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На пути к медицински признанным технологиям для широкомасштабных банков стволовых клеток: разработки и методы

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Towards a Medically Approved Technology for Large-Scale Stem Cell Banks: Tools and Methods

The importance, of the development of stem cell cryobanking has increased recently with an augmentation of stem cell research and its therapeutic applications. The development of therapies is, among other things, limited by high sensitivity of stem cells to freezing-thawing procedures. Thus, new approaches are needed for preservation and related evaluation methods, as well as new technologies for long term storage of large numbers of stem cells. Here we present selected recent improvements of stem cell cryopreservation, e.g. for freezing of adherent human embryonic stem cells using gel-like matrices. We report the application and performance of novel micro-system-based cryosubstrates and devices and describe new evaluation methods and the results of a thermal stress cycle study.

Key-words: human embryonic stem cells, cryopreservation, stem cell banking, cryomicroscopy, cryotechnique, scanning electron microscopy.

В настоящее время возросла важность развития криобанков стволовых клеток в связи с их расширенным изучением и терапевтическим применением. Однако, наряду с другими факторами, вышеуказанная терапия ограничена высокой чувствительностью стволовых клеток к процедурам замораживания-оттаивания. Необходимы как новые подходы к криоконсервированию и связанным с ним методам оценки, так и новые технологии для долгосрочного хранения большого количества стволовых клеток. В настоящей работе мы представляем некоторые улучшенные методы криоконсервирования стволовых клеток, например замораживание эмбриональных стволовых клеток человека с использованием гелеобразного матрикса. Мы представляем результаты применения разработанных на базе микросистемной техники новых криосубстратов и устройств, а также описываем новые методы оценки и результаты изучения циклов температурного стресса.

Ключевые слова: эмбриональные стволовые клетки человека, криоконсервирование, банк стволовых клеток, криомикроскопия, криогенная техника, сканирующая электронная микроскопия.

Наразі зросла важливість розвитку кріобанків стовбурових клітин у зв'язку з їх розширеним вивченням і терапевтичним застосуванням. Але водночас з іншими факторами вищезгадана терапія обмежена високою чутливістю стовбурових клітин до процедур заморожування-відтавання. Необхідні як нові підходи до кріоконсервування та повязаних з ним методам оцінки, так і нові технології для довгострокового зберігання великої кількості стовбурових клітин. В цій роботі ми представляємо деякі покращені методи кріоконсервування стовбурових клітин, наприклад заморожування ембріональних стовбурових клітин людини з використанням гелеподібного матриксу. Ми представляємо результати застосування розроблених на базі мікросистемної техніки нових кріосубстратів та приладів, а також описуємо нові методи оцінки і результати вивчення циклів температурного стресу.

Ключові слова: ембріональні стовбурові клітини людини, кріоконсервування, банк стовбурових клітин, кріомікроскопія, кріогенна техніка, скануюча електронна мікроскопія.

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Interest in stem cell transplantation for therapy of degenerative disorders has intensified recently. These therapies need a reliable and safe supply of high-quality stem cells or stem cell-derived progenitors. Long term preservation of stem cells without loss of vitality and functionality can only be achieved by low temperature conservation, so-called cryopreservation with storage over liquid nitrogen (for review see Hunt, 2007 [12]). Current cryobanking relies on storing sources of stem cells such as umbilical cord blood [29], but the reliable banking of defined, well-characterized stem cells (both somatic and embryonic) and conditions for secure outgrowth are still in the stage of infancy. The general problems of cryobiology create specific problems for sensitive primary stem cells. For example, it is known that damage occurs during freezing and thawing from ice crystallisation and recrystallisation. To protect cells from these processes and solution effects, most current cryopreservation methods control freeze/thaw velocity and use cryoprotectants that influence the water content of cells. Slow freezing and rapid warming to balance cell dehydration [25] is the most effective freezing procedure at the moment. There are two main groups of cryoprotectants: penetrative and non-penetrative [5, 26]. Among penetrative cryoprotective agents (CPA), dimethyl sulfoxide (Me_2SO) [27] is still the most important and effective (other include glycol, ethylene glycol etc.) [23]. Some non-penetrative CPA such as hydroxyethyl starch (HES) or polyethylene glycol can induce glass-like solidification, so-called vitrification, eliminating ice crystal formation [4, 26]. Both types of CPA are used for cryopreservation of stem cells [7–11, 14, 15, 19, 31, 32, 36, 37, 39]. However, at present robust, optimised cryopreservation protocols, especially for human embryonic stem cells (hESCs) are still not achieved. We lack imaging technology; require the development and evaluation of optimal freezing media, compounds and CPA; need to establish validation methods and need to optimise new approaches for freezing, using two- and three-dimensional structures including scaffolds and encapsulation, etc [12, 43].

For future cryobanking it is essential to create new cryosubstrate platforms for most types of cells and tissues. These substrates have to provide the best biophysical parameters for freezing; have to be suitable for storage of high numbers of objects; must be cell specific, informative for users, work flow specific and data sensitive. This can be achieved using modern production processes, like micro-system technology, a novel tool in cryopreservation technology [13, 41].

Cryobanking of stem cells requires safe long-term storage without marked loss of post-thaw viability and functionality [35]. However, there are no available systems for investigating the influence of changes in temperature and other parameters on long-term storage of cells. This and other above indicated problems are focus points of this paper.

