Stem cells within established cancer cell lines: an impact on in vitro experiments

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Abstract

Cancer stem cells (CSCs) are described as cells within a tumor that are able to indefinite self-renewal, form tumors when transplanted in vivo, differentiate into multiple lineages, and express genes such as OCT3/4, SOX2, KLF4, NANOG. Although these traits of CSCs are commonly accepted, there is still a lot of controversy regarding these cells. There are very few methods which allow to obtain these cells, like separation based on surface markers, presence of side population, sphere forming assays, and aldefluor assays. This paper seeks the confirmation of CSCs presence in cancer cell lines such as: breast, prostate, pancreatic, liver, brain, and cervical. Nowadays, researchers use two models of cell culture: established cancer cell lines (ECCLs) and primary cell culture. A major problem with these models is that tumors in organism evolve and cell cultures represent only small fragment of tumor development. Since CSCs were found, there exist high hopes of revealing new therapies targeting CSCs. However, the appearance of new populations with the ability to induce tumors should pour a bucket of water to create a cure for cancer.

Introduction

The recent concerns concerning the connection between stem cells and neoplasms were present in the second half of the 19th century by Virchow and Cohnheim. Virchow’s conclusions were based on similarities between teratocarcinomas and embryonic tissue. Cohenheim’s hypothesis was that carcinoma is stem cell disorder which came from his interpretation of karyotypic chromosomal differences between epithelial and mesenchymal tumors. Next, in 1937, Furth and Kahn found evidence of existence of tumor stem cells by showing that leukemia could be transferred by a single cell in a mouse model. In the 1970’s it was discovered that only a small population (0.0001-1%) of tumor cells could induce a tumor in vivo.3 The turning point in the study of cancer stem cells (CSCs) was Bonnet and Dick experiment in which they proved that cells isolation based on CD34 and CD38 markers allows to obtain cells which differentiate and proliferate, showing the potential for self-renewal expected from the leukemic stem cell.4 From that moment CSCs were found in many tumors such as: prostate, skin, colon, brain. Several of created theories, suggest that CSCs can evade current therapies and lead to recurrence of disease.5 In order to develop effective cancer therapy, it is essential to characterize CSCs and find ways to kill them.19 It seems obvious that new therapies should focus on targeting and killing these cells. Human cancer cell lines have been used to test the functions of oncogenes and tumor suppressor genes for many decades now. In fact, most potential anti-cancer therapies are first tested in established cell lines. However, recent researches question the usefulness of established cell lines for studying cancer stem cells.13 Lately tumor initiating cells (TIC) were found in many established cancer cell lines.12,14 Nevertheless, there is still a lot of controversy regarding CSCs, e.g. Do stem cells really exist within established cancer cell lines? How big is a population of stem cells within cell line? Which methods of isolation are the best? What properties of normal stem cells they possess and what is the stemness of cancer stem cells?

Cancer stem cells hypothesis

Cancer stem cells (CSCs) do not refer to the stem cell of cancer origin, they are rather defined as cancer cells capable to initiate a tumor that replicates the original tumor when transplanted.11 However, it is still unclear how CSCs arise, although there are two controversial theories. The first is that adult stem cells in various tissues could be transformed into malignancies through multiple steps resembling carcinogenesis, during which a vast number of genes are involved. The second one is that differentiation of transformed malignant cells results in the production of cancer stem cells. In fact, both hypothesis have been challenged to be firmly supported by empirical evidences. Recent reports demonstrated that the pluripotent stem cells could be generated from somatic cells by defined factors, including OCT4, SOX2, C-MYC, KLF4, NANOG, and LIN28, and that the cells with epithelial-mesenchymal transition also have the characteristics of stem cells.15~17 But it is possible that both mechanisms described above are true and the method of CSCs development depends on the type of cancer.11 In literature CSCs are described as cells within a tumor that are able to indefinite self-renewal, form tumors when transplanted in vivo, differentiate into multiple lineages, and express genes such as OCT3/4, SOX2, KLF4, NANOG.16,17 A peculiar capacity of many normal stem cell populations is their relatively high expression of ATP-binding drug transporters (ABC) which can protect the normal stem cells from cytotoxic agents. Such a property of CSCs models would explain the persistence of resistant tumor cell populations after chemotherapy. Although these traits of CSCs are commonly accepted, there is still a lot of controversy over the molecular markers. It is well known that on the CSCs surface, markers characteristic for normal stem cells (CD133, CD44) are present. But it has been proved that cells without these specific markers (e.g. CD133) can also induce tumors in immunodeficient mice.18 Such information should change thinking about CSCs as homogenous cell populations which can be easily targeted in human body or within cell line. Since CSCs were found, there exist high hopes of revealing new therapies based on killing these cells. However, the appearance of new populations with the ability to induce tumors should pour a bucket of water to create a cure for cancer.

