Cell Tissue Bank
https://doi.org/10.1007/s10561-019-09780-9

FULL LENGTH PAPER

Effect of different cryopreservation regimens on Ehrlich carcinoma growth

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Received: 4 March 2019 / Accepted: 20 June 2019

Abstract The freezing rate is a decisive factor in determining the purpose of using low temperatures, i.e., for cryoablation or cryobanking of tumor cells. The research aim was to determine effect of different cryopreservation regimens on Ehrlich carcinoma (EC) growth in vivo and subpopulation composition of the formed ascites. The previously cryopreserved with slow and rapid rates EC cells were cultured in peritoneal cavity (PC) of mice for 7 days. Absolute number of cells in the PC, the subpopulation composition of tumor with flow cytometry using CD44 and CD24 markers were determined. Immediately after warming, a significant redistribution of EC subpopulation composition with a decreased content of the most tumorigenic $CD44^{high}$ cells after both freezing regimens was found. Culturing in vivo for 7 days contributed to the restoration of EC subpopulation composition, but with some a decrease in the tumor growth intensity when slow cooling was used. Rapid cooling contributed to significant inhibition of tumor growth with a reduced number of $CD44^{+}$ and increased $CD24^{+}$ cells. None of the cryopreservation regimens resulted in a complete elimination of tumorigenic $CD44^{high}$ tumor cells. The freezing rate determines the preservation of the subpopulation composition of the EC and intensity of its growth in vivo.

Keywords Ehrlich carcinoma · Tumor · Cryopreservation · Biobanking · Cryodestruction

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Introduction

Improvement of the methods of treating cancer is impossible without experimental studies using different tumor cell lines. The existing techniques of their long-term storage are first of all focused on the inoculation of cells in vitro. However, available experimental data suggest that a long-term culturing of persistent cell lines leads to a severe and irreversible loss of their important biological properties, namely: (1) enhancement or inhibition of expression of a number of genes that determine tumorigenic properties (Pandita et al. 2004; De Hamer et al. 2007); (2) decreased metastatic and migratory capacity (Vescovi et al. 2006); (3) distorted functioning of regulatory signaling pathways, supporting the undifferentiated state of tumor cells (Sasai et al. 2006; Clement et al. 2007). These properties are not restored, even when these cells undergo either heterotropic or orthotopic xenotransplantation.

Published online: 25 June 2019

Cryopreservation is an alternative way of long-term preservation of tumor cells to be further used for testing the new antitumor drugs, creation of vaccines as immunotherapeutic agents. In cryopreservation of tumor cells for biobanking, an extreme attention should be paid to the restoration of their tumorigenic potential, which is a generalized characteristic of preserving the cell structure at various levels of its organization. Therefore, the problem remains to develop such cryopreservation regimens for tumor cells, which are capable of preserving their structural and functional properties in a "native" form, i.e., prior to cryoeffect.

The use of ultralow temperatures in oncology also involves solving an important and completely opposite task, i.e. the possibility of cryoeradication of tumor, which foresees a maximal inactivation of the tumor site, minimizing its ability to metastasize. That is, in this case, the cold effect is performed in such a way as to ensure the maximum death of tumor cells. Cryodestruction of small breast tumors is believed to be a very effective and aesthetically attractive method of treating this most widespread cancer among the female population (Edwards et al. 2004; Nurko et al. 2005; Simmons et al. 2016). However, clinical data suggest the possible stimulation of oncological process following local cryodestruction of the tumor (Yamashita et al. 1982; Shibata et al. 1998). It is indicated that this may be due to a "special" response to cryopreservation of the subpopulation of cancer stem cells (CSCs) (Safranchuk et al. 2008; Hol'tsev et al. 2011) as the main functional unit of malignant tissues of any histogenesis (Al-Hajj et al. 2003; De Francesco et al. 2018). In view of this, it is relevant to optimize the conditions for cryoablation, in particular, the rate of cell cooling, cryopreservation duration, final temperature of cooling, etc.

The basic principles of cryobiology suggest that, by varying the cryopreservation conditions, including the cooling rate, it is possible to solve dualistic problems when preserving or destroying the cells of different histogenesis. Thus, the optimal freezing point of a biological object is considered to be such that overcooling is so insignificant that intracellular crystals are not formed, and the harmful effects of dehydration of cells do not manifest themselves to a large extent (Pegg 2015; Fuller et al. 2017). During slow cooling, a gradual formation of extracellular ice occurs with a decrease in the volume of cells because of their dehydration. In this case, the cell damage is associated with the "effects of the solution" against the background of an increased intracellular salt concentration (Fuller et al. 2017). During rapid cooling, on the contrary, the appearance and growth of intracellular ice are observed, that leads to cell damage (Ostankov 2007).