New micro-system based devices for cell and tissue cryopreservation

The development of stem cell therapies requires new approaches to cell freezing and storage equipment. The cell containers of current cryopreservation technology are plastic cryovials with a minimum volume of 1 ml. These are suitable for ~10^7 cells and the whole volume must be thawed at once for use. New micro-system (MST) based devices for cryopreservation and cryobanking of single- and multi-cellular systems can be useful in modern cryobiotechnology [41]. The main principles of those devices are miniaturisation, modulation and storage of information about cryopreserved objects on a chip directly connected to the cryosubstrate [13, 41]. Using those devices it was found that Me_2SO concentration can be reduced to ~2% without adversely affecting post-thaw viability, this is consistent with recent literature [24, 40, 41]. One type of new cryocontainer is shown in Fig. 1a. It consists of 30 wells, each of 25 µl. It can be closed either with a heat-sealing film or by a snap on lid and is suitable when large numbers of samples have to be stored in a small space. After filling with cells, CPA can be added by a micropipette robot (Fig. 1b) capable of accurately dispensing nano-liter volumes. Generally, these substrates and automatic CPA addition allow Me_2SO concentrations to be reduced from the conventional 10% to ~2% [41]. Some cell types such as mouse fibroblasts [40, 41], epithelial tumour cells [24] show higher post-thaw vitalities when frozen in these containers rather than in conventional cryovials. These containers are not limited to cell suspensions and have also been used successfully to freeze multicellular systems such as Langerhans' islets and tumour spheroids [18] The first cryopreservation of CEpan3b human adult stem cells in micro-cryosubstrates is presented in this paper. CEpan3b cells were isolated from pancreatic acini (for details see Kruse *et al.* [21, 22]). Cells were cultured in DMEM (Invitrogen, Paisley, UK) supplemented with 10% FBS Gold (PAA, Austria) and 1000 U/ml Penicillin/Streptomycin (Invitrogen, Paisley, UK) under standard conditions (37°C, 5% CO_2). Cells were sub-cultured so that they remained in exponential growth phase. Harvesting was done by trypsination (Trypsin/EDTA; Invitrogen, Paisley, UK). For cryopreservation a cell density of 0.5–1×10^6 cells/ml was calculated by counting the cells in a haemocytometer. Cell viability was assed by ethidium bromide (EB) and fluorescein diacetate (FDA) [1]. FDA is able to cross plasma membrane, in metabolic cells it is hydrolysed and free fluorescein accumulates inside the

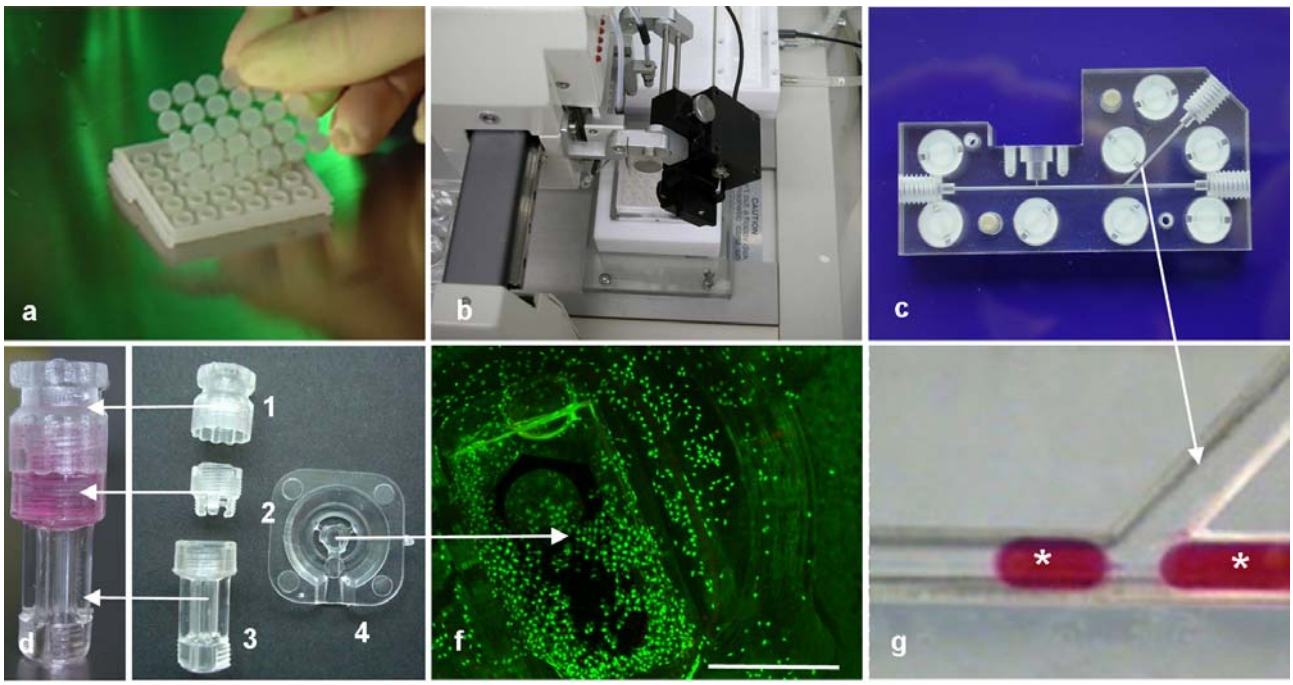

Fig. 1. New micro-system based devices for cryopreservation: a – IBMT-micro-cryosubstrate; b – micropipetting nanoplotter for automatic addition of cryoprotectants; d, e and f – CellProm micro-cryosubstrate: 1 – cap, 2 – retainer, 3 – socket extension, 4 – frame for carrier insert; f – CellProm carrier insert with cultured cells; c and g – integrated cryoprotectant dispenser for generation of compartments with cells and automatic CPA addition in a polymer-chip; c – polymer-chip; g – high speed frames of generation of compartments (dark grey) with multicellular spheroids (asterisk); scale bar in figure 1f 1000 μm.

cells (green fluorescence). EB is only able to pass damaged plasma membranes and intercalate into DNA (red emission).

A pipette robot (GeSim, Rossendorf, Germany) added Me_2SO (WAK-Chemie, Germany) to cells in the wells of miniaturised cryosubstrates (Fig. 1a) for a total volume of 25μl. CPA was manually added to cryovials with a total volume of 1ml. Me_2SO concentrations of 0.5 to 10% were used. Cell suspensions containing Me_2SO were incubated for 30 min at 4°C before freezing with 1°C/min rate to –80°C in a computer controlled freezing device (Kryo10-MRIII, Planer Systems, UK). Until thawing, samples were stored in vapour phase of liquid nitrogen. Thawing was achieved by placing cryovials into a 37°C water bath for approximately 3 min. Micro-cryosubstrates were placed for 5min into an incubator. Cell vitality was immediately assed by double staining with EB/FDA. Vitality was calculated as: 100% × number of living cells/(number of living cells + number of dead cells). Fig. 2 shows the post-thaw vitality with standard deviation of CEpan3b cells frozen in miniaturised cryosubstrates and cryovials with varying concentrations of Me_2SO. For concentrations between 2% and 10% vitalities ranged from 77% (±15%) to 90% (±5%). The vitalities obtained for both systems were comparable for all experimental trials. However, on decreasing Me_2SO concentration to 1% and 0.5% vitality dropped to 45% and 16–26% respectively. Experiments were repeated for all concentrations except 5% four times, the 5% samples were repeated for twelve times. Our results demonstrate vitalities of more than 80% at 2% Me_2SO (Fig. 2). However, in this case there was no no marked effect of container geometry.