Isolation of cancer stem cells

Since CSCs were found in Acute Myeloid Leukemia (AML) by Bonnet and Dick, many investigators tried to improve the existing method of isolation. An enormous amount of effort was put into this procedure because the isolation of cells is a crucial step in every project and there are very few methods which allow obtaining these cells, like using surface markers, side population, sphere forming assays, and aldefluor assays.

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Surface markers

Bonnet et al., proved that it is possible to isolate population of cells which can induce tumor by using CD44 and CD38 surface markers.4 Other studies have also shown that this method is useful in the isolation of stem cells from cancer cell lines (Table 1).19,20 Even if surface markers are a serviceable tool, it is necessary to remember about the disadvantages of this method. It is unclear, however, whether those expression patterns can be used for the isolation of pure cultures or just to enrich tumor stem cells. Many of the surface proteins used, are not specific, meaning that they are also expressed on normal stem cells and tumor-initiating cells of other tumor types. Additionally, identification using antibodies can lead to false-positive results due to non-specific cross-reactivities.3

Side population

The side population method is widely used now. The principles of this technique are based on the ability of stem cells and cancer stem cells to exclude vital dyes such as Hoechst 33342 or Rhodamine 123. These cells are capable of expressing transmembrane transporters, such as the ATP-binding cassette protein, ABC transporter ABCG2/BCRP1 (breast cancer resistance protein 1).28 Moreover, the ABC transporters contribute to drug resistance in cancers (Table 2).37 A number of research groups have found that some established cancer cell lines, which have been maintained in cultures for decades are able to self-renewal in culture, are resistant to anticancer drugs including Mitoxantrone, and can form tumors when transplanted in vivo.10 There are two major problems concerning it. Firstly, toxicity: since it has been demonstrated that Hoechst interferes with C2C12 cells, as long as the dye is present in the nucleus, more and more researchers have found that Hoechst staining can affect cell differentiation. Secondly, instrumentation: Hoechst isolation technique uses Aldehyde Dehydrogenase – 1 (ALDH1) as a marker. ALDH1 is a detoxifying enzyme that oxidizes intracellular aldehydes and thereby confers resistance to alkylating agents. In fact, the detoxification capacity of ALDH1, by protecting SCs against oxidative insult, might underlie the well-recognized longevity of stem cells. ALDH1 also converts retinol into retinoic acid, a modulator of cell proliferation, which may also modulate stem cells proliferation.49 Recent studies showed that this method is valid for neuronal stem cells, hematopoietic stem cells and progenitor cells isolation.39,41 It was also reported that ALDH1 activity was used to isolate cancer stem cells from head and neck squamous cancer, colon cancer, and breast cancer.40,42,43 Cells isolated from mice brain by this technique showed signs of capability to self-renewal and the ability to generate neurospheres and neuroepithelial stem-like cells.44 Although results were promising, it is necessary to remember about certain limitations. For example, the stem cells population identified by using the aldefluor assay is probably heterogeneous, and needs to be dissected using additional markers such as CD44 and CD133.36

Aldefluor assay

Aldefluor assay is a new and promising method for both cancer stem cells (CSCs) and normal stem cells (SCs) isolation. This technique uses Aldehyde Dehydrogenase – 1 (ALDH1) as a marker. ALDH1 is a detoxifying enzyme that oxidizes intracellular aldehydes and thereby confers resistance to alkylating agents. In fact, the detoxification capacity of ALDH1, by protecting SCs against oxidative insult, might underlie the well-recognized longevity of stem cells. ALDH1 also converts retinol into retinoic acid, a modulator of cell proliferation, which may also modulate stem cells proliferation.49 Recent studies showed that this method is valid for neuronal stem cells, hematopoietic stem cells and progenitor cells isolation.39,41 It was also reported that ALDH1 activity was used to isolate cancer stem cells from head and neck squamous cancer, colon cancer, and breast cancer.40,42,43 Cells isolated from mice brain by this technique showed signs of capability to self-renewal and the ability to generate neurospheres and neuroepithelial stem-like cells.44 Although results were promising, it is necessary to remember about certain limitations. For example, the stem cells population identified by using the aldefluor assay is probably heterogeneous, and needs to be dissected using additional markers such as CD44 and CD133.36

