The contribution of donated human embryos suitable for the production of embryonic stem cells to increase the quality of life: Selection and preparation of embryos in the Czech Republic

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Abstract

Background. Human embryonic stem cells (hESCs) have the unique ability to differentiate into any cell type in the human body and to proliferate indefinitely. Cell therapies involving hESC have shown very promising results for the treatment of certain diseases and confirmed the safety of hESC-derived cells for humans. They are used in cell therapy, mainly in targeted therapy of diseases that are currently incurable.

Objectives. The aim of this study was the derivation of clinical-grade hESCs usable in drug development, non-native medicine and cell therapy.

Materials and methods. Embryos were thawed, cultivated to the blastocyst stage if necessary, and assisted hatching was subsequently performed. Embryoblasts were mechanically isolated using narrow needles. Each line was kept as a separate batch. The derived hESCs were cultured under hypoxic culture conditions (5% O_2, 5% CO_2, 37°C) in a NutriStem® hPSC XF Medium with a daily medium change.

Results. From January 2018 to July 2020, 138 selected clients were asked for consent to donate embryos, of whom 52 did not respond, 19 terminated the storage of their embryos and 29 extended the storage. Only 38 clients (27.5%) agreed to donate embryos for the derivation of hESCs. At the same time, personal communication with clients took place and another 17 embryo donors were recruited. A total of 160 embryos from 55 donors aged 26–42 years were collected. The embryos were frozen at the blastocyst (33.1%) or morula (46.3%) stage. After the preparation of 64 embryos, embryoblasts were isolated and cultured. Finally, 7 hESC lines were obtained, 4 research-grade and 3 clinical-grade, the first in the Czech Republic.

Conclusions. We established a current good manufacturing practice (cGMP)-defined xeno-free and feeder-free system for the derivation, culture and banking of clinical-grade hESC lines that are suitable for preclinical and clinical trials. The quality control testing with criteria concerning sterility, safety and characterization according to cGMP ensured the clinical-grade quality of hESC lines.

Key words: stem cells, IVF, human, embryo, hESC

Background

Stem cell research is a promising field of medical science. Contemporary medicine is increasingly dealing with the problems of chronic diseases or diseases for which there is no permanent cure. The potential of stem cells is enormous because the replacement of damaged cells with newly created ones would provide solutions to a whole range of diseases and economic issues associated with healthcare in the 21st century.

Freedom of research, protection of life, new treatment methods, economic disputes, and ethics are the topics colliding in the discussion. Due to different cultural backgrounds and the level of research, nearly every country has a different approach to the topic. This is demonstrated by the differences in legislation regulating the acquisition of stem cells and research concerning them.[1]

Human embryonic stem cells (hESCs), contrary to somatic stem cells, have a unique ability to differentiate into every cell type of the human body. This ability makes them a tremendous cell source for regenerative medicine. Moreover, pluripotent hESCs have an unlimited self-renewal capacity. These features are used in cell therapy to replace missing or damaged cells in the human body. The goal is to enable targeted therapy for currently incurable diseases such as diabetes mellitus,[2] spinal cord injury or Parkinson's disease.[3] Therapeutic approaches using hESCs bring promising results, especially in age-related macular degeneration.[4] However, there are several technical problems. One of the most important issues is to avoid possible teratoma/tumor formation that can arise from all redundant undifferentiated stem cells.[5] For prevention of differentiation to tumor cells, the residual ESCs need to be eliminated using magnetic or fluorescence-activated cell sorting (MACS or FACS)[6] or with antibodies against undifferentiated hESCs.[7] Another problem with the clinical application of ESCs is the allogenic immune rejection of the hESC cells by the recipient. Current immune suppression systems effectively prevent allogenic immune rejection but persistent use of immune suppressants is toxic for patients and increases the risk of infection or cancer, mainly in patients with cytomegalovirus or herpes virus.[8] Despite these shortcomings, treatments using ESC cells are promising for the therapy of a wide range of diseases. The derivation of clinical-grade quality hESC lines enables their application in preclinical and clinical studies.

