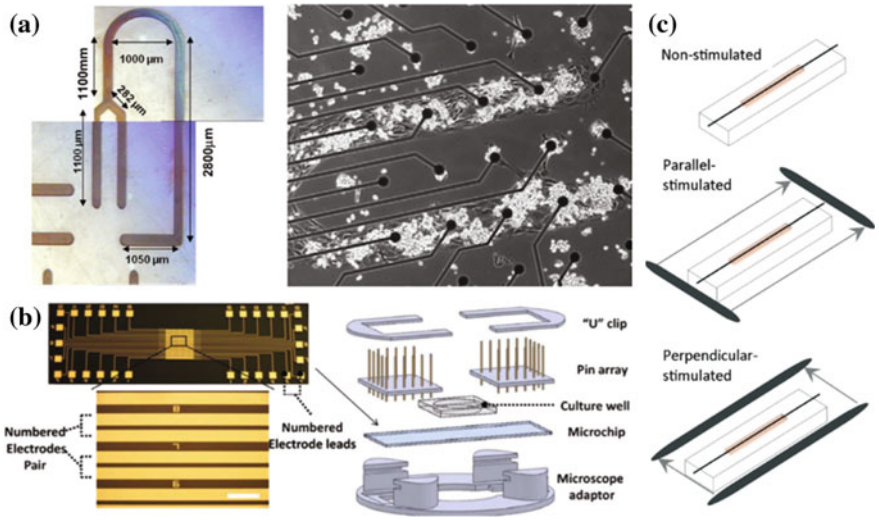

Fig. 8.9 Examples of the microsystems integrated with electrodes with different geometries. **a** A pattern design and dimensions (electrode distance 200 µm) on microelectrode arrays (MEA) for cardiomyocyte stimulation. Reprinted with permission from Natarajan et al. (2011). Copyright 2011 Elsevier. **b** Micropatterned interdigitated gold electrodes on a glass slide. Reprinted with permission from Zhang et al. (2013b). Copyright 2013 Royal Society of Chemistry. **c** Black carbon rods in the microsystem connected to an external stimulator. They provided either parallel or perpendicular electrical field stimulation on cardiac cells. Reprinted with permission from Xiao et al. (2014). Copyright 2013 Royal Society of Chemistry

Table 8.2 Electrical stimulation of the heart cells in the microsystems

Cell line	Electrode material	Electrode geometry	Work parameters	Culture type	References
Neonatal rat ventricular myocytes	Carbon graphite, titanium, stainless steel, titanium nitride-coated titanium	Two rods 4 cm in length, placed 1 cm apart	Amplitude: 1–6 V cm^{-1} Duration: 0.25–10 ms Frequency: 1, 3, 5 Hz	3D collagen scaffolds, static conditions	Tandon et al. (2011)
Neonatal rat cardiomyocytes	Gold	Two rods, placed 1 cm apart, oriented either parallel or perpendicular to the cultured cells	Amplitude: 1.15 V cm^{-1} Duration: 1 ms Frequency: 1 Hz	2D monolayer cell culture	Au et al. (2009)
Rat cardiac myocytes	Gold	Array of interdigitated microelectrodes (comb-like geometry)	Duration: 8 ms Frequency: 1 Hz	Single cells	Zhang et al. (2013b)
Neonatal rat cardiomyocytes	Carbon	Two parallel rods	Amplitude: 6 V cm^{-1} Duration: 1 ms Frequency: 0.2 Hz	3D multicellular culture	Boudou et al. (2012)
Neonatal rat ventricular myocytes	Platinum	Array of electrodes—microcombs	Bipolar square pulse: 10–15 V Duration: 10 ms Frequency: 2 Hz	3D multicellular culture	Agarwal et al. (2013)
Neonatal rat cardiomyocytes		Planar 10 μm electrodes, 200 μm separation, microelectrode arrays (MEA) type	Bipolar pulse: 500 mV Duration: 1 ms Frequency: 2 Hz	2D monolayer cell culture	Natarajan et al. (2011)
Neonatal rat cardiac cells	Carbon	Two integrated rods	Intensity: 74.4 mA cm^{-2} Duration: 2 ms Frequency: 1 Hz	3D multicellular culture	Barash et al. (2010)

(continued)

Table 8.2 (continued)

Cell line	Electrode material	Electrode geometry	Work parameters	Culture type	References
Neonatal rat cardiac cells	Carbon	Two parallel rods	Duration: 2 ms Frequency: 10 kHz	3D scaffolds	Maidhof et al. (2012)
Neonatal rat cardiomyocytes	Carbon	Two rods placed 2 cm apart	Amplitude: 3.5–4 V cm^{-1} Duration: 1 ms Frequency: 1.2 Hz	3D multicellular culture	Xiao et al. (2014)
Adult rabbit ventricular myocytes	Platinum	Two types of microelectrodes into the arrays: a pair of 20 μm wide and 1.5 mm long line electrodes (200 μm apart)	Amplitude: >50 V cm^{-1} Duration: 1–2 ms Frequency: 1 Hz	Single cells	Cheng et al. (2010)

an influence on the phenotype and functions of CMs. Several reports based on 2D cultures have been presented in the literature (Agarwal et al. 2013; Au et al. 2009; Cheng et al. 2010; Natarajan et al. 2011). Single adult rabbit ventricular myocytes isolated from the left ventricle were cultured and stimulated in a microsystem consisting of 15 microwells (Cheng et al. 2010). Each microwell was integrated with a set of five microelectrodes (two Pt stimulating electrodes, a Pt working electrode, a Pt counter electrode, and an Ag/AgCl reference electrode). Au et al. (2009) developed a microfluidic platform for CM culture and their electrical stimulation. The microsystem made of polystyrene (PS) was used for investigation of neonatal rat CMs. The microsystem consisted of microgrooves and microridges with a precisely defined depth (400 nm), width (0.5 or 3 μm wide grooves and 0.5 or 1 μm wide ridges), and periodicity (1 and 4 μm). Smooth polystyrene surfaces were used as control samples. The designed microsystem was integrated with two gold electrodes. The electrodes were oriented in such a way that they were placed either parallelly or perpendicularly to the microgrooves. Such an electrode arrangement enabled the investigation of the dependence between topographical factor and electrical signal. The cells were treated with symmetric biphasic pulses (electrical field of 1.15 V cm^{-1}, a frequency of 1 Hz, a duration of 1 ms) for 7 days. Sarcomere α-actin staining showed that such stimulation caused elongation and alignment of the cells along the microgrooves. It was also observed that cellular orientation was greatly determined by the topographical signals. Electrical field stimulation further enhanced cardiomyocyte elongation, when microgrooves were oriented parallelly to electrical field. The obtained results showed that the presented microsystem can be a useful tool for drug development (tests for verapamil were also performed). Zhang et al. (2013b) presented a microsystem used for electrical stimulation of adult cardiac myocytes (Fig. 8.9b). The developed microsystem was used to study the intercellular electromechanical transduction by measuring the contractile performance of the stimulated and non-stimulated cells. The microsystem was integrated with micropatterned gold electrodes (a width of 200 μm). CMs isolated from the left heart ventricle were exposed to an electric voltage pulse with a frequency of 1 Hz and a duration of 8 ms. The measurements were performed on the cells, which were placed between the two neighboring electrodes. Cell contraction was determined by measuring the change of cell length. Researches demonstrated that the presented microsystem is useful in studying the efficiency of gap junctions in adult cardiac myocytes.

Electrical field can also induce synchronous contractions in spatial cell cultures (Radisic et al. 2004). 3D cultures performed in the microsystems and application of electrical field are recognized as a useful method in regenerative medicine. The *Heart-on-a-chip* systems based on a 3D culture are utilized for investigation of electrical stimulation more and more (Barash et al. 2010; Boudou et al. 2012; Hirt et al. 2014; Lind et al. 2016; Schroer et al. 2017; Xiao et al. 2014). Boudou et al. (2012) developed an interesting microplatform for measurement and manipulation of 3D cardiac cell models using carbon electrodes. Cardiomyocytes isolated from neonatal rat, cultured as cardiac microtissues (CMTs) in collagen and fibrin 3D matrices, were used in the experiments. Two parallel carbon electrodes placed on

both sides of the microplatform were used for cell stimulation. The CMTs were stimulated using biphasic pulses (6 V cm^{-1}, 0.2 Hz, 1 ms). The effect of electrical stimulation on cell alignment and force generation within CMTs was investigated in this study. It was noticed that electrical stimulation improves both the structure and the function of CMTs. It is also important that the developed microplatform can become a potential microtool for monitoring the action of drugs on electrically stimulated 3D cardiac cell models.

The influence of electrical field, and dependence of electrodes arrangement, was investigated in 3D micro-tissue biowires (Xiao et al. 2014). The biowires integrated with carbon rod electrodes were utilized for investigation of primary neonatal rat CMs and human embryonic stem cell-derived CMs (hESC-CMs). Different electrical stimulation (biphasic, rectangular, 1 ms duration, 1.2 Hz, 3.5–4 V cm^{-1}, for 4 days) conditions were applied dependent on a cell type. Rat cardiac biowires were stimulated using both the parallel stimulation chambers (two carbon rods placed 2 cm apart and perpendicular to the biowires) and the perpendicular stimulation chambers (two carbon rods placed 1 cm apart and parallel with the biowires) (Fig. 8.9c). Cell function was analyzed based on the immunostaining of cardiac Troponin T and connexin-43 as well as the mechanical properties of the cells. The obtained results showed that the proposed microdevice can be successfully utilized for investigation of the influence of electrical stimulation on cell functions.

Understanding the role of electrical stimulation in cell metabolic pathways is important in the field of heart tissue engineering. Although many reports based on electrical stimulation of CMs in the microsystems have been presented in the literature, there are still research areas, which have to be deeply studied: e.g., imitation of fully functioning heart tissue, mimicking of CVDs, and regeneration of the CMs based on electric fields (Schroer et al. 2017).

8.4 Monitoring CM Functions

Developing a microsystem especially for culture and investigation of heart cells is associated with selection of methods for analysis of cell functions (Fig. 8.10). Based on the features, which characterize heart cells (especially CMs) parameters such as the expression of cardiac markers, cell contraction/beating, and the level of calcium ions, are often determined in the *Heart-on-a-chip* systems. Besides the above-mentioned assays, cell proliferation, cell viability as well as cell morphology are studied (Mordwinkin et al. 2013). To monitor the CM functions listed above, various instrumental (electrochemical and optical) techniques are utilized.