A second container that allows cell manipulation and cryopreservation is shown in Fig 1e. This includes

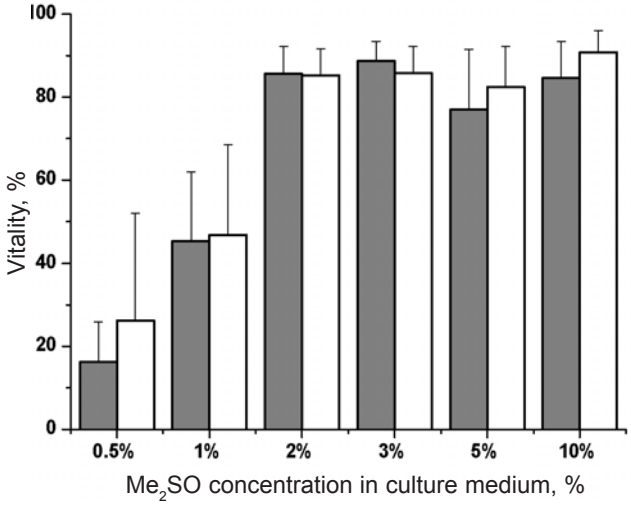

Fig. 2. Post-thaw vitalities of the human adult stem cell line CEpan3b. Cells were frozen in IBMT miniaturised cryosubstrates (grey bars) and commercial 1ml cryovials (white bars). Post thaw vitality was assayed by double staining with fluorescein diacetate and ethidium bromide and was calculated as percentage of living cells divided through total cell number. Experiment were repeated four times (twelve times for 5% DMSO). The error bars are standard deviations.

a small glass carrier suitable for the culture of adherent cells (Fig. 1f) and for microscopy. In addition, the carrier can be transported magnetically for various process steps.

Also a new MST-based polymer chip with an integrated dispenser for generation of compartments containing cells or multi-cellular spheroids has been created recently. Fig.1c and g shows a simple fluidic system that incorporates a CPA dispenser. As spheroids are transported in small compartments of medium (right to left in Fig. 1g) the CPA is added automatically with very rapid mixing. In this system automatic addition of cryoprotectants in nano-liter volume was realized with a micro-fluidic pump. The creating of compartments with multi-cellular spheroids is visible in Fig.1g (Fig. 1g; spheroids marked with asterisk). This picture is a part of a high speed video of this process. One compartment consists of only 65 nl (Fig. 1g) and can be frozen in an computer controlled freezing device. First experiments with this system showed promising results with rat pancreatic islets as a new approach for cryobanking. There are also cryocontainers (of 100–2000 ml capacity) that incorporate flash memory chips for the storage of sample-related data and identification. With suitable electronic infrastructure, these memories can be interrogated while at liquid nitrogen temperature [13]. This close association of data and sample greatly reduces the possibility of book-keeping errors and gives the ability to locate a particular sample without opening the cryotank.

The presented cryosubstrates together with new electronic infrastructure for cryorepository and new cryopreservation methods [42] open new perspectives for medical relevant cell preservation, especially, for stem cell cryopreservation.

Validation technology for freezing procedures

Successful medical application of cryopreserved stem cells is not possible without validation of freezing/thawing procedures. It is necessary to observe and understand ice crystallization processes with and without different cryoprotectants. Cryomicroscopy, using a conventional microscope connected with freezing/thawing system and recording camera, is a powerful tool in this field [2, 20]. A high speed camera [3] allows visualization of very fast intra- or extracellular ice front propagation. A similar set up was recently used for studies of ice propagation in and around adherent endothelial cells on micropatterned substrates [33]. We have used high speed cryomicroscopy for the first time to analyse ice crystallization with CPA addition in multicellular systems [43]. Here we extend these experiments and have firstly used high-speed video cryomicroscopy with embryonic stem cells and encapsulated pancreatic islets. Our system uses a modified microscope (Nikon Eclipse 80i, Nikon, Japan). The cryostage (MDS 600; Linkam, England) is connected to a temperature controller (TMS 94), a pump (all Linkam) and a Dewar for supply of liquid nitrogen. The observation window is purged by dry nitrogen gas. Images are recorded with a digital camera (Pixelink, Germany) connected to the Linksys32 software or a high-speed camera (SpeedCam Visario LT400, Weinberger, Switzerland) capable of up to 4000 frames/sec.

H1 embryonic stem cells, obtained from WiCell (Madison, WI, USA), were cultured onto Mitomycin C inactivated embryonic mouse fibroblasts strain CF-1 (Millipore, Billerica, MA) and passaged under standard conditions recommended from WiCell (Introduction to human embryonic stem cell culture methods; Part IV – splitting human embryonic stem cells, January 2003). Culture medium was modified by the addition of 100 U/ml penicillin and 100 μg/ml streptomycin (all medium components Invitrogen, UK). H1 colonies were picked from culture plates and incubated in culture medium, then transferred into cryopreservation solution for high-speed cryomicroscopy. The cryopreservation solutions were 285 mM trehalose (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) + 5mM KCl (Merck, Darmstadt, Germany) + 5mM histidine (Merck, Darmstadt, Germany) or culture medium + 30% FBS (final concentration 27%) + 10% Me_2SO. Colonies were kept in these media for 5–10 min before freezing. The freezing rate was 10°C/min from 4°C to –80°C. Samples were thawed at 40°C/min. For cryomicroscopy of encapsulated pancreatic islets, the islets were isolated from young Sprague-Dawley rats (CD-rats) (for details see [34]) and encapsulated with 0,65% NT-alginate, dissolved in 0,9% NaCl, using an encapsulation machine described in Zimmermann *et al*. 2005 [42]. After encapsulation, islets were cultivated overnight in an incubator at 37°C, washed once with culture medium supplemented with 7% Me_2SO added by micro-pipette robot (GeSiM, Rossendorf, Germany) at 4°C and incubated at 4°C for 30 min before freezing. For cryomicroscopy, samples were frozen with at 1°C/min from 4°C to –80°C and thawed at 40°C/min.