Objectives

The aim of this study was the derivation of clinical-grade hESCs, usable in drug development, non-native medicine and cell therapy. The use of these methods will increase the quality of life of treated individuals.[9–11]

The task of the clinical part of the project was to select and ensure a sufficient number of donated embryos. Research activities using human embryos in the Czech Republic are regulated by Act No. 227/2006 Coll. on Research on Human Embryonic Stem Cells and Related Activities and Act No. 373/2011 Coll. on Specific Health Services.[12,13]

The methods of isolation and cultivation of hESCs are subject to strict regulations issued by the Ministry of Education, Youth and Sports of the Czech Republic (Ministerstvo školství, mládeže a tělovýchovy České republiky (MŠMT)), and its approval (No. MŠMT-2922/2020-14) was a precondition for initiating our project. A condition for the successful implementation of the project was an interdisciplinary team managing the medical, technological, and instrumental procedures and protocols necessary for the proposed research.

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethical Committee of the University Hospital Brno, Czech Republic, on June 26, 2017 (approval No. 16/2017). Written informed consent was obtained from all participants included in the study.

Materials and methods

The derivation of hESCs must be performed in accordance to the legislation of the Czech Republic and the European Union. An informed consent form was developed by us for the donors regarding the donation of discarded embryos that are not suitable for in vitro fertilization treatment according to Directive 2004/23/EC. Center of Assisted Reproduction (CAR) of the University Hospital Brno was involved in oocyte collection; culture and cryopreservation of embryos; communication with clients; and ensuring informed consent of embryo donors. A handover protocol and transfer of the thawed embryos with the original numerical code were developed in cooperation with the Cell and Tissue Engineering Facility (CTEF) of St. Anne's University Hospital Brno. Before the embryos were handed over to the CTEF, they were thawed and cultivated to the blastocyst stage if necessary. Subsequently, assisted hatching was performed.

Embryo selection aspects

Embryo selection began by reviewing CAR laboratory protocols for the cryopreservation of embryos. It was necessary to check the addresses of the clients, including a relatively complex tracing of the storage fee, and the current number and stage of the embryos. A basic database of potential donors was created and included contact details of patients, patient age, date of freezing, number and stage of embryos, number of embryos in each tube, and information on genetic testing.

Creation of the informed consent form required cooperation with a lawyer and approval by the Ethical

Committee of the University Hospital Brno and the Faculty of Medicine of Masaryk University, Brno. Then, the Czech and English versions were prepared. A letter with basic information and a request for a decision on how to handle the cryopreserved embryos was sent together with a return envelope. Subsequent communication took place through telephone calls, e-mails, letters, and personal meetings.

Under Act No. 373/2011 Coll. on Specific Health Services, embryos can be treated as follows:

1) stored for further use by the recipient, i.e., the storage of embryos is extended at the request of both partners;

2) used for research on human embryonic stem cells with a granted informed consent to their usage;

3) destroyed with written consent (upon consent, the embryos are subsequently thawed and destroyed).

Embryo preparation

Embryos intended for the specified date of transfer to CTEF were thawed using Warm Cleave or Warm Blast media (Vitrolife, Västra Frölunda, Sweden) depending on the stages at which they had been frozen.[14] For the earlier stages, we performed cultivation to the blastocyst stage in medium (Blastocyst Medium; Cook Medical, Bloomington, USA) on the premises of the CAR of the University Hospital Brno embryological laboratory. After reaching the blastocyst stage, assisted hatching was performed using a laser (OCTAX NaviLase; Vitrolife) (Fig. 1).