The examples of *Heart-on-a-chip* systems described in previous sections showed that they are appropriate tools to perform both heart cell cultures and cardiotoxicity studies. Analysis of growth and proliferation of the CMs are mainly studied in the microsystems used for cell culture, toxicity assays, and electrical stimulation. The proliferation and viability of the cells can be determined by differential staining with fluorescent dyes (e.g., Calcein AM and Propidium iodide and

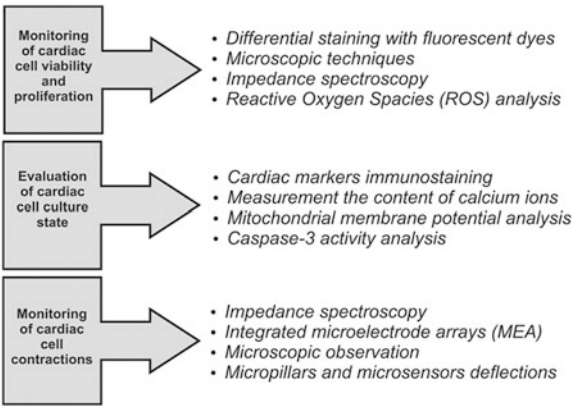

Fig. 8.10 Most common methods used for monitoring cardiac cell viability, proliferation, and contractions in the microfluidic systems

Ethidium homodimer-1) and microscopic observations (e.g., confocal microscopy, optical and fluorescence microscopy) (Ghiaseddin et al. 2017; Marsano et al. 2016; Ren et al. 2013). Besides that, cell proliferation and CM maturation after an external stimulus can be determined by analyzing the expression of cardiac markers. Expression of typical cardiac markers and expression of structural proteins such as sarcomere α-actin, cardiac troponin I, cardiac troponin T, connexin-43, myosin heavy chain 6 (MYH6), and myosin light chain 2a are the most often monitored in heart cellular models (Belaguli et al. 2000; Serena et al. 2009; Tandon et al. 2010). These markers are determined by immunofluorescent staining with a fluorochrome (most often Alexa Fluor family). Grosberg et al. (2011) evaluated cell structure by immunofluorescent staining with Alexa Fluor 488-conjugated Phalloidin (F-actin, green dye) and Alexa Fluor 594-conjugated clone EA-53 (sarcomeric α-actin, red dye). Agarwal et al. (2013) proposed similar immunostaining for analyzing the formation of the anisotropic monolayer of CMs. They used immunostaining with Alexa Fluor 633-conjugated Phalloidin (F-actin, red dye) and Alexa Fluor 546-conjugated clone EA-53 (sarcomeric α-actin red dye). To determine the expression of cardiac markers, similar parameters were used by other research groups.

Cyclic voltammetry can also be utilized to determine viability and proliferation of cardiac cells cultured in the microsystems (Cheah et al. 2010). Such a technique allows the amount of reactive oxygen species to be measured in real-time. The calcium ion level in the cells was used for monitoring cardiac cell viability and proliferation. For this purpose, fluorescent dyes can be used (fluo-3 or fluo-4) (Klauke et al. 2003). The amount of calcium ions can be evaluated for normal (healthy) as well as hypoxia-induced cardiac hypertrophy (disease) conditions. Additionally, the mitochondrial membrane potential (using JC-1 indicator) and caspase-3 activity of the cells can be analyzed after an external stimulus. These parameters are used to evaluate culture states (He et al. 2014; Ren et al. 2013). Transmission electron microscopy is also applied to analyze micro-tissue cell models. Such a technique is used, e.g., to determine the morphology and pore

structure of the prepared hydrogels placed in the microsystems (Ghiaseddin et al. 2017). Cell morphology and arrangement are monitored using microscopy techniques. Typical cardiomyocyte structural properties such as glycogen granules, mitochondria, myofibrils, sarcoplasmic reticulum striated sarcomeres, A-bands, I-bands, and Z-lines can be successfully determined using microscopic techniques (Mordwinkin et al. 2013).

In the recent years, impedance spectroscopy (IS) is intensively developing technology for analysis of cell proliferation in the microfluidic systems (Zhang et al. 2016a). IS is a noninvasive electrochemical method, which can be used for real-time monitoring of cell proliferation, viability as well as contraction (Dean et al. 2007; Min et al. 2003; Qiu et al. 2008, 2009). Contraction is the most specific feature of CMs. This parameter is determined using techniques such as: microscopic observation, impedance spectroscopy and MEA techniques, micropillar and microsensor deflections (Marsano et al. 2016; Natarajan et al. 2011; Tanaka et al. 2007). Many of analytical techniques, applied to monitor CM functions in the microsystems, are based on qualitative analysis. Such methods are especially used for spatial cell cultures. Therefore, it is important to elaborate quantitative, repeatable methods, which can be validated and implemented not only for 2D but also for 3D cell cultures in the *Heart-on-a-chip* systems.

8.5 Summary and Perspectives

The *Heart-on-a-chip* systems are used to create in vivo-like culture models. They provide new possibilities in many biological and preclinical studies. Properly designed microstructures of the microsystems provide an opportunity to perform rapid drug screening and analysis of the effects of external stimulation. Thanks to this, new mechanisms and cardiac cell functions can be discovered and can consequently be useful in regenerative medicine. Many examples of heart culture models have been presented in the literature. There are *Heart-on-a-chip* systems for single, monolayer, and spatial cell cultures, in which the cardiotoxicity of different drug groups as well as the influence of external stimulation on cardiac cell cultures were studied.

Because heart cells have specific features (dynamic conditions, stretching and electrical impulses), the *Heart-on-a-chip* systems have to be equipped with the elements, which ensure such properties. This brings many challenges during the development of the microsystems for cardiac cell culture. The most important problem is the origin of the beating cardiomyocytes. To obtain such cells, some embryo and neonatal specimens are utilized. Therefore, cells coming from animals are the most often used. Human cardiac cells from adults are also investigated; however, they are often characterized by the lack of the beating. Therefore, beating stem cell-derived cardiomyocytes (SC-CMs) have been increasingly cultured. Although a number of the microsystems for heart cell cultures (e.g., for single, 2D, and 3D cell cultures) is developed, they present not fully functioning heart model.

The research, in which these cardiac models are used, still is in the early stage. However, the combination of different solutions, the microsystems and the culture methods, proposed by many research groups and described in this chapter, could improve investigation based on the *Heart-on-a-chip* systems and could implement such microsystems in a personalized medicine in the future. Some aspects should still be investigated and improved in the microsystems proposed so far. The combination of a whole vascular system with a 3D beating heart cellular model and study physiological and pathological conditions in such a fully mimicked cardiovascular system is a perspective for *Heart-on-a-chip* system developing. The usage of a spatial and vascular model for a detailed investigation of CM regeneration is also an important step which should be developed. So far, microscopic observations are mainly used to evaluate the state of 3D culture in the microsystems. Therefore, the elaboration of quantitative microfluidic methods for examination of biochemical processes in cardiovascular system is strongly important. This approach for *Heart-on-a-chip* study can be based on the usage of a perfusion microsystem enriched with additional structural elements for cellular spatial culture and components for automated analytical measurements (e.g., electrodes, integration with commercially available equipment). Moreover, the usage of a digitally controlled module can allow automated dosage of all fluids, and finally it can increase usefulness of such a microsystem in a personalized medicine.

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Chapter 9
Cardiac Cell Culture Microtechnologies Based on Stem Cells

Anna Kobuszewska, Patrycja Sokolowska and Elzbieta Jastrzebska

9.1 Stem Cell Differentiation in *Lab-on-a-chip* Systems

Regenerative medicine is an alternative method, which can be used to treat cardiovascular diseases (CVDs). Stem cells (SCs) are the main source of biological material used in such therapy. This is because these cells can differentiate into a specific tissue, depending on cell origin and pluripotency (see Chap. 6). Precise control of SC fate is a major challenge to scientists. Stem cell differentiation into cardiomyocytes (CMs) may contribute to the development of an in vitro cardiac model and new methods for the treatment of heart disease. So far, promising results in SC usage for heart regeneration were obtained (Segers et al. 2008). Numerous types of SCs are being investigated nowadays, e.g. bone marrow-derived/circulating progenitor cells (BMP-CSs), mesenchymal stem cells (MSCs), adipose tissue-derived stem cells (ATSCs), endothelial progenitor cells (EPCs), embryonic stem cells (ESCs)-induced pluripotent stem cells (iPSCs) and cardiac stem cells (CSCs) (Emmert et al. 2014; Kawamura et al. 2012). Although many reports based on stem cells have been presented, there are many important issues, which have to be considered (e.g. source of the SCs, low reproducibility and throughput, controllable differentiation) (Discher et al. 2009; Silvestre and Menasché 2015; Ye et al. 2011).

Microscale technologies can provide tools, which allow for improving SC investigation. There are various factors utilized for the determination of SC fate on microscale: biochemical factors (growth factors, vitamins, cytokines, small molecules), physical factors (structure and elasticity of biomaterials, electrical and magnetic fields, thermal gradients), mechanical factors (fluidic shear stress,

A. Kobuszewska · P. Sokolowska · E. Jastrzebska (✉)
The Chair of Medical Biotechnology, Faculty of Chemistry,
Warsaw University of Technology, Warsaw, Poland
e-mail: ejastrzebska@ch.pw.edu.pl

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mechanical strain), cell–cell interactions (co-culture) and cell-biomaterial interactions (Guilak et al. 2009; Higuchi et al. 2017; Hwang et al. 2008; Tzatzalos et al. 2016). The *Lab-on-a-chip* systems are powerful tools, which can complete existing laboratory techniques used for SC differentiation studies. The advantages of the *Lab-on-a-chip* systems were characterized in previous chapters; therefore, here we will focus on benefits which are especially related to SC culture and analysis. First of all, a specific niche with a controllable microenvironment can be created in the microsystems (Duinen et al. 2015; Park et al. 2010). Because of this, SCs can be cultured in conditions close to in vivo and is suitable for cell differentiation. The microsystems made of poly(dimethylsiloxane) (PDMS) are most often fabricated for SC culture and differentiation (Ertl et al. 2014). However, depending on the differentiation method such microsystems are integrated with various components for improving SC operating. For example, other materials used as differentiation factors (nanofibers, microgrooves, hydrogels) are integrated into the microsystems (Higuchi et al. 2017; Tomecka et al. 2017). Hydrogels play an increasingly important role in creating the spatial cell arrangement (three-dimensional, 3D) (Perez et al. 2016). The extracellular matrix (ECM) provided by hydrogels can also influence SC differentiation. These materials determine cell attachment and alignment as well as provide physiologically relevant stiffness, which can influence SC differentiation (Mathur et al. 2016). Various studies with hydrogels based on CMs and SCs have been presented in the literature, e.g. analysis of cell contraction forces (Zimmermann et al. 2006), drug cytotoxicity (Schaafm et al. 2011) and heart disease simulation (Hinson et al. 2015). The combination of both microfluidic systems and hydrogels could be a new approach for SC study; however, it is still a significant challenge for researchers.

The microsystems are also integrated with micropumps, microvalves and electrodes (Zhang et al. 2007). This allows various differential conditions to be created (Zhang and Austin 2012). Thanks to this, defined shear stress conditions, a stable electrical force and precise control of biochemical factor delivery to the SCs are possible to be obtained in microscale (Pavesi et al. 2015; Stoppel et al. 2016). For instance, microarrays have been fabricated for the investigation of protein-protein interaction and to define the properties of the SC microenvironment (Lutolf and Blau 2009). Diffusion of biochemical growth factors of the micropatterned cells and their differentiation have also been investigated (Peerani et al. 2007). The microsystems have also been used for precise control of single cell or high-throughput analysis. It is important to mention the possibility of minimizing the amount of the cells, culture medium and chemical reagent in the experiments performed in the microsystems. It is especially important for the studies performed with SCs.