Fig. 3 shows how the ice front is structured, distorted and redirected when passing the H1 colonies and capsules. Furthermore, Fig. 3(a–c) illustrates that when cryomedium contains Me_2SO, cells in the colony darken. This means that cells are frozen and the light refracted. In contrast, cells tend to vitrify rather than freeze if the extracellular cryoprotectant trehalose (isoosmolar solution) is used (Fig 3(d–f)). Though, devitrification occurs upon thawing, accompanied by the so-called "flash out" effect (data not shown).

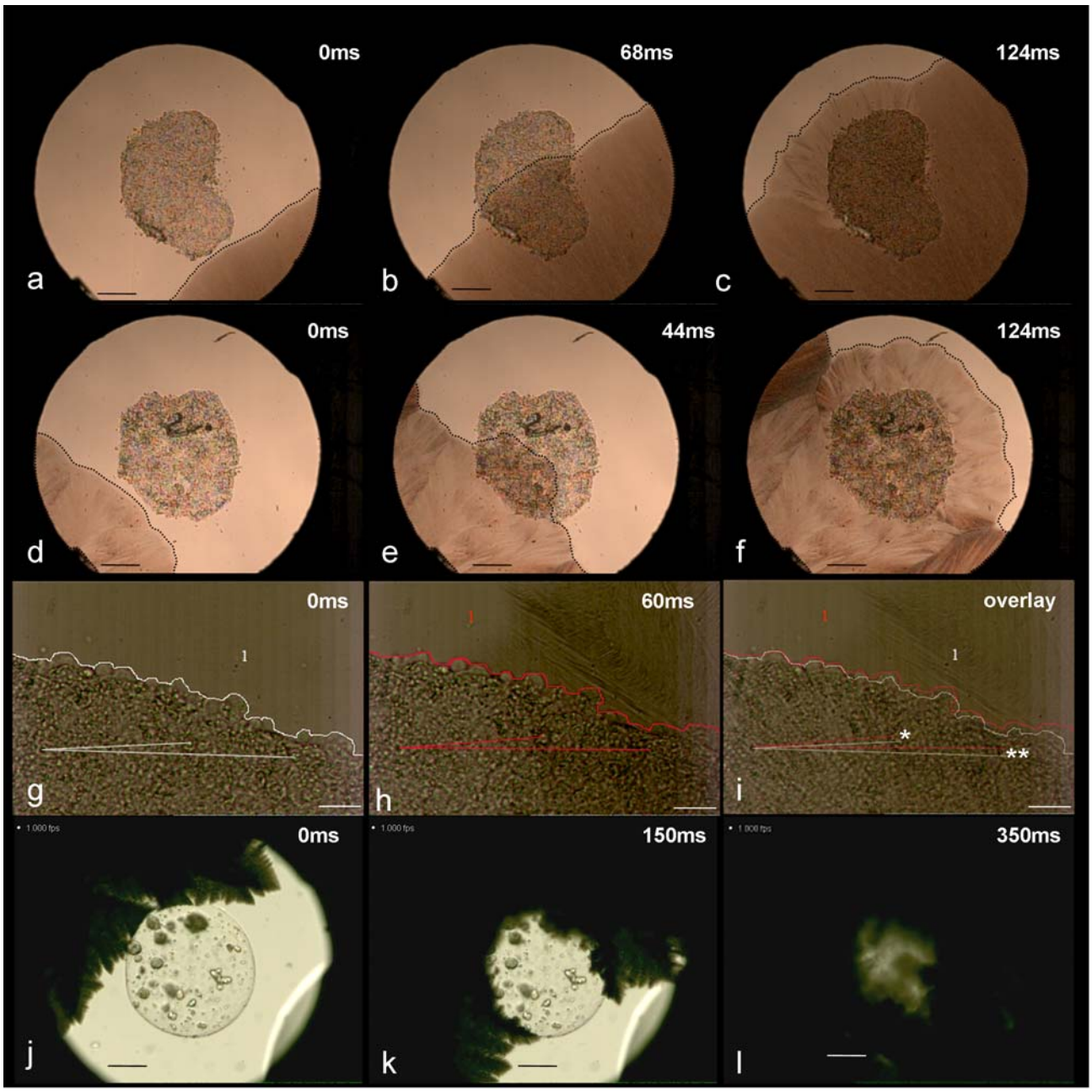

Fig. 3. High speed cryomicroscopy of freezing situations in different systems a–c: H1 colony frozen in cryo medium + 10% Me_2SO. In frame b you can see the ice front (visualised by a black dashed line) grows over the colony. In c, the ice is structured between the front and the colony and the cells freeze (become dark). d–f: H1 colony in isoosmolar trehalose solution. In frame d ice front (visualised by a black dashed line) accelerates when reaching the colony (e) and the ice is structured everywhere (f). g–i: H1 colony frozen in cryomedium + 10% Me_2SO. The upper line in pictures g and h illustrating the border of the colony. The lines within the colony are drawn between two cells. The number in the external medium displays a particle which is moved by the coming ice front for ~313 µm. Frame i is an overlay of g and h where you can see how the border is minimally changed (in a wave manner). The up move of the grey line compared to the white line is at the point of one asterisk ~16µm and at the two ~21µm. j–l: islets of Langerhans encapsulated in alginate; cryopreservation solution: culture medium + 7% Me_2SO. Crystallisation starts in the extracellular medium and is retarded by the alginate bead. Scale bars a–f and j–l: 200 µm. Scale bars g–i: 100 µm

Moreover, Fig. 3(g–i) shows the impact of the ice front hitting the cell colony. Before this strike, the colony is moved in a wave like manner. In these pictures only very small changes are visible due to the way of presentation. So, there seems to be a mechanical stress placed on the cells.

By encapsulation in an alginate matrix, the cells (pancreatic islets) are more protected against the ice front. As shown in pictures k and l the ice front goes around the capsule and freezing of the capsule takes place later (data not shown).

