

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 March 2003 (06.03.2003)

PCT

(10) International Publication Number
WO 03/018783 A1

(51) International Patent Classification⁷: **C12N 5/06**,
13/00, A61B 17/435, 18/20, B23K 26/00

SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VN, YU, ZA, ZM, ZW.

(21) International Application Number: PCT/IN02/00168

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(22) International Filing Date: 20 August 2002 (20.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/314,323 23 August 2001 (23.08.2001) US

Declarations under Rule 4.17:

- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)
- of inventorship (Rule 4.17(iv)) for US only

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Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ISOLATION OF INNER CELL MASS FOR THE ESTABLISHMENT OF HUMAN EMBRYONIC STEM CELL (hESC) LINES

(57) Abstract: A method for isolating an inner cell mass comprising the steps of immobilizing a blastocyst stage embryo having a zona pellucida, trophoctoderm, and inner cell mass, creating an aperture in the blastocyst stage embryo by laser ablation, and removing the inner cell mass from the blastocyst stage embryo through the aperture. The aperture is through the zona pellucida and the trophoctoderm. The laser ablation is achieved using non-contact diode laser. The inner cell mass removed from the blastocyst stage embryo is used to establish human Embryonic Stem Cell lines.



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Title

Isolation of inner cell mass for the establishment of human embryonic stem cell (hESC) lines.

5 Related Application:

This application claims priority to the provisional application Sr No 60/314,323 filed on August 23, 2001 in USA.

Field of the Invention:

- 10 The present invention relates to a method of isolation of inner cell mass (ICM) derived from blastocyst stage mammalian embryo for establishing human embryonic stem cell (hESC) lines, using a non-contact diode laser technique.

Background of the invention

- 15 The isolation of human stem cells offers the promise of a remarkable array of novel therapeutics. Biologic therapies derived from such cells through tissue regeneration and repairs as well as through targeted delivery of genetic material are expected to be effective in the treatment of a wide range of medical conditions. Efforts to analyze and assess the safety of using human stem cells in the clinical setting are vitally important to
- 20 this endeavor.

Embryonic stem (ES) cells are the special kind of cells that can both duplicate themselves (self renew) and produce differentiated functionally specialized cell types. These stem cells are capable of becoming almost all of the specialized cells of the body

and thus, may have the potential to generate replacement cells for a broad array of tissues and organs such as heart, pancreas, nervous tissue, muscle, cartilage and the like.

5 Stem cells have the capacity to divide and proliferate indefinitely in culture. Scientists use these two properties of stem cells to produce seemingly limitless supplies of most human cell types from stem cells, paving the way for the treatment of diseases by cell replacement. In fact, cell therapy has the potential to treat any disease that is associated with cell dysfunction or damage including stroke, diabetes, heart attack, spinal cord
10 injury, cancer and AIDS. The potential of manipulation of stem cells to repair or replace diseased or damaged tissue has generated a great deal of excitement in the scientific, medical and/ biotechnology investment communities.

ES cells from various mammalian embryos have been successfully grown in the
15 laboratory. Evans and Kaufman (1981) and Martin (1981) showed that it is possible to derive permanent lines of embryonic cells directly from mouse blastocysts. Thomson et al., (1995 and 1996) successfully derived permanent cell lines from rhesus and marmoset monkeys. Pluripotent cell lines have also been derived from pre-implantation embryos of several domestic and laboratory animal species such as bovines (Evans et
20 al., 1990) Porcine (Evans et al., 1990, Notarianni et al., 1990), Sheep and goat (Meinecke-Tillmann and Meinecke, 1996, Notarianni et al., 1991), rabbit (Giles et al., 1993, Graves et al., 1993) Mink (Sukoyan et al., 1992) rat (Iannaccona et al., 1994) and Hamster (Doetschman et al., 1988). Recently, Thomson et al (1998) and Reubinoff et al

(2000) have reported the derivation of human ES cell lines. These human ES cells resemble the rhesus monkey ES cell lines.

ES cells are found in the ICM of the human blastocyst, an early stage of the developing embryo lasting from the 4th to 7th day after fertilization. The blastocyst is the stage of embryonic development prior to implantation that contains two types of cells viz.

1. Trophectoderm: outer layer which gives extra embryonic membranes.
- 10 2. Inner cell mass (ICM): which forms the embryo proper.

In normal embryonic development, ES cells disappear after the 7th day and begin to form the three embryonic tissue layers. ES cells extracted from the ICM during the blastocyst stage, however, can be cultured in the laboratory and under the right conditions proliferate indefinitely. ES cells growing in this undifferentiated state retain the potential to differentiate into cells of all three embryonic tissue layers. Ultimately, the cells of the inner cell mass give rise to all the embryonic tissues. It is at this stage of embryogenesis, near the end of first week of development, that ES cells can be derived from the ICM of the blastocyst.

20

The ability to isolate ES cells from blastocysts and grow them in culture seems to depend in large part on the integrity and condition of the blastocyst from which the cells are derived. In short, the blastocyst that is large and has distinct inner cell mass tend to yield ES cells most efficiently. Several methods have been used for isolation of inner

cell mass (ICM) for the establishment of embryonic stem cell lines. Most common methods are as follows:

1. Natural hatching of the blastocyst:

5 In this procedure blastocyst is allowed to hatch naturally after plating on the feeder layer. The hatching of the blastocyst usually takes place on day 6. The inner cell mass (ICM) of the hatched blastocyst develops an outgrowth. This outgrowth is removed mechanically and is subsequently grown for establishing embryonic stem cell lines. However, this procedure has few disadvantages. Firstly, Trophectoderm cells proliferate very fast in
10 the given culture conditions and many a times, suppress the outgrowth of inner cell mass. Secondly, while removing the outgrowth of the inner cell mass mechanically, there is a chance of isolating trophectoderm cells. Thirdly, the percentage of blastocysts hatching spontaneously in humans is very low.