Differentiation methods investigated in *Lab-on-a-chip* systems and the fate of SCs are shown in Fig. 9.1. SC differentiation into neurons, granular cells (kidney), osteoblasts, hepatocytes, endothelial cells, adipocytes as well as cardiomyocytes have been reported (Figallo et al. 2007; Jeon et al. 2014; Ju et al. 2008; Kim et al. 2014; Ni et al. 2008; Pavesi et al. 2015; Wang et al. 2014; Villa-Diaz et al. 2009; Yang et al. 2015, 2017).

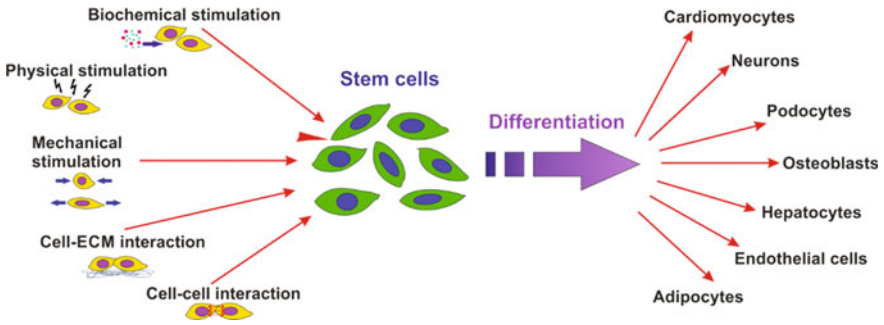

Fig. 9.1 Methods of SC stimulations, which can be used to obtain specific cell lineages in *Lab-on-a-chip* systems

Because SCs can potentially be useful for heart regeneration, SC differentiation into cardiomyocytes have been investigated more and more in the microsystems. In recent years, a few review papers about *Heart-on-a-chip* based on stem cells have been published (Ghafar-Zadeh et al. 2011; Jastrzebska et al. 2016; Yang and Ma 2012). However, the use of SCs for *Heart-on-a-chip* is still in the initial phase. SCs are often differentiated in macroscale and afterwards they are seeded in the microsystems and cultured under flow conditions. To differentiate SCs into cardiac cells, three main methods are used: biochemical, mechanical and physical stimulation. The effect of SC differentiation into CMs is investigated by expression studies of specific cardiac cell markers. For this purpose, immunofluorescence staining of the cytoskeletal proteins is performed. Cardiac troponin T (cTnT), α-sarcomeric actin, connexix43, β-myosin heavy chain (β-MHC), Nkx2 are the most common cardiac-specific markers (Qureshi et al. 2012). Specific cardiac gene expressions of, e.g. α-MHC, ANF (atrial natriuretic factor), BNP (B-type natriuretic peptide), MYL2 (myosin light chain 2), MYL7, MYH6 (myosin heavy chain 6), MYH7 are also defined using a polymerase chain reaction (PCR) (Chen et al. 2009; Pavesi et al. 2015). Additionally, the induced stem cell-derived cardiomyocytes (SC-CMs) could contract. Therefore, the amplitude of cell contraction is measured to identify SC differentiation.

A review of the recent literature shows that microscale technologies enable the creation of in vivo-like models of SCs. The *Lab-on-a-chip* systems have been successfully used for SC culture, SC differentiation, cytotoxicity assays as well as investigation of heart regenerationRegeneration using SCs. Examples and detailed characterization of these microsystems are presented in the next sections of this chapter.

9.2 Biochemical Stimulation

Soluble chemical factors are used for SC differentiation into specific cell types. Such factors are introduced to the cells by their supplementation in a culture medium and incubation with the cell over a period of time. Biochemical factors can influence the fate of the cell, e.g.: cell viability, proliferation, self-renewal and differentiation. Factors such as bone morphogenic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), the Wingless/INT proteins (WNTs) have been used to differentiate SCs into CMs (Dimarakis et al. 2006; Uzel et al. 2014). The fibroblast growth factor (FGF) is also commonly used. The FGF family consists of a large and diverse group of small polypeptide growth factors, which play a crucial role in cardiac differentiation (Kawai et al. 2004; Kofidis et al. 2004; Rosenblatt-Velin et al. 2005). Additionally, the transforming growth factor beta (TGFβ) and 5-azacytidine (5-AZA) have been reported as factors utilized for cardiac SC stimulation (Cheng et al. 2016; Tabar and Studer 2014). The heart is capable of three TGFβ isoform expressions which exhibit a specific function in cardiac differentiation (Jeon et al. 2014; Kumar and Sun 2005). Kumar and Sun investigated the effect of TGFβ isoforms on ESC differentiation. Significant cardiac differentiation and an increase in beating cells were observed for ESC incubation only with TGF-β2 isoform. Most of the presented factors were investigated in the microfluidic systems. The biochemical methods used for SC differentiation into CMs in *Lab-on-a-chip* systems are summarized in Table 9.1.

The *Lab-on-a-chip* systems can be used for SC stimulation using controlled dosage (concentration, exposition time) of the biochemical factors. Both two-dimensional (2D) and three-dimensional (3D) culture models of SCs have been presented in the literature (Goβmann et al. 2016; Moya et al. 2013; Wan et al. 2011). However, cardiomyocytes derived from SCs have been investigated more often than SC differentiation. An interesting solution was presented by Goβmann et al. (2016). They developed a new device/method, called Cell Drum technology, for mechanical tension analysis of the cells (Fig. 9.2a). Each Cell Drum well contains an ultra-thin (ca. 3.0 μm) circular membrane made of PDMS. Human-induced pluripotent stem cells-derived cardiomyocytes (hiPS-CMs) were obtained (in macroscale) from human skin fibroblasts differentiated by the usage of Yamanaka factors (Oct4, Sox2, cMyc, Klf4) (Takahashi et al. 2007). Both a monolayer and a thin tissue culture were obtained on Cell Drum membrane. Next, the cells regular beating induced membrane deflection, which was monitored using a pressure sensor. The obtained culture models of hiPS-CMs were used for investigation of selective agonists and antagonists of both calcium (S-Bay K8644 and verapamil) and potassium (veratridine and lidocaine) channels. It was found that the beating cell imitated in vivo human heart tissue. Additionally, the tested drugs influenced cell contraction. The developed heart mimicking system can be used for easy and fast pharmacological analysis of cardiac drugs. It should be noted that cardiotoxicity assays are based on cell contraction and the main feature of heart

Table 9.1 Biochemical stimulations

Cells	Biochemical factor	Type of culture	Conditions	Effect	Cardiac marker	References
Human iPSC	Activin A, BMP-4	3D	EBs, perfusion	Vascularized cardiac microtissue	Cardiac troponin T (cTnT)	Moya et al. (2013)
Mouse ESC	BMP-2	2D, 3D	EBs, perfusion	Cardiac differentiation	α-myosin heavy chain (α-MHC)	Wan et al. (2011)
BM-hMSC	VEGF, Ang-1, TGF-β1	3D	Co-culture with ECs	Human vascular network	α-smooth muscle actin (α-SMA)	Jeon et al. (2014)
Human iPSCs	Yamanaka factors	2D	Static	Cardiac differentiation	α-MHC	Goβmann et al. (2016)
Human PSC	ROCK inhibitor Y-27632	3D	EBs	Cardiac microwires (CMWs)	ANF, BNP, MYL2, MYL7, MYH6, MYH7	Thavandiran et al. (2013)
Human iPSC	B27 minus insulin supplement, CHIR99021 WNT inhibitor	3D	Perfusion	Cardiac differentiation	sarcomeric α-actin, DAPI	Mathur et al. (2015)
Human ESC	BMP-4, bFGF, VEGF, IWP-2	3D	Perfusion	Cardiac differentiation	sarcomeric α-actin, cTnT	Xiao et al. (2014)
Human iPSC	WNT inhibitors: Y-27632, CHIR99021, IWP-2, B27 minus insulin	3D	CBs, perfusion	Cardiac differentiation	cTnT, sarcomeric α-actin, α-MHC	Bergström et al. (2015)
ESC	Mixture of 50% EB and 50% EGM-2 medium	3D	EBs, perfusion	Endothelial cells differentiation	PECAM	Lee et al. (2011a, b)

EBs Embryoid bodies, *BMP* Bone morphogenic protein, *Ang-1* Angiopoietin 1, *VEGF* Vascular endothelial growth factor, *TGF-β1* Transforming growth factor β1, *bFGF* Basic fibroblast growth factor, *EGM-2* Endothelial growth medium-2, *PECAM* Anti-platelet endothelial cell adhesion molecule, *α-MHC* α-myosin heavy chain, *IWP-2* Inhibitor of Wnt production-2, *WNT* Signalling pathways, *2D* Two-dimensional, *3D* Three-dimensional, *ANF* Atrial natriuretic factor, *BNP* B-type natriuretic peptide, *MYL2* Myosin light chain 2, *MYL7* Myosin light chain 7, *MYH6* Myosin heavy chain 6, *MYH7* Myosin heavy chain 7

cells. Therefore, the utilization of the Cell Drum technology could have a high impact on heart regeneration research.

Microwell/systems technologies are increasingly used to culture and differentiate ESCs (Lee et al. 2011a, b; Mohr et al. 2006; Wan et al. 2011; Xiao et al. 2014). These cells have a specific property, i.e. they are able to create 3D structures called embryoid bodies (EBs). Conventional methods such as dissociated suspension, a methyl cellulose culture, a hanging drop culture, a spinner flask and a bioreactor culture are still often used to obtain EBs as the first step in ESC differentiation (Moya et al. 2013; Thavandiran et al. 2013). EBs can spontaneously differentiate into various cell types. Therefore, the culture medium should be supplemented with factors (e.g. LIF—leukaemia inhibitory factor), which maintain the undifferentiated cell form. These factors should be removed, and differential biochemical agents should be added in the culture medium to differentiate EBs into a specific cell type. It is also important that the size and shape of EBs influence the fate of the cells (Hwang et al. 2008). The utilization of the microtechnology gives the possibility to precisely EB size manipulation. EB dimensions are determined by the geometry and size of the microwells. A microsystem with ca. 200 microwells was proposed as a new and a promising method for creation of EBs (Miyamoto et al. 2016). It was investigated that how different densities of the SCs influence EBs formation. Different EB fates were noticed depending on the initial size of EBs. Large EBs were differentiated into hepatic and cardiac cells, whereas small EBs promoted vascular differentiation. The proposed microplatform could be used as high-throughput technique for EBs formation and SC fate study.

A controllable maintenance of undifferentiated EBs and a precise control of their fate is a big challenge for the scientists. The ESC culture method proposed by Mohr et al. (2006) should be underlined. They presented a microwell system for 3D long-term human ESC culture and EB formation. It was proven that microwell systems allowed undifferentiated ESCs to be obtained for several weeks. Thanks to this, such methods can increase the effectiveness and the reproducibility of undifferentiated cell cultures. Moreover, both controllable ESC differentiation via forming EBs and drug cytotoxicity evaluation could be performed in this microsystem.