Stem cells in breast cancer cell lines

The first breast cancer stem cells were found in solid tumor by Al Hajji et al., who used CD44+ and CD24– phenotype and showed that hundred of these cells could form tumors in

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Drug transported by protein</th>
<th>Type of cancer where ABC pomp is present</th>
<th>Table 2. The most important multidrug resistance protein belonging to ABC family.</th>
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<tr>
<td>ABCA2</td>
<td>ABCA2</td>
<td>Estramustine</td>
<td>Small cell lung cancer</td>
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<tr>
<td>ABCB1</td>
<td>PGP/MDR</td>
<td>Doxorubicin, etoposide, vinblastine, paclitaxel</td>
<td>Breast cancer,6 ovarian cancer41</td>
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<tr>
<td>ABCC1</td>
<td>MRP1</td>
<td>Doxorubicin, daunorubicin, vincristine, etoposide, camtothecin,</td>
<td>Neuroblastoma46</td>
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<td>ABCC2</td>
<td>MRP2</td>
<td>Vinblastine, cisplatin, doxorubicin, methotrexate</td>
<td>Non-small lung cancer</td>
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<tr>
<td>ABCC3</td>
<td>MRP3</td>
<td>Methotrexate, etoposide</td>
<td>Lung cancer44</td>
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<tr>
<td>ABCC4</td>
<td>MRP4</td>
<td>6-mercaptopurine, 6-thioguanine, methotrexate and its metabolites</td>
<td>Breast cancer45</td>
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<tr>
<td>ABCC5</td>
<td>MRP5</td>
<td>6-mercaptopurine, 6-thioguanine, methotrexate and its metabolites</td>
<td>Non-small lung cancer</td>
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<tr>
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<td>MRP8</td>
<td>5-fluorouracil</td>
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<tr>
<td>ABCG2</td>
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<td>Mitoxantrone, topotecan, doxorubicin, daunorubicin, irinotecan,</td>
<td>Colorectal cancer8</td>
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<td></td>
<td>methotrexate, imatinib</td>
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Table 1. Cancer stem cell markers.
mic mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors. Although the analysis of clinical samples is traditionally thought to provide more relevant data, it turned out that cell lines adapt to in vitro culture and can potentially no longer resemble their primary counterparts. However, it has been demonstrated that, despite the ability to grow in vitro, breast cancer cell lines possess similar molecular and genetic signatures to the tumors from which they have been derived. Recent studies showed that CD44+/CD24− phenotype does not allow to distinguish tumorigenic cells from non-tumorigenic cells. The SUM149 line which has only 5% of CD44+/CD24−, exhibits a similar tumorigenic potential as SUM159 and SUM319 lines which have more than 90% of CD44+/CD24−. So, it seems that there is a smaller connection between CD44+/CD24− phenotype and CSCs in breast cancer cell lines than it used to be thought. That is why additional marker should be used. Fillmore and Kuperwasser, proposed epithelial surface antigen (ESA) and they used flow cytometry to detect CD44+/CD24/ESA- cells. The percentage of these cells ranges from 0.1-0.5% in luminal lines, such as MCF7 and SUM225, to about 2.5% in the basal and mixed cell lines SUM149, SUM159, SUM315, and MDA.MB.231. Most importantly, these cells exhibit properties of self-renewal in vitro, tumors formation from very few cells, slow divisions, and selective resistance to chemotherapy. A similar experiment was made by Charafae-Jauffret et al., who tried to isolate CSCs from thirty three breast cancer cell lines by using the aldefluor assay. They managed to isolate CSCs from twenty three out of thirty three cell lines. The aldefluor-positive population (Table 3), ranged from 0.2% to nearly 100%. Moreover these cells were able to generate tumors, self-renewal, and recapitulate the phenotypic heterogeneity of the initial tumor when injected to NOD/SCID mice. Apart from results described above there are findings which suggest that the use of additional markers such as ESA and ALDH1 may not be sufficient to identify all cancer stem cell populations in breast cancer cell lines and that there is a need for a further study of this issue.

