Chapter 1

History of Cell Culture

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Additional information is available at the end of the chapter

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Abstract

From the ancient Romans, through the Middle Ages, to the late of the nineteenth century, the Aristotelian doctrine of spontaneous generation was one of the most basic laws. Even the invention of the microscope and investigations of Leeuwenhoek and Hook did not disprove the Aritostelian doctrine. Finally, in the eighteenth century, the spontaneous generation doctrine was laid by Louis Pasteur. Moreover, in the first decade of the eighteenth century, nucleus was observed in plant and animal tissues, and Virchow and other scientists presented the view that cells are formed via scission of preexisting cells. In the first decade of the twentieth century, Ross Harrison developed the first techniques of cell culture in vitro, and Burrows and Carrel improved Harrison's cell cultures. In mid-twentieth century, the basic principles for plant and animal cell cultures in vitro were developed, and human diploid cell lines were established. On the basis of knowledge about the cell cycle and gene expression regulation, the first therapeutic proteins were produced using mammalian cell cultures. The end of twentieth century and early twenty-first century brought the progress in 3-D cell culture technology and created the possibility of the tissue engineering and the regenerative medicine development.

Keywords: spontaneous generation, Harrison's hanging drop culture method, HeLa cell line, Hayflick limit, cell culture history

1. Introduction

At the present time, animal and human cell cultures are significant tools widely used in many branches of live science. Different variants of cell culture found application in modeling diseases, IVF technology, stem cell and cancer research, monoclonal antibody production, regenerative medicine and therapeutic protein production. All those different scientific approaches would not be possible without some crucial discoveries that had been made over the centuries from Aristotelian spontaneous generation doctrine through Pasteur's experiments and Carrel's cell culture to large-scale cultures for therapeutic proteins production and vision of

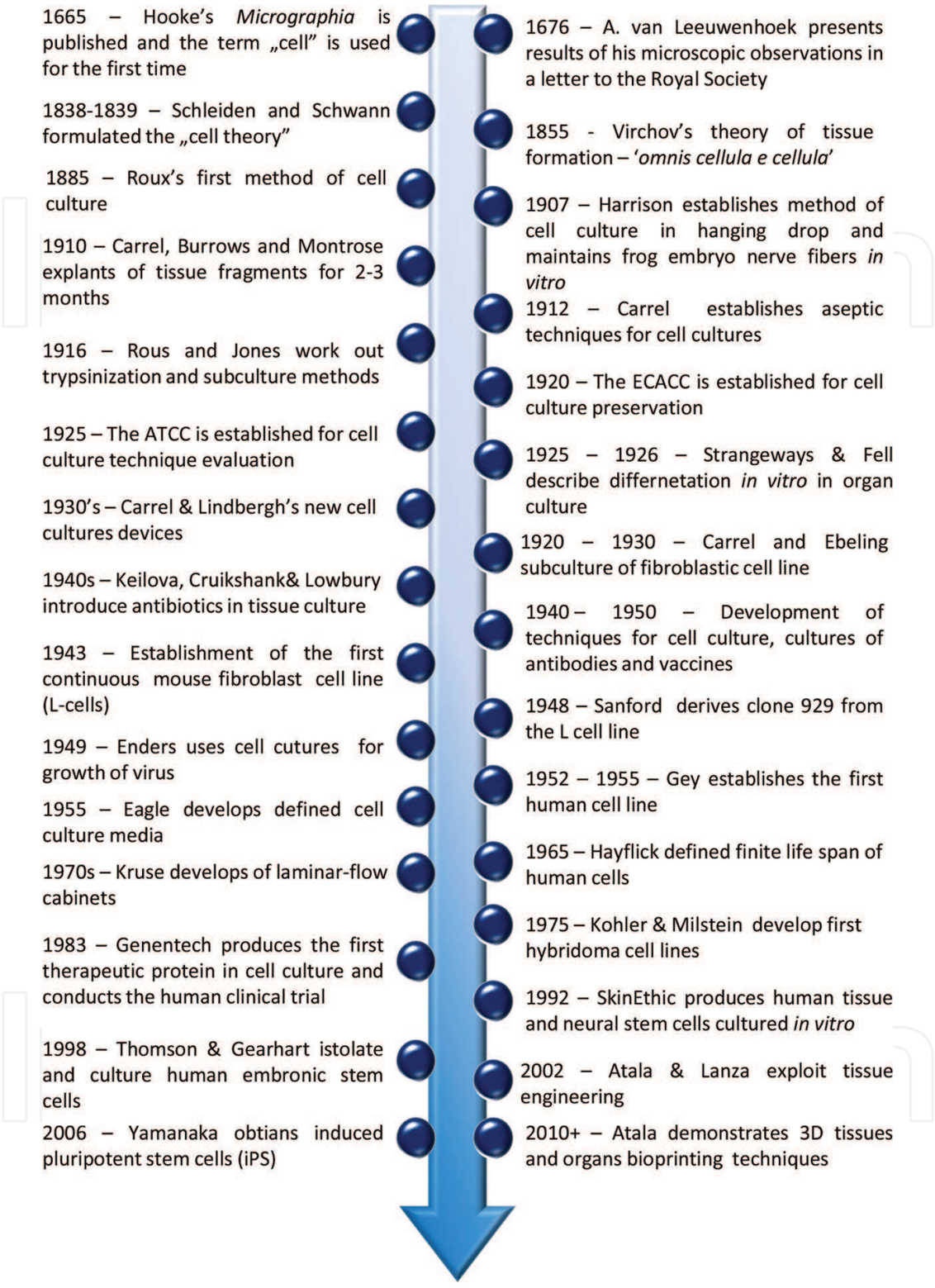

Figure 1. Timeline: key milestone in cell cultures.

the future of regenerative medicine and in situ bioprinting of wounds. The main milestones in cell cultures are presented in proposed chapter (see **Figure 1**).

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2. Live under the microscope

The development of biological sciences would not have been possible without one of the greatest inventions—microscopes. In the sixteenth and seventeenth centuries—two countries—the Netherlands and Italy played a crucial role in constructing and using microscopes and telescopes. In the Netherlands, around 1590, Hans Janssen and his son invented a compound microscope—constructed of two convex lenses. In the early 1600s (about 1610), the great Galileo Galilei (1564–1642) constructed several simple microscopes and telescopes, which he called as "occhialino." The term "microscope" was used for the first time in 1625 by the Italian physician Giovanni Faber [1].

The first publication, in which Petrus Borellus described the use of microscope in medicine, was written in 1653. He presented 100 microscopic observations and applications (e.g., removing ingrowing eyelashes invisible with the naked eye). In 1646, Athanasius Kircher (1601–1680), a Jesuit priest, described that "in the blood of fever patients a number of things might be discovered." Kicher showed later (in 1658) that maggots and other living creatures (some of them he called microscopic "worms") occurred and developed in decaying tissues [1–3]. Two other microscopists—Swammerdam (1637–1680) in 1667 and Malpighi (1628–1694)-characterized red blood cells [1,2]. In Bologna, another scientist, Joseph Campini, illustrated the first use of the microscope in the clinical examination of a wound on the leg of a patient [1, 4].

At the beginning of the seventeenth century, two inventors—Robert Hook (1635–1702) and Antonie van Leeuwenhoek (1632–1732)-made an unusual discovery. Both of them made their first observations of life under the microscope and made the previously invisible microscopic world real [3].

The English physicist, Hooke, published in 1665 the first important work on microscope construction, its components and microscopic observations. In his *Micrographia*, he illustrated microscopic structures of many biological samples (e.g., insects, plants, sponges, bryozoans, fossils), as observed through microscopes and described the microscopic units. The "cells" or "pores", as he called small compartments of a slice of cork (thickened walls of dead cells) were chosen to refer to these microscopic units. Although Robert Hooke used the term "cell" differently compared to the later biologists, the today's term "cell" comes directly from Hooke's *Micrographia* [3, 5, 6].

In 1676, the Royal Society (RS) received a letter from Antonie van Leeuwenhoek, in which the microscopist had described his exciting discoveries—observations and records of small living particles. These microorganisms, which Leeuwenhoek called "animalcules," were mainly protozoa and bacteria [3, 4]. Implementation of his scientific project was inspired by the Hooke's bestseller, *Mircographia*. He started by handcrafting lenses and constructing microscopes (he was known to make over 500 microscopes). The Leeuwenhoek's single lens microscopes were smaller than magnifying glasses (3–4 inches long) but were capable of 270× magnifications or even more (while the Hooke's microscopes could only achieve magnifications of about 50×), with clear and bright images. The observed specimen was mounted in

front of the lens on a spiked screw [4]. Leeuwenhoek began his microscopic observations with insect samples (e.g., parts of bees) and continued with observations of spirogyra, vorticella, protozoa and motile bacteria (e.g., from the human mouth). He also examined many human and animal tissue samples, and he described blood cells (for the first time illustrated in his *Arcana* in 1695), sperm cells (he called semen "sperm animals"), skeletal muscle fibres, epithelial cells, teeth and circulatory system structures. He was the first to use histological staining (he stained muscle tissue with saffron) and described most of his observations (most of them involved microorganisms) in 560 letters to the Royal Society (RS) during his lifetime, and thus, he became the "Father of Microbiology" [1, 3, 4, 7].

Since Leeuwenhoek's invention microscopes have been one of the most fundamental tools, particularly, in the biological sciences, but also in clinical pathology and medical diagnosis [8]. In the twentieth century, many discoveries have been made in the field of life sciences, due to modern microscopy techniques. In 1941, Fritz Zernike constructed first phase contrast microscope. Another invention was a microscopic differential interference contrast technique (phase contrast) evolved by Georges Nomarski. Invention of fluorescence and confocal microscopy revolutionized life sciences. Confocal scanning microscopy gives possibility to examine fixed or alive biological specimens. This technique allows the selective and specific detection and visualization of molecules at small concentrations with good signal-to-background ratio [8]. Technique of confocal microscopy was evolved by Marvin Minsky in 1957 [9]. Confocal scanning microscopy technique is based on the restriction of photodetection to light originating from the focal point, whereas in fluorescence microscopy, the entire sample is excited indiscriminately, where the fluorescent photons arise from out-of-focus fluorofores. The optical sectioning gives three-dimensional microscopic reconstruction of biological samples. For photodamge and photobleaching reduction, the confocal microscopy was improved by the use of spinning disk scanners that were based on the disk invented by Nipkow (in 1884). Use of many pinholes enhances detection of the fluorescence and reduces excitation [8].