So far research indicates that cardiomyogenic differentiation is higher in microscale cultures than in conventional well plates. Moreover, the culture type has influence on SC fate (Wan et al. 2011). An example of a developed PDMS microsystem used for both 2D cultures (coated with 0.1% gelatin) and for 3D cultures (EBs were mixed with collagen I) is shown in (Fig. 9.2b). The usage of a perfusable cardiac biowire, which mimics 3D functional cardiac tissue, provides a new approach for the investigation of heart functions (Xiao et al. 2014). A microplatform made of PDMS and glass plates enabled 3D cardiac cell culture. The microsystem consisted of a drug reservoir, a connecting channel and a biowire bioreactor (Fig. 9.2c). 3D microtissues were generated using human ESC-derived cardiomyocytes (hESC-CMs) and primary neonatal rat cardiomyocytes. hESC-CMs were obtained through EB differentiation with BMP-4, bFGF, activin, VEGF, inhibitor of WNT production-2 (IWP2) using macroscale. The hESC-CMs and neonatal rat CMs (suspended in collagen type I) were seeded in the microfabricated

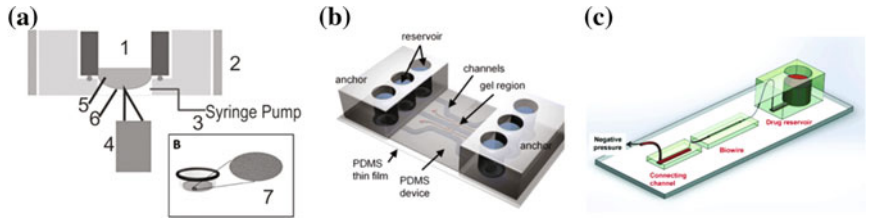

Fig. 9.2 **a** A scheme of Cell Drum tissue analyser: (1) cylindrical Cell Drum culture medium container, (2) ring heater, (3) air pump, (4) laser triangulation sensor, (5) culture medium, (6) membrane and cell monolayer, (7) cell monolayer. Reprinted from Goβmann et al. (2016). Open Access. **b** A scheme of PDMS microsystem, which contains three microchannels for a 2D cell cultures and two gel microchannels for a 3D cultures. Reprinted with permission from Wan et al. (2011). Copyright 2011 Springer. **c** A scheme of the perfusion microdevice integrated with a drug reservoir, a connecting channel and a biowire bioreactor. Reprinted with permission from Xiao et al. (2014). Copyright 2013 Royal Society of Chemistry

biowire platform and cultured for 14 days. The microplatform was integrated with electrodes (carbon rods), which were used for electrical stimulation (biphasic rectangular signal, 3.5–4 V cm^{-1}, 1 ms duration, 1.2 Hz, 4 days stimulation) of the cultured cells. To characterize cardiac biowires, cardiac markers were immunostained (connexin 43, cTnT, α-actin, F-actin fibres). Such a model was also used for in vitro testing of different compounds. Nitric oxide (NO) and donor sodium nitroprusside (SNP) were investigated in the fabricated platform. The developed microplatform was successfully used for 3D cultures (as biowires) of rat and SC-delivered cardiomyocytes. Such an in vivo-like model can allow a unique opportunity to test drug cytotoxicity as well as to analyse proliferation, interaction and physiology of CMs and SCs. In the future, it can be a good technique not only for study of SC-CMs but also for SC differentiation and analysis of SC influence on heart cell regeneration.

iPSCs are the next kind of stem cells, which are also used to obtain cardiomyocytes. However, iPSCs are the most often differentiated into beating cardiomyocytes (iPSC-CSs) by the usage of biochemial factors and AggreWell or Matrigel-coated plates in macroscale. Next, the beating iPSC-CSs are studied in the microsystems (Bergström et al. 2015; Mathur et al. 2015; Moya et al. 2013). The simulation of a whole cardiovascular system based on iPSC-CMs has been presented by Moya et al. (2013). The geometry of the PDMS/glass microsystem was designed in such a way that a perfusion vascular system was mimicked. It consisted of two fluidic microchannels separated by a central microchannel, in which 12 linear-arranged microchambers were placed (Fig. 9.3a). The beating iPSC-CM organoids were seeded with endothelial colony-forming cells (ECFCs)-derived ECs and normal lung fibroblast in the developed microsystem. A co-culture of the organoids and the cells was performed within the next 28 days. It was observed that during this time a vessel network was created. The obtained 3D model of vascularized cardiac tissue can be used to analyse cardiocytotoxicity of new compounds and heart regeneration using SCs.

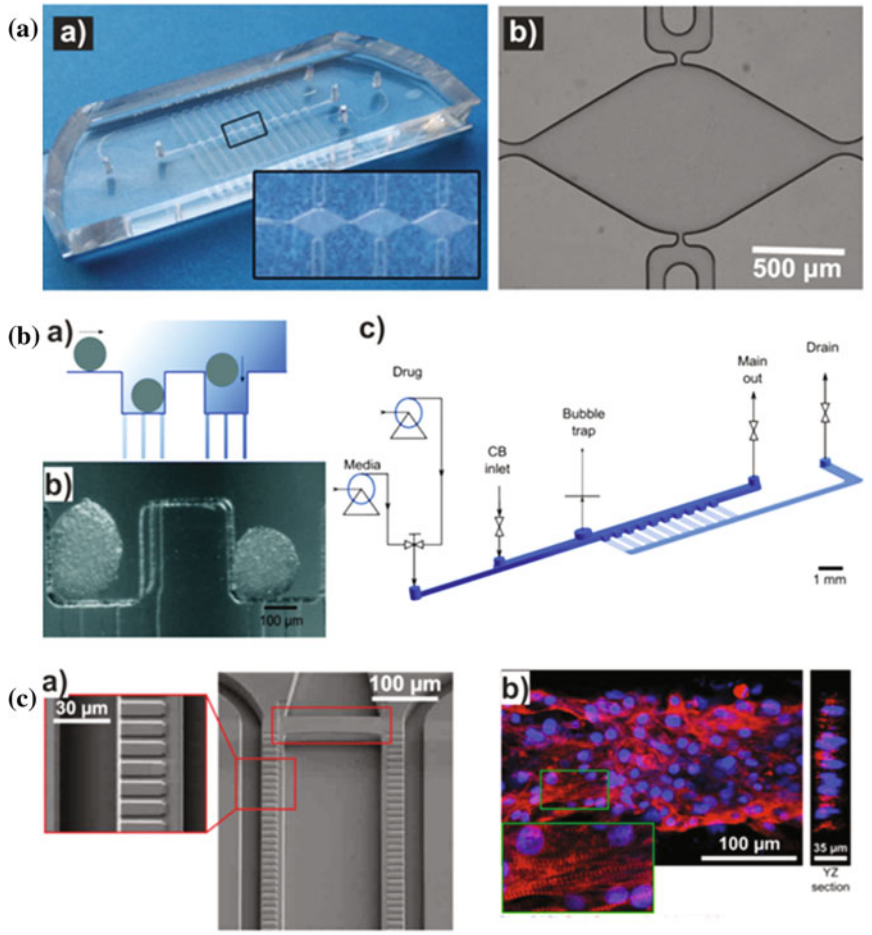

Fig. 9.3 **a** a. Fabricated PDMS-based microfluidic system for 3D cell culture. b. A view of one daisy-chained microchamber. Reprinted from Moya et al. (2013). Open Access. **b** a. A scheme of introducing cardiac bodies into the microsystem. b. A view of cardiac bodies seeded inside the niches. c. Scheme of the fabricated microfluidic system. Reprinted with permission from Bergström et al. (2015). Copyright 2015 Royal Society of Chemistry. **c** a. A view of the fabricated microfluidic system with culture microchamber and two-sided microchannels separated by two rows of pillars. b. A confocal fluorescent microscopy of the cardiac microtissue in the microphysiological system (MPS). Inset shows the view of the sarcomeric α-actin (red) and DAPI (blue) staining. Reprinted from Mathur et al. (2015) Open Access

Other microsystems for study of beating iPSC-CMs have been developed in the next years. However, they were mainly used for mimicking of heart functions (not vascularization) and drug cardiotoxicity analysis. Bergström et al. (2015) presented a PDMS-based microfluidic system to analyse cardiotoxicity on cardiac bodies (CBs) derived from iPSCs. The possibility to study the beating of single CB is the

main advantage of this research. The microsystem consisted of the main channel connected with two inlets: for CBs seeding and culture medium perfusion (Fig. 9.3b). Ten microchambers used for CB cultures were placed along the main channel. Additionally, each microchamber was connected with three drainage channels. The beating CBs were seeded in the microchambers and incubated with different concentrations of doxorubicin, verapamil and quinidine. In turn, Mathur et al. (2015) presented a cardiac microphysiological system (MPS) with a different geometry. The designed microsystem consisted of a central cell chamber, two-sided media channels and arrays of connecting microchannels (Fig. 9.3c).

3D cardiac cultures of the beating hiPSC-CMs (obtained in macroscale by biochemical stimulation with B27 insulin and WNT inhibitor) were formed in a MPS. Next, the cytotoxicity of four different compounds, i.e. isoproterenol, E-403, verapamil and metoprolol was investigated. It was proven that MPS is an appropriate tool to use for 3D cardiac tissue culture and cytotoxicity analysis. Cell beating frequency was used to investigate cardiotoxicity of drug substances in the microsystems. Because it is the non-invasive method, cardiotoxicity analysis in real-time could be performed.

As it was mentioned before, SC differentiation into CMs is often performed in macroscale and next the SC-delivered CMs are cultured and investigated in *Lab-on-a-chip* systems. There is one report about SC differentiation into CMs using the biochemical method performed in microscale. Jeon et al. (2014) presented SC differentiation in a PDMS microfluidic system. The geometry of the microsystem is shown in Fig. 9.4. It consisted of a central microchannel for 3D cell culture and two-sided microchannels for media perfusion. Bone marrow-derived human mesenchymal stem cells (BM-hMSC) and vein endothelial cells (HUVECs) mixed with each other and with fibrinogen were cultured in the central channel. To induce vascularisation, the culture medium was supplemented with: 50 ng ml^{-1} VEGF, 50 ng ml^{-1} VEGF with 100 ng ml^{-1} Ang-1 (angiopoietin) or 50 ng ml^{-1} VEGF with 1 ng ml^{-1} TGF-β1. Additionally, HUVECs were cultured as a monolayer in the side microchannels. The level of vascularization was monitored by immunostaining α-smooth muscle actin (α-SMA). It was found that, α-SMA in a vascular network significantly increased after the addition of TGF-β1 and Ang-1. The presented microsystem can be successfully used for creation of a 3D functional and a perfusable

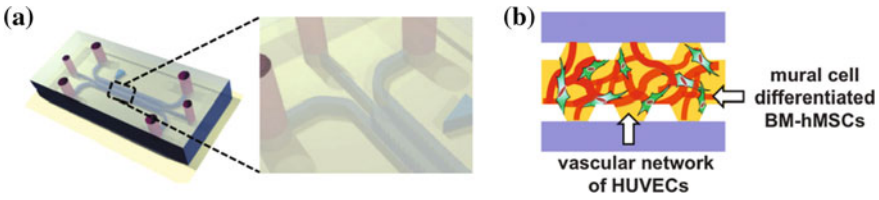

Fig. 9.4 **a** A scheme of the microfluidic systems for 3D cell cultures. **b** A scheme of 3D perfusable microvascular network with endothelial cells (HUVECs) (red) and BM-hMSCs (green) Reprinted with permission from Jeon et al. (2014). Copyright 2014 Royal Society of Chemistry

microvascular network and analysis of SC differentiation. Moreover, this research can have a huge impact on the investigation of heart regeneration in the microsystems.

