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RESEARCH ARTICLE

OPTIMIZED METHOD FOR USING EMBRYONIC MICROENVIRONMENT TO REPROGRAM CANCER STEM CELLS

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ABSTRACT

Purpose: The embryonic microenvironment contains many properties that have not yet been fully explored. Our aim in this study is to report an optimized and efficient method that enables investigating the effects of the secretome of pluripotent embryonic stem cells on cancer stem cells.
Material and Methods: The study is performed with a chimeric model consisted of mouse blastocysts, non cancer stem cells and human prostate cancer stem cells. Ovulation induced mice were used for blastocyst collection. DU145 prostate cancer cell line was separated into non cancer and cancer stem cells using cancer stem cell biomarker expressions by fluorescent activated cell sorting. Human prostate cancer stem cells and non cancer stem cells were microinjected into 4-day blastocyst culture in vitro by intracytoplasmic sperm injection.
Results: Chimeric models provide us great convenience in basic oncological studies. In this study, using a chimeric model, we were able to study the secretome of mouse embryonic stem cells and their effect on cancer stem cells. The method is efficient and yield promising result; and could be used to study the effects on other cells as well.
Conclusion: The embryonic stem cell microenvironment is suggested to have a great regenerative capacity, nowadays, the center of attraction for cancer research studies. Ethical issues restrict the human embryo studies, however, mimicking the in vivo human microenvironment with 3D cell cultures or bioprinting are now possible. Finally, optimization of new methods including 3D cell cultures with human cell lines will be a great opportunity for better understanding the reprogramming notion.

Keywords: embryonic stem cells, cancer stem cells, induced pluripotent stem cells, microenvironment, secretome, intracytoplasmic sperm injection

INTRODUCTION

Cancer stem cells (CSC) are the small population of cancer cells and have been identified as the culprits of tumor invasion, metastasis, drug resistance, and relapse. Invasion/migration patterns, signaling pathways and gene expression profiles of cancer cells are similar to embryonic stem cells (ESCs). Embryonic stem cells have special properties of pluripotency and self-renewal and can transform into approximately 200 cell types when stimulated with the required signals (1). Their remarkable plasticity puts them in the center of interest of tissue engineering and regenerative medicine (2,3). ESCs are obtained by isolation of cells from inner cell mass (ICM) of primordial embryo. Specialized immunological and mechanical methods are used; followed by incubation with various growth-factor containing medium (4). Two important features that make these cells as the focus of attention in regenerative medicine can be defined as their ability to proliferate without differentiation by the self-renewal process and their potential of forming specialized cell types when induced for differentiation (5,6).

During early development, ESCs and the embryonic microenvironment share many characteristics that are important for pluripotency, self-renewal and differentiation with tumor cells and the tumoral milieu such as cell invasion/migration, common signaling pathways and gene expression (7). These common features raise the possibility that the embryonic microenvironment can be used to elucidate the molecular mechanisms involved in cancer development. In addition, the microenvironment is known to be a key regulator in tumor progression and metastasis, as well as having a regulatory role in ESC fate in early embryonic development. It is known that the embryonic microenvironment does not permit tumor development, as early developmental signals innately suppress the expression of proto-oncogenes (8). Likewise, the oncogenic characteristics of cancer cells have also been demonstrated to be suppressed in embryonic stem cell cultures that imitate the early embryonic environment (8). These observations may suggest that cancer cells can be reprogrammed in the presence of an environment that maintains the appropriate regulatory mechanisms required for a normal cellular phenotype.