The fluorescent microscopy was also revolutionized by the two-photon microscopy invention. In this technique, two-photon excitation is applied, that means that using ultrafast laser (infrared) is possible to obtain locally very high photon concentration that occurs only at the focal point of the microscope. The two low-energy photons excite together a chromophore (only at the scan plane) and generate fluorescence. Use of infrared results in lowering the light scattering cross section of living tissues, which gives possibility to examine fluorophores deep in living samples. In contrast to confocal microscopy, the two-photon microscopy ensures that the problem of photodamage and photobleaching is reduced, but disadvantage of that method is worse spatial resolution in comparison with confocal microscopes [8]. In the 1990s, Stefan Hell developed super-resolution fluorescence microscopy technique and gave the scientists possibility to examine structures of the size of a few nanometers.

Immunofluorescene techniques with the new fluorescence molecules (immunofluorescence reagents, organic dyes, quantum dots) and discovery of fluorescent proteins (e.g., GFP) and use of confocal microscopy made new possibilities to examine biological specimens [10, 11]. For example, confocal microscopy allows the live-cell imaging (time-laps microscopy) to monitor cell movements, cell and tissue structures in one (1-D), two (2-D), three (3-D) spatial

dimensions or 4D—(3D × time) [12]. The variant of live-cell imaging techniques—fluorescence loss in photobleaching (FLIP)-utilizes repeated photobleaching that can be used to assess the continuity of membrane of endoplasmic reticulum or Goligi apparatus. Fluorescence resonance energy transfer (FRET) technique gives opportunity to display interactions between two molecular species. The energy transfer from fluorescent "donor" to fluorescent "acceptor" is possible when fluorofores are in nanometer proximity [12].

Using fluorescent dyes, it is possible to label live or death cell nuclei, for example, SYTO59 or SYTO61 for live cells, DAPI, popidium iodide (**Figure 2**), Sytox Green or T0-Pro-3 for the nuclei of death cells [12], and fluorescently labeled antibodies used for, for example, HeLa cell mitoses with anti-tubulin staning [13], anti-cytokeratin staining (**Figure 2**).

Cellular junction identification is based on detection of structural components and proteins that are associated with those components. For studying cell adhesion and cellular junctions monoclonal, polyclonal antibodies labeled with conjugates for visualization of the target cellular structures are used for gap junction-Connexin-40, CX40; Connexin-43, CX43; pannaxin (1, 2) for synapses; for tight junctions (TJ)—claudins, occludins, JAMs (junctional adhesion molecules) and CRB1 (human Crumbs homolog 1); for adherents junctions—cadherin-catenin-actin modules; for desmosomes and hemidesmosomes—cadherins (desmoglesins and desmocllins) and intergins [15–17].

Mentioned techniques can be used for determination of ion concentration, for example, pH, Ca^{2+}, K^{+}, Na^{+}, O_2, in biological systems (for example within cells) [18]. Many of fluorescence probes are ion indicators with a different fluorescence lifetime (τ_f) of the free form of probes and the form bound to ions. This property allows to selective and quantitative imaging of several different ions (pH, Ca^{2+}, K^{+}, Na^{+}) in the same time. The intracellular pH determination is commonly analyzed using c-SNAFL-1 fluorescence probe. Different values of τ_f for bounded and unbounded form of fluorophore and different emission spectra are measured. The Ca^{2+} determination can be performed with the [Ca^{2+}]-sensitive probe Fluo-3 or indo-1. The Fluo-3

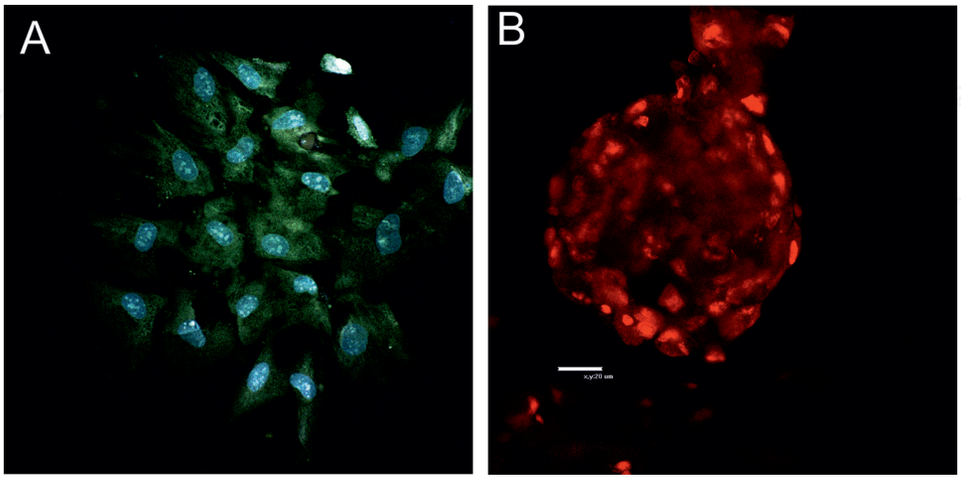

Figure 2. Bovine mammary epithelial cells immunostained against cytokeratins and DAPI-stained nuclei (confocal laser scanning microscopy, 400×) (A). Dome structures stained with propidium iodide (confocal laser scanning microscopy; magnification, 600×) (B) [14].

reacts on the presence of Ca^{2+} ions, the higher intensity of fluorescence, the higher Ca^{2+} concentration [18], whereas the O_2 concentration analysis is based on reduction of τ_f that can be also used for imaging purposes [18].

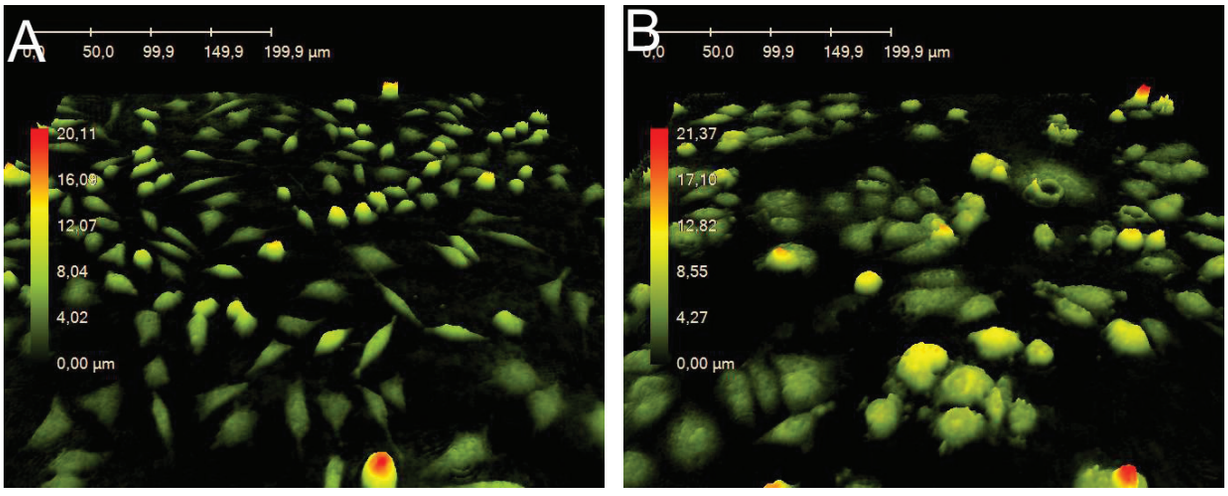

Figure 3. Cell cultures visualized using label-free holographic microscopy. L929 fibroblast cell line, 200× (A). MCF-7 adenocarcinoma cell line, 200× (B) [unpublished, Luzny and Jedrzejczak-Silicka].

Using modern microscopy technique gives the possibility to study cell structures, motility of cells and organelles, cell-cell communication and membrane potential in single cells. Microscopic techniques found important application in biomedical field (e.g., confocal endomicroscopy, oftalmology) [19, 20].

The live cells in vitro and in vivo imaging techniques accelerate drug discovery. Real-time imagine provides analysis of drug response upon target activity and pathophysiology and results in higher clinical predictivity [21]. Based on in vitro model, the monitoring of cellular phenotypes within complex samples such as co-cultures, 3-D culture models, is now possible. Cell attachment, migration (velocity, direction), vesicle formation, angiogenesis, stem cell differentiation can be recorded using automated imaging platforms [21, 22].

Some of them are based on the label-free phase holographic microscopy. In this technique, the low-power (635 nm) red diode laser divided into two beams—reference and an object beam—that passes through the unlabeled cell cultures on T-flask surface merged together can be recorded as the hologram imagines (**Figure 3**) [23–25].

Another microscope—the atomic force microscopy (AFM)-gives unique possibility to visualize structure, topography (**Figure 4**) and examine mechanical properties of cells (e.g., adhesion force distribution, cells stiffness—Young's modulus as a biomarker of the relative metastatic potential).This method is a variant of scanning probe microscopy that demonstrated better resolution, than the optical diffraction limit [26].

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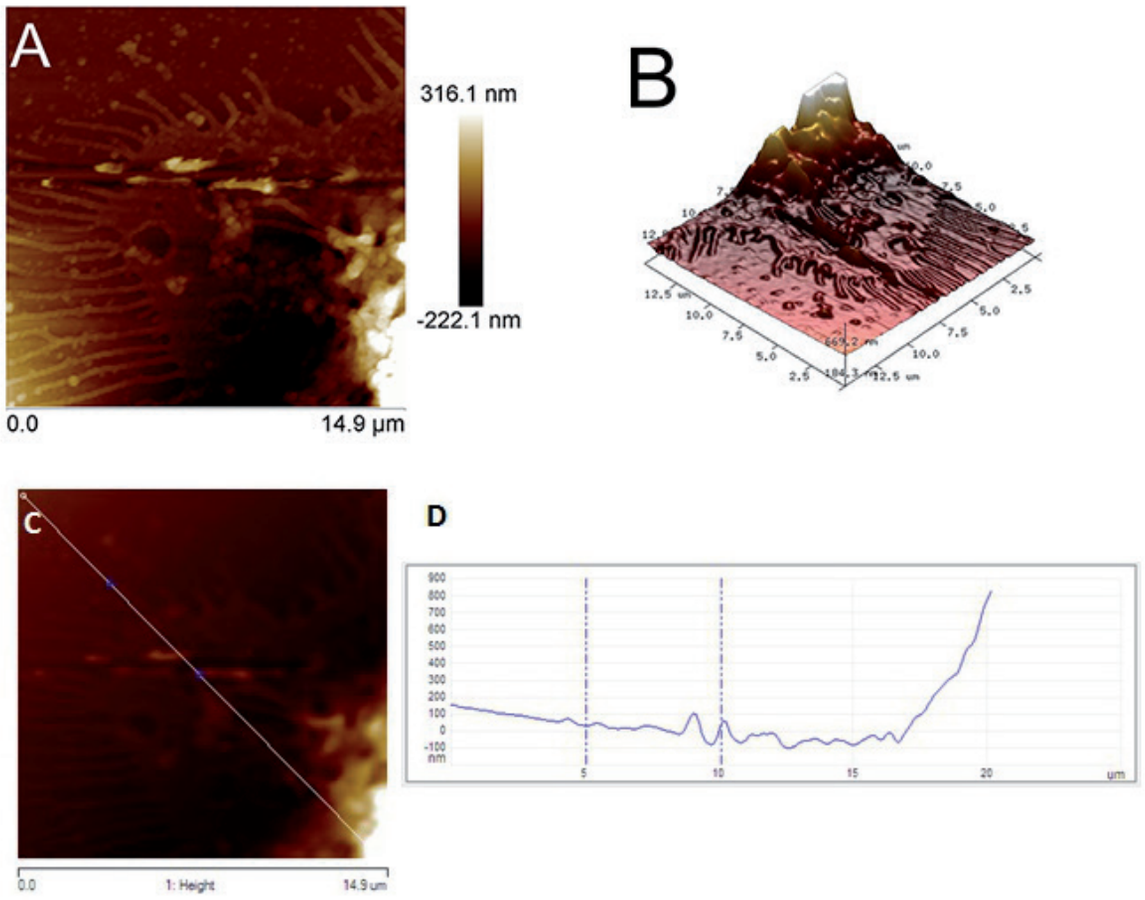

Figure 4. The peripheral MAC-T—bovine mammary epithelial cell margin recorded using atomic-force microscopy (AFM). AFM height image (A). AFM height image—3-D image (B). MAC-T cell height measurements (C and D) [unpublished, Jedrzejczak-Silicka].