For human embryonic stem cells, the whole colony freezes by addition of Me_2SO (Fig. 3(a–c). This effect has already been clearly shown for pancreatic islets; the growth of extra-cellular ice occurs first and intracellular ice forms later [2]. If frozen in a solution

containing isoosmolar trehalose, the colony seems to be vitrified. Vitrification is a very successful tool for cryopreservation of human embryonic stem cells [32, 39]. But vitrification cannot really be proven by microscopy.

Not only the ice structure is affected by the colony, but also the colony seems to be mechanically pushed by the extracellular ice formation (Fig. 3(g–i)). This stress might be a main reason for poor cryopreservation results with cells frozen adherent to a substrate. The ice front might push the cells and possibly disrupt the monolayer. For cell aggregates in suspension the ice front might be detrimental in another way, it possibly causes injuries. An alginate bead as a first surface for the ice crystals to arrive might be a solution (Fig. 3(j–k). With cryomicroscopy we cannot see how ice crystals are located inside the alginate beads and inside the colonies. A high resolution method such as freeze-substitution electron microscopy must be used applied.

Long-term stability of stored cells

Modern cryobanks store large numbers of objects long-term in or over liquid nitrogen and there are situations in which the temperature rises above –130°C, the glass transition temperature of water. Vysekantsev *et al*. [35] demonstrated for three cell lines and for yeast and bacteria that a cyclic temperature change up to 100°C for mammalian cells leads to a significant loss of post-thaw viability. These experiments show clearly that those influences have to be investigated for different cell lines and types. Therefore, we have built up a system in which those effects can be studied without any side effects like transportation. The self-made cycling device (Fig. 4) consists of a sample chamber within an elevator system in a vacuum isolated liquid nitrogen storage vessel. The level of liquid nitrogen is automatically maintained through connection to a liquid nitrogen storage tank. A vertical temperature gradient is generated between liquid nitrogen at the bottom and an electrical heat source at the top of a closed copper cylinder. A controlled elevator system adjusts the height of the cell sample to achieve the desired temperature. The sample temperature is controlled through two sensors one at the top of the sample chamber and one directly on top of the sample. Temperature data were logged in a computer file. For long-term stability evaluation L929 mouse fibroblasts and the human cancer cell line PC3 (both DSMZ, Braunschweig, Germany) were cultured and harvested under standard conditions. L929 cells were cultured in DMEM supplemented with 10% FBS and 0.1 mg/ml gentamycin. PC3 cells were cultivated in a 1:1 mixture of RPMI 1640 and Ham's F12, supplemented with 10% FBS and 0.1 mg/ml gentamycin (all PAN, Aidenbach, Germany). After incubation for 30min at 4°C in cryomedium (culture medium containing 5% Me_2SO) cells were frozen in IBMT miniaturised cryosubstrates in a self-made freezing device at 1°C/min from 4°C to –80°C. After freezing, samples were stored overnight in liquid nitrogen before cycling. Cell samples were inserted into the cycling device. One control group remained in liquid nitrogen; another was left at –80°C. After cycling, samples were stored for a minimum of one hour at –196°C before thawing and vitality testing.

Our study of the warming speeds at different places in a cryorack showed clearly the need of investigation of repeated warming effects on deep frozen cells. On pulling out a cryorack from the tank, warming up to –80°C occurs within 3 min and after approximately 12 min a temperature of –20°C was reached (data not shown). After cycling the cell samples 12 times from liquid nitrogen to about –80°C with holding at each temperature for twelve hours (Fig. 5a), we could not find a loss in post-thaw vitalities for different mammalian cell lines compared to the control stored at –196°C over the whole period (Fig. 5b). Fig. 5b shows post thaw vitalities with standard deviation. Experiments were repeated for four times. Using this system we did not decrease cell viability by 12 temperature cycles to –90°C. This contrasts with the findings of Vysekantsev *et al.* [35]. Additionally, Galmes *et al.* [6] showed that storage at a constant –80°C for up to 10 years did not influence haematopoietic cell viability.

Improvement of cryopreservation of adherent stem cells using gel-like matrix

Cryopreservation of hESCs is a field of great interest despite many difficulties and unsolved problems. It is known that these cells do not survive well the freeze/thaw cycle if dispersed to single cell level

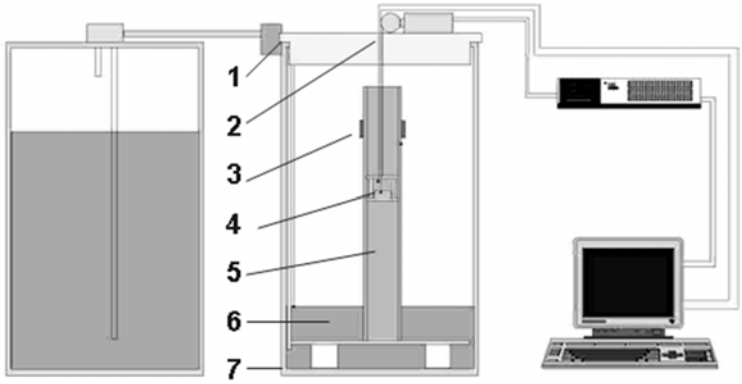

Fig. 4. Schematic of the cycling system. A copper cylinder (5) with a heater (3) at the top is placed into a storage tank (7); with a drive (2) and an elevator system the sample chamber containing two temperature sensors (4) can be moved to a height giving the desired temperature; the liquid nitrogen (6) level is automatically maintained (1) throughout experiments.