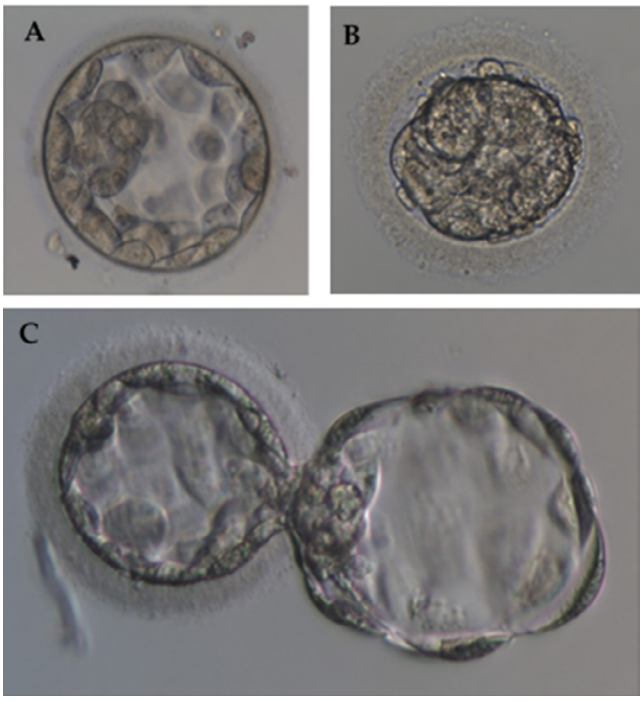

Fig. 1. Preparation of the donated embryos prior to isolation of embryoblasts and cultivation of human embryonic stem cells (hESC) lines. A. A morula stage embryo after thawing; B. A blastocyst after cultivation; C. A blastocyst after assisted hatching

Embryo transfer and embryoblast isolation at CTEF

The prepared embryos in the hatched blastocyst stage were placed in the transport medium and transferred to the CTEF laboratory using a temperature-controlled transport incubator at 37°C (ICT-P portable incubator; Falc Intruments, Treviglio, Italy).[15] Immediately afterwards, the embryoblast was isolated, followed by cultivation of the hESC line 8.

The embryoblast was mechanically isolated using a microscope (Nikon Eclipse Ti; Nikon Corp., Tokyo, Japan) with attached micromanipulators (Eppendorf, Hamburg, Germany) and an oil and air microinjector (CellTram® 4r Air/Oil; Eppendorf). Prior to the isolation of the embryoblast, the blastocyst was placed in medium (Sydney IVF Gamete Buffer; Cook Medical) covered with culture oil (Sydney IVF Culture Oil; Cook Medical) using denudation micropipettes (Microtech IVF, Brno, Czech Republic). A biopsy and fixation micropipette (Microtech IVF) was used for subsequent isolation. Each embryoblast was isolated separately and aseptically, and detailed records were kept describing the derivation and all subsequent preparation steps.

Derivation of hESCs

The derivation of hESCs was carried out in the cleanrooms of the CTEF department in purity class A against the background of purity class B, according to current good manufacturing practice (cGMP). The manufacturing process was undertaken according to standard operating procedures; microbiological and particle monitoring was performed in cleanrooms. Each line was kept as a separate batch, which was controlled during production and as a final product by quality control staff for sterility, quality, safety, and other critical parameters. An analytical certificate was created for each final product, i.e., the hESC line in clinical-grade quality.

Embryoblast was mechanically isolated using narrow needles. The derived hESCs were cultured under hypoxic culture conditions (5% O_2, 5% CO_2, 37°C) in NutriStem® hPSC XF Medium (Biological Indutries, Kibbutz Beit-Haemek, Israel) with a daily medium change.

Results

Between January 2018 and July 2020, 138 suitable clients whose embryos were frozen in the years 2014–2017| were asked for consent to donate embryos. Of those, 52 (37.7%) did not respond, 19 (13.8%) terminated the embryo storage and 29 (21.0%) extended the storage (Table 1, column n1). Only 38 clients (27.5%) agreed for the use of their embryos for the derivation of hESCs.

At the same time, personal communication with suitable CAR clients took place. Out of 22 personally contacted couples at CAR, with embryos frozen between January 2018 and

Table 1. Composition of embryo donors contacted between January 2018 and July 2020

Embryo donors	Contacted						Personal communication			
	n1	2013	2014	2015	2016	2017	n2	2018	2019	2020
Number of donors	138	3	27	29	34	45	22	8	9	5
No reply	52	2	13	10	12	15	–	–	–	–
Terminated storage	19	1	4	7	5	2	–	–	–	–
Extended storage	29	0	4	3	8	14	–	–	–	–
Embryos donated for research	38	0	6	9	9	14	17	6	7	4
Frozen embryos/year										
Total number of embryos	107	0	15	27	20	45	53	16	26	11
Embryos/patient	2.8	0	2.5	3.0	2,2	3.2	3.1	2.7	3.7	2.8

Table 2. Composition of the stages of the frozen donated embryos in 2014–2020

Stage of frozen embryos	n	%
Blastocyst	53	33.1
Morula	74	46.3
10-cell	6	3.8
8-cell	13	8,1
6-cell	6	3.8
5-cell	1	0.6
4-cell	5	3.1
3-cell	1	0.6
Pronuclei	1	0.6
Total	160	100.0

Table 3. Genetic testing results of donated embryos

Genetic state	n	%
Euploid embryo	5	17.2
Mosaic	2	6.9
Aneuploid embryo	16	55.2
Translocation	4	13.8
PGT-M (*SURF1* gene)	2	6.9
Total	29	100.0

PGT-M – preimplantation genetic testing for monogenic disorders.