3. The end of spontaneous generation

From the ancient Romans, through the Middle Ages, to the late of the nineteenth century, the Aristotelian doctrine of spontaneous generation was one of the most basic laws in biological sciences [3]. This idea was presented for the first time by Aristotle in his History of Animals, where he described the generation of insects from animal flesh, mud, and other organic and inorganic matter [27]. According to this thesis, non-living matter (water, land or hay) bears the potential to generate spontaneously different and complex organisms. For example, in the seventeenth century literature, recipes for mice were known—the mixture of old shirts and wheat placed in a jar for 21 days produced mice [28, 29]. Even the invention of the microscope and investigations of Leeuwenhoek and Hook did not refute the Aritostelian doctrine. The existence of micro-organism—protozoa, and unicellular living organisms was a specific link between the inanimate substance and living organisms, and perversely, supported the spontaneous generation doctrine [3].

The first attempt to verify the idea of spontaneous generation was made by an Italian physician Francesco Redi (1626–1697). In 1668, Redi tested his hypothesis (described in the "*Experiments on the Generation of Insects*") that maggots did not arise spontaneously in decaying tissues

(rotting meat) but developed from fly eggs. In his experiment, Redi prepared three variants of flasks with meat [27, 28]; some flasks were open to the air, some were completely sealed and the third variant was covered only with a gauze. According to his expectations, maggots appeared only in completely open flasks, in which the flies laid their eggs. Although Redi's experiment proved that maggots did not appear in meat if flies were kept away by the seal or gauze, the idea of spontaneous generation was still strongly believed. Even Redi believed that spontaneous generation was possible under some circumstances, for example, insects—gall flies—were generated by abnormal growths of plant (galls) itself [27–30].

This theory was disproved by the Italian naturalist Lazzaro Spallanzani (1729–1799) in the mid-eighteenth century. In his masterful experiments, he showed that an organism was derived from another living organism(s), and he confirmed that there was a gap between inaminate matter and living organisms [3]. He repeated the experiments of English priest and biologist John Turberville Needham. In 1745, Needham started his experimentation after reading about Leewenhoek's animalcules in the letter to RS [31]. He observed the growth of microorganisms in chicken broth placed in the sealed flask and heated for 30 min [31]. This result seemed to validate the Aristotelian doctrine of spontaneous generation; Spallanzani was intrigued, but not convinced, and suggested that microorganisms had not appeared spontaneously after the boiling process but had entered the broth from the air before the flask was sealed [28]. He found significant errors in Needham's experiments and modified previous technique on the basis of his own several hundred experiments. He placed the broth in the flask, sealed the flask, created a partial vacuum and then boiled the broth [28]. The results of the experiments clearly demonstrated that the infusoria (a class of aquatic microorganisms, including primarily the organisms which now are classify as Protista) did not generate spontaneously in sterilized flasks [27]. His assiduity earned him success in disproving the validity of the theory of spontaneous generation, but even supporters of empirical evidence of spontaneous generation argued that he had only proven that spontaneous generation could not occur without air [28, 31, 32].

Finally, the spontaneous generation doctrine was laid by Louis Pasteur (1822–1895) [3]. Between 1860 and 1862, the young French chemist performed a lengthy series of experiments that were a variation of Needham and Spallanzani methods. His experiments focused on the development of microbes in the previously boiled infusions. Pasteur' s experiments were performed in a series of flasks with their necks heated in a flame and drawn out into a long "S" shape, like a "swan neck." The "swan-necked flasks" were prepared after flasks had being filled with the pre-boiled infusion (liquid was heated to 100°C and boiled for several minutes) [30, 33]. Air could enter to such a flask, but not micro-organisms. When Pasteur tilted the flask, the broth reached the lowest point in the neck and airborne microorganisms could have settled by the gravity. In addition, when the neck was broken off, the dust particles entered the flasks [28, 30]. The effect of this action was rapidly visible—the yeast/sugar water infusion became cloudy with microbes. Based on the obtained results, Pasteur concluded that microbes and their germs were carried out with the dust particles. When the dust was excluded, the infusoria was not altered. This experiment not only refuted the theory of spontaneous generation, and proved that the living matter can only arise from pre-existing life, but also demonstrated that micro-organisms are omnipresent—even in the air [28, 30]. Even

after the presentation of Pasteur's result, some of his opponents suggested that his experiment proved only that dust was necessary for spontaneous generation [30]. But what would have happened if Pauster had tried his famous experiment with the "swan-necked flasks" and boiled hay infusion? About a decade later, it was found that the hay bacillus—*Bacillus subtilis*—produced heat-resistant endospores; therefore, the result of the experiment could have been different, and the discussion about the theory of spontaneous generation might have not had ended [30].

The results obtained by Pauster were validated in practice by Lister (1827–1912). Pauster's conclusions about the prevalence of microorganisms in the air were taken into account in his pioneering antiseptic surgical procedures [34]. Lister suggested that microorganisms caused infection and gangrene similarly to Pasteur's fermentation process [36, 37]. He prevented wound infections in his patients using spray (Richardson's hand spray) and a solution of carbolic acid as an antibacterial agent. Lister later used in his aseptic methods a large hand-operated tripod to achieve germ-free conditions [34, 35]. Thanks to this technique, the end of the nineteenth century was the beginning of aseptic surgery and also symbolically the end of the Aristotelian doctrine.

4. The cell theory

At the same time, when the great debate about the spontaneous generation was held, other observations were made focusing on cell components and organization of the living matter. In the first decade of the eighteenth century, nucleus was probably observed in plant and animal tissues, but the first description of nuclei in epithelial cells was made by Felice Fontana (1730–1805) and published in 1781 in the *Traitésur le Vénin de la Vipére* [3, 37]. The term "nucleus" (literally "little nut" in Latin) was introduced 50 years later, in 1831, by the distinguished Scottish botanist Robert Brown (1773–1858). On the basis of his microscopic observations of orchid leaves, Brown found that this structure is essential in living cells. He described the nucleus as "a single circular areola generally somewhat more opaque than, the membrane of the cell, is observable…. This areola, or nucleus of the cell, as perhaps it might be termed, is not confined to the epidermis, being also found not only in the pubescence of surface, particularly when jointed, as in *Cypripedium*, but in many cases in the parenchyma or internal cells of the tissue" [3, 38–40]. In his publication (1827), Brown also described the first observation of Brownian Movements in *Clarkia pulchella* pollen [39, 41].

Technical improvements in microscope constructions helped in 1838 the botanist Matthias Schleiden (1804–1881) and in 1839 the zoologist Theodor Schwann (1810–1882) to formulate the "cell theory" [3, 42]. They suggested that every organism and every structural element of plant and animal tissues are formed of cells. Schleiden studied the structure of plant tissues and concluded that all plant structural elements are composed of cells or their products. He also properly noticed that the "increase in the size and number of cells is responsible for growth" [43]. Although his atomistic conclusion of the "cell theory" was proper, the reminiscence of the "spontaneous generation" doctrine influenced his theory of "free cell formation." According to this theory, the formation of a nucleus of "crystallization" within the cytoblast was the first

phase of cell generation. Subsequent to nucleus formation was the enlargement of condensed material leading to the formation of a new cell [3]. This theory of "free cell formation" was rejected by scientists of that time—Robert Remark, Rudolf Virchow, Albert Kölliker [3].

A year later, Schwann examined animal tissues and also observed that "the elementary parts of all tissues are formed from cells" and that "there is one universal principle of development for the elementary parts of organisms…and this principle is in the formation of cells" [3, 44]. He argued that even "the highly differentiated organisms (plants and animals)…are the formation of cells." In his Untersuchungen, he wrote that "the tissues of animals are formed of cells. The globules of lymph, pus and mucus are cells with their walls distinct and isolated from each other. Horny (squamous) tissues are cells with distinct walls, but united into coherent tissues; bone and cartilage are formed of cells whose walls have coalesced; fibrous tissue and tendon are cells which have split into fibres; and muscle, nerves and capillary vessels are cells of which both the walls and cavities have coalesced" [43, 44]. Schwann defined that a cell has three essential elements—nucleus, a fluid content and a wall (or membrane) [43, 45, 46]. Other fundamental principle formulated by Schwann (partially as a token of gratitude to his colleague Schleiden) determined that the "cells arise inside and near other cells by differentiation of a homogenous primary substance called the "cytoblastema" in a process analogous to crystallization" [47, 48]. After Schwann's conclusion pertaining to morphological units of tissues and organs, two histopathological atlas texts were published by Julius Vogel and Herman Lebert. Eventually, the formulation of the "cell theory" provoked the scientist in the nineteenth century to verify the Aristotelian doctrine and accepted the "cell theory" as a scientific fact [42].