152. Microsurgery:

Another method of isolation of inner cell mass is mechanical aspiration called microsurgery. In this process, the blastocyst is held by the holding pipette using micromanipulator system and positioned in such a way that the inner cells mass (ICM) is at 9 O'Clock position. The inner cell mass (ICM) is aspirated using a biopsy needle
20 which is beveled shape and is inserted into the blastocoel cavity. This procedure too is disadvantageous as the possibility to isolate the complete inner cell mass is low and many a time cells get disintegrated. It is a very tedious procedure and may cause severe damage to the embryo. The operation at the cellular level requires tools with micrometer precision, thereby minimizing damage and contamination.

3. Immunosurgery:

This is a commonly used procedure to isolate inner cell mass (ICM). The inner cell mass (ICM) is isolated by complement mediated lysis. In this procedure, the blastocyst is exposed either to acid tyrode solution or pronase enzyme solution in order to remove the zona pellucida (shell) of blastocyst. The zona free embryo is then exposed to human surface antibody for about 30 min to one hour. This is followed by exposure of embryos to guinea pig complement in order to lyse the trophectoderm. This complement mediated lysed trophectoderm cells are removed from inner cell mass (ICM) by repeated mechanical pipetting with a finely drawn Pasteur pipette. All the embryonic stem cell lines reported currently in the literature have been derived by this method. However, this method has several disadvantages. Firstly, the embryo is exposed for a long time to acid tyrode or pronase causing deleterious effects on embryo, thereby reducing the viability of embryos proper. Secondly, it is time consuming procedure as it takes about 1.5 to 2.0 hours. (Narula et al.,1996). Thirdly, the yield of inner cell mass (ICM) per blastocyst is low. Fourthly, critical storage conditions are required for antibody and complement used in the process. Lastly, it involves the risk of transmission of virus and bacteria of animal origin to humans, as animal derived antibodies and complement are used in the process. In this process, two animal sera are used. One is rabbit antihuman antiserum and the other is guinea pig complement sera.

The human cell lines studied to date are mainly derived by using a method of immunosurgery, where animal based antisera and complement was used.

Other possible disadvantages of the existing cell lines are as follows:-

1 Use of feeder cells for culturing the human embryonic stem cell (hESC) lines
produces mixed cell population that require the Embryonic stem cells (ESC) to be
separated from feeder cell components and this impairs scale up.

5

2. Embryonic stem cells (ESC) get contaminated by transcripts from feeder cells
and cannot be used on a commercial scale. It can be used only for research
purposes.

10 Geron established a procedure where human Embryonic Stem Cell (hESC) line was
cultured in the absence of feeder cells (XU et.al 2001). The hESC were cultured on an
extracellular matrix in a conditioned medium and expanded in this growth environment in
an undifferentiated state. The hESC contained no xenogenic components of cancerous
origin from other cells in the culture. Also, the production of hESC cells and their
15 derivatives were more suited for commercial production. In this process, there was no
need to produce feeder cells on an ongoing basis to support the culture, and the
passaging of cells could be done mechanically. However, the main disadvantage of this
procedure is that the inner cell mass (ICM) is isolated by immunosurgery method,
wherein the initial derivation of Embryonic Stem Cells is carried out using feeder layer
20 containing xenogenic components. This raises the issue of possible contamination with
animal origin viruses and bacteria.

In order to simplify the procedure of inner cell mass isolation and to make it safe, the
scientists of the present invention have come out with a novel method of isolation of the

inner cell mass using a non-contact laser, wherein, the use of animal based antisera and complement have been eliminated.

Use of Laser technique in Assisted Reproduction:

- 5 With the advent of assisted reproductive technologies (ART), several methods have been used for improving fertilization, facilitating blastocyst hatching (Cohen et al, 1990) and performing blastomere biopsy (Tarin and Handyside, 1993). Commonly used methods are chemical (Gordon and Talansky, 1986), mechanical (Depypere et al., 1988) and laser (Feichtinger et al., 1992) so as to produce holes in the zona pellucida (Gordon,
- 10 1988). Recently, an infrared 1.48 μm diode laser beam focused through a microscope objective was shown to allow rapid, easy and non-touch microdrilling of mouse and human zona pellucida and high degree of accuracy was maintained under conventional culture conditions (Rink et al., 1994). The drilling effect was shown due to a highly localized heat-dependent disruption of the zona pellucida glycoprotein matrix (Rink et al.,
- 15 1996). Contrary to the detrimental effect on compacted mouse embryos induced by the 308 nm xenon-chlorine excimer laser (Neev et al., 1993), the drilling process in the infrared region did not affect embryo survival in mice (Germond et al., 1995) or in humans (Antinori et al., 1994).
- 20 Currently, lasers are being investigated as a tool to aid fertilization and in assisted hatching. Recent reports show that use of 1.48 μm diode laser for microdrilling mouse zona pellucida is highly safe and does not affect neuro-anatomical and neurochemical properties in mice and also improves fertilization (Germond et al., 1996). Obruca and colleagues first reported the success of laser-assisted hatching in human IVF in 1994. In

this study, a 20- to 30-micron hole was made in the zona pellucida (ZP) when the embryos were at the two- to four-cell stage, and embryos were transferred immediately. Patients with previous IVF failures from two separate centers were included in this study. There was a higher implantation rate per embryo in the laser-assisted hatching group
5 (14.4%) versus the control group (6%). Pregnancy rates per transfer were also improved (40% versus 16.2%).