**Stem cells in prostate cancer cell lines**

Prostate cancer is the most common in the western world. The early detection of it, can be cured by surgery and radiation therapy. In advanced stages androgen ablation therapy is used, but in most cases patients die because of metastases. The presence of stem cells in prostate cancer cell lines would confirm their usefulness as a model during the development of new anticancer therapies. Patrawala et al., studied the tumorigenicity of ABCG2* (an ATP-binding cassette transporter associated with multidrug resistance) cells. Out of six prostate cancer cell lines (LNCaP, LAPC9, Du145, PC3, PPC-1) only LAPC9 contained a small population of ABCG2- cells. A hundred of these cells were able to induce tumors in NOD/SCID mice when thousands of LAPC9 ABCG2- cells could not do so. Furthermore, ABCG2+ cells possess properties such as self-renewal, expression of stemness genes, and an ability to give rise to non-side population cells. The fact that the side populations can be heterogeneous and the higher tumorigenicity of ABCG2+ cells may result from combined effects of several other populations should be taken under consideration. CD133 and CD44 markers were useful during CSCs isolation from tumor samples and the CD133 surface marker was used by Pfeiffer and Shalken to identify CSCs in prostate cancer cell lines (Du145, 22Rv1, LACP-4, DuCaP, LNCaP and PC-3). In five cell lines there was no detectable CD133+ population, only Du145 cell line had a small subset of these cells. Surprisingly, there was no difference in colony-forming assay between CD133- and CD133+ populations. It was assumed, that CD133 is not a good marker for CSCs in prostate cancer cell lines and a similar suggestion was proposed by Bisson and Prowse, who found out that CD133 selection by fluorescence-activated cell sorting (FACS) failed to enrich C4-2B cells with sphere forming ability. However, it is not clear whether CD133 is a marker or not, because Wei et al. isolated the CD44+ integrinα2β1+ cells to enrich CD133+ cells from the Du145 cell line which had self-renewal capacity, formed sphere-like clones similar to brain cancer stem cells. Regardless of results obtained from the CD133 molecule, investigators managed to isolate CSCs from prostate cancer cell lines by a sphere-formation assay. PC-3, Du145, LNCaP, VCaP, 22Rv1, LACP-4, DuCaP cell lines contained small subset of self-renewing cells with abilities to form spheres, characteristic for stem cell growth. Furthermore, these cells express the putative stem cell markers α2-integrin and BCRP (breast cancer resistance protein). All these results showed that prostate cancer cell lines have populations of CSCs but it is necessary to reveal new markers for the CSCs isolation from these cell lines.

**Stem cells in pancreatic cancer cell lines**

Three pancreatic cell lines PANC-1, PSN-1, and CFPAC-1 were capable to form spheres in cancer stem cells medium. In order to further characterization, the expression of CD24, CD44, ESA, and CD133 markers were examined. However, this phenotype was not sufficient to distinguish the sphere-capable from the sphere-incapable cells and an additional marker needed to be used. Adding CD44v6, a protein marker of metastasis in pancreatic cancer, allowed to separate the sphere-capable from the sphere-incapable cells. PanC-1 cells expressed higher levels of CD24, CD44, ESA, CD133 and CD44v6 markers than cells cultured in the adherent standard cell conditions (RPMI + 10% FBS). Moreover, cells isolated from pancreatic cancer cell lines cultured in cancer stem cells medium demonstrated a self-renewal capability and multipotentiality, which strongly suggest, that these cancer cell lines contain stem cells. More evidence for stem cells in pancreatic cancer cell lines were provided by Dębinski and Krauss, who worked on BxPC-3 and Panc0327 cells. To isolate slow-cycling cells they used a label retention method. Cells were labeled with the long lipophilic tracer dye (DiI). Cells obtained by this technique displayed multiple cancer stem cells properties. They were able to re-establish colonies of tumor tissue that were visually indistinguishable from the preselected cell population and possessed an increased invasive and metastatic potential. Also DiI+ cells had an increased tumor formation ability and could form tumors in severe combined immunodeficiency (SCID) mice. Moreover, both cell lines had population which expressed CD24, CD133, and CD44 surface markers. This data strongly suggest that BxPC-3 and Panc0327 cell lines contain subset of cancer stem cells (CSCs). Results for BxPC-3 are even more plausible if we take Yao et al. work under consideration. They used Hoechst 33342 staining and FACS analysis to isolate CSCs from BxPC-3, CFPAC-1, Mia PaCa-2, Panc-1, and SW1990 cell lines. The SP cells exhibited an increased tumorigenic ability following in vitro transplantation into BALB/C nude mice and an increased chemoresistance due to in vitro