9.3 Physical Stimulation

Physical cues have been recognized as critical factors during SC differentiation into specific cell types. Structure, topography and elasticity of the biomaterials as well as electrical fields are physical factors which are often used to determine SC fate. These parameters are characterized in the next sections.

9.3.1 Surface and Structural Stimulation

Elasticity/stiffness of the materials may determine, especially in 2D cultures, cell adhesion, morphology and phenotype. It was also proven that the topography of culture surface and extracellular matrix (ECM) influence SC differentiation (Engler et al. 2006; Flaim et al. 2008; Solanki et al. 2010). These factors affect gene and protein expressions which finally improve growth, differentiation and maturation of SCs (Murphy et al. 2014; Pek et al. 2010). Various studies based on surface stiffness and topography (among others on PDMS, polyacrylamide, glass, polystyrene—PS) were performed in macroscale. Surface properties can be changed by micropatterning. In this case, the materials are coated with different proteins (e.g. collagen, poly-L-lysine, fibronectin). So far, studies have shown that micropatterning influences cell morphology, migration, functionality, cytoskeletal structure and nuclear shape.

The combination of surface stimulation (microgrooves) and bioprinting technique is a promising approach to study SC differentiation in CMs. Such a solution has been proposed by Bhuthalingam et al. (2015). Different microgrooves (linear, concentric circles and sinusoidal wave—S-Shaped) were patterned in polystyrene films (Fig. 9.5a). Human MSC suspension was prepared in bioink solution, which contained gelatin dissolved in Dulbecco's modified Eagle's medium (DMEM). Then, the cellularized bioink was printed on the polystyrene surfaces containing different grooves. A higher expression of GATA4 (cardiomyocyte marker) was observed on S-shaped patterns than on other geometries. The results showed that the geometry of the microgroove had a high impact on SC fate. Moreover, the bioprinting is a controlled method, which could enhance MSC differentiation. The proposed bioprinting technique can be useful for further investigation of different SC types. Other methods of surface stimulation, presented in the literature, were mainly focused on the study of alignment and maturation of stem cell-derived cardiomyocytes (obtained using biochemical stimulation in macroscale). For example, Salic et al. (2014) tested an increase of sarcomere expression and alignment of human embryonic stem cell-derived cardiomyocytes (hESC-CMs) on 2D

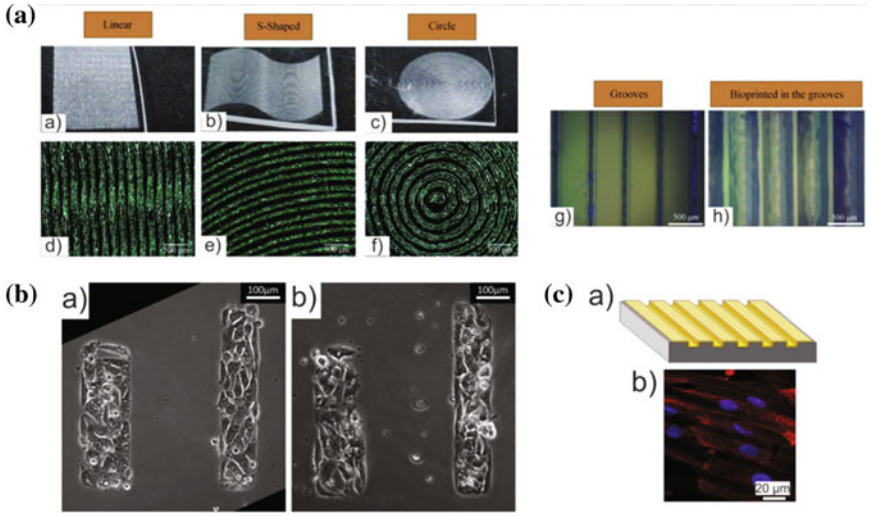

Fig. 9.5 **a** Polystyrene films with micropatterned grooves: linear (a), sinusoidal wave—S-shaped (b), concentric circles (c) and grooves (g, h). The cells were visualized using fluorescein diacetate (FDA) and DAPI staining (d–f). Reprinted from Bhuthalingam et al. (2015). Open Access. **b** A 2-day culture (a) and 3-day (b) culture of hESC-CMs seeded onto the micropatterned grooves with different dimensions. Reprinted with permission from Salic et al. (2014). Copyright 2014 Elsevier. **c** A scheme of a microgrooved flexible scaffold (a). A view of the sarcomeric α-actin (red) and DAPI (blue) immunostaining (b) Reprinted from Rao et al. (2013). Open Access

micropatterned surfaces with specific geometries (Fig. 9.5b). Surfaces with different rectangular geometries (areas ranging from 2.500 μm^2 to 160.000 μm^2) were utilized in the experiments. For this purpose, non-adherent poly(ethylene glycol) (PEG) regions were micropatterned and ECM proteins (Matrigel and fibronectin) were put on gold-coated glass slides. Fibronectin-coated microgrooved performed in PDMS were also utilized for investigation of cellular alignment and calcium (Ca^{2+}) cycling of iPSC-CMs (Rao et al. 2013). The scheme of the microgrooved substrate is shown in Fig. 9.5c. It was found that cell alignment and parallel organization of sarcomeres were mainly dependent on the groove width. The above studies also showed that the modified substrates influence Ca^{2+} cycling in SC-CMs. The proposed highly aligned cell model may be useful in pharmacological studies and understanding how surface geometry influences SC-CMs maturation. In the future, the developed surfaces with microgrooves can be applied to study SC differentiation and heart cell regeneration.

Dimension and geometry of microstructures also influence the fate of SCs and EBs. An interesting geometry of the microsystem (i.e. tent-like structure) was used to study cardiogenesis (Tanaka and Fujita 2015). A PDMS-based microsystem consisted of a microchannel layer, a microchamber layer with a membrane and a cylindrical block on the membrane (Fig. 9.6). The proposed structure was supposed to perform analysis of cell beating based on periodical oscillation of fluid in the

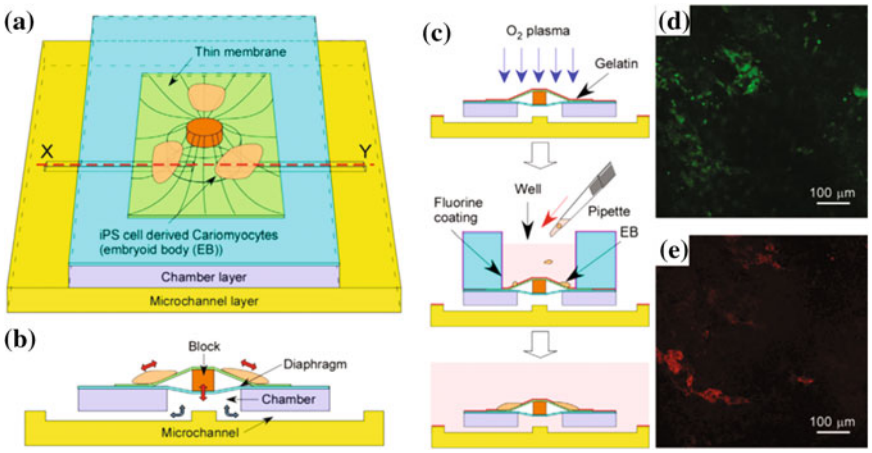

Fig. 9.6 **a** A scheme of microdevice for iPSC differentiation into cardiomyocytes on the tent-like structure. **b** Cross-sectional view along line X–Y. **c** A scheme of method of attaching EBs onto the microchip, which was O_2 plasma and gelatin treated. **d**, **f** Fluorescent microscopy of anti-cardiac myosin heavy chains antibody (green) and anti-cardiac troponin I antibody (red) immunostaining. Reprinted with permission from Tanaka and Fujita (2015). Copyright 2015 Elsevier

microchannel connected to the membrane. EBs-derived iPSCs were cultured in the microsystem. EBs were attached to a thin PDMS tent-like membrane. Periodical oscillation of fluid in a microchannel and cell beating was observed 14 days after cell seeding. SC differentiation was also proven by immunostaining MHC and cardiac troponin I.

3D polymeric scaffolds, including microspheres, porous forms and nanofibers are also used to culture and differentiate SCs in microscale (Ghafar-Zadeh et al. 2011). Polymer scaffolds next to the hydrogels are physical stimulations, in which no external actuation is used to differentiate SCs (Hoffman 2012; Zuppinger 2016). Nutrients in 3D hydrogel cultures are uniformly distributed to all cells (Annabi et al. 2013; Ghiaasedin et al. 2017). In turn, scaffolds create rigid networks and structures with higher physical stiffness than hydrogels. Functional nanofibers scaffolds produced by electrospinning are meaningful in many SC differentiations. The nanofibers can mimic the native ECM fibres (Alamein et al. 2015; Bianco et al. 2009; Heydarkhan-Hagvall et al. 2008; Liu et al. 2014; Tomecka et al. 2017; Wang et al. 2013). Thanks to this, SC proliferation and interaction with ECM-like nanostructures can be investigated (Luo et al. 2015). For example, collagen (type I)—grafted polyethersulfone (PES) nanofiber matrix was used to culture mouse ESCs. The results showed that ESCs cultured on a PES nanofiber matrix were undifferentiated during the whole culture, whereas cell proliferation was increased. It indicated that a collagen-grafted PES nanofiber matrix can be used to maintain undifferentiated forms of SCs as well as to differentiate SCs under precise control (Hashemi et al. 2011).