July 2020, another 17 embryo donors were recruited (77.3%) (Table 1, column n2). A significantly higher proportion of donors from directly contacted clients compared to the group contacted by mail (27.5%) were influenced in their decision by a personal discussion with the selected group of couples, especially in cases with genetically examined embryos unsuitable for transfer (6 pairs, 27.3%). The remaining donors were recruited from clients who did not consider cryopreservation of all embryos (6 pairs, 27.3%) or wanted to terminate their embryos (10 pairs, 45.4%). The time lag, and the development of an opinion on the need for medical research can also have a significant impact on the total number of recruited embryos. Following personal communication with 22 couples, cryopreservation was terminated in 5 cases (22.7%) by thawing and disposal of embryos. A total of 160 embryos were obtained from 55 donors aged 26–42 years. Most often, the embryos were frozen at blastocyst (53 embryos (33.1%)) or morula (74 embryos (46.3%)) stages. The composition of embryo stages is presented in Table 2. The number of frozen embryos in each tube was 1 or 2 according to the patient's wishes. Our recommended standard is 1 embryo per tube and 61.2% of embryos (98 tubes) were frozen individually in our group. The other 62 (38.8%) embryos were frozen in pairs (31 patients).

Of the 29 genetically examined embryos, there were 5 euploid embryos (17.2%), 2 mosaic embryos (7.9%), 16 aneuploid embryos (55.2%), and 6 embryos with a translocation or carrying a monogenic defect (20.7%) (Table 3). The relatively small number of genetically examined embryos from the whole group (18.1%) is due to predominance of healthy embryos in the whole group of donated embryos. Even so, this share is higher than the average at the CAR of the University Hospital Brno. In 2021, 64 embryos were transferred to the CTEF for further processing, from which 7 hESC lines were obtained: 3 clinical-grade lines (MUCG01, MUCG02 and MUCG03) and 4 research-grade lines (MUES 10, MUES 11, MUES 12, and MUES 13) (Fig. 2). Laminin 521 (BioLamina AB, Sundybyberg, Sweden) was used in combination with NutriStem hPSC XF Medium (Biological Industries), human serum albumin and E-cadherin for mechanical derivation. The hESCs were cultured on Laminin 521 in the NutriStem hPSC XF Medium. The isolated lines were frozen according to the procedure described by Souralova et al.[16]

After thawing, the cell lines were tested using a cell viability test. Cell attachment was examined 2 days after thawing by counting the number of colonies. Growth was assessed by a change in confluency between days 2 and 5 after thawing. The cells were counted, and viability was measured during the first passage after thawing using cell counter Countess III (Thermo Fisher Scientific, Waltham, USA). The isolated hESC lines underwent mycoplasma examination, karyotype determination and genetic analyses, and the pluripotency markers were established using flow cytometry and immunocytochemistry.[16]