The effect of microenvironment contents (secretome) on CSCs is still not fully understood. The secretome encompasses the secretion of bioactive molecules and extracellular vesicles (EVs) with significant paracrine activity and includes exosomes, microvesicles, membrane particles, peptides, and small proteins like cytokines (9). Exosomes are the smallest member of the extracellular vesicles, varying in size from 30 to 100 nm, and are active in many biological events such as cell-cell interaction, signal transmission, communication, and transport of cellular molecules (10). It has been revealed that exosomes have contributions such as creating a microenvironment that will trigger tumor formation, angiogenesis, metastasis by changing the adhesion properties, motility and invasion of target cells, and drug resistance (11). What makes exosomes interesting when compared to other EVs, is the RNA cargo (mRNA and miRNA) they carry (12). Exosomes, which play a role in cell-cell interaction, can change the function of the cell through its RNA cargo when transferred to the recipient cell (13,14). Especially considering cancer cells gaining epithelial-mesenchymal transition (EMT) properties. EMT, which is defined as the cells losing their epithelial properties and gaining mesenchymal properties, is required for metastasis (15). With this transition, cells gain the ability to invade and migrate (16).

In the light of all this information, the question of whether the reprogramming feature of the embryonic microenvironment can be utilized for cancer treatment comes to mind. In this article we discuss the reprogramming feature of the embryonic microenvironment and the optimized microinjection method that we used to investigate the embryonic stem cell reprogramming capacity on cancer cells. We microinjected cancer (stem) cells into the blastocyst stage of a mouse embryo. Because of the ethical issues, it is not possible to study directly in human model. Therefore, our studies were conducted in mouse-human chimeric models. Human prostate CSCs were isolated for microinjection into mouse blastocyst ICM by intracytoplasmic sperm injection (ICSI) as detailed below. Alternatively, İPS cells and stereomicroscopy can be used for microinjection instead of the ICSI method. The method that we have optimized and report in this manuscript can be efficiently used in research to investigate ESCs reprogramming effect on CSCs and non-CSCs.

MATERIAL AND METHODS

CD133+ CD44+/low CSC isolation

CD133+ CD44+/low CSCs from DU145 prostate cancer cells were isolated by fluorescent activated cell sorting (FACS). Cells were treated with 0.25%

Trypsin-EDTA and observed with a microscope that all cells were separated from the flask surface. RPMI 1640 medium (with 10% FBS) was added for trypsin inactivation. The suspension was centrifuged at 1000 rpm for 10 minutes. After removing the supernatant, PBS was added and vortexed. 10 µl of the suspension was taken and cells were counted with trypan blue. Then, the cell suspension was centrifuged at 1000 rpm for 5 minutes, suspended in sterile PBS (with 5% BSA) and collected in tubes by passing through filter-caps. CD44-APC (Miltenyi Biotec, UK) for 10µl/10 6 cells and CD133-PE (Miltenyi Biotech, UK) for 10µl/10 6 cells were added to the suspension, incubated for 10 minutes at +4o C by vortexing and centrifuged at 1000 rpm. Cells resuspended in sterile PBS were separated by FACS into two separate tubes as prostate CSCs (or sorting) expressing CD133+ CD44+/low surface markers and the remainder non-CSC (or non-sorting) separated by flow cytometry analyzer.

Ovulation Induction and Blastocyst Obtention in Mice

An ethical approval was obtained from the Animal Experiments Local Ethics Committee of Ege University, Izmir, Turkey (Ethics Committee No: E-99166796-050.06.04-622902-259, date:29.03.2022) for the experimental applications.

To induce superovulation in CD1 female mice, 5 IU (International Unit) pregnant mare serum gonadotropin (PMSG) was administered intraperitoneally. Folligon (Intervet, USA) containing 1000 IU PMSG was diluted in PBS (pH 7.4) with a final concentration of 50 IU/ml under sterile conditions and stored at -800 C in the freezer to be used later. 0.1 ml PMSG (50 IU/ml) was injected intraperitoneally into female mice housed in a 14-hour light – 10-hour dark cycle.

Ovulation induction was performed by intraperitoneal administration of 5 IU human chorionic gonadotropine (hCG) at 48 hours following PMSG administration. Pregnyl (Organon, USA) containing 5000 IU hCG was diluted in PBS (pH 7.4) with a final concentration of 50 IU/ml under sterile conditions and stored at -800 C in the freezer to be used later. 0.1 ml of pregnyl (50 IU/ml) was administered intraperitoneally to female mice housed in a 14-hour light – 10-hour dark cycle. Each hCG-treated female mouse was housed in individual cages with a CD1 breeding male mouse. The next morning, whether mating took place was evaluated according to the presence of vaginal plugs in the vagina of female mice. Female mice with vaginal plugs were used for embryo isolation. Embryos at the two-cell stage were obtained by flushing the oviduct 46 hours after hCG administration. Washing after 115 hours was performed and blastocysts were collected.