Many studies based on regulation of SC fate on nanofiber scaffolds have been discussed in the literature. Nanofibers were utilized to induce SC differentiation into, e.g. neural cells, chondrogenic cells and osteoblasts (Li et al. 2012; Smith et al. 2010). 3D-shaped polymeric scaffolds are also used for SC differentiation into CMs. Ghasemi-Mobarakeh et al. (2014) stimulated EBs delivered from ESCs on Polycaprolactone (PCL)/gelatin nanofibrous scaffolds for 5 days. The presence of CMs was determined by immunostaining cardiac markers (α-sarcomeric actin and conexin43) and cell beating. EB differentiation into CMs was also tested on collagen/Matrigel scaffolds (Zhou et al. 2010). It was found that mouse ESCs seeded onto 3D scaffolds, aggregated and formed EBs. To induce cardiac differentiation, 0.1 mg/ml ascorbic acid was supplemented into the culture medium for 7 days. Beating CMs were observed 7 days after EB seeding; however, synchronous contraction was noticed on the 19th day of culture. Cardiac markers such as cTnT, anti-cytokeratin 18 antibody (CK18), anti-murine antibody (CD31) and nestin were determined to prove SC differentiation into CMs. The obtained results showed that collagen/Matrigel scaffolds can be successfully used to create EBs and ESCs differentiation into CMs. PCL/gelatin and collagen/Matrigel scaffolds can also be useful to investigate the mechanism of SC differentiation and human myocardium regeneration after infarction. It should be noted that the scaffold composition can have an important role in CS differentiation. A new approach was proposed by Yang et al. (2016). They tested how tunnelling nanotubes (TNTs) integrated with a PDMS biochip influences stem cell and cardiomyocyte communication. A novel biological process, unidirectional mitochondrial transfer, mediated by heterotypic TNT connections was discovered. These results could be a base for further research of cardiomyocyte regeneration using SCs.

The next technique, which enables 3D culture of the cells, is the microsphere method. Microspheres can be used to precisely deliver SCs into the damaged tissue. Moreover, growth factors and therapeutic molecules can be placed inside the microspheres. The usage of encapsulated and differentiated SCs can be an effective method for CVD treatment. Trimethyl ammonium-coated polystyrene microspheres were used for 3D culture and differentiation of human ESCs (Phillips et al. 2008). ESCs have the capacity to differentiate towards pancreatic, neuronal and cardiomyocyte cells. The utility of microspheres in CVD treatment is also studied in vivo. Microspheres made of alginate were used for encapsulation of human MSCs and transplantation into rats with ischaemia-reperfusion myocardial infarction (Yu et al. 2010). Alginate was used as a non-toxic and semi-permeable material. The alginate microspheres with the encapsulated MSCs are shown in Fig. 9.7. Properties of the fabricated microspheres improve cell attachment and growth on the injured heart. The effects of treatment after microsphere introduction into the rats were investigated by echocardiography and immunostaining of the histopathological preparation. The results showed that the encapsulated MSCs into microspheres induced angiogenesis and heart regenerationRegeneration.

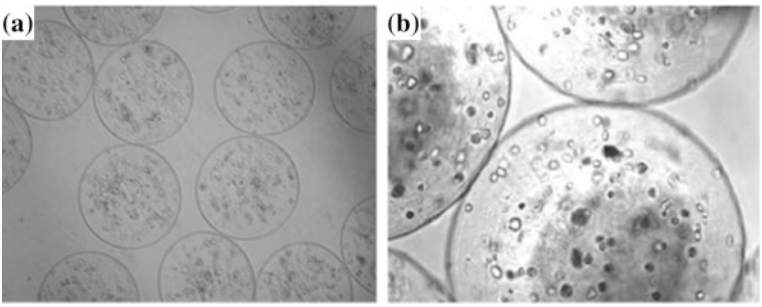

Fig. 9.7 Human mesenchymal stem cells two days after encapsulation into alginate microbeads. Magnification 4× (**a**) and 10× (**b**). Reprinted with permission from Yu et al. (2010). Copyright 2010 Elsevier

9.3.2 Electrical Stimulation

Electrical field can play an important role in many biological and medical applications. The tests to prove the usage of electrical field for the treatment of various diseases are also performed. The mechanisms of electrical stimulation have been investigated for many years and so far they are not understood in many cases (Ghafar-Zadeh et al. 2011). A knowledge of electrical field functions for heart stimulation could have an important meaning for the study of heart properties under normal and pathological conditions. Many reports based on electrical stimulations in macroscale have been presented in the literature (Barash et al. 2010; Kujala et al. 2012; Maidhof et al. 2012). Microtechnology is investigated as an effective method for electrical manipulation of SCs and differentiation of SCs into cardiac cells. Electrodes used for stimulation of SCs in the microsystems are fabricated from the same materials such electrodes for CM stimulation (see Chap. 8).

The type of electrical signal, cell culture (single cell, monolayer and 3D cultures) and a culture system have an influence on SC differentiation into cardiac cells (Tandon et al. 2009). A chamber with shielded electrodes, flasks and dishes integrated with electrodes and customized microchamber can be utilized for electrical stimulations. SC differentiation after electrical field action can be determined by analysing contractile cell activity, cell elongation, cell morphology, the force of cell contraction, electrical cell activity and gene expression. The parameters which can influence electrical stimulation for cardiac tissue engineering are shown in Fig. 9.8.

Biphasic square pulses have most often been reported as electrical signal used for SC differentiation into CMs. For example, Pavesi et al. (2015) developed a PDMS-based microdevice useful for mechanical and electrical stimulation of human BM-MSCs. The microdevice consisted of three layers: a pneumatic layer for mechanical stimulation, a fluidic layer for the SC culture and a conductive layer for electrical stimulation (Fig. 9.9a). An interesting technique was used to fabricate the conductive layer. A mixture of carbon nanotubes (CNTs) and PDMS was deposited on the silicon wafer. Biphasic square pulses (5 V cm^{-1}, 1 Hz, 1 ms) were

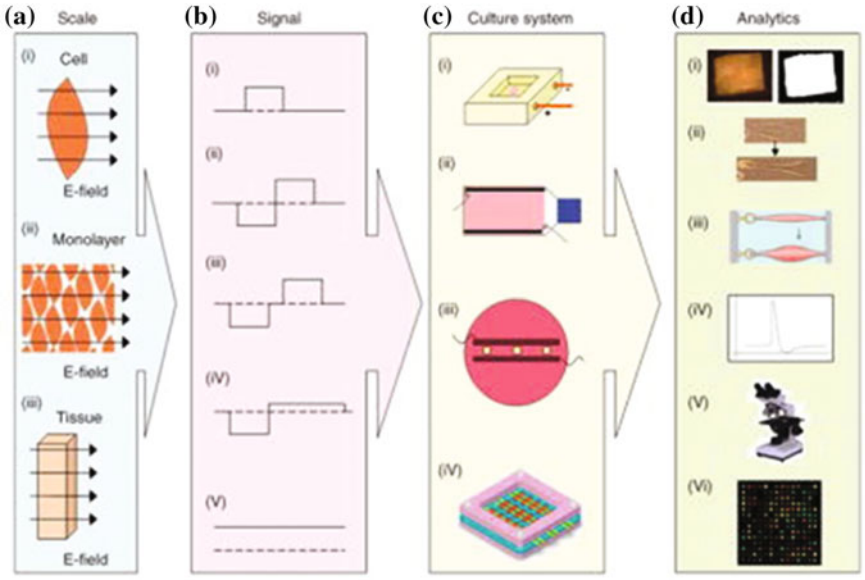

Fig. 9.8 Overview of the parameters which can influence electrical stimulation during stem cell differentiation into cardiomyocytes including: **a** scale (single cell (i), monolayer (ii), 3D culture (iii)), **b** the electrical signal monophasic pulses (i), charge-balanced biphasic pulses (ii), charge-balanced biphasic pulses with interphase delay (iii) charge-balanced biphasic pulses with slow reversal (iv), direct current (v), **c** the culture system (a chamber with shielded electrodes (i), a T-flask (ii), a Petri-dish (iii), a customized chamber (iv) and **d** the analytics performed to evaluate contractile activity (i), elongation (ii), force of concretion (iii), electrical activity (iv), morphology (v) and gene expression (vi) Reprinted with permission from Tandon et al. (2009). Copyright 2009 Nature Publishing Group

generated 24 h after SC seeding in the microdevice. An efficiency of human BM-MSC differentiation into CMs was evaluated on the 14th day of the culture. For this purpose, specific cardiac markers (MYH7, Nkx2.5, cTnT, MEF2C—myocyte enhancer factor 2C and TUBB—tubulin beta) were defined using quantitative real-time polymerase chain reactions (qRT-PCR). High expression of the markers mentioned above was noticed. Not only biphasic but also monophasic square pulses were utilized for SC differentiation in microscale. An example of a PDMS-based microdevice used for electrical stimulation of SCs using monophasic square pulses is shown in Fig. 9.9b (Tandon et al. 2010). It was found that such stimulation enhanced SC proliferation, elongation and perpendicular orientation to the electrodes.

They can be fabricated using some wires/rods (Serena et al. 2009; Thavandiran et al. 2013) or planar electrodes patterned on the substrate (Tandon et al. 2010; Zhou et al. 2016). A PDMS micro-bioreactor with 4 × 4 wells was integrated with electrodes made of different rods is shown in Fig. 9.9c (Serena et al. 2009). 10 cm × 1.3 mm diameter 304 stainless steel, titanium and titanium nitride-coated

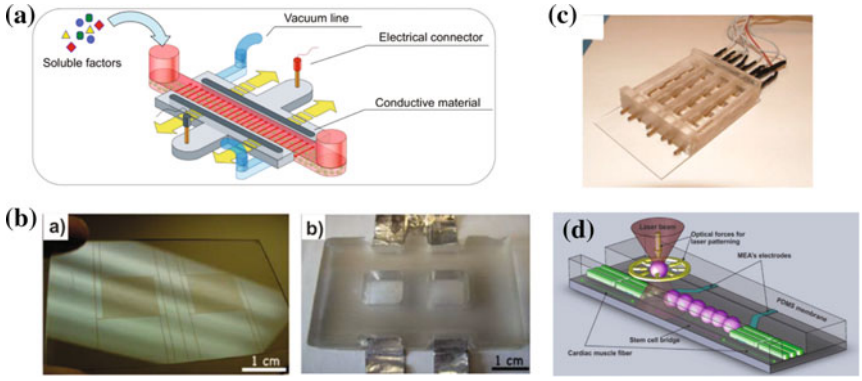

Fig. 9.9 **a** A scheme of the microfluidic platform containing central channel (red, to provide culture medium), the pneumatic channels (light blue, to perform mechanical stimulation) and the electrical layer with two conductive regions composed of carbon nanotubes and polydimethylosiloxane (light grey). Red arrows represent the electric field. Reprinted from Pavesi et al. (2015). Open Access. **b** Views of the slide with electrode arrays (a) and polydimethylosiloxane layer with two culture wells (b) Reprinted with permission from Tandon et al. (2009). Copyright 2009 Royal Society of Chemistry. **c** A view of the polydimethylosiloxane-based bioreactor for electrical stimulation with 16 culture wells. Reprinted with permission from Serena et al. (2009). Copyright 2009 Elsevier. **d** A scheme of the microfluidic system. Stem cells (pink spheres) were laser-patterned to form a bridge connecting two separated muscle fibres (green). Reprinted with permission from Ma et al. (2012). Copyright 2011 Royal Society of Chemistry

titanium rods were integrated with three independent microdevices. Such microsystems were utilized for EB differentiation studies. As it was mentioned before, the mechanisms responsible for electrical field-induced therapy are still not fully known. Serena et al. (2009) investigated that the intracellular reactive oxygen species (ROS) could take part in SC differentiation into CMs. Therefore, the effect of H_2O_2 (hydrogen peroxide) on the SCs was additionally investigated to prove this hypothesis. It was found that stainless steel electrodes induced the highest cardiac differentiation. These electrodes and 1 nM of H_2O_2 generated a comparable ROS level. Based on these results, the authors proved that electrical stimulation can differentiate SCs into CMs based on mechanisms associated with intracellular ROS generation.