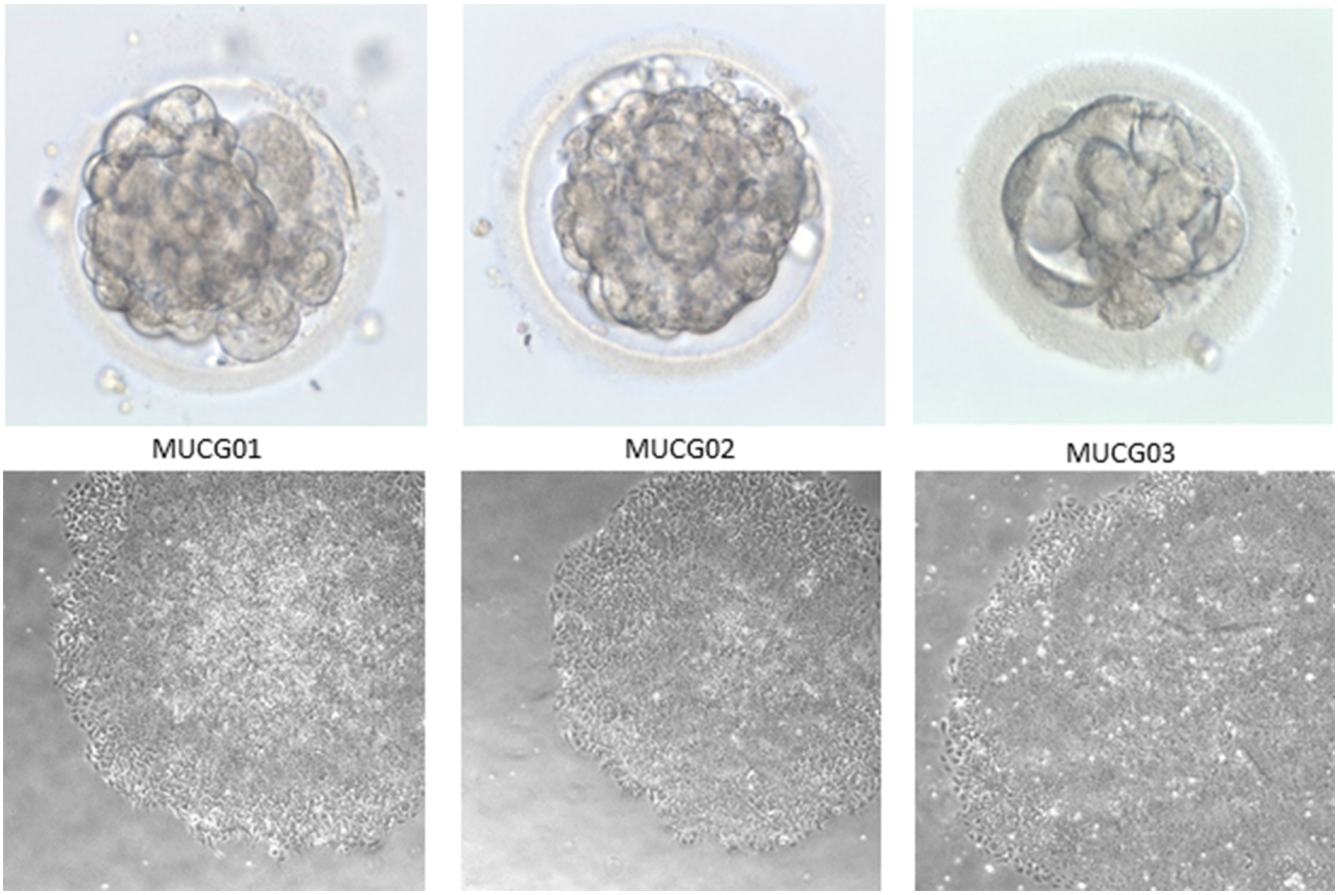

Fig. 2. Isolated embryos and corresponding derived human embryonic stem cells (hESC) clinical-grade lines MUCG01-03

Discussion

Research on stem cells of an adult organism usually does not raise ethical problems. Use of fetal tissues is mostly acceptable, or even completely legal in countries where abortion is permitted. A problem arises with the acquisition of stem cells from human embryos, which brings several questions based mainly on moral and religious principles. International Consortium on Stem Cells, Ethics and Law divides countries into 4 groups according to the extent to which their national legislation restricts stem cell research. The groups are 1) countries with a liberal policy; 2) countries with a compromise liberal policy; 3) countries with a compromise restrictive policy; and 4) countries with a restrictive policy.[1] The Czech Republic is among the states with a compromise liberal policy.

Research on hESC is controversial in some countries because it involves the destruction of human early embryos. Several people believe that human life begins at conception and that an embryo is a person.[17] On the other hand, many excess early human embryos are unnecessarily stored or discarded without further use. Senator Hatch says: "I believe that human life begins in the womb, not in a Petri dish or refrigerator… To me, the morality of the situation dictates that these embryos, which are routinely discarded, be used to improve and save lives. The tragedy would be in not using these embryos to save lives when the alternative is that they would be discarded".[18] We agree with this opinion and do not see a problem with the use of hESCs for research purposes to bring more effective therapeutic methods for incurable diseases. In these cases, we talk about embryos that are on the verge of being either discarded or used for research, i.e., about isolation of embryonic cells in vitro conditions. This means a continuous monolayer of embryonic cells, which does not resemble a human embryo or a person. In this study, we worked only with unwanted embryos and these embryos were primarily produced for infertility therapy and are not desired anymore, perhaps because the reproductive wish of the parents has been either fulfilled or abandoned.