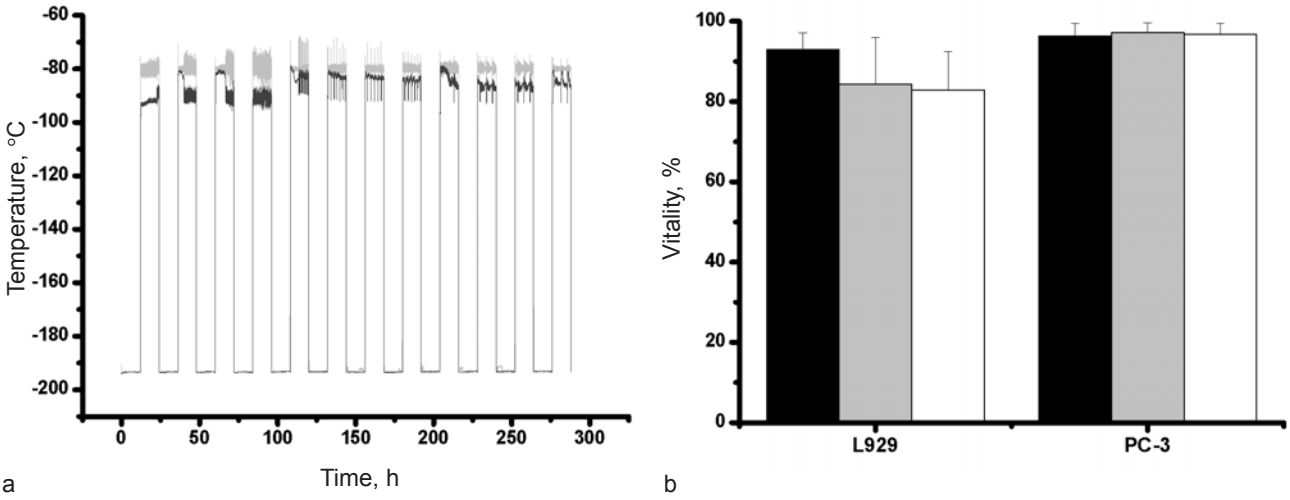

Fig. 5. Graph a displays the temperature profile measured during one trial of sample cycling, temperature measured at the top of the sample chamber (light grey line) and directly on the samples (dark grey line).
In b the white bars show the post-thaw vitalities after twelve cycles from –196 to –80°C. The black bars are the controls constantly held at –80°C. The grey bars are the controls stored at –196°C. Experiments were repeated four times. Error bars are standard deviation.

[8]. Slow freezing protocols, usually with relatively high amounts of Me_2SO (in the range of 10%) showed only poor survival and functionality rates of 10–36% [7, 15, 19]. An improvement has been reported by addition of the extracellular matrix protein type IV collagen [19] or through addition of trehalose to freezing and thawing medium [37], so that 48% recovery of undifferentiated cells could be achieved. Also some promising results have been reported from Ware *et al.* with rates in the range of 80% [36] if cells were frozen with slow rates in small volumes of 250 µl. These high levels of post-thaw viability and functionality are usually only reached if vitrification is used [31, 32, 39]. Unfortunately, this method is very time and labour intensive and cannot be automated, sterile cryostored over liquid nitrogen and is therefore not suitable for bulk cryopreservation.

Another promising approach is to freeze cells in their native state that means adherent to their culture surface with or without feeder cells [9–11, 14]. Ji *et al.* [14] reported good results if a gel layer was used. They showed a recovery of up to 82%. Heng *et al.* demonstrated that despite good vitalities directly after thawing, human embryonic stem cells died within hours due to apoptosis [9–11]. Addition of apoptosis inhibitor to the freeze and thaw medium can improve cryopreservation [11]. A solution to these problems would be important in cell replacement therapy. In this field very promising studies have been reported [28, 30].

In this paper we report a successful cryopreservation of adherent human embryonic stem cells using a gel-like matrix. H1 embryonic stem cells were cultured as described above. ECM gel (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was diluted in Dulbecco‘s modified Eagle‘s medium (Invitrogen, Paisley, UK) to a final concentration of approximately 0.5 mg/ml. Non-adhesive Petri dishes were covered with ECM gel and dried 30 minutes. Feeder cell suspension was added in a density 1×10^5 cells/ml onto ECM covered dishes. Inactivated feeder cells were cultured for 1–3 days on gel before adding H1 cells. H1 were harvested by collagenase type IV treatment and cultured for 2–3 days on ECM + feeder cells. Before cryopreservation a second gel layer (diluted 1:10) was drawn on the samples. ECM was added for 30 min at room temperature above the cells. Remaining liquid was discarded and cryopreservation solution (culture medium + 15% heat inactivated FBS, containing 10% ME_2SO) was added drop by drop to the cells in a total volume of 0.6 ml per Petri dish (diameter 3.5 cm). After incubation for 30 min at 4°C, samples were frozen to –80°C at 1°C/min in a computer controlled freezing device (SYLAB, Neupurkersdorf, Austria). Cells were stored until thawing in –80°C freezer. A control (non-frozen) sample was stained with FDA/EB and pictures were taken for an imaging based vitality evaluation method. Samples were thawed in a 37°C water bath, special care was taken that no water came inside the cell samples. The samples were stained directly, 24 h and 48 h after thawing. After that the same samples were prepared for scan-ning electron microscopy (SEM). Petri dishes with cells were wshed with 0.25M Hepes (PAN, Aidenbach, Germany) buffer solution, fixed overnight at 4°C with 2% glutaraldehyde (Agar Scientific, Essex, UK) in sodium cacodylate buffer (Agar Scientific, Essex, UK) and treated with 2% osmium tetroxide (Roth, Karlsruhe, Germany), 1% tannic acid (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and 1% uranyl acetate (TED PELLA INC, Redding, CA) in water (PAN, Aidenbach, Germany) as previously described [16] and