A planar microelectrode array technology (MEA) with integrated recording and stimulation electrodes is also used to stimulate cells (Chen et al. 2009). MEA platforms enable precisely localized current to be injected into the cell culture and differentiated cell properties to be detected in the same culture chamber. A potential impact in regenerative medicine could have a MEA-based microsystem presented by Ma et al. (2012). MEA-based biochip was utilized to mimic the cardiac model. First, rat CMs were seeded on a biochip to form muscle fibres. After 4 days, small gaps (120 μm length) were created to mimic a myocardial infarction. Next, rat mesenchymal stem cells from bone marrow (rMSCs-BM) were laser-patterned into the gaps to create a bridge between cardiomyocytes and rMSCs-MB (Fig. 9.9d).

Fibroblasts laser-patterned in the gaps were used as control samples. The electrical conductivity of SCs and fibroblasts was determined using MEA. Additionally, cardiac markers were immunostained (sarcomeric α-actin and connexin 43). It was found that SCs-CMs bridges showed higher and more stable conduction through the gap junction than CMs-fibroblasts interactions. The above results proved that SCs can be a promising method for heart regeneration, and the proposed microchip can be successfully used for cell monitoring using MEA.

The electrodes integrated with the microsystems are used not only for stimulation but also for electrical conduction monitoring. MEA and electrical impedance spectroscopy (EIS) are used to measure SCs after their differentiations (Ma et al. 2012; Zhou et al. 2016). Zhou et al. (2016) presented a microsystem for quantitative analysis of the changes in electrical parameters of mouse ESCs at different stages of differentiation. A 0 h—undifferentiated state, 24 h—transition state and 48 h fully-differentiated state of SCs were investigated in the microsystem. EIS was used as non-invasive, label-free and real-time analysis of cell conditions based on their dielectric properties and biological structure. The microfluidic device composed of PDMS, and glass with patterned titanium and gold electrodes was used for hydrodynamic trapping of single mouse ESC (Fig. 9.10a). The differences between SC stage and magnitude of the cell impedance were noticed. Cell impedance increased with an increase in cell size and SC differentiation state. A label-free cell cytometry can also be used to distinguish differentiated and undifferentiated SCs (Fig. 9.10b). For this purpose, a microsystem consisting of a PDMS layer and a glass slide integrated with two platinum electrodes and a Si3N4 passivation layer was tested (Myers et al. 2013). Undifferentiated human iPSC- and human iPSC-derived CMs were successfully distinguished based on the recording of extracellular field potential (FP) signals from suspended cells in flow. Such a technique can be useful for investigating differentiation stages of SCs and for optimizing SC stimulation parameters (Table 9.2).

9.4 Mechanical Stimulation

9.4.1 Mechanical Strain

Cardiac cells are continuously exposed to a variety of mechanical stimulations under native conditions, i.e. action of muscle forces, gravity or blood flow. Moreover, the interactions between the cells are crucial for their stretching (Gupta et al. 2010). The above-mentioned forces regulate cellular physiology and functions. Therefore, mechanical forces are mimicked in the experiments performed in vitro. Thanks to this, cardiac cells can be cultured under conditions similar to in vivo. It was proven that mechanical stimulations can enhance SC differentiation into CMs. Cell stretching has been simulated both in macroscale and the microsystems (Gwak et al. 2008; Pavesi et al. 2015; Ruan et al. 2015; Simmons et al.

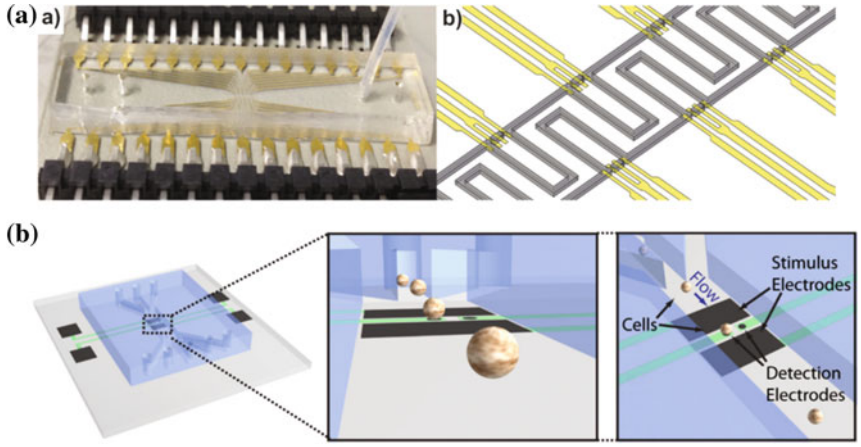

Fig. 9.10 **a** A view of the fabricated microsystem (a). Three-dimensional scheme of the trapping channels (grey) and electrodes (yellow) (b). Reprinted from Zhou et al. (2016). Open Access. **b** A scheme of the electrophysiology-activated cell cytometry system composed of microfluidic flow chamber integrated with microelectrode and the measurement simulation, Reprinted with permission from Myers et al. (2013). Copyright 2012 Royal Society of Chemistry

2012). 2D and 3D mechanical strains have been reported in the literature (Marsano et al. 2016; Mummery et al. 2012; Pavesi et al. 2015; Shimko and Claycomb 2008). A deformable and elastic substrate (membrane) is exposed to 2D strains with controllable magnitude and frequency. 2D strain can be uniaxial (exerted along one axis), biaxial (exerted in two directions) and equiaxial (exerted in all directions). Air pressure microchannels are most often used to deform a thin membrane, on which cells are cultured. SCs 2D or 3D cultured on elastic membranes can be exposed on cyclic stretch. Range value during the stimulation equalled 5–20% strain, 1–3 Hz. The cells were often stimulated for 1 or 2 days (Clause et al. 2009; Kurpinski et al. 2006; Park et al. 2004). However, long-strain stimulation (7 days or 2 weeks) was also investigated (Gwak et al. 2008; Shimko and Calycomb 2008; Zimmerman et al. 2006).

Marsano et al. (2016) designed a *Heart-on-a-chip* platform to generate mature and highly functional micro-engineered cardiac tissues (μECTs). The microsystem was composed of three PDMS-based layers. The top and bottom layers had two rows of micropillars, which created a central microchannel and two-sided microchannels. The top and bottom PDMS layers were separated by a thin PDMS membrane. Cyclic uniaxial strains (10–15%) were generated in the microsystem because of their integration with an electronically controlled pressure regulator system. 3D culture of neonatal rat CMs and human iPSC-CMs suspended in a fibrin gel matrix were performed in a culture microchannel. Stretching of 3D cultured cells was monitored using a microscope and analysed based on ImageJ software. Immunofluorescence staining was used to detect cardiac markers: troponin I, connexin43 and sarcomeric α-actin. It was found that 5 days after cell

Table 9.2 Electrical stimulations

Cells	Type of electrode	Type of culture	Conditions	Effect	Cardiac marker	Refs.
Human BM-MSCs	CNTs	2D	Biphasic square pulses, 5 V/cm, 1 ms duration, 1 Hz,	Cardiac differentiation	MYH7, NKX2.5, TNNT2, MEF2C, TUBB, actin cytoskeleton changes	Pavesi et al. (2015)
Human ESCs	Electrodes made of 304 stainless steel and titanium and titanium nitride rods.	3D	EBs, single electrical fields pulse, 1 V/mm, 1–90 s duration and continuous stimulation, 1 V/mm, 5 ms duration, 5 Hz	Cardiac differentiation	Cardiac Troponin T (cTnT)	Serena et al. (2009)
Rat MSCs-BM	ITO electrodes	3D, cell bridges on muscle fibres	–	Cardiac differentiation	Sarcomeric α-actin, connexin43	Ma et al. (2012)
Mouse ESCs	Co-planar electrodes made of titanium and gold	Single cell	100 mV, 100 Hz–20 MHz	Cardiac differentiation	Impedance measurements	Zhou et al. (2016)
Murine ESCs	MEA 6 × 6 array of platinum electrodes	2D	EBs, electric current: 10, 30, 60 μA; 10 ms duration, 1, 2, 4 Hz	Cardiac differentiation	β-MHC, cTnT	Chen et al. (2009)
Human iPSCs	Pt/Ti electrodes	3D Cell clusters	Cytometry measurement; sequence of twenty 0.4 ms wide pulses at 0.1 s interval	Differentiated iPSC-derived CM and undifferentiated iPSCs distinguish	Differential voltage signal	Myers et al. (2013)

(continued)

Table 9.2 (continued)

Cells	Type of electrode	Type of culture	Conditions	Effect	Cardiac marker	Refs.
Human hASCs	ITO-coated glass surface	2D	Square monophasic pulses, 20 mV, 2 ms duration, 1 Hz	Cardiac differentiation	Connexin43	Tandon et al. (2010)

CNTs Carbon nanotubes, *ITO* Indium tin oxide, *MEA* Multielectrode array, *2D* Two-dimensional, *3D* Three-dimensional, *EBs* Embryoid bodies, *β-MHC* β myosin heavy chain, *MYH7* Myosin heavy chain 7, *NKX2.5* Homeobox protein Nkx-2.5, *TNNT2* Gene, which provides instructions for producing a cardiac troponin T, *MEF2C* Myocyte enhancer factor 2C and *TUBB* Tubulin beta

injection, expression of cardiac markers increased. The fabricated microsystem enabled not only generation of 3D cardiac microtissue but also controllable mechanical stimulation and quantitative analysis of cell culture.

The mechanical strain with the usage of additional factors can enhance cardiac differentiation of SCs. Different factors (e.g. biochemical factors, electrical fields and mechanical stimulations) are often simultaneously used to precisely control the fate of SCs in the microsystems. Surprising results were obtained in a hybrid PDMS-hydrogel microfluidic platform fabricated by (Wan et al. 2011). They used both biochemical stimulation and controllable uniaxial cyclic stretching for induction of cardiogenesis. A 4-day cultured EBs were stretched at 10% strain and 1 Hz frequency for 24 h. Expression of α-MHC was analysed to evaluate the effectiveness of SC differentiation. Surprisingly, the expression of α-MHC was lower after uniaxial cyclic stretching. This indicated that mechanical stimulation can be stopped and reduced the SC differentiation into CSs. The usage of an additional factor most often enhances cardiogenesis. However, cell type, kind of stimulation and value of the used parameters have impact on a degree of SC differentiation. Therefore, such a type of research should still be performed.

It should be noted that a microsystem for SC culture under fast changes in gas partial pressure and cyclic stretching was also developed (Campillo et al. 2016). The PDMS-based microsystem consists of a thin membrane for rat bone marrow-derived mesenchymal stem cell (BM-MSC) culture. Such culture was exposured to hypoxia conditions and cyclic stretch. The proposed microsystem can be a useful tool for investigation of SC response to hypoxia and stretch. Moreover, it has promising application in regenerative and personalized medicine in the future.