| Table 3. The percentage of aldefluor-positive cells found in each breast cell line. |
|-------------------------------------------------|---------------------------------|------------------|------------------|------------------|------------------|------------------|
| **Results**                                     | **Breast cancer cell line**     |
| ALDEFLUOR-negative                              | T-47D, SUM185, ZR-75-20, MA-134, SUM190, SUM44, ZR-75-B, |
| ALDEFLUOR-positive 0-1%                         | MCF7, SUM225, MDA-MB-231, HS5787, MCF10A, BT-20 |
| ALDEFLUOR-positive 1-5%                         | SUM229, S68, MDA-MB-453, ZR-75-1, HCC1800, MDA-MB-436, MDA-MB-147, HCC1877, HCC1954, BrCa-MZ-01 |
| ALDEFLUOR-positive 5-99%                        | SUM149, SUM159, 194A1, SK-BR-7, HME1, |
| ALDEFLUOR-positive 100%                         | SK-BR-3, HCC38 |

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Stem cells in liver cancer cell lines

Understanding the mechanism underlying carcinogenesis within the liver, can help to reveal new therapies against liver malignancies. Isolating CSCs from hepatocellular carcinoma (HCC) cell lines using different methods. Few researchers tried to identify CSCs by population assay with Hoechst 33342 dye. They managed to identify CSCs in Huh7, Hep3B, PLC/PRF/5, HCCLM3, MHCC97-L, MHCC97-H using this technique. There was a significant difference in SP proportion for example: Shi et al., identified 28.7% of SP cells in HCCLM3 line and only 0.9% in Hep3B (comparatively to Haraguchi et al., and Chiba et al. findings), but all the SP showed similar self-renewal capability, clonogenicity and the ability to induce tumors in NOD/SCID mice.66 Transplantation of as few as 1000 SP cells from Huh7 cell line induced tumors, whereas an injection of thousands of non-SP cells failed to initiate tumors in mice model. Moreover, these cell lines expressed such genes as ABCG2, ABCB1, CEA-CAM6 which are responsible for chemoresistance.67-69 Suetsgu et al., examined three liver cancer cell lines (Huh7, hepatoblastoma cell line HepG2 and human fetal hepatocyte cell line Hc) for the presence of CD133+ cells. Only in the Huh7 cell line a population of CD133+ was found. CD133+ cells were more tumorigenic than CD133− cells and exhibited mature hepatocyte markers on lower level (glutamine synthetase and cytome E430 343).69 CD133− cells obtained by Ma et al. from Huh7, PLC/PRF/5 and HepG2 lines had not only the ability to form tumors in vivo, and high colony-forming efficiency but also could proliferate into non-hepatocyte-like, and angiomyogenic-like lineages.71 The CD133 molecule is not the only one used to identify CSCs in HCC cell lines. Recent studies validate CD90 as a marker for CSCs in some HCC cell lines. Yang et al., used this molecule to obtain tumorigenic cells from HepG2, Hep3B, PLC, Huh7, MHCC97-L, and MHCC96-H cell lines.72 There were differences among cell lines in the tumorigenic potential but in all these cell lines CD90− cells were found. Five hundred of MHCC97-L and MHCC97-H cells were able to induce tumor in NOD/SCID mice, whereas over thousand of cells from HepG2, Hep3B, PLC, Huh7 lines were needed to get the same results.73 The evidences of the presence of CSCs seems to be obvious but methods used to isolate them should be further studied. CD133 could not be found in all HCC cell lines and CD90 gave excellent results in the identification of tumorigenic cells but it is necessary to provide data about other characteristic traits of CSCs in these cells.