In a separate study, Er:YAG laser was used to thin the ZP of embryos derived from patients undergoing repeated IVF. Using a laser for thinning the ZP, embryologists are
10 able to achieve accurate reduction of the ZP by 50%, which is very difficult with acidic Tyrode's solution. Presence of Acid Tyrode's solution near the embryo may also be detrimental. The rate of clinical pregnancies in the laser-hatched group was 42.7%, as compared to 23.1% in the control unhatched group. Since this data looked promising, the indication of laser-assisted hatching was extended. Women undergoing IVF for the
15 first time yielded 39.6% clinical pregnancy rate in the laser-treated group versus a 19% rate in the control unhatched group (Parikh et al 1996).

During the last decade there has been ongoing research on the isolation of inner cell mass (ICM), as it is useful in establishing embryonic stem cell lines which in turn have
20 the ability to develop into most of the specialized cells in the human body including blood, skin, muscle and nerve cells. They also have the capacity to divide and proliferate indefinitely in culture.

The present invention involves the isolation of inner cell mass (ICM), using laser ablation technique without undergoing the cumbersome procedure of immunosurgery. Hence, in the present invention, the use of animal derived antibodies or sera are eliminated and the procedure is safe, simple, rapid and commercially viable.

5

The present invention, obviates the shortcomings associated with the conventional methods of isolation of inner cell mass (ICM). The inner cell mass (ICM) isolated by the present invention is found to be intact without causing any destruction or damage to the cells. The present invention thus provides a quick reliable and non-invasive method for
10 isolation of inner cell mass (ICM). It also completely ruptures the trophectoderm thereby minimizing the contamination of inner cell mass (ICM), thus ensuring the purity of inner cell mass (ICM).

REFERENCES

- 15 1. Antinori S, Versaci C, Fuhrberg P et al (1994). Seventeen live birth after the use of erbium-yttrium aluminum garnet laser in the treatment of male factor infertility. Hum Reprod. 9 : 1891-1896.
- 20 2. Cohen J, Elsner C, Kort H et al (1990). Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod . 5 : 7-13

3. Depypere HT, McLaughlin KJ, Seamark RF et al (1988). Comparison of zona cutting and zona drilling as techniques for assisted fertilization in the mouse. *J. Reprod Fertil.* 84 : 205-211.
- 5 4. Doetschman T, Williams P and Maeda N (1988) Establishment of hamster blastocyst derived embryonic stem (ES) cell. *Developmental Biology* 127: 224-227.
- 10 5. Evans MJ and Kaufman MH (1981). Establishment in culture of pluripotential cells from mouse embryo. *Nature* 292: 154-156.
6. Evans MJ, Notarianni E, Laurie S and Moor RM (1990) Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocyst. *Theriogenology* 33: 125-128.
- 15 7. Feichtinger W, Strohmer H, Fuhrberg P et al (1992). Photoablation of oocyte zona pellucida by erbium-yag laser for in-vitro fertilization in severe male infertility. *Lancet.* 339, 811.
- 20 8. Gordon JW (1988). Use of micromanipulation for increasing the efficiency of mammalian fertilization in vitro. *Ann. N.Y. Acad.Sci.* 541 : 601-613.
9. Gordon JW and Talansky BE (1986). Assisted fertilization by zona drilling : a mouse model for correction of oligospermia. *J. Exp Zool.* 239: 347-354.

10. Germond M, Nocera D, Senn A, Rink K. et al (1995). Microdissection of mouse and human zona pellucida using 1.48 microns diode laser beam: efficacy and safety of the procedure. *Fertil Steril.* 64: 604-611.
- 5
11. Germond M, Nocera D, Senn A, Rink A et al (1996). Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 micron diode laser beam. *Hum Reprod.* 11: 1043-1048
- 10 12. Giles JR, Yang X, Mark X and Foot RH (1993). Pluripotency of cultured rabbit inner cell mass cells detected by isozyme analysis and eye pigmentation of fetus following injection into blastocysts or morula. *Molecular Reproduction and Development* 36: 130-138.
- 15 13. Graves KH and Moreadith RW (1993). Derivation and characterization of putative pluripotential embryonic stem cells from pre-implantation rabbit embryo. *Molecular reproduction and Development* 36: 424-433.
- 20 14. Iannaccone PM, Taborn GU, Garton RL et al (1994). Pluripotent embryonic stem cells from the rat are capable of producing chimeras. *Developmental Biology* 163:288-292.