9.4.2 Shear Stress

The effect of shear stress on endothelial cells (ECs) and CMs has been investigated by many research groups. Although SC fate under exposure to shear stress has not been very well investigated, there is research being performed with conventional methods and the microfluidic systems. Shear stress in macroscale was successfully used to differentiate SCs into the cells of the cardiovascular system: CMs (Huang et al. 2010), vascular smooth muscle cells (Huang et al. 2005) and endothelial cells (Metallo et al. 2008; Wu et al. 2008). Microscale allows for a creation of controllable flow of culture medium and other biochemical factors. Thanks to this, SC fate can be investigated under controllable conditions. Due to the fact that the microstructures with various dimensions can be developed, different values of shear stress can be exposed to SCs. The effect of shear stress on various SCs was investigated in the microfluidic systems (Jeon et al. 2014; Kang et al. 2010; Toh and Voldman 2010; Xiao et al. 2014). A shear stress value in the range of 1–20 dyn cm^{-1} was utilized to stimulate cells. SC differentiation into CMs was investigated in a simple geometry of a microsystem (Villa-Diaz et al. 2009). For this purpose, human ESCs were cultured under dynamic and static conditions in the microfluidic

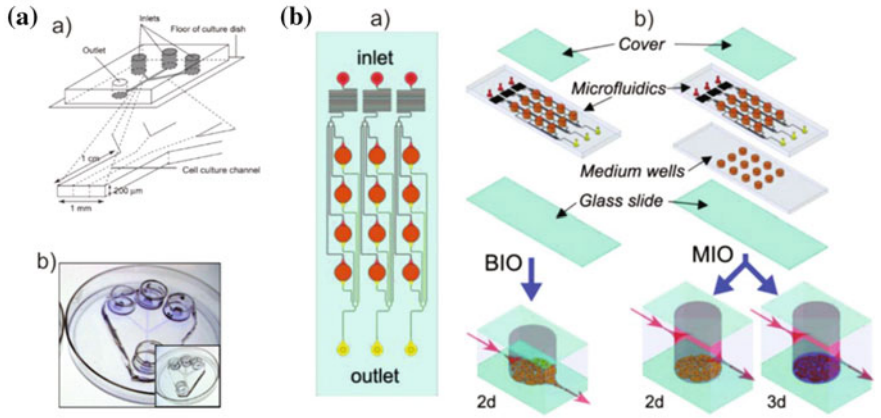

Fig. 9.11 **a** A scheme of the designed microfluidic system with three inlets microchannels converting in the cell culture microchannel (a) A view of the fabricated microsystem (b) Reprinted with permission from Villa-Diaz et al. (2009). Copyright 2009 Royal Society of Chemistry. **b** Scheme of the micro-bioreactor array, containing 12 independent cell culture microwells (a). A scheme of the two configuration used in the experiments: BIO (a bottom inlet/outlet) and MIO (a middle inlet/outlet). While BIO and MIO configuration allowed for two-dimensional cell culture, the usage of hydrogel in MIO configuration allowed three-dimensional cell culture (b). Reprinted with permission from Figallo et al. (2007). Copyright 2007 Royal Society of Chemistry

system for 48 h. The PDMS-based microsystem consisted of three inlet microchannels, which merge in a main cell culture microchannel (Fig. 9.11a).

In this case, non-significant differences were observed between static and perfusion conditions. It should be noted that the type of cell culture and value of shear stress have been crucial in SC differentiation into cardiac cells. Figallo et al. (2007) presented a PDMS micro-bioreactor array (MBA) for study SC differentiation based on culture and conditions types. The microsystem consisted of twelve independent culture wells (Fig. 9.11b). The MBA platform enabled both 2D culture (the cells attached to the substrate) and 3D culture (the cells were encapsulated in a hydrogel) of human ESCs. Additionally, two types of experiments were performed in this microsystem (a) BIO configuration—culture medium flowed directly over the attached cells (b) MIO configuration—a culture medium flows above the main chamber with the cells cultured in a monolayer (2D) or encapsulated in hydrogel (3D). hESC differentiation was investigated in this microsystem under both static and dynamic conditions for 4 days. To induce differentiation, hESCs were additionally cultured with a medium containing human VEGF. It was noticed that MIO configuration more closely imitated the native environment and reduced hydrodynamic stress. Cardiac differentiation was evaluated by immunostaining α-SMA. It was noticed that higher shear stress influences higher vascular differentiation.

9.5 Challenges

There are other methods, which can be applied in the microfluidic systems to differentiate SCs into CMs. It was proven that optical, magnetic, ultrasonic and thermal stimulations can influence the fate of SCs and maturation (Guess et al. 2014; Jenkins et al. 2010; Lucchetta et al. 2005; Oberti et al. 2010). However, these methods were initially tested in macroscale. Therefore, there are many issues, which have to be deeply investigated before using these methods for cardiogenesis in microscale. The usage of graphene and their forms for differentiation is present in the literature as a promising technique in regeneration therapy. Biological studies based on graphene (G) and their forms: graphene oxide (GO) and its reduced form (rGO) have been rapidly growing in the last few years (Charlier et al. 2008; Sun et al. 2008). Graphene and rGO are hydrophobic, not very soluble in water and also require surfactants or surface modifications for biological applications. GO is hydrophilic and can be dispersed in water to form stable colloids (Zhang et al. 2010). Graphene, GO and rGO can be used in gene therapy and photodynamic therapy, especially as drug carriers (Matteini et al. 2014). Moreover, graphene materials have mainly been explored for a construction of matrices and biosensor components in tissue engineering. Study of the cytotoxicity and the influence of graphene and its form to differentiate SCs is a relatively new area of research. There are several reports describing the process of SC differentiation using graphene and GO. These materials were often used to differentiate MSCs into cell lineages such as adipocytes, osteoblasts and chondrocytes (Bitounis et al. 2013; Yoon et al. 2014). However, in these studies, graphene or GO was used to modify the surface of cell growth to improve cell attachment to the culture surface, proliferation and differentiation. Additional factors, most often biochemical, were used for differentiation. Lee et al. investigated the effects of G and GO substrates on the adipogenic and osteogenic differentiation of MSCs (Lee et al. 2011a, b). In order to determine the role of G and GO in differentiation and proliferation, MSCs were seeded on PDMS coated with graphene and GO and PDMS layers with the culture medium. They noticed that MSCs seeded on PDMS were round and poorly attached as compared to G and GO. Although GO is hydrophilic like PDMS, due to the presence of oxygenated groups, it can bind to serum proteins via electrostatic interactions. Moreover, it was proved that MSCs is differentiated into osteoblasts at low cell density (3000 cells per cm^2) in a medium with ascorbate (0.2 mM), dexamethasone (10^{-8} M) and β—glycerolphosphate (10 mM). After twelve days of osteogenic induction, a 7-fold increase in the extent of mineralization in the MSCs cultured on G compared to those cultured on PDMS has been proven (Lee et al. 2011a, b). There are also reports based on graphene differentiation of SCs into CMs. Lee et al. reported that graphene at least partially enhances cardiomyogenic differentiation of hESC. (Lee et al. 2014) hESC cultures were performed on: (1) Matrigel-coated glass, (2) Vitronectin (VN)-coated glass and (3) VN-coated graphene for 21 days. In this study, cardiac mesodermal gene expressions were determined by a qRT-PCR assay. The total RNA was extracted from the

differentiated hESCs on days 4, 7, 14 and 21 and reverse-transcribed into cDNA. On the 14th day, it was noted that the graphene group showed an increase in the cardiac mesodermal gene (MESP1) and mesodermal gene (T and M-CAD) expression compared to the glass and Matrigel groups. Moreover, higher gene expression of cardiomyogenic proteins (α-MHC, β-MHC, MLC2a, cTnT), transcriptional factors (NKX2-5, GATA4, and MEF2C) and gap junction proteins (Connexin43) in the graphene group than the Matrigel and the glass groups at 14th and 21st days was observed.

Apart from graphene, GO was studied in adhesion, proliferation and differentiation of mouse myoblasts—C2C12 cells (Ku and Park 2013). The authors have proven that myogenic differentiation was enhanced by GO, as evidenced by the analysis of myogenic protein expression, multinucleate myotube formation and the expression of differentiation genes (MyoD, myogenin, Troponin T and MHC). According to the results, myoblasts grew well on the graphene oxide surface. The morphology of the cells was analysed by cytoskeleton staining after a 1-day culture. Real-time PCR confirmed that gene expression levels were the highest on GO sheets. Although there are reports describing the usage of graphene and GO to SC differentiation into SMs, the experiments in microscale are not performed. So far, the investigation has shown that graphene and its forms can be combined with PDMS—the most commonly used material for *Lab-on-a-chip* system fabrication. Thus, previous research has indicated that the studies based on graphene and its forms in microscale will be a promising method for investigation of SC differentiation into cardiac cells.

9.6 Summary and Perspectives

Regenerative medicine is a promising method, which can be used to treat CVDs. Although advanced research based on heart regeneration is being performed, it needs to be investigated further. The *Lab-on-a-chip* systems are fabricated to obtain conditions similar to the native environment. The microsystems are used to differentiate SCs using biochemical, physical (electrical, structural, surface) and mechanical (strain, shear stress) factors. However, the SC differentiation is most often started in macroscale (for example using biochemical factors) and next the cells are additionally stimulated in microscale. It should be noted that stem cells-derived cardiomyocytes (SC-CMs) are most often differentiated in the microsystems. There are few studies performed in the microchips, in which SC types, e.g. ESCs, MSCs and iPSCs were stimulated. This seems to be important to investigate how full microscale conditions influence cardiogenesis of SCs.

There are key signalling parameters, which enhance cell growth and enable the mimicking of native myocardium in the microsystems. They are: dynamic conditions, stretching, spatial and parallel cell arrangement (by the usage of hydrogels, scaffolds or nanofibers) and electrical field. To obtain a highly effective cardiogenesis, the above-mentioned features should be provided in the microsystems

dedicated for SC culture. It should be noted that SC research is most often performed on 2D than 3D cultures. On the other hand, it is known that spatial and parallel cell arrangement enhance the expression of cardiac markers. Therefore, to obtain a high efficient of cardiogenesis, three-dimensional SC culture should be maintained in the microsystems. Thanks to this, a native myocardium could be mimicked in SC culture model and enhance SC differentiation into CMs more than in research performed so far. A fully functionalized microsystem mimicking heart features is a perspective of cardiac culture microtechnologies based on stem cells. Moreover, there are only a few studies based on CVD mimicking and CM regeneration. Therefore, a diseased heart model maintained with a defined vascular system seems to be important to test cell regeneration by the usage of SCs. The optimized CM/SC model could be used to investigate SC differentiation using techniques potentially useful for cardiogenesis (e.g. graphene, magnetic and thermal stimulations). The combination of advanced microfluidic technologies with a diseased heart model and SC differentiation methods would become highly crucial *Heart-on-a-chip* systems for personalized medicine and therapeutic.

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