Embryo Collection and In Vitro Embryo Culture

Vaginal plug detected females were euthanized by cervical dislocation after anesthetization of 10mg/kg xlasine and 60mg/kg ketamine hydrochloride combined intramuscularly approximately 46 hours after mating. The ampulla part of the oviduct was washed in 30 mm petri dishes with a thin injector tip, allowing the embryos to pass into the external medium heated to 370 C. Embryo culture medium was put into the incubator one night before and gassed. 20 µl microdrops of this medium were formed in 30 mm sterile petri dishes in a laminar flow cabinet and embryos were placed in these drops to be cultured in an incubator.

Co-culture and Microinjection into the Blastocyst

For co-culture experiments, a group of embryos were incubated in embryo culture medium with DU145 CD133+ CD44 +/low CSCs in 6-well plates. The second group was incubated together with nonCSCs in 6-well plates containing embryo culture medium. An average of 100,000 cells were cocultured with 60 embryos/well for 4 days. After the embryos reached the blastocyst stage, the embryos and cells were collected separately and suspended in RNALater Stabilization Solution (Invitrogen) at +4o C. CSCs and non-CSCs incubated with embryo culture medium were used as negative control group. ICSI is a method of microinjection of sperm into oocyte in vitro. CSCs and non-CSCs were injected into blastocysts using the micromanipulator device. Early blastocysts obtained by culture were divided into two groups; (i) 1-2 CSCs and (ii) 1-2 non-CSCs and were injected into the ICM of the blastocysts by micromanipulator. Cell inoculation was performed by microinjection into the ICM of >80 embryos for both groups. After micromanipulation, the embryos were washed, placed in blastocyst culture medium (G2 plus, Vitrolife, Sweden) which had been gassed in a 5% CO 2 incubator the night before and incubated in 37C°, 5% CO2, 95% humidity incubator. Because the viability of both embryos and cancer stem cells/ non cancer stem cells medium concentration is optimized with 1/2, 1/3, and 1/4 dilutions. Embryos that were

checked for viability at 24 and 48 hours were stored in RNALater Stabilization Solution (Invitrogen) with a high viability rate (70-80%).

iPSC 3D Cell Culture

Fibroblasts were transfected with pCXLE-hOct3/4-shp53, pCXLE-hSK, pCXLE-hUL, and pCXWBEBNA1 plasmids for reprogramming factor induction, using Neon Transfection System. Fibroblasts were treated with 3 μM EPZ004777 (DOT1L inhibitor) to increase reprogramming efficiency for 6 days. On day 7, transfected fibroblasts were transferred onto MEF-feeder cells and maintained in human ESC medium containing DMEM/F-12 with %20 KOSR (Thermo Fisher Scientific), 1% Penicillin/Streptomycin, 1% Non-essential amino acids, 1% L-glutamine, 0.1 mM β-mercaptoethanol, 10 ng/ml FGF. iPSC colony formation was observed under microscope until single colonies were formed. iPSCs were passaged with a 1:6 ratio with ReleSR (Stem Cell Technologies) every week and maintained on MEFs at 37o C, 5% CO 2 without ROCK inhibitor (17,18).