15. Martin GR (1981) Isolation of pluripotent cell lines from early mouse embryos cultured in medium conditioned with teratocarcinoma stem cells. Proceeding of National Academy of Sciences USA 72: 1441-1445.
- 5 16. Meinecke-Tillmann S and Meinecke B (1996). Isolation of ES like cell lines from ovine and caprine pre-implantation embryo. J Animal Breeding and Genetics 113: 413-426.
17. Narula A, Taneja, Totey SM (1996) Morphological cells to trophectoderm and
10 inner cell mass of in vitro fertilized and parthenogenetically developed buffalo embryo: the effect of IGF-I. Mol. Reprod. Dev. 44(3):343-51.
18. Neev J, Gonzales A, Lucciardi F et al (1993). Opening of the mouse zona
pellucida by laser without a micromanipulator. Hum Reprod . 8 : 939-944.
- 15 19. Obruca A, Strohmer H, Sakkas D (1994). Use of laser in assisted fertilization and hatching. Hum Reprod. 9:1723-1726.
20. Parikh FR, Kamat SA, Nadkarni S et al (1996). Assisted hatching in an in vitro
20 fertilization program. J Reprod Fertil Suppl 50: 121-125.
21. Reubinoff BE, Per MF, Fong CY, Trounson A and Bongso A (2000) Embryonic stem cell lines from human blastocysts: Somatic differentiation in vivo. Nat Biotechnol. 18: 299-304.

22. Rink K, Delacretaz G, Salathe RP et al (1994). Proceedings SPIE. 2134A, 412-422.
- 5 23. Rink K, Delacretaz G, Salathe RP et al (1996). Non-contact microdrilling of mouse zona pellucida with an objective delivered 1.48 microns diode laser. Lasers Surg Med. 18:52-62.
24. Sukoyan MA, Golublitsa AN, Zhelezova AI et al (1992) Isolation and cultivation of
10 blastocyst derived stem cell lines from American Mink. Molecular Reproduction and Development 33: 418-431.
25. Tarin JJ and Handyside AH (1993). Embryo biopsy strategies for preimplantation diagnosis. Fertil Steril 59:943-952.
- 15 26. Thomson JA, Itskovitz-Eldor J, Shapiro SS. et al. (1998). Embryonic stem cell lines derived from human blastocyst. Science 282 : 1145-1147.
27. Thomson JA, Kalishman J, Golos TG et al (1996). Pluripotent cell line derived
20 from common marmoset blastocyst. Biology of Reproduction. 55: 254-259

Objects of the Invention

1. It is an object of the present invention, to develop a process of isolation of inner cell mass, using laser ablation technique, without undergoing the cumbersome procedure of immunosurgery.
5
2. It is another object of the present invention to isolate ICM using laser ablation technique without using any animal generated antibodies and sera, thereby preventing the possibility of transmission of animal organism to human and thus can safely be used on commercial scale.
10
3. It is another object of the present invention to isolate inner cell mass (ICM) from blastocyst stage of a mammalian embryo using a non-contact diode laser.
- 15 4. It is another object of the present invention to isolate inner cell mass (ICM) by simple, shorter and easily feasible way without affecting/destroying the inner cell mass (ICM) .
- 20 5. It is still another object of the present invention to ensure the purity of inner cell mass (ICM) by rupturing completely trophectoderm thereby minimising the contamination of inner cell mass (ICM).

6. It is still another object of the present invention to isolate inner cell mass (ICM) of high yield and purity as compared to the inner cell mass (ICM) isolated by the conventional methods.

5 These and other objects of the invention will become more readily apparent from the ensuing description.

Details of Invention:

The present invention relates to isolation of inner cell mass, using laser ablation of zona
10 pellucida (ZP) and trophectoderm (TE) and aspiration of inner cell mass for establishing embryonic stem cell lines. In the present invention, the non contact diode laser used is highly accurate and reliable tool for cellular microsurgery. The system incorporates the latest in fiber optic technology to provide the most compact laser system currently available. The 1.48 μm diode non-contact Saturn Laser System is mounted/implanted
15 via the epifluorescence port to inverted microscope fitted with micromanipulators. A pilot laser is used to target the main ablation laser and a series of LEDs inform the user when the laser is primed and is ready to fire. A two-second-operation window is used to reduce the possibility of accidentally firing the laser. The spot diameter of the laser can be varied according to the hole size required.

20

Couples undergoing in vitro fertilization (IVF) treatment voluntarily donate surplus human embryos. These embryos are used for research purposes after taking the written, voluntary consent from these couples. In the present invention, blastocyst stage embryos are taken for the isolation of inner cell mass. The blastocyst is placed in a

35mm petridish in a 50 micro litre droplets of Ca⁺⁺/Mg⁺⁺ free embryo biopsy medium and is covered with mineral oil. The micromanipulator is set up to perform the embryo biopsy procedure. The blastocyst is placed in embryo biopsy medium and the petridish containing the blastocyst is placed on the heating stage of the microscope. The

5 blastocyst is positioned at the center of the field. The blastocyst is immobilized on to the holding pipette in such a way that the inner cell mass is at 3 O' Clock position. The zona pellucida and trophectoderm close to inner cell mass is positioned on the aiming spot of the laser beam. A small portion of zona pellucida and trophectoderm is laser ablated. Biopsy pipette is then gently inserted through the hole in the zona pellucida and

10 trophectoderm and the inner cell mass is gently aspirated. After isolation of the complete inner cell mass, the cells are given several washes with embryonic stem cell (ESC) medium. The cells are then plated on to feeder layer with embryonic stem cell medium for establishing embryonic stem cell lines. The embryonic stem cells were then characterized for cell surface markers such as SSEA-1, SSEA-3, SSEA-4, TRA-1-60,

15 TRA-1-81, OCT-4 and alkaline phosphatase. The embryonic stem cell lines are also karyotyped.

a) Development of blastocyst in vitro:

Institutional Ethics Committee approval has been obtained before initiation of this study.