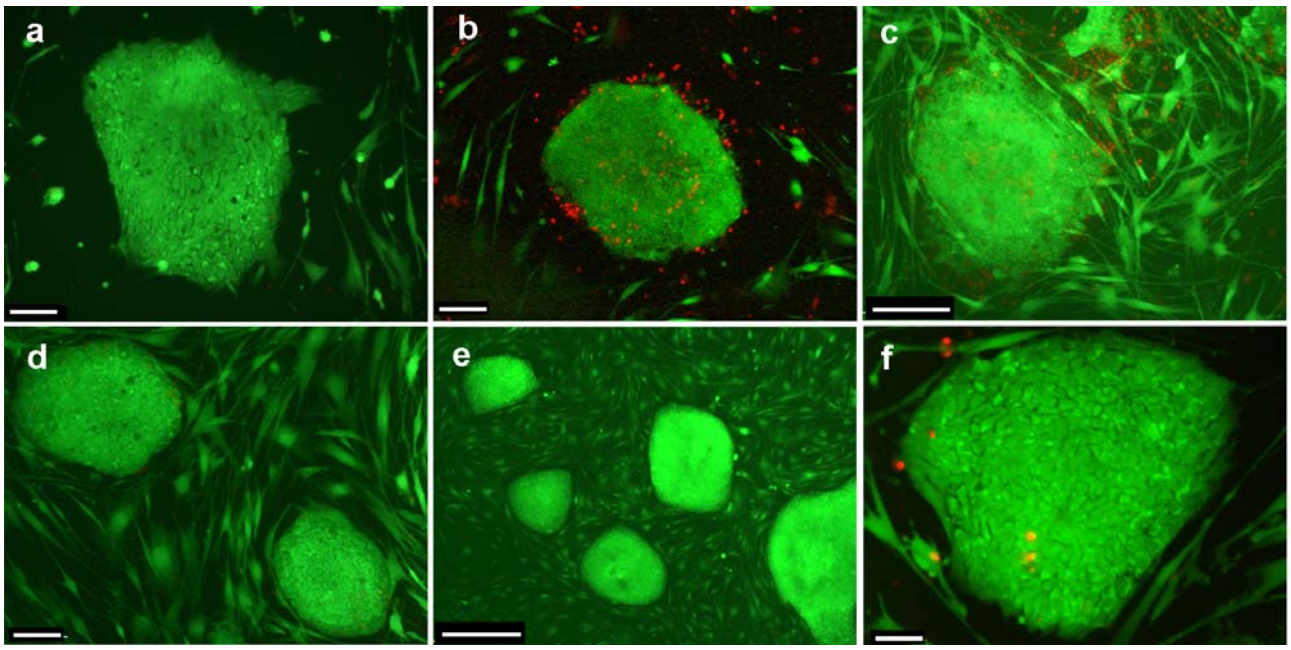

Fig. 6. Representative images of vitality testing of H1 stem cell colonies with feeder cells frozen with ECM gel before (a), immediately (b), 24h (c), 48h (d) after cryopreservation. and 3 days after post thawing cultivation (e, f). Scale bars are 100 μm, exept in in e, which is 500 μm.

modified [17]. Then the cells were dehydrated in increasing series of ethnol, dried in automated Polaron Range critical point dryer CPD-7501 (Quorum Technologies Ltd.) and prepared for SEM. Samples were examined in a field emission scanning electron microscope FESEM XL30 (Phillips, USA) using secondary electron (SE) modes with 10 kV accelerating voltage and 10 mm working distance.

Before freezing most of the cell colonies of different sizes were vital and attached to the substrate (Fig. 6a). After thawing approximately 30% hESCs colonies were detached from the substrate, but more than 85% of the remaining H1 embryonic stem cells were vital even 24 and about 95% of cells 48 h later (Fig. 6b, c, d; Fig. 7). 3 days after thawing the H1 colonies were harvested by collagenase (see above) and successful re-cultivated (Fig. 6e, f).

SEM of the same objects has shown that H1 cell colonies were attached directly to ECM gel before (Fig. 8a, b, c) and after (Fig. 8, d–l) cryopreservation. The colonies in the non-frozen control showed close cell-cell contacts and their surfaces were covered with microvilli (Fig. 8b, c). Sometimes the colonies in control were partly detached from the substrate (perhaps, during SEM preparation) and numerous filaments and blebs were visible on the reverse side (Fig. 8a, insert). Most of the stem cells had immediately (Fig. 8(d–f)), 24 h (Fig. 8(g–i)) and 48 h (Fig. 8(j–l)) after cryostorage a surface relief comparable with the non-frozen control (Fig. 8b, c). However, some cells were detached immediately after thawing (Fig. 8e, arrows). In several colonies we saw a smoothing of cell surface, a sign of low temperature stress reaction (Fig. 8f, double arrow). 24 h after thawing we have observed also the development of cell damages, (Fig.8, h, asterisks) as well as absolutely smooth damaged cells with maceration and holes on the surfaces (Fig. 8h, double asterisks). The whole recovery of cell surface relief can be observed 48 hours after thawing (Fig. 8j, k, l).

It is known that hESCs can be grown in culture as colonies on feeder cells and can remain non-differentiated or be differentiated in a desired way, e.g. cardiac differentiation [28, 30]. There are three ways to cryopreserve hESCs. Slow freezing of dispersed colonies is usually not successful and needs high levels of cryoprotectant [14, 15, 19]. Vitrification is a powerful tool but because of the already mentioned limitations not

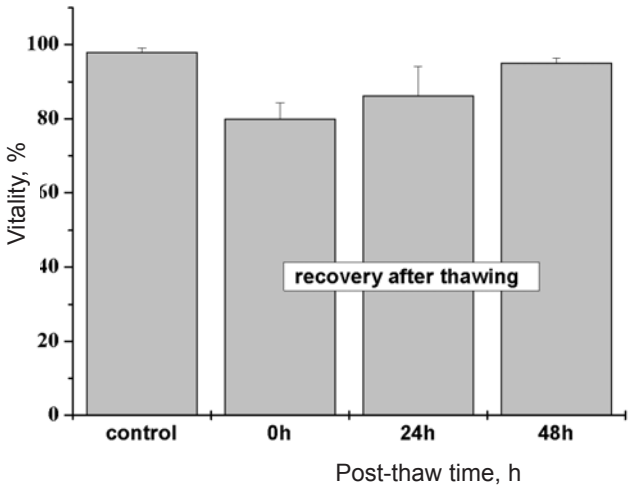

Fig. 7. Vitality testing for H1 stem cell colonies after cryopreservation using ECM gel and serum-contained medium with 10% Me_2SO.