ESC 3D cell culture

ICM of blastocyst stage embryos are used to generate hESCs. These unique cells can continuously proliferate and differentiate in to all three embryonic germ layers. After the ICM isolation embryos are destructed and this raises ethical debates in the world (16). An innovative alternate approach has been generating hESC lines from single blastomeres biopsied from patient embryos as is routinely done during preimplantation genetic testing. Chung et al. successfully derived 5 human ESC lines from a single blastomere biopsied from individual eight cell embryos (17). The single blastomere was co-cultured with the parental biopsied embryo for up to 24 hours before moving to blastocyst medium containing fibronectin and laminin. The biopsied parental embryo remained available for clinical use after cultivation to the blastocyst stage. The presence of laminin was noted to be critical for formation of ESC-like aggregates and prevention of trophectoderm-like vesicles. During stem cell derivation in serum-free media, inclusion of FGF increased cloning efficiency, sustained cell proliferation and was necessary to prevent differentiation of hESC (19).

hESC were cultured either on growth factor-reduced Matrigel-coated (Corning) dishes or on irradiated CF-1 mouse embryonic fibroblasts (MEFs) (AMS Biotechnology). 1.6% Matrigel solution in DMEM/F12 was incubated for 2 hours at room temperature. When on Matrigel, hESCs were cultured in mTeSR1 (StemCell Technologies), with medium changed every day. When cultured on MEFs, hESCs were cultured in 'Primed medium', consisting of DMEM F12 (Thermo Fisher Scientific) supplemented with 100 mM b-mercaptoethanol (Thermo Fisher Scientific), penicillin–streptomycin (Thermo Fisher Scientific), GlutaMAX (Thermo Fisher Scientific), MEM nonessential amino acids (Thermo Fisher Scientific), and 20% v/v KnockOut Serum Replacement (Thermo Fisher Scientific). This was supplemented with 12 ng ml1 bFGF2 (Stem Cell Institute) before use. hESCs were passaged using StemPro Accutase (Thermo Fisher Scientific), which was added for 3 min at 37˚C, before being diluted in DMEM/F12 and centrifuged. Cells were then plated in their appropriate medium supplemented with 10 mM ROCK inhibitor Y-27632 (STEMCELL Technologies). ROCK inhibitor was removed after (20).

RESULTS

Reprograming of Cancer cells by a Chimera Model

We optimized an efficient method for ESC reprogramming of cancer (stem) cells by microinjection into 3D cell culture. Chimeric animal models were used for understanding the reprogramming effect of pluripotent mouse ESC secrotome on human prostate CSCs. Retrieval of animals and blastocyst after ovulation induction require manipulation experience. However, our study group was successful after a few trials in obtaining blastocysts and preserving their viability for 3-4 days. Cancer stem cells were co-cultured after isolation (Figure 1). Both embryos and cancer stem cells/ non-cancer stem cells were maintained in 1/2 RPMI 1640 medium (with 10% FBS) and G2 plus, Vitrolife. In we observed that the viability of co-cultured cells increased to 3-4 days with this protocol (Figure2). In

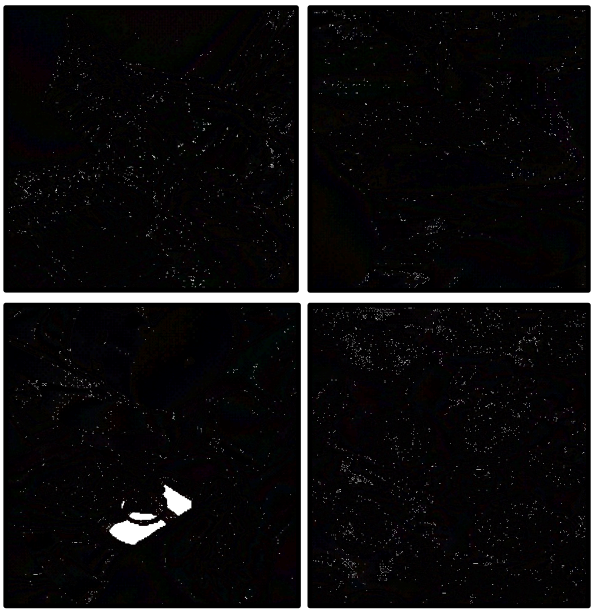

Figure 1. Procedures for obtaining embriyosu from mice.