Meanwhile, Virchow and other scientists presented the view that cells are formed *via* scission of preexisting cells. Virchow formulated (influenced by Remark) an aphorism—*omnis cellula e cellula* (published in 1855)—that became crucial in the theory of tissue formation and part of the biogenic law. He stated in 1858 that "where a cell exists, there must have been a preexisting cell, just as the animal arises only from an animal and a plant only from a plant" [3, 49, 50]. The cell was described as the fundamental unit of life, but also the basal element of pathological processes. In his publication—*Cellularpathologie*—he created a pathogenic concept that all diseases are the result of changes in normal cells [3, 49]. In the publication of *Cellular Pathology* (1863), Lecture III is titled "Physiological and Pathological Tissues" and in it Rudolf Virchow tried to explain problem of the pathological tissues, called by him "neoplasm." In his work he stated that "…every pathological structure has a physiological prototype, and that no form of morbid growth arises which cannot in its elements be tracked back to some model which had previously maintained an independent state in the economy." In that statement the physiological prototype is healthy or normal state. The diseases state as opposed to normal state was described as "…a physiological type can be found for every pathological formation, and it is just as possible to discover such types for the elements of cancer…." The transition from the healthy to the neoplastic state is the effect of mutation (now we use the term—somatic mutation). In his work, the term "histological substitution" was used to describe that can occur in diseased conditions—"…a given tissue is replace to another; but even when this new tissue is produced from the previously existing one, the new formation may deviate more or less from the original type. Therefore, there is a great chasm between

physiological and pathological substitutions, or at least between the physiological and certain forms of the pathological ones." In 1859, Virchow advanced his theory that abnormal changes in the cells derived by common descent from a "germ cell" could lead to a disease such as cancer. The "pathological substitution" and cancers Virchow indentified histologically as lines of cells with "bad behaviour" and presented the human diseases as a result of "civil war between cells" [51].

On the contrary, Louis Pasteur developed the germ theory of diseases. Pasteur's theory was rejected in its entirety by Virchow who was convinced that the diseased tissue was caused by changes within healthy cells, but not from invasion of other organisms [49]. Virchow tried to understand the nature and origin of cancer, and some of his theories were correct; nevertheless, Pasteur was also right about the causality of diseases [45, 49, 51].

5. Harrison's hanging drop technique and Dr. Carell's immortal cells

In the late nineteenth century, Wilhelm Roux (185–1924) demonstrated that it is possible to maintain living cells (of the neural plate of chick embryos) outside the body in saline buffer for a few days [52].

At the same time, Leo Loeb (1869–1959) evaluated a technique called "tissue culture within the body." In this technique, Loeb was able to culture cells from inside and outside body tissues. For example, he placed skin fragments of guinea pig embryo in agar and coagulated serum, then grafted them into adult animals. Using this procedure, Loeb obtained reproduction of mitotic epithelial cells. This technique was not strictly considered as a classical cell and tissue culture, due to grafting tissues and fluids in living animals [52, 53].

The American embryologist Ross Granville Harrison (1870–1959) developed the first techniques of cell culture in vitro in the first decade of the twentieth century [52–56]. In Harrison's experiments (1907–1910, at the Yale University), small pieces of living frog embryonic tissue were isolated and grew outside the body. He placed the tissue in a solution of lymph on a coverslip, inverted the material on a glass slide with a depression in it and maintained the explanted tissue in a hanging drop [54, 57]. Harrison's method, although adapted from microbiological technique and used for bacteria studies (invented by Robert Koch in the 1880s and first used for anthrax bacilli growth), was successfully applied to cell cultures [52, 54]. Harrison's experimentations made the cell life "visible." In his research article "Observations on the Living Developing Nerve Fiber," he described a method of maintaining nerve cells and was able to monitor fiber development [52, 58–60]. He noted "the development of the nerve fibers by independent growth from cells outside the body" [59]. The development of the nerve fiber was a continuous process from a single cell, in parts from chain of cells, or progressed within plasmatic bridges that remained between embryonic cells after their division [54, 60]. The use of a clotted lymph and special technique helped Harrison in the presentation of nerve outgrowth from tissue explants into the medium, but unfortunately, Harrison's observations were time-limited by rapid bacterial contaminations. For that reason, Harrison introduced aseptic techniques in working with cell cultures. The glassware was flamed,

chirurgical equipment (e.g., needles, scissors and forceps) was boiled, and the cloths and filter papers were autoclaved. Aseptic technique made it possible to obtain sterile preparations that could be maintained in vitro for over five weeks. Due to changes in sterile tissue preparation, Harrison was able to report various stages of cell development in a continuous manner over time. Drawings of observed nerve fibers were made with a camera lucida [54]. Thanks to the development of his technique, Harrison shed light on enormous possibilities of cell and tissue culture application not only as a tool in bacteriology, embryology, physiology or histology studies, but also the production of monoclonal antibodies, vaccines and drugs [52].

In 1910, Montrose Burrows (1884–1947) visited Harrison at Yale and adapted the method of hanging drop cell culture to his experimental requirements [58, 59]. Burrows worked with warm blood tissues, in which the chicken plasma clot was used [54]. Plasma was much easier to obtain and was more homogenous in quality, and thus, the preparation process was more reliable [52]. Then, together with Alexis Carrel (1873–1944) at the Rockefeller Institute for Medical Research in New York, they established cell cultures of embryonic and adult tissues (connective, periosteum, cartilage, bone, bone marrow, skin, kidneys and thyroid gland) of many species (e.g., dog, cat, chicken, guinea pig, rat) that could be maintained in vitro, due to the "plasmatic media"—fresh plasma from the same source as the tissues [52, 61, 62]. Burrows and Carrel evaluated other culture media composed of diluted plasma with different salt and serum solutions [52, 63]. Using complex media, they were able to subculture and maintain cultures for several months. They worked not only with normal adult mammalian tissues, but also with cancerous tissues. Those changes distinguished Burrows and Carrel's cultures from Harrison's and gave them the possibility to introduce the idea of continuous culture—obtaining new cultures from the old ones, without establishing primary cultures from new tissue explants [54, 64]. The results obtained by Carrel and Barrows were published in the Journal of the American Medical Association in 1910, and the term "tissue culture" was defined for the first time in 1911 as "a plasmatic medium inoculated with small fragments of living tissues." The introduced term "tissue culture" described also the growth and reproduction outside the body [54, 60].

In January, 1912, Carrel and his coworkers developed the first "cell line" derived from the fragments of explanted chicken embryo heart [52, 61]. This cell line was subcultured hundreds of times, and after the initial contamination outbreak, it was continued by Arthur Ebeling in Carrel's laboratory. This cell line was maintained by washing with Ringer's solution and medium changes [65, 66]. Due to the rigorous aseptic techniques, this is one of the most famous cell lines (described in many articles, e.g., cell line birthday was celebrated annually in the New York World Telegram); it was maintained until 1946 when the cell line culture was finally terminated, 2 years after Carrel's death [61]. Carrel's cell line was a phenomenon for scientists. Indefinite growth of Carrel's cells was evident, and it was defined that cells could live indefinitely except for some lethal circumstances [67, 68]. Problems with obtaining indefinite growth of cells were attributed to the inadequacies of the technique. In 1956, Haff and Swim described cell aging in vitro, but they stated that their failure to obtain an immortal cell line was caused by deficiencies in the culture medium [67, 69].

The success of Alexis Carrel and his laboratory was not only possible due to the rigorous aseptic techniques, but also due to the development of first practical cell culture flasks (in 1923),

which were called "D flasks". This culture flask (also called a D-3.5 flask) had a diameter of 3.5 cm and was made of PYREX glass. New cell culture flasks allowed to culture cells in a larger medium volume and made culture maintenance much easier [61].

In 1938, Carrel published the book "The culture of organs," in which he presented the cultivation techniques of whole organs. Carrel started a collaboration with Charles A. Lindbergh in 1930. They worked on the process of organ perfusion, such as whole heart perfusions of cats and kittens. Organ perfusion was carried out through the aorta with Tyrode's solution supplemented with 50% serum at 37°C [62, 65, 66].

6. Evaluation of cell culture techniques and establishing principles in cell culture maintaining

The early of twentieth century was the time when the basic principles for plant and animal cell cultures in vitro were developed [70]. Evaluation of cell culture knowledge was possible not only due to hanging drop culture technique. The significant impact on cell culture development had introduction of the aseptic techniques and Rous and Jones tissue trypsinization technique [70–72]. Rous and his colleague found that use of trypsin solution results in obtaining single cell suspension and cells detachment for subculture. The 3% trypsin solution was used successfully for plasma digestion and did not damage most cells. When 5% trypsin solution was tested, obtained cells were dead [72]. Until then, cultures were obtained from the tissue explants, and use of trypsin facilitated procedure of obtaining homogenous cell strains [73].

6.1. Cell line cultures development

The first cell line—the "L" cell line—was established by Earle in 1948. This cell line was derived from subcutaneous mouse tissue [70, 71] and displayed quite different morphology from the origin of tissue [70].

In 50s and 60s, another diploid cell lines were developed—HeLa (by Gay, see subsection 7) MRC-5 (by Jacobs) and WI-38 (by Hayflick and Moorhead) from human tissue and Vero (Verde—French for green and RenO—French for kidney) cell line obtained from simian tissue [70, 74]. The examples of the earliest derived cell lines are presented in **Table 1**.

The establishment of cell lines gives possibilities to determine differences between cell lines culture and the primary cell cultures. The primary cell cultures are obtained directly from the tissues or organs and are considered primary until the first passage (subculture). The primary cell cultures are mainly initiated from normal or malignant adult tissues and embryonic tissues. The population of cells in primary cultures prepared by tissue disruption (using enzymatic or/and physical methods) is mixed and contains different cell types. This type of culture is used in many areas such as physiology and cellular metabolism, cytogenetics, pharmacology or tissue engineering [70, 76]. Subculture technique allowed researches to obtain cell lines by serial subculture cells from primary cell cultures. The cell lines established from normal tissues display finite growth (see Section 6.3—Hayflick phenomenon). In the contrary, cell lines obtained from cancerous tissues were able to indefinitely proliferation. The indefinitely

Name	Species and tissue	Morphology	Author and year of origin
L929	Mouse connective tissue	Fibroblast	Earle, 1948
HeLa	Human cervix	Epithelial	Gay, 1951
CHO	Chinese Hamster ovary	Epithelial-like	Puck, 1957
MDCK	Canine kidney	Epithelial	Madin and Darby, 1958
WI-38	Human lung	Fibroblast	Hayflick, 1961
BHK-21	Syrian Hamster kidney	Fibroblast	Macpherson and Stoker, 1961
Vero	African Green Monkey kidney	Epithelial	Yasumura and Kawakita, 1962
NIH 3T3	Mouse embryo	Fibroblast	Todaro and Green, 1962
MCR-5	Human lung	Fibroblast	Jacobs, 1966
SH-SY5Y	Human neuroblastoma	Neuroblast	Biedler, 1970

Table 1. Commonly used cell lines [75].

proliferation of cells from normal tissues was also described and was in general the result of spontaneous transformation (see Section 8). Different cell lines are commonly used in many valuable studies, but use of cell lines also has same disadvantages and limitations, especially in drug development. The main disadvantages and limitation of using cell lines are listed below:

- The genetic aberrations of cell lines related with increasing passage numbers,
- The genotypic and phenotypic drift in continuous cultures, especially deposited in cell banks for many years,
- The cell line response toward the tested drug might be different form patient response toward the same drugs,
- Different microenvironments of the original tumor and cancer cell cultures (2D and 3D),
- Cross-contamination of cell cultures with HeLa cell line (it was reported that a large number of cancer cell lines are cross-contaminated),
- Culture conditions can change the morphology, the gene expression and several cellular pathways,
- Infections with mycoplasma that can change the culture properties,
- Difficulty in the establishment of long-term cancer cell lines of certain types of tumors,
- Cell culture environment is different from that of the original tumor,
- Loss of the natural heterogeneity of the tumor or tissue [77, 78].