20 Prior written consent was taken from individual donor for the donation of surplus embryos for this study after completion of infertility treatment.

Protocol generally used for infertility patients for obtaining viable embryo is as follows:

The ovarian superovulation began with GnRH agonist analog suppression daily starting in the mid-luteal phase and administered in doses of 500-900 mg for about 9-12 days. Ovarian stimulation was started after adequate ovarian suppression with human menopausal gonadotropins (hMG) or recombinant follicle stimulating hormone (FSH) (Gonal-F, Recagon) in appropriate doses depending on the age and ovarian volume. The dose was also adjusted as necessary to produce controlled ovarian stimulation. Serum beta-estradiol (E2) measurements were carried out as required. Vaginal ultrasound was performed daily from cycle day 7 onward. Human Chorionic gonadotropin 5000-10000 I.U. was administered when three or more follicles were at least 17 mm in largest diameter. Transvaginal aspiration was performed 34-36 h later. Oocytes were then subjected to intracytoplasmic sperm injection.

A glass holding pipette 40-60 μm in diameter was used to secure the egg. Motile sperm were placed in a drop of polyvinyl pyrrolidone (PVP) solution and overlaid with mineral oil. An injection needle with an outer diameter of roughly 5-6 μm and inner diameter 3-4 μm was used to pierce the zona pellucida at about 3 O' Clock position. The selected spermatozoon was immobilized by cutting the tail with the injection micropipette. The holding pipette secured the oocyte and spermatozoon was injected directly into the center of the oocyte.

20

Oocytes were checked after 16-18 hours of culture for fertilization. At this point the fertilized oocyte had pro-nuclei (also called one cell embryo). One-cell embryos were then transferred into pre-equilibrated fresh ISM-1 medium and incubated at 37 ° C in a 5% CO₂ in air. The next day embryos were transferred into ISM-2 medium. Every

alternate day embryos were transferred into fresh ISM-2 medium. From day 5 onward embryos were checked for the blastocyst development. After the treatment is over, the surplus blastocysts were donated by the couples for this research work.

5 **b) Setting up of the Laser:**

The present invention relates to describing a unique method for inner cell mass isolation for establishment of embryonic stem cells using the non-contact diode laser. The laser is highly accurate and reliable tool for cellular microsurgery. The system incorporates the latest in fiber optic technology to provide the most compact laser system currently
10 available. The 1.48 μm diode non-contact Saturn Laser System was mounted via the epifluorescence port to Zeiss inverted microscope fitted with micromanipulators.

A pilot laser was used to target the main ablation laser and a series of LED's inform the user when the laser is primed and ready to discharge the laser beam. A two-second-
15 operation window was used to reduce the possibility of accidentally firing the laser. The spot diameter of the laser can be varied according to the ablation size required.

c) Laser Ablation and isolation of inner cell mass.

The blastocyst stage embryo was individually placed in a 50 μl drop of biopsy medium
20 (Ca ⁺⁺/Mg ⁺⁺ free) in a 35-mm petri dish. The embryo was immobilized on to the holding pipette in such a way that the inner cell mass remained at 3 O' Clock position and the zona pellucida and trophectoderm close to inner cell mass positioned on the aiming spot. A continuous 1.48 μm diode laser was used to aperture the Zona Pellucida (ZP), which is a glycoprotein layer protecting the oocyte. At this wavelength, the hole was

induced by a local thermo-dissolution of the glycoprotein matrix. Once the zona pellucida was dissolved, trophectoderm cells were ablated by giving 3 pulses to cause photolysis. After ablation of both zona pellucida and trophectoderm, the aspiration pipette was introduced through laser-ablated hole and ICM was removed by gentle aspiration, having an internal diameter of 30-35 microns.

d) Culturing of human Embryonic Stem Cells (hESC)

Prior to culturing, the aspirated ICM was washed thoroughly in ES medium, which medium was found to be preferred for isolation of embryonic stem cell lines.

10 Given below is the procedure when the invention was carried out using feeder layer. In this process, the inner cell mass was cultured in 96 well plate in the presence of mouse inactivated embryonic fibroblast feeder layer. Embryonic fibroblast feeder layer was preferably obtained from 12.5 to 13.5 day old C57BL/6 mice or C57BL/6XSJL F-1 mice or out bred CD1 mice or from human amniotic fluid and used as a feeder layer.

15 Embryonic fibroblast feeder layer was inactivated by gamma irradiation (3500 rads). The mouse embryonic fibroblast feeder layer was cultured on 0.5% gelatin coated plate with ES medium consisting of Dulbecco's modified Eagle's medium without Sodium pyruvate with high glucose contain (70-90%), Fetal bovine serum (10-30%), beta-mercaptoethanol (0.1mM), non-essential amino acids (1%), L- Glutamine 2 mM, basic

20 fibroblast growth factor (4 ng/ml). Inner cell mass was then plated on mouse inactivated embryonic fibroblast. After 4-7 days, ICM derived masses were removed from outgrowth with sterile fire polished pipette and were dissociated mechanically and plated on fresh feeder cells. Further dissociation was carried out with 0.5% trypsin-EDTA supplemented with 1% chicken serum.

Established cell lines were karyotyped and characterized for several surface markers such as SSEA-1, SSEA-3, SSEA-4, OCT-4, Alkaline phosphatase, TRA-1-81, TRA-1-60 as described by Thomson et al., (1998), Reubinoff et al., (2000).