Investigations on cryopreservation of tumor cells have a long history. The researchers focused on the effect of cooling rates on viability of frozen-thawed cells. So, for example, Tatsutani K discovered a significantly reduced survival of cells of human primary prostatic adenocarcinoma cell line named ND-1 at a cooling rate of less than 5 °C/min and above 25 °C/min to a final temperature of − 40 °C (Tatsutani et al. 1996). At the same time, McGrath JJ did not reveal a statistically significant difference in viability of HeLa cells cooled down to − 20 °C at a rate of 5, 20 and 30 °C/min (McGrath et al. 1975). Bischof noted a slight difference in the survival rate of AT-1 rat prostate cancer cells at cooling rates of 5, 10 and 50 °C/min, and a moderate decrease in this index with an increase in cooling rate up to 100 °C/min (Bischof et al. 1997). Santos using a slow freezing rate (2 °C/min to − 180 °C) and a cryoprotective medium consisting of 6% glycerol with 3.0% Tris buffer, 1.8% citric acid, 1.3% D-fructose and 20% egg yolk, managed to obtain 85.2% of viable Ehrlich carcinoma (EC) cells (trypan blue staining) after thawing (Santos et al. 2015). Other authors have found that the EC cells can be successfully cryopreserved in ascitic fluid, without the use of classical cryoprotectants at a cooling rate of 15 °C/min down to − 80° C (Kiricuta et al. 1979). The resulting cells were kept at − 80 °C for 3 days, warmed and evaluated in vitro and in vivo. The viability of the thawed cells was 50% (trypan blue staining). After injection of cryopreserved EC to mice, the formation of 10 ml of ascites took place in four weeks, while the use of native cells resulted in the accumulation of a similar volume of ascites twice as fast, that is, in just two weeks. Authors pay attention to the fact that when using a very slow cooling rate (0.4 °C/min), the frozen-thawed cells generally lost their ability to proliferate. In some studies, the use of a two-stage freezing program was proposed: 1 °C/min from room temperature down to − 80 °C, from − 80° C down to − 196 °C at a rate of 300–400 °C/min and ascitic fluid was applied as a cryoprotectant, which allowed to obtain 65% viable cells (trypan blue

staining), to maintain their proliferative and metabolic activity (Fedets 1987). The direct immersion of samples into liquid nitrogen (300–400 °C/min) resulted in the death of 92% of EC cells (preservation of 8.1%) with a significant decrease in their functional potential. Thus, both rapid and slow cooling rates are not optimal for maintaining the viability and functional capacity of EC cells. It should be noted that not all the tumor cells are similar in their functional potential. The ascitic form of EC, a model of human breast cancer (Ozaslan et al. 2011), has been found to consist of undifferentiated cells, which, however, are in a hierarchical subordination and differ in their ability to form new tumors (Goltsev et al. 2017a, b). In particular, in studying the tumorigenic potential of different subpopulations of EC cells, it has been established that the $CD44^{-}$ fraction has a low tumorigenic activity in comparison with the $CD44^{+}$ fraction that has an increased morbidity due to the presence of CSCs with a high expression of the CD44 marker ($CD44^{high}$) (Goltsev et al. 2017a).

This is consistent with the classical data reported by Al-Hajj M and confirmed by a number of authors who have proven that the highest morbidity of human breast cancer is inherent in a minor $CD44^{+}/CD24^{-/low}$ subpopulation, which has virtually infinite self-renewal capacity and moreover it is chemo- and radioresistant (Al-Hajj et al. 2003; Koren and Bentires-Alj 2015). The very this subpopulation, in addition to a self-renewal, has the ability to form less differentiated progenitors, which make up the bulk of the tumor. Hence, heterogeneous tumors are seen as a complicated ecosystem in which even a small subpopulation (CSCs) can affect the growth of an entire tumor, and thereby actively support tumorogenesis. Therefore, the question remains unanswered: which of the subpopulations of tumorigenic cells in heterogeneous tumor pool are cryosensitive and which are cryoresistant? In other words, how their tumorigenic activity changes after cryopreservation if either slow or rapid freezing is used?