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6.2. Cell culture conditions

On the basis of different experiments on cell cultured in vitro, the conditions and physico-chemical properties of environment for the growth and maintenance of human and animal cell cultures were established (see **Table 2**).

In the 1920s, composition of salt solutions was formulated specifically for cell cultures, for example, Pannett and Compton (1924), Gay (1936), Earle (1943) or Hanks salts (1948). Establishing formulas of salt solutions was the first step to define cell cultures requirements. The scientists indentified the most needed components for cellular metabolism, such as amino acids, salts, vitamins, hormones and glucose. Between 1932 and 1962, about 60 chemically defined media were worked out [80], for example, Morgan, Morton and Parker develop media199, and Earle and his coworkers worked out protein free media for L cell culture. In that time, EM medium—Essential Medium, and DMEM—Dulbecco Modified Eagle's Medium, were also develop with essential and nonessential amino acids [71]. Media were also divided into two types of media that were worked out—media for long-term and short-cultivation, for example, Trowell's medium T8 (1959) for organ culture [80, 81]. In 50s and 60s, different scales (small and large scale) of cell cultures were worked out. The large-scale cell culture development has allowed the creation of Salk vaccine for polio infection. The polio virus was cultured in simian and human kidney cells [70].

Nowadays, cell culture media are usually supplemented with the antibiotics, but first effect of antibiotics on cell cultured in vitro was established in the 1940s. Herrell and coworkers found that the different preparation of penicillin exhibited toxic action on mitosis due to some impurities in penicillin preparation. In the comparison with penicillin G, it was practically harmless for cells [82, 83]. Keilova presented in her work influence of streptomycin directly on the explants of heart, aorta and frontal bone of the chick embryo [84]. It was also found by Lawrence that in higher concentration, antibiotics (including penicillin, streptomycin, tetracycline and neomycin) affected not only migration of epithelium around skin explants, but also in some concentrations caused respiratory damage or necrotic changes [83]. In other study, Krueger analyzed effect of streptomycin on protein synthesis in mammalian cells and found that this antibiotic altered the in vitro synthesis of antibody to phage MS-2 in spleen and lymph node cells from immunized rabbits [85].

For protection or for cleaning up the cell cultures, combining of antibiotics with specific antisera or chemical can be used [86]. For fungus or yeast antifungal agents, for example, Amphotericin B (Fungizone) and Nystatnin can help to prevent their growth but will not eliminate them [86]. Mycoplasmas are theoretically not susceptible to common antibiotics such as penicillin and its analogues. Some studies report that several bacteriostatic antimicrobial agents inhibit the growth of mycoplasmas, but not eradicate the contaminants. On the other hand, using of antibiotics causes antibiotic-resistant strains development [87]. The antibiotics such as aminoglycosides and lincosamides are highly effective in mycoplasma elimination. It was also found that tetracyclines and quinolones are highly effective against mycoplasmas. The quinolones—ciprofloxacin, enrofloxacin—are commercially available as mycoplasma removal agent (MRA). Other product—BM-Cyclin—contains the macrolide tiamulin and the tetracyclineminocycline [88, 89].

Factors	Characteristic
Growth substrates	The surface for cell adhesion, growth, proliferation that determine also cellular secretion activity of cells. Earlier the glass surface was widely used, now in most of laboratories plastic (usually polystyrene) labware is used for typical monolayer cultures. The surface of that cell culture vessels can be enhanced by coating with proteins, such as collagen, gelatin, laminin, fibronectin that are components of extracellular matrix. For that purpose also polymers can be used, for example, poly-L-lysine or other commercial matrices [52, 54, 79]
Media and other components	
Media	Are composed of two main components: a basal nutrient medium and supplements. The balanced salt solution, for example, DPBS, HBSS, EBSS, form basis of complex media. The supplements complete media with nutrients, proteins, amino acids, buffering system and vitamins. The most popular media are Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimal Essential Medium (EMEM), Medium 199 (M199), Roswell Park Memorial Institute (RPMI–1640) or Lebovitz Medium (L-15) [73]
Amino acids and vitamins	The amino acids essential for growth and cell proliferation, for example, cysteine, L-glutamine and tyrosine. For proper metabolism, cells require B vitamins (especially presence of B12 is essential), choline, folic acid, inositol, biotin [52, 73, 75]
Ions and trace elements	The major ions—Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, PO_4^{3-}, SO_4^{2-}, HCO_3^-—affect osmolarity of culture media. Trace elements such as zinc, copper, selenium and tricarboxylic acids intermediates are used in cultures madia [52, 75]
Carbohydrates and organic supplements	Glucose is mainly used as an energy source [33], but in some cell types galactose, mammose, fructose or maltose can be used [56, 59]. Other sources of carbon provide nucleosides. The culture media can be also supplemented with pyruvate, lipids (cholesterol, steroids, fatty acids), citric acids intermediates [52, 75, 79, 80]
Serum	Serum is a complex mixture of proteins, source of minerals, lipids, hormones, and growth and adhesion factors. Fetal bovine serum (FBS) and newborn calf serum (NCS) are most common. For more specific cultures human, horse or rabbit sera are used [52, 73]
Antibiotics and antimycotic solutions	Antibiotics and antifungal with laminar flow hoods reduced the frequency of contamination. In cell cultures most often penicillin streptomycin solutions are used. As the antimycotic agents the kanamycin or amphotericin B are applied [52, 73, 79]
Growth factors and hormones	Hormones and growth factors are used especially in serum-free media. Those factors are ensured cellular growth, division, and differentiation. The most popular are fibroblastic growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) or platelet derived growth factor (PDGF). In the group of hormones the most common are hydrocortisol and insulin [52, 73, 79]

Factors	Characteristic
Physico-chemical properties of cultures in vitro	
Potential hydrogen (pH)	For animal and human cells a pH was determined in the range of 7.0 -7.4. Some differences can be noticed for transformed cells (7.0–7.4), and in some cases cells require higher pH levels, for example, normal fibroblasts (7.4–7.7). In the range of 6.5–7.0 cells stop growing, and between pH 6.0–6.5 cells losing their viability. The pH level can be checked by presence of phenol red in culture medium [52, 73, 79]
Oxygen	The oxygen, as a part of the gas phase is required for adequate cell physiology, function, and differentiation. The oxygen requirements are depend on the type of cells. In general, low concentrations of oxygen are used and depend on the dissolved oxygen in culture media. The higher concentration of oxygen can inhibit cell growth and metabolism. In some cases transformed cells can be anaerobic [52, 73, 79]
Carbon dioxide and bicarbonate	The buffering system is essential to maintain proper pH. For establishing physiological pH for cells CO_2 is dissolved in the culture medium. Carbon dioxide establishes equilibrium with HCO_3^- ions. The bicarbonate buffers not only show low toxicity, but also help in glucose metabolism. The other buffering system include use of HEPES buffer, but is was found that this system is toxic to some type of cells [52, 73, 79]
Temperature	Generally most of cell lines are maintained at 37°C (earlier called "warm-blood animal" temperature), but temperature is determined by origin of tissue, for example, lower temperature is usually used for skin and testicles cell cultures [52]
Osmolarity	Cells exhibit rather wide tolerance to osmotic pressure. This factor can influence on growth and cell function. In general osmolatiry should be similar to the natural tissue environment. The osmolarites between 260 mOsm/kg and 320 ± 10 mOsm/kg are applicable [52, 73, 79]
Viscosity	The important factor for cell suspension cultured in stirred vessels or when cells are dissociated after trypsinization [52]

Table 2. The cell cultures environment [71, 73, 75, 79, 80].

Besides, the culture media requirements, physiochemical conditions for cell cultures should be properly complied. Firstly, incubator was used by Robert Koch in his microbiological studies in the second half of the nineteenth century. Incubators were also used by Virchow, Pasteur or Pettenkofer in their pioneering studies [90]. Use of incubators for cell cultures was recommended by Carrel and Burrows. Working with cell cultures of "warm-blood" animals, they needed to maintain proper culture temperature [67]. Earlier, some of scientists use only warm media to work with in vitro cultures, but this method was very unsatisfactorily. The CO_2 incubators became widely available commercially by the 1960s [91]. Today, cell culture is maintained in automated incubators that ensure proper environmental conditions—temperature,

humidity and gaseous atmosphere (see **Table 2**). Most mammal cell cultures require temperature of 37°C, CO_2 in the range of 5–10% and relative humidity (RH) of 95% to minimize media evaporation and condensation [92, 93].

Development of the animal and human cell cultures would not be possible without combination of techniques that prevent cell cultures form bacterial, fungal contaminations. The early safety cabinet dedicated to microbiological researches was, for the first time, presented in 1909 by the W. K. Mulford Pharmaceutical Co., Glenolden, Pa. The first safety cabined (a ventilated hood) was designed to prevent infection with *Mycobacterium tuberculosis* during the preparation of tuberculin. The earliest publication describing microbiological cabinets was released in 1943. Van den Ende built a safety cabinet using an electric furnace to create inward airflow and to incinerate the exhaust air. In the 1960s, the laminar flow clean room, with either horizontal or vertical airflow, was developed. The clean room environment was used in the pharmaceutical industry and hospitals. The clean rooms ensured the flow of filtered air over the technician and the work material. The particles present in air were trapped in the HEPA filter, although clean rooms that fulfilled their functions were very expensive and could not be relocated when it was needed. Thus, the class II safety cabinets were developed. The principle purpose of the class II safety cabinet (laminar flow hoods, biology safety cabinets, BSCs) is the effective protection of personnel, the environment and the experiment [94, 95], for example, biological material during cell culture establishing, cells subculturing from micro-organisms. Nowadays, the laminar flow hood is equipped with a HEPA (high-efficiency particle) filter that removes particles from air that blows into the hood. The cabinets are also equipped with ultraviolet light (UVC, with wavelengths between 290 and 200 nm) that sterilize work surface of the hood [92, 96]. The UV light is used due its physical properties that are effective germicide and vircide. The UV light affects DNA by forming adducts of pyrimidine bases [96]. The main principles to avoid problems of contamination are as follows:

- Good aseptic technique—working with a biological safety cabinets (BSC), use of sterilized equipment, plasticware, glassware,
- To prevent cell cultures contamination, the copper CO_2 incubators can be used, due to inhibition the growth of many different microorganisms (e.g., bacteria, fungi, algae, yeast). The copper ions disrupt key proteins and proteins essential to microbial life [97],
- Mycoplasma testing (monthly) using, for example, PCR-based kits, DNA fluorochrome staining (**Figure 5**), autoradiography, ELISA, immunofluorescence, biochemical assays,
- Routine microscopic culture observations for microbial and yeast detection,
- Use of routine antibiotics should be avoided, and using antibiotics might cause selection of the resistant microbial strains,
- Regular filtering of culture media using 0.2 μm filters for protection against bacteria and fungi, and 0.1 μm filters to remove mycoplasma,
- Avoiding chemical contamination by testing all new lots of reagents—media, sera, trypsin, water,

- Use of medical grade gases rather than industrial grade gas mixture that may contain toxic impurities, for example, carbon monoxide,
- To avoid cross-contamination by other cell lines, monitoring cell culture program should be incorporated, for example, karyotyping, electrophoresis and isoenzyme analysis, detection of markers using immunological or biochemical techniques, DNA fingerprinting [86, 97, 98].