5

Examples

The following examples are intended to illustrate the invention but do not limit the scope thereof.

10 Example I:

Total of 24 blastocyst stage human embryos were used for the isolation of inner cell mass. Embryos were washed several times in blastocyst culture medium (ISM-2 medium, Medicult, Denmark). Individual blastocyst was then placed in the 50 μ l drop of $\text{Ca}^{++}/\text{Mg}^{++}$ free embryo biopsy medium (EB 10 medium, Scandinavian). Micro drops
15 were covered with mineral oil. Micromanipulator was set up. A glass holding pipette with outer diameter 75 μ m and inner diameter 15 μ m was used to secure the embryo. Biopsy pipette with an outer diameter of roughly 49 μ m and inner diameter 35 μ m was used for aspiration of inner cell mass. A pilot laser was used to target the main ablation laser. Embryo was immobilized on to the holding pipette in such a way that inner cell mass
20 remained at 3 O' clock position and the zona pellucida and trophectoderm close to inner cell mass positioned to the aiming spot. The hole was induced by a local thermo-dissolution of the zona. Trophectoderm cells were ablated by giving 3 pulses to cause photolysis. After ablation of both the zona pellucida and trophectoderm, the biopsy pipette was introduced through laser ablated hole and inner cell mass was removed.

Inner cell mass was then washed several times in ES medium and placed in 96 well dish in the presence or absence of feeder cells. The following data is presented in the tabular form.

- 5 Table 1: Summary of hESC lines developed using Laser ablation Technique of the present invention with the use of mouse feeder cells.

No. of blastocyst used for laser ablation	Total inner cell mass removed	With mouse feeder cells	
		No. of ICM used	No of ES cell lines established
24	18	14	4

Similarly, an experiment was conducted with conventional method of isolation of inner cell mass i.e. using immunosurgery and may be reported as follows:

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Example 2:

The objective was to determine efficiency of isolation of inner cell mass with conventional method i.e. immunosurgery and compared with newly invented laser ablated method.

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21 blastocyst stage human embryo were used for isolation of inner cell mass. Embryos were washed several times with blastocyst culture medium (ISM-2 medium) and followed by ES medium. Individual blastocyst stage embryo was then placed in 50 μ l microdrops of 1:50 anti-human antibody (Sigma) for 30 minutes at 37 o C and 5% CO₂ in

air. Blastocyst stage embryos were then washed four times after incubation with ES medium. Blastocysts were then again placed in 50 μ l of microdrops of guinea pig complement at the concentration of 1:10 for 10 minutes at 37°C and 5% CO₂ in air. After incubation blastocyst stage embryos were washed several times in ES medium using
 5 fine bore glass pipette in order to remove trophectoderm. Isolated inner cell mass was then washed with ES medium and cultured in 96 well plate in the presence or absence of feeder cells. Data are presented in the table:

10

Table 2: Summary of hESC lines developed using immunosurgery with / without the use of mouse feeder cells.					
No. of blastocyst used for laser ablation	Total Inner cell mass removed	With mouse feeder cells		Without mouse feeder cells	
		No. of ICM used	No of ES cell lines established	No. of ICM used	No. of ES cell lines established
21	14	12	3	2	0

15

Although the isolation of inner cell mass using both the methods did not show any significant difference, however, of isolation of inner cell mass by laser ablation has distinct advantage. This method will eliminate the use of antibodies and sera of animal origin. Isolation of inner cell mass by laser ablation method can be further cultured in the presence or absence of feeder layer. However, culturing of inner cell mass in a feeder free condition will further eliminate the possibilities of contamination of ES cell lines with animal viruses or bacteria and can be commercially utilized for human transplantation

studies. In the current experiments, efforts were made to establish ES cell line in the absence of feeder cells.

Detailed description of the preferred embodiments:

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A preferred embodiment of the invention is illustrated in the accompanying photomicrographs

Fig 1(a) to 1(g) of the present invention, pertains to the isolation of inner cell mass (ICM) from the blastocyst of one embryo and Fig 2(a) to 2(g) pertains to the isolation of inner cell mass (ICM) from the blastocyst of another embryo. Fig 3,4,and 5 pertains to culturing of ICM on feeder cells at different stages.

Fig 1. (a) shows a photomicrograph of human blastocyst, secured with glass holding pipette such that the ICM is at 3 O' Clock position.

Fig 1 (b) shows a photomicrograph wherein part of zona pellucida and trophectoderm ablated with laser (arrow).

Fig 1 (c) shows a photomicrograph of aspiration pipette close to the blastocyst following zona and trophectoderm ablation.

Fig 1 (d) shows a photomicrograph of beginning of aspiration of ICM with aspiration pipette.

Fig 1 (e) shows a photomicrograph of large portion of ICM in the aspiration pipette during aspiration process.

5 Fig 1 (f) shows a photomicrograph of the ICM after removing from the blastocyst.

Fig 1 (g) shows a photomicrograph of the remaining trophectoderm and zona pellucida remaining after ICM isolation.

10 Fig 2 (a) shows a photomicrograph of another human blastocyst, secured with glass holding pipette such that the ICM is at 3 O' Clock position.

Fig 2 (b) shows a photomicrograph of slight protrusion of inner cell mass after zona and trophectoderm is laser ablated.

15

Fig 2 (c) shows a photomicrograph of the aspiration pipette being position close to the ICM after ablating the zona and neighboring trophectoderm cells with laser.