Thus, the research aim was to determine the effect of different cryopreservation conditions on Ehrlich carcinoma growth in vivo and subpopulation composition of the formed ascites.

Materials and methods

Animals

Experiments were performed in 8-month-old female BALB/c mice, kept under standard animal house conditions of the Institute for Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. The research was performed in accordance with the Law of Ukraine "On the Protection of Animals Against Cruelty" (No. 3447-IV, 2006), with meeting the requirements of the Institute's Bioethics Committee, which are in accordance with the provisions of the "European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg 1986).

Culturing of EC cells in vivo

Ehrlich carcinoma cells were grown in peritoneal cavity (PC) of the BALB/c mice. Intraperitoneal inoculations were performed in the left lower quadrant of abdominal wall at a dose of 3×10^6 cells/mouse in 0.3 ml of physiological solution. At day 7 after intraperitoneal inoculation of EC cells the animals were withdrawn from the experiment under a light ethereal anesthetic. Using a syringe, washed with a sterile physiological solution with heparin through needle No. 10, the formed ascites was obtained. The native EC cells obtained in such a way were the control.

Cryopreservation of EC cells

Part of the suspension of EC cells with a concentration of 5×10^7 ml was cryopreserved in the 1.8 ml vials (Nunc, USA). The freezing medium was ascitic fluid without the use of traditional cryoprotectants. The cells were frozen using the UOP-06 freezer, produced by the Special Constructing and Designing Bureau of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine.

Cryopreservation modes

Regimen 1 (R1)—slow cooling—represented the cooling at a rate of 1 °C/min down to − 80 °C,

followed by immersion into liquid nitrogen (Hol'tsev et al. 2011);

Regimen 2 (R2)—rapid cooling—was the cooling at a rate of 300 °C/min down to − 196 °C (direct immersion into liquid nitrogen) (Fedets1987).

The samples were warmed in a water bath at 40–41 °C for 45–50 s, with constant shuttling of the vials until the solid phase disappeared. Immediately after freeze-thawing in vitro, the cells in the Goryaev's chamber were counted, their viability was assessed using the trypan blue dye exclusion test (Sigma, USA), and a phenotypic assessment of the subunit composition of the EC by flow cytometry was performed.

To determine the intensity (Int) of tumor growth in vivo, formed by cryopreserved with R1 and R2 regimens EC cells, they were administered intraperitoneally to the experimental animals at a dose of 3×10^6 cells/mouse and cultured for 7 days, and then there were determined:

- *Absolute number of cells (ANC)* by the formula:
  ANC = vol × number of cells,
  where vol is the volume of ascites accumulated in the PC, number of cells is the count of EC cells, found in the Goryaev's chamber.
- *Intensity of tumor growth by the formula:*
  Int = ((ANC (cryo)/ANC (cont)) × 100%,
  where ANC (cont) is the absolute number of the EC cells in the control group PC; ANC (cryo) is the absolute number of EC cells in the PC of animals, which tumors were induced by the administration of cryopreserved EC cells;
- *Subpopulation composition* of *the formed ascites* was determiend by phenotypic markers with flow cytometry. The phenotypic estimation of the EC subpopulation was performed with FACS Calibur (Becton Dickson, USA) using the monoclonal antibodies (BD Biosciences, USA) to CD44 (FITC) (No. 553133, clone IM7), as well as CD24 (PE) (No. 553262, clone M1/69). Samples were used as control with the addition of labeled FITC and PE monoclonal antibodies of the same isotypes (BD Biosciences, USA), (No. 553988, clone A95-1 and No. 553989, clone A95-1), as well as antibodies to the marker being studied. Double immunostaining of cells was performed using CD44 (FITC) and CD24 (PE) monoclonal antibodies. The cells with an average CD44 marker fluorescence value of more than 10^3 (logarithmic) were classified as $CD44^{high}$-subpopulations. Analysis of the results obtained with flow cytometer was performed using the "WinMDi 2.9" software (Joseph Trotter, La Jolla, USA).

Statistical analysis

The Mann–Whitney U-criterion was used to determine the statistical significance of the differences in continuous variables when comparing between the groups with multiple (more than two) comparisons Kruskal–Wallis ANOVA tests using Excel (Microsoft, USA) and Statistics 8 (StatSoft, USA) software.

Results

Results of the analysis of number of EC cells and their viability using slow (R1) and rapid (R2) cooling indicate that R1 ensured the preservation of both studied parameters at a higher level if compared to R2 (Fig. 1).