Stem cells in brain tumor cell lines

Kondo et al., used Hoechst 33342 dye for SP isolation from rat glioma C6 cell line and rat neuroblastoma B104 cell line.74 The C6 cells as well as the B104 cells contained 0.4% of SP cells. Furthermore, the SP cells have self-renewal capacity, and proliferate into heterogeneous population. Also the C6 SP cells can form neurospheres and produce neurons, which have NSCs (neuronal stem cells) properties. Finally, they produce tumors in nude mice with high efficiency, whereas the non-SP C6 cells do not.75 Zheng et al. and Shen et al., also found cells with stem cell properties in the C6 glioma cell line but there is a huge difference between their results and results presented by Kondo et al.13,23 They used tumor formation assay instead of the SP method and found out that C6 line is mostly composed from CSCs (Zheng et al. suggested 100% and Shen et al. suggested 80%). Moreover, it seems that Hoechst 33342 is very harmful for CSCs in C6 glioma line because after two hours of incubation with Hoechst 33342, there was only 1.8% of CD133+ cells with stem cell properties instead of 88.5%.13,76 These three works showed, that mistakes can be made very easily during researches on stem cells in established cancer cell lines, and that in fact the nature of these cells is still not known. Cruz et al., provided possible explanation to such differences and controversies in experimental findings connected with the fact that all glioma cells have stem cell properties, but their phenotype varies depending on the environmental conditions.77 The SP cells were also found in other glioma cell lines such as SK-MK-1, U87MG, U375MG, KNS42, and U251.75 The percentage of the SP cells ranged from 0.1% for the U251 cell line to 2.8% for the SK-MG-1 cell line. The SK-MG-1 cell line was chosen for further investigation. The SP cells from SK-MG-1 are capable of self-renewal, and generation of both, SP and non-SP cells, whereas few SP cells were generated from the non-SP cells. The SP cells also showed a multi-lineage differentiation potential, they could produce glial and neuronal-lineage cells simultaneously under different conditions. The SP cells formed spheres in neuron-specific medium and had a significant ability to proliferate in vitro as well as grow into xenografted brain tumors in vivo. These results suggest that SP cells from SKMG-1 cell line possess stem cell properties, like self-renewal, multi-lineage differentiation potential, and tumorigenicity.75
The goal of the primary cultures is their uniqueness. Primary cultures come from certain unique individual. Thanks to the techniques of culture, propagation, freezing and storing of cells, the primary cultures offer a possibility of performing repeatable tests on the material derived from the patient. It is very difficult to find in a laboratory conditions, the model reflecting the process of stem cells or progenitor cells maturation and differentiation. It seems, that the primary cultures in vitro can be such a simplified model illustrating the existing dependencies between stem cells and differentiated cells.85,86

The isolated cells are known to lose most functional differentiation when separated and placed in cell culture.67 The cellular identity is not lost permanently, we can make cells remember many of their original tissue specific traits by controlling the microenvironment.88 There are now much evidences that the microenvironment regulates the specificity of tissue and significantly contributes to tumorigenesis. It has to be remembered that the role of the environment in the genes expression is very important. In glioma cells for example, the expression of gene in primary cultures of tumor cells is much closer to in vivo tumors than to established cancer cell lines.89 What sounds surprisingly is that cancer cells for unknown reasons, grow much less than normal cells.90 The tumor cells when cultured to generate a cell line, are characterized with an initially slow overall growth. Loss of a microenvironment of normal cells can lead to described phenomena. The use of standardized growth conditions, the effect of the culture protocol, and the loss of cell-cell interactions can also evoke slowing growth ratio.17