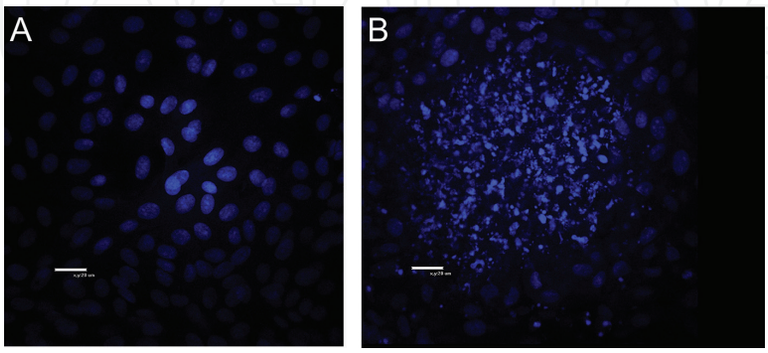

Figure 5. Photomicrographs (400×) of bovine mammary epithelial cells stained with DAPI dye. The clean culture (A) [14] and the infected culture (B) [unpublished, Jedrzejczak-Silicka].

6.3. Hayflick phenomenon

In 1961, Leonard Hayflick (1928) and Paul Moorhead defined the finite life span of normal human cells. Hayflick inspired by Carrel's observations started research on the possible viral etiology of human cancer [99, 100]. Firstly (in 1958), normal human embryonic cells were exposed to cancer-cell extracts. Hayflick expected that normal cells would change and display cancer-like properties, but normal cells did not grow any longer. Hayflick thought that he made a mistake in culture medium composition, glassware cleaning or other technical procedures. A few years later (in 1961), when working with the cytogenetist Paul Moorhead, he performed a series of experiments that validated Carrel's theory. In their work, they demonstrated that normal human fibroblasts doubled a finite number of times, stopped dividing and entered the phase III phenomenon. Hayflick divided the time of primary cell culture into three phases [99, 100]. Phase I—"or the primary cell culture that terminates with the formation of the first confluent sheet." Phase II—"is characterized by luxuriant growth necessitating many subcultivations" [99]. This phase takes about ten months, and the cells in this phase are termed "cell strains" [99, 100]. Finally, the cell strain enters Phase III. In phase III, the cells stop dividing, and the cell strain is lost after a finite period of time. On the basis of these experiments, Hayflick argued that normal cells have a finite capacity to replicate as opposed to cancer cells (e.g., HeLa cell line) that are immortal and display indefinite growth [99, 100].

Hayflick and Moorhead findings revised Carrel's idea of cellular immortality. Due to evidence of defined life span of normal cells, Dr. Witkowski conducted his own private investigations to find the answer to the phenomenon of Dr. Carrel's immortal cells. In his publication, he

presented three theories to explain why the culture obtained by Carrel and his coworkers was maintained in vitro for 34 years. The first hypothesis presented the "cell transformation theory." It is known that transformation can occur spontaneously and can also be induced by oncogenic viruses, but it is also established that spontaneous transformations occur particularly often in murine cell cultures and are extremely rare in chick cells. The transformed cells usually display changes in morphology and behavior, but Carrel's cells were described as being unchanged in appearance. Thus, Witkowski raised a question—"could the 'immortal' cells have been a spontaneously transformed cell line?" [67]. The second theory concerns cell contamination. The "immortal strain" was cultured using embryo extract, and Hayflick also noted that periods of intense cell growth corresponded to the occasions on which embryo extract was incorporated in the culture medium [67, 99]. Hayflick suggested that embryo extract contained living cells, and those cells grew and gave the impression that the original cells were stimulated by the extract. The question is Could Alexis Carrel replenish his cultures with "young" cells? [67]. The third theory was the "re-stocking" theory. It was suggested that the "immortal cells" could originate from intentionally replenished cell culture population by Carrel's technicians [67]. The presented theories tried to explain the phenomenon of Carrel's culture.

7. Immortality of HeLa cell line

In 1951, Henrietta Lacks was diagnosed with aggressive adenocarcinoma of the cervix by Dr. Jones at Johns Hopkins Hospital in Baltimore. After cervical biopsy, the samples were send to Dr. George Gay (1917–1994)—director of the Tissue Culture Laboratory [52]. His assistant, Mary Kubicek, first noticed that the cells remained viable in a nutrient solution of chicken plasma [101, 102]. She placed Lacks' specimen into the culture medium and cultured in roller tubes. Established cell cultures grew robustly, were durable and divided every 20 h. This cell line was called HeLa (derived from patient's name—Henrietta Lacks), but for years, HeLa cells were also interpreted as originating from Harriet Lane or Helen Larsen [71, 101, 102]. This situation was associated with confidential information about the originator of HeLa cells, and it was until the Obstetrics and Gynecology named Henrietta Lack as the HeLa cell source in 1971 [101–104].

In 1952, Dr. Gay and his coworkers published the results of 1-year HeLa cultures. They stated that they had established and maintained "continuous roller-tube cultures for almost a year" [105]. It was demonstrated that the cells of HeLa line grew in various media—in chicken plasma medium, bovine embryo extract and human placental cord serum [102, 105]. HeLa cell line established by Gay gave Jonas Salk and John Enders possibilities to develop poliovirus cultures in a non-nervous tissue system [71, 101, 102]. The poliomyelitis virus was successfully propagated in HeLa cell cultures by Dr. Gay [102]. HeLa cell line was cultured in almost all known culture media and was rapidly distributed to the laboratories in the United States and other countries to scientists who were interested in cancer studies. HeLa cell line was also distributed to pharmaceutical companies, and thus, HeLa cells became the most popular and valuable resource for cancer studies [96, 102].

The most famous cell line was studied intensively, in particular the mechanisms that made it so aggressive. Currently, it is known that HeLa cells were infected with human papillomavirus 18 responsible for protein synthesis that degrades the protein of the p53 tumor suppressor gene [101]. HPV18-positive HeLa cells displayed changes in microRNA expression [102]. It was also found that HeLa cells had a mutation within Lacks' HLA supergene family on chromosome 6 [101]. In 2013, HeLa genome was fully sequenced and published without the knowledge of Lack's family (later, the family has endorsed restricted access to HeLa genome data) [103]. Groups from the European Molecular Biology Laboratory and the Institute of Human Genetics (Heidelberg, Germany) determined that the insertion of HPV18 was located on chromosome 8 [106, 107]. This result was consistent with previous studies, but additionally, nine putative viral integration sites were found. It was also discovered that four of the HeLa chromosomes had been shattered and reassembled into highly rearranged chromosomes. The term "chromothripsis" was introduced to define the described phenomenon, and it was found to be associated with 2–3% of all cancers. The presence of chromothripsis was also confirmed especially in chromosome 11 [106, 107]. Other rearrangements were observed on chromosomes 5, 19 and X. The chromothripsis process is also manifested in a high number of CN (copy number along the genome) switches, high interconnectivity and alternations between a low number (2–3) of CN states [106–110]. The comparison of transcriptomes of HeLa with normalized gene expression levels of 16 tissues (from Illumina Human BodyMap 2.0) showed that 1907 genes, of which 805 genes were protein-coding, were more highly expressed in HeLa cells. Finally, 23,966 genes, of which 5593 were protein-coding, were not found to expressed in HeLa cells [107].

In the light of the results presented by Landry and his coworkers, the suggestion of the biologist Van Valen made in 1991 that HeLa cells have become new species—*Helacyton gartleri*—as a result of countless cell passages, viral infections or other cell line contaminants seem to take on a new importance [102, 111, 112].

8. Immortalization of cells and first monoclonal antibodies

In cell cultures, the transformation may occur spontaneously, and immortal cell populations were observed in many laboratories from the early 1940s to the early 1960s [113]. Immortal cells arise spontaneously from normal cells, and murine cell cultures are especially prone to that process [67, 117]. Cell cultures can be transformed by oncogenic viruses, for example, SV40 [115, 116] or by radiation (x irradiation) [114, 117] and chemical carcinogens, for example, methylocholanthracene [64, 118]. Hayflick defined the immortality term as a"life form capable of indefinite survival in conditions where no changes have occurred in molecular composition from some arbitrary beginning" [119].

Cell culture observations in the fifties brought the conclusions that cells derived from, for example, skin and muscle exhibit contact inhibition of growth. Other findings were made for cells infected with the Rous sarcoma virus. In that case, cell growth was not arrested, and it was the first evidence of cells' transformation by oncogenic retroviruses. The dense focus assay was widely used to describe oncogenic activity and indicated that the transformed

cells displayed the ability to continue proliferate even after they reached confluence. In studies focused on the transformed cells, it was also noted that those cells were able to form a multilayer on top of normal cells. It was also argued that the loss of contact inhibition was correlated with tumorigenicity. The transformed cells were able to anchorage-independent growth and proliferate in the absence of serum in medium [120]. Working with SV40, scientists developed a model-transforming virus. SV40 was used for transformations of many different animal and human cell cultures, for example, 3T3 cell line was established [117, 120, 121]. The mechanisms that play a crucial role in immortalization and transformation are not very well defined, but several cell lines provide evidence that telomere maintenance, pRB and p53 tumor suppressor protein pathways are important in these processes [120].

The first hybrid mammalian cells were obtained via viral fusion in human and mouse cells in 1965 by Harris and Watkins. In their work, they demonstrated that fusion of cells of different species was possible [122]. Using a new technique of UV inactivation, Harris and Watkins obtained heterokaryons from human HeLa cells and Ehrlich ascites tumor cells from mice [123].