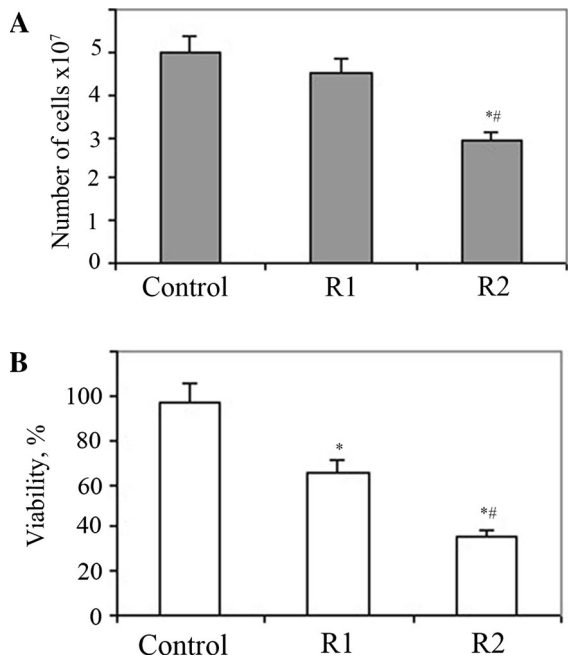

Fig. 1 Number of EC cells (**a**) and their viability (**b**) prior to and immediately after cryopreservation with various regimens. *Notes* *—difference is statistically significant if compared with similar indices of the control; #—if compared with the similar indices for the cells cryopreserved with R1 ($P < 0.05$)

At the next stage of the research, phenotypic estimation of EC cells was performed prior to and immediately after their cryopreservation under different regimens. Total population of EC cells of 7 days of development (the control) comprised the subpopulations with the following phenotypic markers: $CD44^{high}$—0.37 ± 0.02%, $CD44^{+}CD24^{-}$—3.78 ± 0.21%, $CD44^{+}CD24^{+}$—2.89 ± 0.22%, $CD44^{-}CD24^{+}$—5.33 ± 0.42% (Fig. 2).

Warming and subsequent washing of cryopreserved EC cells resulted in redistribution of the tumor subpopulation composition. The degree of these changes significantly depended on a freezing regimen. First of all, it is worth noting that the $CD44^{high}$ cell content was significantly lowered after both cryopreservation regimens, that was 2.5 times after R1 and 3.4 times after R2. An even more striking difference in the performance of the two selected freezing regimens was observed in reducing the concentration of $CD44^{+}CD24^{-}$ cells, namely in 1.6 times after R1 and in 6.4 times after R2 (Fig. 2).

The CD44 molecule is known to be one of the most characteristic markers of CSCs responsible for their self-sustainability and proliferation (Ricardo et al. 2011; Louderbough and Schroeder 2011). The least differentiated CSCs of the mammary gland are CD44 positive cells and they do not express the CD24 receptor (Al-Hajj et al. 2003; Wicha 2008; Jaggupilli and Elkord 2012). The high ratio of CD44/CD24 in tumor has been established to directly correlate with an increased proliferation and tumor genesis, which is confirmed by the enhanced formation of mammospheres in xenogeneic models of immunodeficient mice (Li et al. 2017). Assuming their crucial role in tumor growth, it should be emphasized that the conditions, implemented with slow cooling, are more favorable for the preservation of $CD44^{+}CD24^{-}$ cells, although they did not ensure a complete preservation of this subpopulation.

Decisively, the slow cooling rate (R1) contributed to a rise (in respect of the control) in the number of subpopulations that expressed on their surface CD24 marker ($CD44^{+}CD24^{+}$ та $CD44^{-}CD24^{+}$) against the background of decreasing the quantitative content of $CD44^{+}CD24^{-}$ cells (Fig. 2).

The most stringent conditions for cryopreservation, implemented with R2 led to a statistically significant decrease in the number of all the subpopulations that were detected ($CD44^{+}CD24^{-}$—6.4 times, $CD44^{+-}CD24^{+}$—2.7 times, $CD44^{-}CD24^{+}$ in almost 3.8 times relative to the control). The possible redistribution due to a complete destruction of cells at R2 (a decreased number of cells in the sample made almost 65%) (see Fig. 1) is not excluded, in this mode the loss of both CD44 and CD24 receptors was very likely.