The primary cultures have many disadvantages, most of all the difficulties associated with their establishment, the differences between the cell cultures derived from similar tissues coming from different organisms and the necessity of experiential assessment of cell viability.85,86 In the in vitro studies there are often the established cell lines used, both transformed and cancer. The transformed and tumor cell lines, if omitting the possibility of occurring the genetic drift and contingent viral transfection, are characterized by a stable phenotype of cells. The cancer cells in established cancer cell lines do not age in the in vitro conditions.91,92 It is thought that the established cell lines are burdened with an error arising from the lack of diversity in terms of cell aging and differentiation. Immortalized and tumor cell lines do not undergo the process of replicative senescence, which is an equivalent to the aging process of cells in the in vivo conditions.93 The primary cultures give the opportunity to study the impact of age heterogeneous cells population on the growing colonies of cells in vitro. The microscopic observation of living cells shows that at the time of establishing the primary culture, colonies of epithelial cells grow only in the places with clusters of cells, which proliferate rapidly, providing the confluence on the surface of culture dish. This observation applies to many epithelial cells, such as the epithelium of human prostate, urinary bladder of rabbit and rat, human epidermal and melanocytes.93,94,95 As a rule, setting of the primary epithelial culture is failed, when in a suspension of digested cells only a single cell are observed. The morphology of cells in the primary cultures at the time of their establishment, tends to reflect on the stem cells importance. In the clusters of cells that giving rise to the primary cultures, the progenitor cells are found.96 They can be combined, e.g. with fragments of basement membrane and contain the other cells forming a niche of normal stem cells. These results are consistent with the assumptions in other works.97,98 Stem cells give rise to epithelial cell cultures in vitro, whereas differentiated cells have inferior properties in this regard. Each digested and prepared for in vitro culture tissue must have a certain number of progenitor cells, whose potential determines an appropriate number of divisions. The proliferation of stem cells gives beginning to the colony of intensively and long-dividing cells (holoclones). The primary culture containing holoclones develops properly, and can be used for regeneration. Cells with a low ability to proliferate form colonies of paraclines. These colonies probably do not contain stem cells.99-101

The proper understanding of in vitro models, used for research purposes, should be connected with the degree of cells differentiation heterogeneity. It is considered that examining the influence of different substances on one type of cells, seemingly in the in vitro studies, there is in fact an influence of these substances on the heterogeneous group of cells with varying mark of differentiation and proliferative potential, different receptors expression and resistance to drugs examined. In spite of such looking, the in vitro cultures resemble very simplified model of tissue conditions. All the experiments on immortalized cell lines do not often give an essential results, and may even contain misleading information. Such experiments only inform us about the overall toxicity of the substance, drug or other agent to all cells. Results obtained from homogenous and heterogeneous in vitro culture models will be different. The example is an influence of doxazosin (α1-receptor antagonist), which induces apoptosis in the prostate epithelial cells. Doxazosin induces apoptosis in the epithelial cells and prostate stroma in vivo and in vitro.102 The primary culture of the prostate epithelium is composed of stem cells and differentiated cells. In the highest concentration of doxazosin (80 µM), the percentage of apoptotic cells among the primary epithelial culture cells was 50. The same concentration of doxazosin causes that only 10% of prostate epithelium stem cells were in the phase of apoptosis. The same complex at the same concentration works in a different way on whole population of cells, on stem cells, as well as differentiated cells.103 Stem cells and differentiated cells show different sensitivity to different cytotoxic agents, which induce apoptosis. It is easy to notice, how without a separate analysis of stem cells and differentiated cells the incorrect conclusions can be made.

Heterogeneous model of established cancer cell line: implications

The proper information from in vitro cytotoxic tests had been obtained only when the primary cultures were analyzed. Primary cultures show heterogeneity in the range of differentiation state of cells. It is impossible to see such differences in the analysis with the use of homogenous (from assumption) cancer cell lines or the primary cultures, not separated on stem cells and differentiated cells.89 The presented model of the heterogeneous primary culture of normal cells has its counterpart in the tumor cell culture. Among the cell lines derived from tumors, the populations of stem cells were found in the case of prostate, breast, skin, liver, pancreas, and other cancers. As a result, there can be assumed that in the cancer cell lines heterogeneity in terms of differentiation is also observed.104,105,106 The cancer also have a population of stem cells that is responsible for their growth, and other tumor characteristic biological properties.107,108 The mechanisms responsible for cell resistance against medicines, and other substances, vary between stem cells and differentiated cells.109-112 The analysis of the results of in vitro cytotoxicity tests should be based on separate analysis describing the cytotoxicity for stem cells and differentiated cells. The model of the heterogeneous cancer cell line is still a simplified model and needs to be developed. This model partially explains discrepancies between results obtained from in vitro experiments and clinical practice. With the rapidly developing techniques of cell isola-
Heterogeneity within established cancer cell lines is not just a theoretical model, but it can be used in studies of the effects of substances in vitro, probably helping to predict real drug action in vivo.19,114

Advantages and disadvantages of primary cultures and established cancer cell lines

It is well known, that validity of a model depends on how it resembles original tumor. Nowadays, in laboratory conditions two models of cell culture are used: established cancer cell lines (ECCLs) and primary cell cultures. A major problem with these models is that tumors in organism evolve.