Firstly, monoclonal antibodies were produced by Georges Köhler and Cesar Milstein in 1984 [52]. They described derivation of a number of culture cell lines that were able to secrete anti-sheep red blood cell (SRBC) antibodies. Cell fusions were obtained using mouse myeloma and mouse spleen cells from an immunized donor. For cell fusion, two myeloma cell lines derived from BALB/c mice were used. The P1Bu1 cell line was resistant to 5-bromo-2′-deoxyuridine and did not grow in the HAT selective medium. Thus, the cell line secreted a myeloma protein—IgG2A. The second cell line was P3-X63Ag8, derived from P3 cells resistant to 8-azaguanine, and did not grow in HAT medium. The P3-X63Ag8 secreted MOPC 21–IgG1 (κ). Cell fusion was performed using an inactivated Sendai virus. The karyotype of hybrid cells (after 4 months) was lower than the sum of the two parental cell lines. After cell fusion by Sendai virus, the cells of P3-X63Ag8 line were able to growth in HAT medium and secreted immunoglobulins that contained MOPC 21 protein [124, 125].

Through research conducted by Köhler and Milstein, medicine and science obtained monoclonal antibodies as a very useful tool for research and diagnosis that can be used in the treatment of different diseases, for example, rheumatoid arthritis, cancer, cardiovascular diseases, transplantations or infectious diseases [126–128]. For this reason, in 1984, The Nobel Assembly of Karolinska Institutet decided to award the Nobel Prize in Physiology or Medicine to Niels K. Jerne, Georges J.F. Köhler and César Milstein for theories related to "the specificity in development and control of the immune system" and the discovery of "the principle for production of monoclonal antibodies" [128].

9. Production of therapeutic proteins in mammalian cell cultures

On the basis of knowledge about the cell cycle and gene expression regulation, in 1986, the first therapeutic protein—recombinant tissue-type plasminogen activator (tPA, Activase; Genentech, San Francisco, CA, USA)—was obtained in the culture of immortalized Chinese hamster (*Cricetulus griseus*) ovary (CHO) cell line [129, 130]. In addition, many other recom-

binant protein pharmaceuticals are expressed in CHO cells and other cell lines, such as mouse myeloma (NS0), baby hamster kidney (BHK) or human embryo kidney (HEK-293). It was estimated that about 60–70% of all recombinant proteins are produced in mammalian cells [129, 131, 132]. Cells used in the recombinant protein synthesis maintain a recombinant gene (with key transcriptional regulatory elements) and a selection gene. The most popular genes for selection process are dihydrofolatereductase (DHFR), which encodes an enzyme involved in nucleotide metabolism and glutamine synthetase (GS) responsible for the expression of an enzyme that catalyzes the synthesis of glutamine from glutamate and ammonia [129].

The production of recombinant proteins in mammalian cells can be performed in two main forms: adherent cell cultures and suspension cell cultures [129]. The example of adherent cells widely used in protein production is the CHO cell line [133]. This immortalized cell line was established by Dr. Puck in his laboratory (at the Eleanor Roosevelt Institute for Cancer Research) in 1957. For establishing primary cell cultures, 0.1 g of ovary tissue of Chinese hamster was used. After the trypsinization process, cell culture was described as predominantly of fibroblast type, with a near diploid karyotype (only about 1% of the cell population had a different number of chromosomes, more or less than 2n = 22). This small difference in diploid character of primary cells is generally rare in primary cells of full diploid karyotype. After some time from establishing the culture, the morphology of cells changed, and it seemed that the cell culture underwent spontaneous immortalization. After further 10 months of the culture, other morphological changes were observed. Recloning of these cells with a modified morphology (from fibroblast-like to more epitheloid) resulted in the cell line known as CHO (or CHO-ori) [134]. The CHO cells were used for the first time in biotechnology after establishing the CHO-DXB11 cell line. This cell line established by Dr. Chasin carries a deletion of one DHFR locus and a missense mutation (T137R) of the second locus. Those changes made the cells totally incapable of the reduction of foliate to dihydrofolate (DHF). This cell line was a system for the production of human tPA in a roller bottle system. The cells are grown attached to the inner wall of the bottle filled with culture medium to 10–30% of its normal volume. The bottles are slowly rotated to assure oxygen supply and to wet the cells [129, 134]. Among other pharmaceutical proteins produced using CHO cultures are Epogen (erythropoietin), ENBREL (a TNF inhibitor) or HERCEPTIN (an anti-HER2 breast cancer antibody) (see **Table 3**) [129, 134, 135].

To scale-up production bioprocesses, adherent cells can also be cultivated in stirred-tank bioreactors. For anchored-dependent cells, (e.g., CHO) polymer microcarriers are used, and follicle-stimulating hormone and virus vaccines were produced this way [129].

The second form of the production of recombinant proteins in mammalian cells is a suspension culture. CHO cells are also capable of growing as a single-cell suspension. Cell lines, such as NS0, BHK, HEK-293 or PER-C6 (human retina-derived), are grown in suspension. Suspension cultures are optimized for a high-density cell culture in the absence of serum or other animal-derived components. In some procedures, the reduction of the temperature (to 30–33°C) and increased osmolarity are used to enhance the production process [129, 136]. Production on a higher scale is possible by using bioreactors. The main types of mammalian cell cultures are batch, fed batch, repeated batch, continuous and perfusion cultures [137].

Biotherapeutic product	Type	Therapeutic use	Manufacturer	FDA approval
Activase	Tissue plazminogen activator	Acute myocardial infraction	Genentech	1987
Epogen	Erythropoietin	Anemia	Amgen	1989
Pulmozyme	Deoxyribonuclease I	Cystic fibrosis	Genentech	1993
Cerezyme	β-Glucocerebrosidase	Gaucher's disease	Genzyme	1994
Avonex	Interferon-β	Relapsing multiple sclerosis	Biogen Idec	1996
Rituxan	Anti-CD20 mAb	Non-Hodgkin's lymphoma	Genentech, Biogen Idec	1997
Follistim	Follicle stimulating hormone (FSH)	Infertility	Serono	1997
Benefix	Factor IX	Hemophilia B	Wyeth	1997
Herceptin	Anti-HER2 mAb	Metastatic Brest cancer	Genentech	1998
Tenecteplase	Tissue plazminogen activator (engineered)	Myocardial infraction	Genentech	2000
ReFacto	Factor VIII	Hemophilia A	Wyeth	2000
Aransp	Erythropoietin (engineered)	Anemia	Amgen	2001
Humira	Anti-TNFα mAb	Rheumatoid arthritis	Abbott	2002
Raptiva	Anti-CD11a mAb	Chronic psoriasis	Genentech	2003
Xolair	Anti-IgE mAb	Moderate/severe asthma	Genentech	2003
Avastin	Anti-VEGF mAb	Metastatic colorectal cancer	Genentech	2004
Luveris	Luteinizing hormone (LH)	Infertility	Serono	2004
Aldurazyme	Laronidase	Mucopolysaccharidosis	Genzyme	2006
Myozyme	α-Gluosidase	Pompe disease	Genzyme	2006
Vectibix	Anti-EGFR mAb	Metastatic colorectal cancer	Amgen	2006
Denosumab	Anti-RANKL mAb	Osteoporosis, giant cell tumor of bone	Amgen	2010
Ipilimumab	Anti-CTLA4 mAb	Melanoma	Bristol-Myers Squibb	2011

Table 3. List of biotherapeutics approved by FDA produced in Chinese Hamster Ovary cell lines [131, 136].

Finally, mammalian cell culture ensures most often consistent glycosylation patterns and relatively homogeneous (in comparison with *E. coli*, yeast, baculovirus expression vector systems, but with minor differences between different species of cell host) [138]. The consistent glycosylation profile maintained between batches is crucial for the recombinant biotherapeutic

protein production, but the extent of glycosylation may decrease over time in a batch culture. The depletion of nutrients (e.g., glucose or glutamine) is the reason of limit for the glycosylation process, and thus, the fedbatch strategies should ensure proper concentrations of key nutrients to avoid their decrease to a critical level that could compromise protein glycosylation. Some studied presented that the pattern of protein glycosylation is dependent on the expression of various glycosyltransferase enzymes that occur in the Golgi of the cell and display different relative activity among species. Those differences can account for significant variations in structure. Complete glycosylation process is usually associated with maximization of two processes—galactosylation and sialylation that usually are incomplete and result in glycan structural variation. An alternative approach involves glycoengineering of the proteins in vitro [139].

10. Phenomenon of induced pluripotent stem cells (iPS)

In 2006, Shinya Yamanaka and his colleagues demonstrated that reprogramming of adult mouse tail-tip fibroblasts toward embryo stem cells by simultaneously induced expression of four transcription factors—Oct3/4, Sox2, Klf4, and c-Myc—was possible. Reprogrammed cells were selected by the presence of *Fbx15* gene expression, which is characteristic of early development and embryo stem cells. The induced pluripotent stem cells (iPSCs) exhibited traits of mouse ES cells but also showed differences in gene expression and chromatin organization in comparison with ES cells [140, 141]. Later, researchers have shown that the selection for Nanog expression after transduction of four factors (Oct3/4, Sox2, Klf4 and c-Myc) resulted in obtaining a more similar population of cells to ES cells. After that experiment, another discovery was made by Yamanaka and his coworkers. They introduced mouse retrovirus receptor into human cells to obtain higher transduction frequency by amphotropic retrovirus. With this procedure, 60% of cells exposed to the retrovirus expressed a reporter gene. Then, the same four genes were introduced into adult human dermal fibroblasts, and the first human iPS was created. The selection was based on morphology and growth characteristics of these cells. It was also found that each clone of iPS carried from three to six retroviral integrations for each of four factors. The very important trait of human iPS was the capacity to form tissues of all three germ layers in tissue cultures and transplantations (into immune-deficient mice) [140]. The authors stated that the reprogramming process is unknown, but they suggested that Oct3/4 and Sox2 upregulated the expression of genes related to pluripotency. These two genes upregulate "stemness" genes in both human and mouse ES cells. On the other hand, c-Myc and KIf4 may alter chromatin structure modifications, thereby granting Oct3/4 and Sox2 access to crucial target genes [140–142]. It has also been found that KIf4 interacts with p300 histone acetyltransferase and plays a role in gene transcription regulation via histone acetylation. The role of c-Myc is the induction of differentiation and apoptosis of human ESC, but the same gene in mice plays a positive role in ES [142].

Other group of scientists—Thomson and his colleagues—demonstrated that pluripotency in human fibroblasts can be obtained by reprogramming of *OCT4* and *SOX2* with *NANOG* and *LIN28* genes in human somatic cells of mesenchymal phenotype [140, 143]. Effective repro-

gramming was also evaluated in primary, genetically unmodified human fibroblasts—IMR90. The IMR90 cells were transduced with a combination of four genes (*OCT4*, *SOX2*, *NANOG* and *LIN28*), and after 12 days, the colonies of cells displayed human ESC morphology. The cells had normal karyotype and were verified by the presence of cell surface markers and genes typical for ESC. The induced cells were able to differentiate into embryoid bodies and teratomas [143].