In full, the change in a functional potential of the EC cells after the application of various cryopreservation conditions against the background of the abovementioned redistribution of the subpopulation composition of tumor can be assessed in vivo. Indeed, in 7 days after the intraperitoneal inoculations of the EC cells, cryopreserved with R1 and R2, in both cases the tumor growth inhibition was observed if compared to the control (Fig. 3), but the intensity of these changes

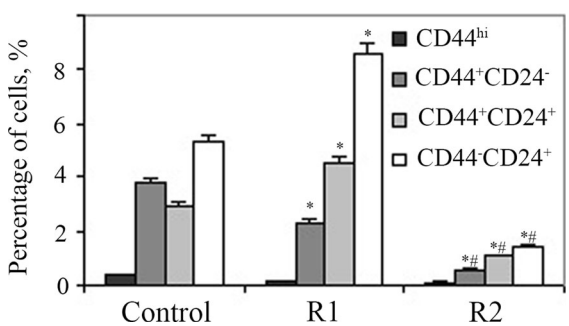

Fig. 2 Subpopulation composition of EC cells, determined in vitro prior to and immediately after cryopreservation with various regimens. *Notes* *—difference is statistically significant if compared with similar indices of the control; #—if compared with the similar indices for the cells cryopreserved with R1 ($P < 0.05$)

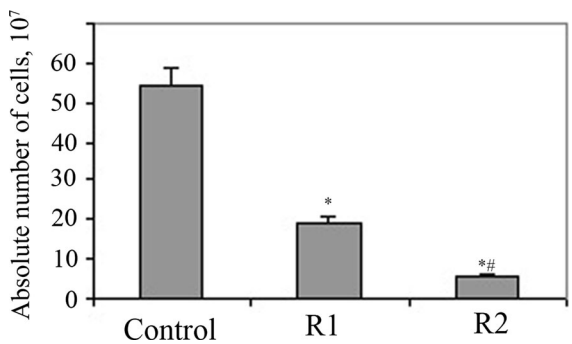

Fig. 3 Absolute number of EC cells in PC after in vivo culturing of cryopreserved cells with various regimens for 7 days. *Notes* *—difference is statistically significant if compared with similar indices of the control; #—if compared with the similar indices for the cells cryopreserved with R1 ($P < 0.05$)

was determined by the freezing regimen. Thus, R1 caused a decrease in an absolute number of EC cells almost 3 times, and R2 did almost ten times of the control level, indicating an inhibition of proliferation of cryopreserved cells (Fig. 3).

After intraperitoneal inoculation of the cells cryopreserved with R2, among those the two-thirds were nonviable (see Fig. 1), in the PC almost ten times less cells were formed if compared with the control (see Fig. 3), which was accompanied by a significant redistribution of the formed subpopulations excluding the most tumorogenic $CD44^{high}$ cells (Fig. 4). There was found a 1,5-fold reduction of the content of $CD44^{+}CD24^{-}$ cells on the background of increased quantity of $CD24^{+}$ cells ($CD44^{+}CD24^{+}$ i $CD44^{-}$-$CD24^{+}$) (Fig. 4a). That is the effect of R2 resulted in the reduced potential of the formation of the less differentiated subpopulations ($CD44^{+}CD24^{-}$) and increased the number of subpopulations, which had advanced differentiation and which expressed CD24-receptor (Fig. 4).

Despite the fact that the cryopreservation of tumor cells with the use of slow cooling (R1) resulted in a decrease in the proliferative potential of the total tumor cell pool in 2.8 times if compared with the control (see Fig. 3), the ratio of all investigated tumor subpopulations was close to the control (Fig. 4b).

Taking into account the fact that during the tumor formation in the PC, such indices as the absolute number of cells and the percentage of content of different subpopulations were simultaneously changed, it was decided to estimate the absolute number of individual subpopulations of cells in the formed ascites. The obtained data confirm that the regimen R1, which led to a 2.8-fold inhibition of tumor growth in general (see Fig. 3), resulted in a two-fold decrease in the absolute amount of each of the studied tumor subpopulations compared with the control (Fig. 5).

A significant decrease in the absolute number of all identified subpopulations of tumor that were formed by cells directly plunged into liquid nitrogen (R2) (see Fig. 3) led to a convincing reduction in the absolute number of different subpopulations of EC cells in the PC (see Fig. 5). The most indicative (if compared with the control) when using R2 was a tenfold decrease in the formation of the number of cells $CD44^{high}$ and a 15-fold decrease in the number of $CD44^{+}CD24^{-}$ cells. This caused the maximum inhibition of EC growth, almost ten times if compared with the control level (see Fig. 3).