Both models have advantages and disadvantages (Table 4). It is easy to notice, that advantages of ECCLs are the simplicity in culturing a reproducible results and their easy access. On the other hand, there are even primary cultures of tumor cells which are not immortal but their genomic stability can be better than it is in ECCLs. Moreover, their gene pattern is very similar to original tumor. It is wisely to be aware of models pros and cons before using them in experiments. It is also important to remember about the optimization of protocols.17

Conclusions

Established cancer cell lines (ECCLs) contain a cancer stem cells (CSCs) population. However, it is necessary to remember that the percentage of CSCs in cell lines, their capability to form tumors, self-renewal potential can vary strongly even among cell lines derived from the same type of cancer. It is believed that CSCs are responsible for metastasis and we hope to reveal new drugs and therapies which will effectively kill all cancer cells but the good in vitro model is essential. It is still not known whether the CSCs identified in ECCLs have the same properties as the CSCs obtained from samples provided by patients. Moreover, the percentage of CSCs in ECCLs is mostly different than the percentage of CSCs in patient samples and we need to take under consideration the evolution of cancer cell lines because of thousands of passages. Despite the fact that the CSCs are in multiple ECCLs it does not mean that they can be easily isolated due to a lot of problems with protocols used to identify CSCs. There are a few methods but all of them are very laborious, time-consuming and flawed. However, without developing reliable techniques of CSCs isolation, it is almost impossible to develop new treatment strategies in experimental and clinical oncology. Heterogeneity within established cancer cell lines will change our view on experimental works in vitro and probably lead to reevaluation of the previous results.

Table 4. Comparison of primary cell cultures and established cell lines traits.

<table>
<thead>
<tr>
<th>Traits</th>
<th>Primary cell cultures</th>
<th>ECCLs</th>
</tr>
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<tbody>
<tr>
<td>Morphology</td>
<td>Distinguishable&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Non-distinguishable&lt;sup&gt;13&lt;/sup&gt;</td>
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<tr>
<td>ABC pomp</td>
<td>/-&lt;sup&gt;19,112&lt;/sup&gt;</td>
<td>+ in CSCs&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oncogenic and anti-oncogenic changes</td>
<td>+,-3&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+,-3&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stages of tumor development</td>
<td>Only one&lt;sup&gt;17&lt;/sup&gt;</td>
<td>Only one&lt;sup&gt;17&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methylation of genes</td>
<td>+,-3&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+,(Very high)&lt;sup&gt;13,18&lt;/sup&gt;</td>
</tr>
<tr>
<td>Loss of differentiation potential</td>
<td>+,-3&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+,(Very high)&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Immortality</td>
<td>+,-4&lt;sup&gt;3&lt;/sup&gt;,&lt;sup&gt;31,32&lt;/sup&gt;</td>
<td>+,-4&lt;sup&gt;16,17&lt;/sup&gt;</td>
</tr>
<tr>
<td>Genetic stability</td>
<td>High&lt;sup&gt;35&lt;/sup&gt;</td>
<td>Low&lt;sup&gt;37&lt;/sup&gt;</td>
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<tr>
<td>Resemblance to tumor original cells</td>
<td>High&lt;sup&gt;98,99&lt;/sup&gt;,&lt;sup&gt;30&lt;/sup&gt;</td>
<td>+,(Medium)&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
<tr>
<td>Repeatability of results</td>
<td>Low&lt;sup&gt;44&lt;/sup&gt;</td>
<td>High&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heterogeneity</td>
<td>High&lt;sup&gt;98,95&lt;/sup&gt;</td>
<td>Low&lt;sup&gt;103,104&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Not stable&lt;sup&gt;63,32&lt;/sup&gt;</td>
<td>Stable&lt;sup&gt;18,106&lt;/sup&gt;</td>
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</tbody>
</table>

References

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50. Patrawala L, Calhoun T, Schneider-Broussard R, et al. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic.


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