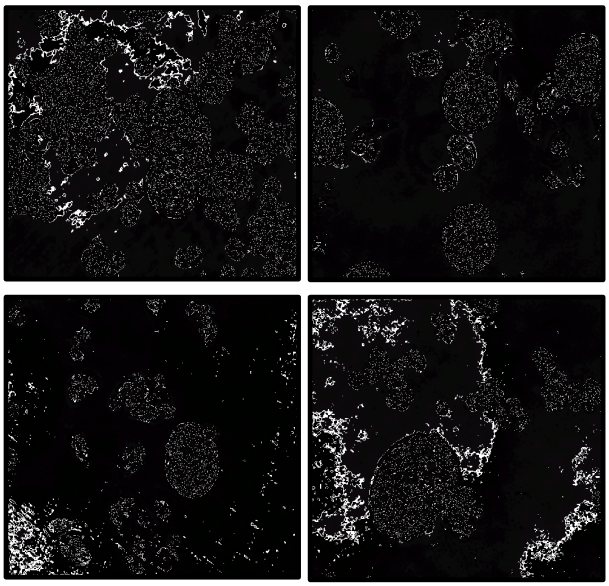

Figure 2. Co-culture of CSCs and embryos.

the ICSI method, 3-4 CSCs were microinjected into the ICM, and their viability was maintained for 36 hours (Figure 3).

Reprograming Cancer Cells in Human Sera

Although chimeras are suitable for much research, cell signaling, molecules, and cellular responses are different in each organism. Therefore, it cannot reflect human physiology 100%. This is especially true for cell signaling pathways. Chimeras are usually used as a model for preliminary studies, but it is an unnatural system when compared to the human body. Such studies in humans are not possible. Therefore, 3D cell culture models with human cell lines are used to produce more relevant results, especially in cell signaling research. ESC and iPSC cultures can be maintained by following cell culture guidelines, paying attention to sterility, and using growth mediums correctly. Injection into 3D culture can easily be performed using ICSI or a stereo microscope. It is important to mark cancer cells with viable cell stains. By following these cells, it will be possible to perform single cell analyzes and evaluate their morphological properties. The most important issue is to provide the right medium to prevent rapid differentiation of cells, cell contamination and microbiological contamination. Hence, data obtained from 3D cell culture models can be used for developing new treatment strategies.

DISCUSSION

Various clinical studies are pointing the benefits of stem cell-based therapy in many diseases. However, hESC-based clinical treatments that involve the destruction of the human embryo also pose many ethical and safety problems. hESCs can be obtained and produced form the inner cell mass of

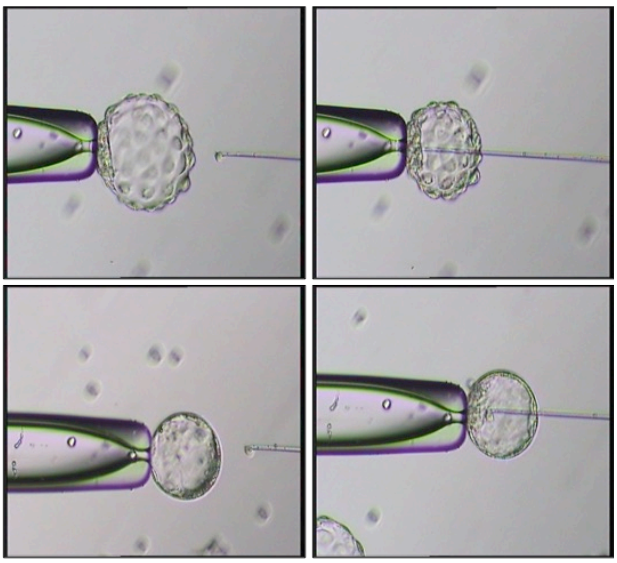

Figure 3. Microinjection of CSCs into the blastocyst by ICSI

preimplantation embryos (21). These cells have the ability to transform into any type of the germ layers as endoderm, mesoderm and ectoderm (22,23). Thus, as mentioned in the previous sections, hESC have an important role in development of new treatment strategies and elucidating early human embryo. This ethical dilemma regarding hESCs, which can be used in the treatment of many diseases, can be seen as an

obstacle to their use for different indications and perhaps to early access to treatment by patients in clinical trials.