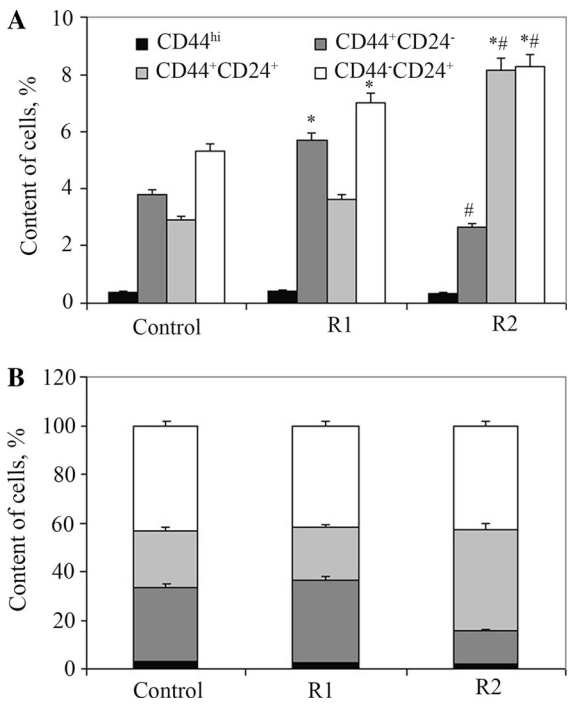

Fig. 4 Subpopulation composition (**a**) and ratio of different phenotypic features of tumor populations (**b**) after in vivo culturing of cryopreserved cells with various regimens for 7 days. *Notes* *—difference is statistically significant if compared with similar indices of the control; #—if compared with the similar indices for the cells cryopreserved with R1 ($P < 0.05$)

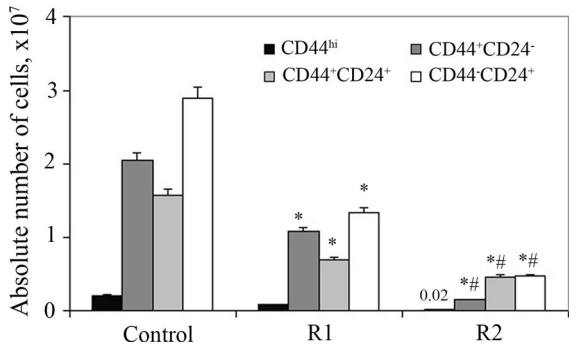

Fig. 5 Absolute number of subpopulations of tumor cells in PC for 7 days of in vivo culturing of cryopreserved with various regimens of EC cells. *Notes* *—difference is statistically significant if compared with similar indices of the control; #—if compared with the similar indices for the cells cryopreserved with R1 ($P < 0.05$)

Discussion

The use of express-evaluations for the EC cell count after cryopreservation has shown that the rapid cooling results in a significant reduction in both the amount and viability of tumor cells. To a large extent, the results match the data of other authors, which indicate the advantage of using a slow cooling rate (2 °C/min to − 180 °C) to improve the preservation of structural and functional characteristics of EC cells (Santos et al. 2015) and rapid cooling (300–400 °C/min) to inhibit completely their functional potential (Fedets 1987).

At the same time, our previous studies have shown that the EC is a heterogeneous population both on phenotypic and functional characteristics (Goltsev et al. 2015, 2017a, b). The individual EC subpopulations are in hierarchical relationships and provide functioning of each other (Goltsev et al. 2017a, b). The effect of cryopreservation factors can lead to the elimination of some subpopulations, which causes an inhibition or enhancement of the development of others (Goltsev et al. 2018). On another hand, it is conceivable that the frozen-thawed cells which appear to be viable through a series of rapid tests, that is, have no disorders of membrane's permeability, implement their function in a "specific" way (Hol'tsev et al. 2011). The reasons for this can be, first of all, damage to the receptor apparatus of a cell,ranged from the shedding of the receptors to changing their conformational structure.

It should be noted that the membrane structures used for phenotypic identification of cells play an essential role in the implementation of their functional potential and are most vulnerable to cold stress (Goltsev et al. 2006; Karimi-Busheri et al. 2013; Munévar et al. 2015). At the same time, they are often subjected to strong post-translational modifications, such as glycosylation, phosphorylation, and lipid fragmentation in non-physiological conditions, which results in modulation of signaling processes, transcription and translation disorders of a number of regulatory proteins, which generally determine the change in a functional status of cryopreserved cells.