A condition for the successful implementation of our project was the creation of a fully operational team, covering medical, technological and instrumental aspects that are necessary for the proposed research and development. These include: 1) proven expertise in hESCs derivation; 2) direct access to cGMP facilities with relevant expertise in advanced therapy medicinal products (ATMP) medical application development; and 3) CAR involvement with expertise in human embryo handling and access to potential donors.[19,20]

Czech Act 227/2006 Coll. on Research on Human Embryonic Stem Cells and Related Activities states that:

– only research carried out on hESC lines is allowed (§2a);

– hESC lines are all hESCs that are stored in cultures or are subsequently stored in cryopreserved form (§2c);

– hESCs are all pluripotent stem cells derived from human preimplantation embryos created extracorporeally (§2b);

– only embryos that are not older than 7 days (without cryopreservation period) can be used (§8/3).

Convention on Human Rights and Biomedicine (In vitro embryo research: Article 18/1 "If the law allows for in vitro embryo research, adequate embryo protection must be provided by law.") does not distinguish between embryos and hESCs; it only requires legal embryo protection within the permitted research. The Explanatory Memorandum to the Human Embryonic Stem Cell Research Act states that "the draft law does not concern embryo stem cell research but only human embryonic stem cell research, in line with the principles of the Convention on Human Rights and Biomedicine".[13]

In previous studies, we have dealt with the issue of thawing embryos in different culture conditions according to stages at which they were frozen.[13,21,22] The oldest embryos suitable for donation for basic research we have are from 1997. The problem is that most donors from this period have ceased communicating with CAR and do not pay for embryo storage. However, not all ethical aspects have been resolved so far, and the Ethical Committee of the University Hospital Brno has not approved the termination of storage for these embryos.

Almost 38% of respondents did not reply to the letter. Approximately 20% of clients responded positively, did not want to lose the embryos and wanted to arrange a transfer date. A relatively large proportion of the contacted clients (14%) wanted to end storage without participating in a research project. For some couples, this information has provoked conflicting reactions and conflicts of opinion between the partners on which option to choose.

In personal communication, the 2 most important reasons for donating embryos for research purposes were mentioned. The 1st was the view that it was better to use the embryos for research than to destroy or waste them. Many respondents were in favor and made positive general statements about the research. The 2nd reason was altruism, expressed as a desire to help other infertile couples and to contribute to the development of scientific and medical knowledge.

There are many reasons for refusing to use stem cells and embryos for research purposes.[12] The use of stem cells is increasing in clinical therapy, from chronic diseases to plastic and esthetic surgery.[23–25] The most common view is that of the embryo as a potential child. Some patients had a strong emotional reaction to the idea of embryo donation for medical research, and others referred to the children that the embryos can become. These reasons are common and similar to those reported by patients in previous studies.[26] In an Australian study, parents of 5-year-old children conceived after in vitro fertilization (IVF), who were asked about the use of frozen embryos for research purposes, often referred to these embryos as siblings of the children born and commented on the psychological implications of manipulating embryos under a microscope.[27]

Limitations of the study

First, we are limited by the number of infertile couples willing to donate redundant embryos for research purposes. Second, due to the ongoing COVID-19 pandemic, personal contact with clients was minimized, with a negative impact on the number of embryos donated for research purposes.

Conclusions

Based on the signed informed consent, a total of 160 donated embryos were obtained from patients. A transfer protocol and embryo transfer methodology were developed. The delivery plan for thawed anonymized embryos included approx. 5 thawed blastocysts per week with assisted hatching. Subsequently, embryos were prepared and 64 embryos were transferred. After their transfer, embryoblasts were isolated and subsequently cultured. Finally, 3 clinical-grade quality hESC lines were obtained, the first created in the Czech Republic, respecting the requirements for ATMP.

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