20 Fig 2 (d) shows a photomicrograph of ICM being aspirated with the aspiration pipette by gentle suction.

Fig 2 (e) shows a photomicrograph of large portion of ICM in the aspiration pipette.

Fig 2 (f) shows a photomicrograph of the ICM after removing from the blastocyst.

Fig 2 (g) shows a photomicrograph of the trophectoderm and zona pellucida left after the isolation of ICM from the blastocyst.

5 Fig 3 (a) shows a photomicrograph of isolated inner cell mass in culture seeded on primary mouse embryonic fibroblast feeder cells (day 3).

Fig 3 (b) shows a photomicrograph of isolated inner cell mass in culture on primary mouse embryonic fibroblast feeder cells (day 7)

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Fig 4 shows a photomicrograph of isolated ICM in culture on the primary mouse embryonic fibroblast feeder cells (day 5) another embryo.

15 Fig 5 shows a photomicrograph of embryonic stem cell line derived from inner cell mass isolated by laser ablation method.

One skilled in the art will appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned therein above. The instant invention has been shown and described herein in what is considered to be the most practical and preferred embodiment. It is recognized, that, departures may be made therefrom within the scope of the invention. It is to understood that the invention is not limited to the particulars disclosed and extends to all equivalents within the scope of the scope of the claims.

20

We Claim

1. A method of isolating inner cell mass (ICM) for establishing human Embryonic Stem Cell (hESC) lines using a non-contact diode laser technique comprising
5 the steps of
 - (i) placing the blastocyst in a 35mm petridish in a 50 mico litre conventional Embryo biopsy medium covered with mineral oil ;
 - 10 (ii) setting up the micromanipulator system consisting of the microscope with the heating stage, tool holders to hold the holding pipette, aspiration pipette and air syringes;
 - 15 (iii) placing the petridish of (i) above, containing the blastocyst on the heating stage of (ii) above and adjusting the blastocyst at the center of the field;
 - (iv) securing the blastocyst with holding pipette at 9 O'clock position by suction through the air syringe in such a way that the inner cell mass (ICM) which is to be isolated is at 3 O' clock position;
 - 20
2. A method of claim 1, further comprising of dissolving zona pellucida, by laser ablation of zona pellucida using 3-5 pulse of 1.48 micro diode laser to create a hole in glycoprotein matrix present in zona pellucida .

3. A method of claim 1, further comprising of removing the trophectoderm cells adjacent to inner cell mass (ICM) by ablation of trophectoderm cells using 1.48 micro diode laser by cell lysis.
- 5 4. A method of claim 1, further comprising of aspiration of inner cell mass (ICM), comprising of the following steps:
- 10 (i) bringing the aspirating pipette close to blastocyst through the hole in the zona pellucida and trophectoderm cells close to inner cell mass (ICM);
- (ii) aspirating the inner cell mass (ICM) into the aspiration pipette by gentle suction through air syringe of claim 4(i) above;
- 15 (iii) the aspirated inner cell mass (ICM) of claim 4(ii) above is slowly released into the culture droplet of claim 5 below;
5. A method of claim 1, further comprising of culturing of Embryonic Stem Cells comprising of the following steps:
- 20 (i) washing the isolated inner cell mass (ICM) several times in micro drops of Embryonic Stem Cell media;

- (ii) placing the inner cell mass (ICM) on the plate, containing feeder layer in the presence of a media , for the establishment of Embryonic Stem Cells;

5 6. A method of claim 1, wherein Embryo biopsy medium is Ca⁺⁺/ Mg⁺⁺ free

7. A method of claim 5 (ii) wherein the media used is Embryonic Stem Cell media.

8. A method of claim 5(ii), wherein the plate used is 0.5% gelatin coated plate.

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9. A method of claim 5, wherein the Embryonic Stem Cell media is a combination of

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(i) Dulbecco's Modified Eagle's Medium (DMEM) without Sodium pyruvate with high glucose content (70-90%);

(ii) Fetal Bovine Serum (10-30%)

(iii) Beta-Mercapto Ethanol (0.1mM)

20

(iv) Non- Essential Amino Acids (1%)

(v) L- Glutamine 2 mM

(vi) Basic Fibroblast Growth Factor (4ng/ml)

10. A method of claim 5(ii), wherein feeder layer is murine or human origin
- 5 11. A method for isolating an inner cell mass comprising the steps of:
- (i) immobilizing a blastocyst stage embryo having a zona pellucida, trophectoderm, and inner cell mass;
- 10 (ii) creating an aperture in the blastocyst stage embryo by laser ablation; and
- (iii) removing the inner cell mass from the blastocyst stage embryo through the aperture.
- 15 12. The method of claim 11, wherein the aperture is through the zona pellucida.
13. The method of claim 11, wherein the aperture is through the zona pellucida and the trophectoderm.
- 20 14. The method of claim 11, wherein the laser ablation is achieved using a non-contact diode laser.
15. The method of claim 14, wherein the non-contact diode laser is a continuous 1.48 μm diode laser.

16. The method of claim 11, wherein the inner cell mass is removed by aspiration using an aspiration pipette introduced through the aperture.

5 17. The method of claim 11, wherein the method is carried out in the absence of animal generated antibodies and sera.