The obtained data indicate that the slow cooling rate contributed to an increase (versus the control) of the relative number of subpopulations expressing CD24 marker on their surface against the background of decreasing the quantitative content of $CD44^+$ cells. The reason for such a different response of the cells to cold stress may consists in the peculiarities of the structure of the CD44 and CD24 receptors (Fig. 6).

The membrane structure, identified as CD24, is known as an extremely short highly compacted protein (not more than 30 amino acid residues), which is characterized with a high thermal resistance, therefore it was defined as a heat-stable antigen (HSA) (Springer et al. 1978). The peculiarity of this receptor structure is the absence of an intracellular domain (Fig. 6). On the contrary, CD44 is a much larger transmembrane protein (341 amino acid residues), which consists of a terminal, transmembrane, and cytosolic domain. The very terminal portion of this complex, phenotypically identified as the CD44 marker, can undergo much more modifications during cryopreservation, in particular by means of shedding both due to mechanical destruction and "ectodomain" way as a result of proteolytic degradation of intracellular domains (Hayashida et al. 2010).

"Ectodomain" shedding is induced by a number of stimulants, including UV light, osmotic pressure, hyperoxidation, and the action of proinflammatory cytokines, in particular interleukinin-1 beta (Takenobu et al. 2003). It is in this way the expression and function of surface receptors are regulated and a wide range of cell functions is modulated (Hayashida et al. 2010). Ectodomain destruction rapidly reduces the level of cell surface protein expression and simultaneously converts membrane-associated proteins into soluble effector, which may be the inductors for triggering other signaling stages. This causes the modulation of cell functions in "non-physiological" conditions, including those after cryopreservation. In addition, cryopreservation factors can cause proteolytic degradation of the cytosolic fragment of the CD44 molecule by activating matrix metalloproteinases (Evdokimova 2008). Indeed, after cryopreservation of heart valve cells Kitagawa T. et al. found a serious disturbance of mitochondrial function in endothelial cells and in fibroblasts as a result of increased activity of MMP-1 and MMP-2 (Kitagawa et al. 2001). The mentioned cryosensitivity of the CD44 marker is consistent with the data on the dependence of its expression level on the surface of red blood cells on the cryopreservation conditions (Zemlianskykh and Babijchuk 2016).

There is no doubt that the redistribution of antigenic spectrum of EC cells after cryopreservation may