Current limitations are a major barrier while developing cell-based clinical therapies and have also a slowing effect on the advancement of hESCs technology. Research on this subject has been contentious because of the ethical controversy surrounding the harvest of hESCs, so, the bulk of studies have tended towards animal models (24).

Besides ethical concerns, safety concerns of hESC-based treatments in humans are also one of the main concerns that should be well defined. Pluripotent characteristics of hESCs; firstly, the plasticity allows hESCs to differentiate into hundreds of different cell types, and then the difficulty of control after in vivo transplantation makes them a double-edged sword (25).

This issue has been solved with previous iPSC derivations, but there are still open questions about the clinical translation of iPSC at the present. The limitless differentiation potential of iPSCs, which can also be used for human reproductive cloning, raises serious ethical concerns on opening up the possibility of creating human animal chimeras and genetically modified human embryos while also posing serious safety concerns about unwanted differentiation and cancerous transformation. Regarding their karyotype, phenotype, telomerase activity, and ability to differentiate, iPSC and hESCs are quite similar. Nevertheless, because their generation is different from hESCs, iPSCs are thought to be ethically superior to hESCs (26).

Patient specific- individual- iPSCs has the ability to differ ceratin cell types which can be accomplished in-vitro. This procedure can provide personalized drug selection and trial of new/innovative drugs. Since iPSC-derived cells are also produced from the patients' somatic cells this approach also avoids the immunological rejection risk of transplanted iPSC-derived cells (27). The advancement of reproductive technology makes it possible to produce gametes from human iPSCs (28). This technique seems to be an opportunity for treatment of infertility, but this opportunity is also associated with very sensitive ethical-legal restrictions like potential exploitation of created embryos, human nuclear transfer, and the risk of altering natural reproduction (28).

When it comes to hESCs, the biggest safety concern with iPSC-based treatment is the potential for teratoma development if patients get iPSC-derived cells containing undifferentiated iPSC. Tumor development and/or unintended iPSC differentiation in a wide range of somatic cells may emerge from unregulated proliferation and differentiation of transplanted undifferentiated iPSCs (29). Therefore, a difficulty for personalized and regenerative medicine is the development of more efficient techniques to produce pure populations of autologous iPSC-derived differentiated cells (30).

As a result, more in-vitro and in vivo animal studies should be performed to create an optimum growth and differentiation technique and similar preclinical safety studies to assess the potential of iPSCs and differentiated cells produced from iPSCs for clinical use in patients.

CONCLUSION

It is of great importance to investigate new signaling pathways and molecules that can be used in cancer treatment and to develop new methods. Benefiting from the regenerative capacity of the stem cell in its natural environment and developing a treatment protocol using cell stabilization in the embryonic period is seen as a new and promising method. This way, which can be briefly summarized as "reprograming", makes it possible to eliminate the malignant potential of cancer cells. While ethical problems limit the study on human embryos, it is possible to mimic the in vivo environment with 3D cell cultures and even bio printable cultures. The human CSC co-culture environment with hESC will give us the chance to reveal different molecular mechanisms in the cancer model we want (prostate, breast, colon, gastric cancer…etc). The chimeric model is a model that can be easily used ethically and offers the opportunity to work with the ESC in its natural environment. However, possible differences in molecular mechanisms will create limitations regarding the development of treatment protocols in human cancers.

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Author contribution: BC. Soner and G. Oktem devised the project. E. Acikgoz and G. Oktem carried out the CSC isolation. Ovulation induction and blastocyst obtention in mice is performed by F. Oltulu and E. Ozcinar, A. Demir and A. Taskiran carried out the embryo collection and in vitro embryo cultures. A. Demir, A. Taskiran and Z. Yuce participated in the co-culture and microinjection into the blastocyst. Z.Yuce and G. Oktem carried out the iPSC and ESC 3D cell culture. BC. Soner conceived of the study and participated in its coordination. G. Oktem participated in the design of the study and performed the statistical analysis. EN. Tavmergen-Goker helped to draft the manuscript. All authors read and approved the final manuscript.

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