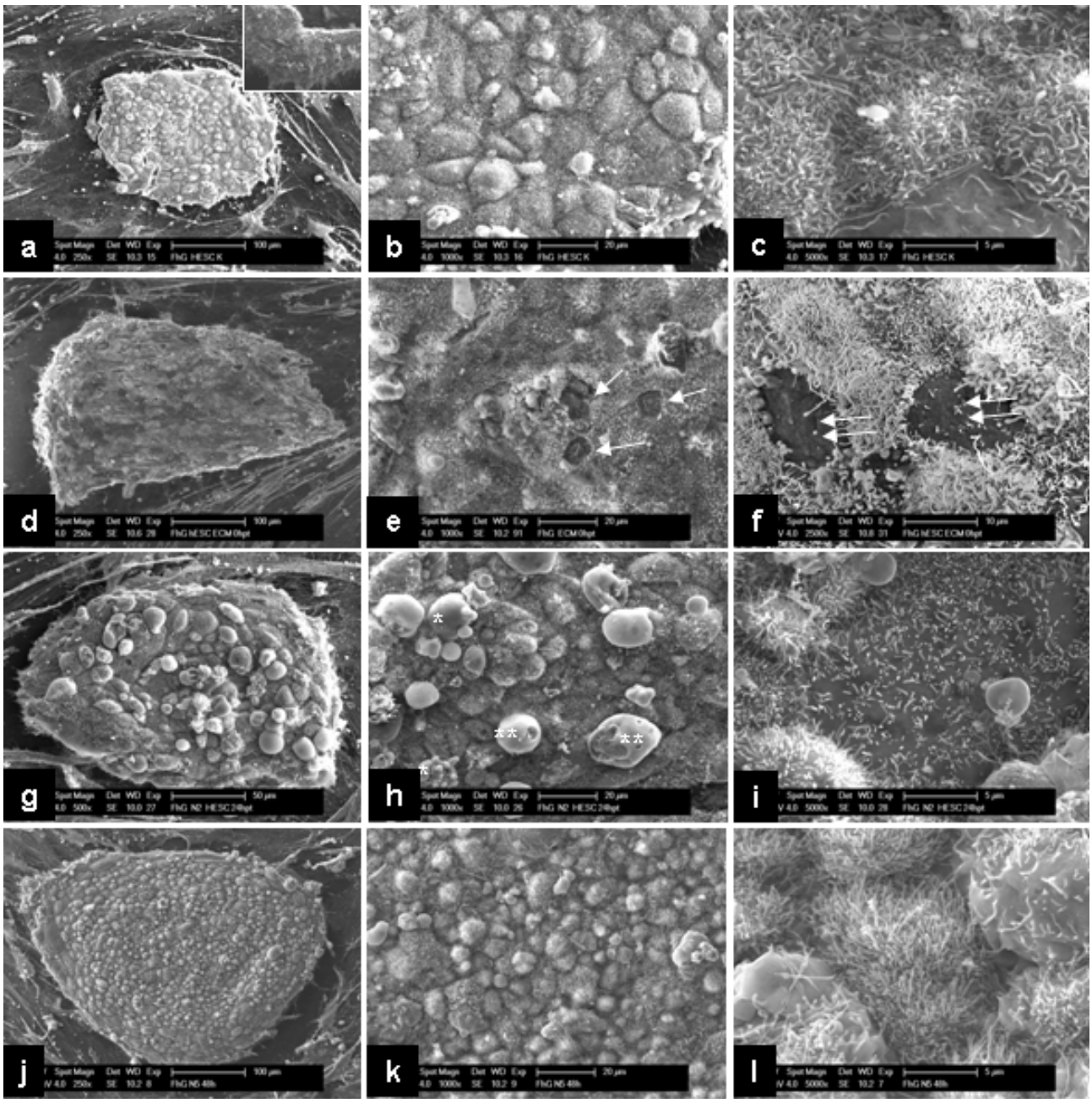

Fig. 8. H1 embryonic stem cells cultured and cryopreserved with serum-containing medium and 10% Me_2SO using ECM gel (a, b, c – control; d, e and f – cells immediately after thawing; g, h and i – 24 h later; j, k and l – 48 h later): a – overview of cell colonies; insert – outside of colony; note of structure similar to the extracellular matrix; b, c – cell surface of control; the embryonic stem cells are covered with microvilli; d, e, f – structure of stem cell colony immediately after thawing: note detachment (e, arrows) and smoothing (f, double arrows) of some cells; g, h, i – blebs on the surface of some cells (asterisks) and appearance of the smooth cells with damaged surfaces (double asterisks) 24h after thawing; j, k, l – whole recovery of microvillous relief of stem cell colony 48 h after thawing. Scale bars are presented in the pictures.

suitable. Cryopreservation of adherent hESCs with or without feeder cells can be an alternative for stem cell storage, especially, due to the reduction of differentiation after thawing [14]. Our design with ECM-coating of plastic surfaces is comparable with Matrigel™[14]. In contrast to the results of Heng *et al.* [9] the first cryopreservation of H1 embryonic stem cells on the ECM gel (Fig. 6(d–f)) was successful. Combined fluorescent and SEM of the same colonies in our study have shown that, immediately after thawing, there is a stress reaction (smoothing of cell surface), but only a little permanent damage. 24 hours after thawing hESCs colonies some damages as well as apoptosis (blebs, Fig. 8h) were developed.

Perhaps, good preservation with ECM-gel is achieved by the following reasons. Firstly, the cells may contract with the ECM and therefore not detach from the substrate. Secondly, filaments of ECM (as in Fig. 8a, insert) may absorb water, thereby avoiding harmful ice crystallisation. This opens up new pers-

pectives for creating novel MST cryosubstrates with different ECM proteins for screening and cryopreservation of embryonic stem cells with optional differentiation after thawing.

Summary and outlook

1. We have presented novel microsystem-based tools and devices for cryopreservation. Comparison with standard cryovials has shown that the reduction of concentration of toxic CPA's is possible and can yield a high viability for adult stem cells.

2. Using new cryoequipment for the study of stability in long-term storage of cells, we have shown a constant viability of cancer cells throughout repeated freezing cycles.

3. We have demonstrated a possibility of precise imaging of freezing processes of multicellular systems by means of high-speed video cryomicroscopy. This method allows new insights in the phase transition mechanisms by freezing of embryonic stem cell colonies. Fluidic simulation and modelling will allow a cell specific freezing procedure design.

4. More than 85% of the adherent H1 hESCs remain vital up to 48 hours after cryopreservation and following re-cultivation using gel-like matrices. This opens new avenues for stem cell cryopreservation.

The new devices and methods will lead to new biobanking procedures and standards [42]. However, the methods for investigation of hESCs recovery after cryopreservation are still missing. In this case technology for parallel long-term video microscopy with high numbers of cells for analysis of cryoinduced hESCs differentiation is needed.

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