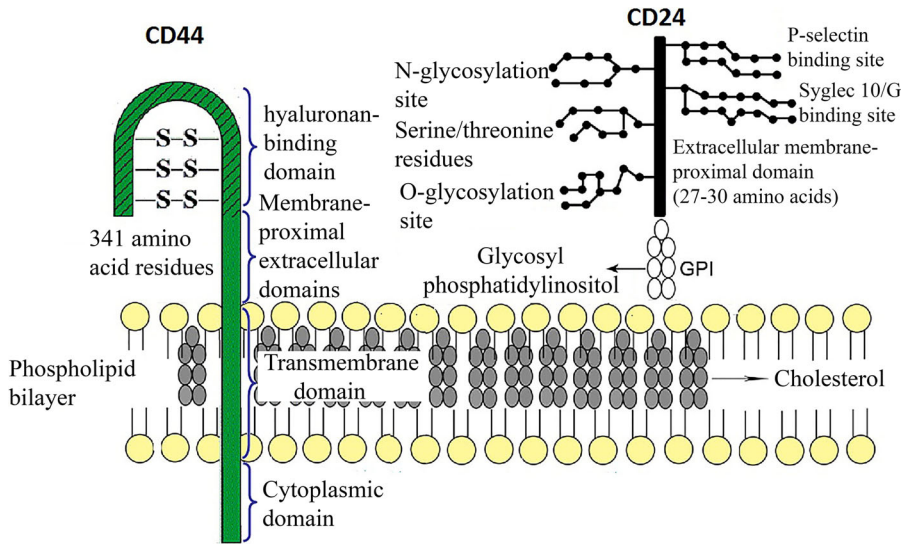

Fig. 6 Schematic representation of CD44 and CD24 receptors

stipulate various tumor growth intensity depending on the cryopreservation conditions. The obtained results demonstrate that the most severe conditions of cryopreservation, implemented at R2 led to a significant loss of both CD44 and CD24 receptors (see Fig. 2). However, after 7 days of culturing, such cells formed in vivo tumors with a reduced proliferative potential (see Fig. 3), wherein the populations with positive CD24 markers dominated against the background of a decrease in the number of $CD44^{+}$ cells (see Fig. 4). This may be above all related to the function of CD24. This antigen is known, in addition to the classical function, to act as a ligand for P-selectin and to provide the adhesion and migration of several types of cells, in particular tumor cells, it can bind to a number of ligands, namely Siglec-G, Hypoxia Inducible Factor-1 (HIF-1), heat shock proteins-HSP 70 and 90, etc. (Ayre and Christian 2016). In this case CD24 functions as a rheostat for modulation of the reactions transduced by partner cell surface receptors. Such partner receptors can determine even the opposite biological effects. For example, in cancer cells, the CD24 receptor can induce both pro- and antiproliferative effects, increase or decrease the ability to metastasis depending on the cancer type (Kristiansen et al. 2004; Ju et al. 2011). Suyama et al. found that CD24 is capable of suppressing the proliferation and invasiveness of breast cancer cells by blocking the Sonic Hedgehog (SHH) pathway, which is one of the main ones of self-sustaining tumor cells. The authors have shown that this is due to the inhibition of the transcription factor STAT1 (Suyama et al. 2016). In this regard, it becomes clear why the EC cells with elevated expression of the marker CD24 have a reduced ability to form tumors.

In addition, the intensity of tumor growth depends on the nature of cooperative interaction of different EC subpopulations (Goltsev et al. 2015, 2017a, b). Quantitative and qualitative characteristics of these subpopulations after the use of different freezing regimens may alter. This assumption was confirmed by attestation of the subpopulation composition of mice PC after 7 days of culturing of cryopreserved EC cells (Fig. 5).

However there are several aspects to consider. Firstly, the intensity of tumor growth, which is directly related to an absolute content of cells in the PC is considered as not only their quantity but also the volume of ascitic fluid. Accumulation of a liquid part of the ascites tumor results from an increased concentration in abdominal cavity of low molecular weight metabolites (lactate) due to the implementation of metabolic function of ascites cells, in particular anaerobic glycolysis (Zykova and Inzhevatkin 2008). This leads to a rise in osmotic pressure inside the abdominal cavity, and the ascitic fluid continues its accumulation as the tumor grows. In her report Fedets OI has shown that cryopreservation can reduce the activity of enzymes of glycolysis in EC cells (Fedets 1987). At the same time, the intensity of anaerobic glycolysis under conditions of rapid freezing decreased by almost 90%, whereas after freezing at a

rate of 1 °C/min it did no more than 30% of the control level. This is in agreement with the data we obtained about a more intense decrease in the absolute number of cells in PC when using R2, i.e., rapid freezing (see Fig. 3).

Thus, varying the cooling rates for the specimens enables the selection of the cryopreservation conditions, which can to a greater or lesser extent ensure the preservation of the tumor-forming potential of the EC cells. Such a potential is maintained to a greater extent when using R1 with a slow cooling rate. But even with this mode to achieve a complete restoration of functional activity of preserved tumor cells still failed. This opens up the prospect of further improvement of methodological approaches to minimize the negative effects of low temperatures and more efficient biobanking of tumor cells. One of them may be the "stabilization" of the EC culture after warming according to the method that involves a three-fold re-culturing to level the effects of low-temperature preservation factors (Goltsev et al. 2012). In general, this method can be recommended to be used for cryobanking of tumor cells. Although the revealed redistribution of the subpopulation composition of the EC cells after cryopreservation does not allow it to be recommended for storage of biopsy samples in order to test the effectiveness of future anti-tumor therapy.

It is worth to note that even regimen R2, which ensures a proper tumor cryodestruction, did not lead to a complete elimination of the most tumorigenic $CD44^+$ cells. In this regard the results obtained with multiple cryopreservation to inhibit the proliferative activity of EC precursor cells (Diabina et al. 2014) may be perspective.

Funding This work was supported by funding from the National Academy of Sciences of Ukraine (No. 2.2.6.110).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval The research was performed in accordance with the Law of Ukraine "On the Protection of Animals Against Cruelty" (No. 3447-IV, 2006), with meeting the requirements of the Institute's Bioethics Committee, which are in accordance with the provisions of the "European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg 1986).

Human and animal rights This paper does not contain any investigations in humans, as well as no authors of this paper performed such studies.

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