Firstly noticed that the differences between iPSC and adult human cells were morphology and growth characteristic. Before 2009, the human iPS cells were described as highly similar to human embryonic stem cells (ES), and those similarities included morphology, proliferation, expression of cell-surface markers, gene expression (with the telomerase expression) and chromatin organization [140]. In 2009, Chin and his colleges [144] presented results obtained from the comparison of three human ESC lines and five iPSC lines. They reported differences in hundreds of genes expression. Deng et al. [145] and Doi et al. [146] reported differences in DNA methylation and indicated that epigenetic memories of donor cells in human iPSC [141].

Induced pluripotent stem cells (IPSCs) are genetically identical to the mature body cells from which they were derived. It was noticed that the same genes are chemically altered in stem cells derived from adult cells, when cells undergo differentiation, and also when the normal cells become cancer cells. The iPSCs display ability to self-renew and differentiate to every type of cells. The difference between adult and iPSCs is subtle. The study that focused on fibroblasts and the pluripotent stem cells into which they were reprogrammed shows that difference was classified as epigenetic (it was described as—what gets copied when the cell divides, although it is not the part of the DNA sequence). It is due chemical change—methylation that is associated with silencing genes. During that study, differentially methylated regions (DMRs) of genes whose expression was changed in the process of being reprogrammed from a parent cell to a stem cell were identified. The process of reprogramming an adult cell to a stem cell involves DMRs and genes. Studies based on cancer cell showed that differently methylated sites were located in cancer cells which matching up with many of the methylated areas that had been implicated during differentiation processes of normal tissues [146]. It was stated that there is the high degree of overlap between the differently methylated regions and genes that are involved in reprogramming fibroblasts into stem cells and also reprogramming a normal cells into a cancer cells [146].

In 2012, the Nobel Prize in Physiology and Medicine was awarded to John B. Guordon (for the discoveries that proved reversible nature of cell specialization) and Shinya Yamanaka (for reprogramming mature mouse cells to immature cells) [147, 148]. Both discoveries are of great importance in many areas of medicine, for example, oncology and regenerative medicine. It was reported that ESC were successfully used in cartilage repair, peripheral nerve repair or cardiac regenerative therapy. Moreover, MSC were used in certain types of therapies, for example, autologous transplantations or hematopoietic disease therapies [148].

11. 3-D bioprinting technology

ECM (extracellular matrix) development allows to obtain cell-cell and ECM-cell interactions in cultures [52]. Using 2-D cell cultures, the researchers were not able to mimic in vivo state.

The classical monolayer cultures have various limitations, for example, loss of tissue specific architecture, cell-cell interactions [56, 57]. The new techniques development helped to improve cell cultures microenvironment, for example, three-dimensional (3D) cell culture models. This technique gave possibility to achieve non-adherent (anchorage-independent) and adherent (anchorage-dependent) cell cultures. For anchorage-independent cultures, the cell aggregation can be achieved by using low-attachment surface dishes and/or coated with agarose and poly-hydroxyethyl methacrylate (pMEMA). The 3-D cultures of the anchorage-dependent cells can be obtained by using porous materials for prefabricated scaffolds that support adherence of cells [52]. The 3-D culture format gives unique possibility to analyze and to understand tumor cell growth, migration, therapy resistance. The culture of multicellular tumor spheroid (MCTS) for anticancer drug screening was developed. For this cell model, chitosan/collagen/alginate (CCA) fibrous scaffold was used, and such 3-D model gave important information about metastatic spread of carcinoma cells [56, 149]. The 3-D culture technique is based on the idea to mimic and has many advantages, but this relatively new and innovative technique displays some limitation and disadvantage that are summarized below:

The advantages are as follows:

- More representative in vitro model that exhibits biochemical and morphological features specific for the in vivo state,
- 3-D culture ensure cell-cell and cell-ECM interactions (mechanical and biochemical signals) that are essential for different processes such as differentiation and proliferation,
- This type of cultures ensure more accurate tissue-specific architecture,
- More accurate for drug and cancer biology experiments [56],
- Different types of 3-D cell culture systems, for example, 3-D spheroids grown on matrix, 3-D spheroids grown within matrix (scaffold-based 3-D culture), 3-D spheroids grown in suspension, scaffold-free 3-D culture [77, 150].

The disadvantages are as follows:

- Some matrices used for 3-D cultures are animal-derived or human-derived and have components (often unwanted such as growth factors or viruses) from that reason implementation for clinical work is difficult (risk the potential transmission of diseases),
- In some 3-D cultures, detachment of cells is difficult,
- Some existing systems fail to mimic the biomechanical characteristics of tissue in vivo represent a static condition [150],
- For scaffold-based culture systems, reproducibility between different batches is unsatisfactory,
- In synthetic scaffolds PEG-based, PEG is cell compatible but inert; cells that are embedded are not able to attach to the matrix without modifications (e.g., RGD-sites covalently attached to PEG hydrogels),

- Difficulties in encapsulated cells recovering (e.g., for isolation of nucleic acids or protein), screening and bioprocessing in 3-D culture systems like imaging tools are difficult, for example, autofluorescence of collagenous scaffold [150].

Progress in 3-D cell culture technology created the possibility of tissue engineering development and enhanced progress in the regenerative medicine [52]. Firstly, tissue-engineered cartilage was developed in nineties, and in 2013, the ear was printed using a hydrogel to form an ear-shaped scaffold and cells that formed cartilage [151]. The 3-D bioprinting technology is one of most intriguing innovation, but the idea of 3D printing is not new. The first description of 3-D printing was made by Charles W. Hull that he called his method "sterolitography" [152–155]. The formation of 3-D scaffolds for biological materials was the first step in the development of that technology. The next step was to evaluate technique that allows to print living cells layer-by-layer into special 3-D scaffolds [153].

The 3-D bioprinting technology depends on many elements such as inkjet, microextrusion and laser-assisted printing. The first inkjets printers used for bioprinting were modified version of widely available 2-D ink-based printers. In the cartridge, the ink was replaced with a biological material, and the paper with a stage with controlled elevator to control of the xyz axis. Now, inkjet printers for bioprinting applications use thermal or acoustic methods to eject drops of bioink onto substrate. The other crucial element of bioprinting is the microextrusion that usually consists of a material-handling system, dispensing system and the stage. The function of the microextrusion printers is to control extrusion of small bead of material, which is deposited onto substrate. The extrusion of material can be controlled by pneumatic or mechanical dispensing systems. The third important factor in organ or tissue printing process is laser-assisted bioprinting (LAB). The LAB device consists of a pulsed laser beam, a focusing system, a "ribbon" that provides transport of material, and substrates for cell-containing material. The materials used in regenerative medicine and 3-D bioprinting are based on natural (e.g., alginate, collagen, chitosan, fibrin) or synthetic polymers (e.g., PEG). Materials should be characterized by good printability, high biocompatibility, known degradation kinetics and byproducts, material biomimicry and proper structural and mechanical attributes [153].

The successful bioprinting process depends on cells selection for tissue or organ printing. Printing organs or tissues requires multiple cell types, for example, the primary functional cells, embryonic and induced pluripotent stem cells. The cells chosen for bioprinting must be robust to survive the printing process, and thus, in many studies, cell lines are used. For example, fibroblast or transformed cell lines are robust enough to shear stress and pressure [153, 155].

The progress in bioprinting manifests in obtained 2-D tissue such as skin, hollow tubes (e.g., blood vessels, trachea), organs as the bladder or solid organs such as the kidney [153].

The organ and tissue printing will not only solve problem of organ transplantation but will give possibilities to use those construct in drug discovery, chemical, biological or toxicological analysis, and cancer research [153, 156]. For example, the cancer 3-D tissue model was obtained for human ovarian cancer (OVCAR-5) cells and normal fibroblast [156].

12. Future of cell cultures

The future of animal cell technology will enlarge its applications, for example, use of viral vectors for gene therapy, vaccine technology, recombinant protein production for therapeutic purposes. Moreover, human cell cultures can also be used for personal therapies—gene therapies, tissue engineering, transplantation of organs. In the future, more human diseases will be treated by new form of therapies based on organ and tissue cultures [74].

Since HeLa established, immoral cancer cell lines are intensively studied as a biological models to investigate cancers biology (e.g., cancer initiation, progression, metastasis, tumor microenvironment and cancer stem cells) and evolved anticancer drugs or alternative form of therapy, for example, hyperthermal therapy, use of nanoparticles. However, many results obtained from the examination of immortal cancer cell lines suggest that cancer cell lines are not representative, due to cancer heterogeneity and drug-resistant tumors occurring in patients [157]. To solve the problems with the present standard therapy ("one treatment fits all"), two elements should be realized. The first one, that is based on the idea—"health is a molecular thing," that focused on genome-based studies and biomarkers analyses that will expand the range of diagnostic, and the cancer patients will be treated with the optimal targeted therapy [157, 158]. For example, form of personalized medicine was presented by Thomas Blankenstein and Wolfgang Uckert (Berlin Institute of Health) who are working on a T-cell therapy (using genetically modified T cells) that specifically targets mutations (mutations that lead to errors in the mechanisms that control cell division) in the genome in order to fight tumors [159]. The second one includes the derivation and short-term culture of primary cells from solid tumors to evaluate or improve personalized cancer therapy [157].

Studies with cancer cell lines give opportunity to understand of tumor biology and allow high-throughput screening for drug development. Although many important investigations were performed using cancer cell lines, the results give limited information and present low clinical correlation. The genetic aberrations of cancer cell lines that are related with increasing passage numbers are one of the reasons why this type of study does not fully represent clinical situation. Thus, primary tumor cell cultures (e.g., 3-D tumor culture derived from solid tumor specimens) can give more accurate information about individual cancer cases and support establishment of clinical setting [157, 160].

Due to specify of cancer cells, different therapeutic strategies should be chosen, for example, monoclonal antibodies, radiotherapy, chemotherapy, small molecule inhibitors, targeted therapies or combinations of two, three forms of treatment. Information about the"specify of cancer cells" is complex and includes not only tumor microenvironment and signalling pathways analyses, but also patient-specific tumor cultures for drug profiling prior to adequate clinical treatment selection [157].

Future medicine will able to use widely stem cells [adult and as well human embryonic stem cells (HESC)] for damage tissue replacement [74]. The idea of Dr. Atala's of in situ bioprinting therapy and the results obtained in that area are promising. It was presented by Albanna and his colleagues that "the skin bioprinter is able to deliver two different types of skin cells and

biomaterials directly on target locations and cover skin wounds and defects." It is possible that in the near future, use of skin bioprinters will be a useful tool in surgical reconstruction and a preferred form of therapy in wound and burns treatment [161].

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