18. A method for establishing human Embryonic Stem Cell lines comprising the steps of:

10

(i) isolating an inner cell mass from a blastocyst stage embryo by creating an aperture in the blastocyst stage embryo by laser ablation, and removing the inner cell mass from the blastocyst stage embryo through the aperture;

15

(ii) culturing the inner cell mass in the presence of an embryonic stem cell medium and a mouse inactivated embryonic fibroblast feeder layer to produce inner cell mass derived masses.

19. The method of claim 18, wherein the embryonic stem cell medium consists essentially of:

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(i) Dulbecco's modified Eagle's medium in an amount from about 70% to about 90% of the embryonic stem cell medium, the Dulbecco's modified

Eagle's medium being without sodium pyruvate with high glucose content;

- 5 (ii) fetal bovine serum in an amount from 10% to 30% of the volume of the embryonic stem cell medium;
- (iii) beta-mercaptoethanol in an amount of about 0.1 micro-mole based on the total moles of the embryonic stem cell medium;
- 10 (iv) non-essential amino acids in an amount of about 1% of the volume of the embryonic stem cell medium;
- (v) L-glutamine in an amount of about 2 micro-moles based on the total moles of the embryonic stem cell medium; and
- 15 (vi) basic fibroblast growth factor in an amount of about 4 nanograms per milliliter of the embryonic stem cell medium.
20. The method of claim 18 further comprising the steps of removing the inner cell mass derived masses, mechanically dissociating the inner cell mass derived masses, and re-plating the mechanically dissociated inner cell mass derived masses on fresh feeder cells.
- 20

21. A method of isolating inner cell mass (ICM) for establishing human Embryonic Stem Cell (hESC) lines using a non-contact diode laser technique substantially as herein described with reference to example and as illustrated in the accompanied photomicrographs.



Fig 1(a)



Fig 1(b)

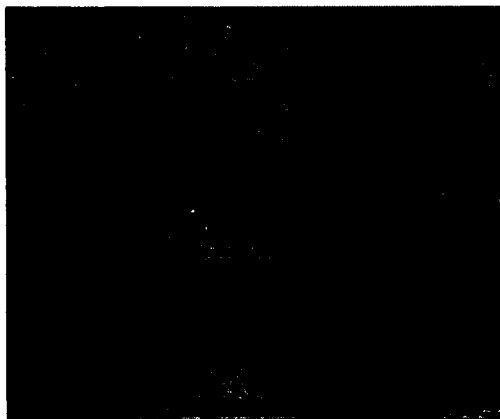


Fig 1(c)

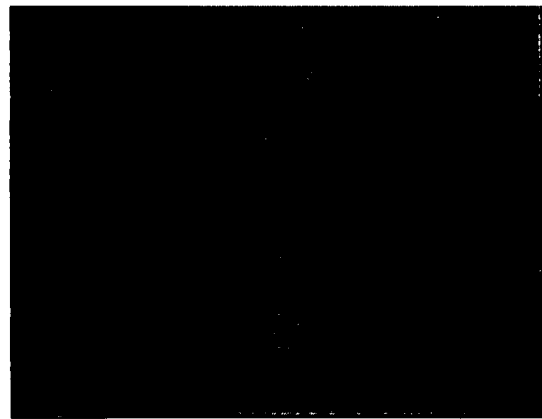


Fig 1(d)

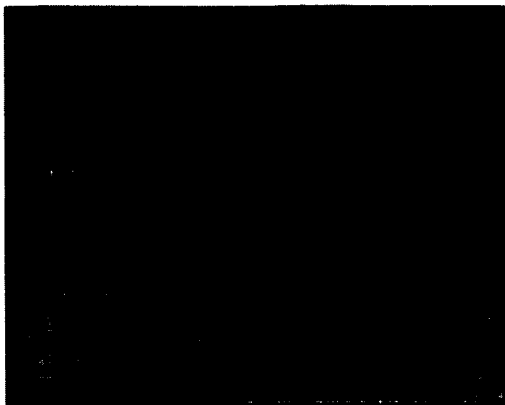


Fig 1(e)

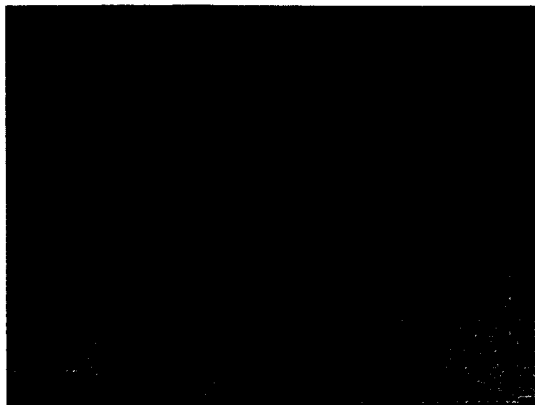


Fig 1(f)

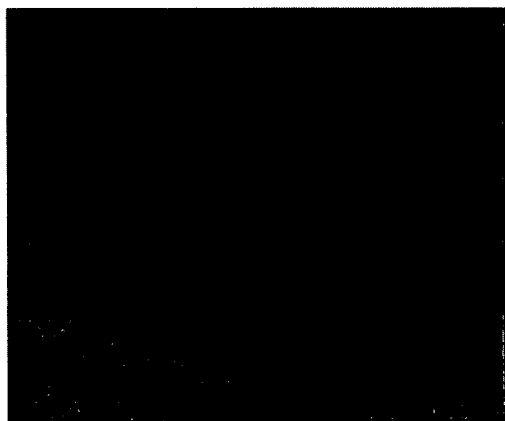


Fig 1(g)

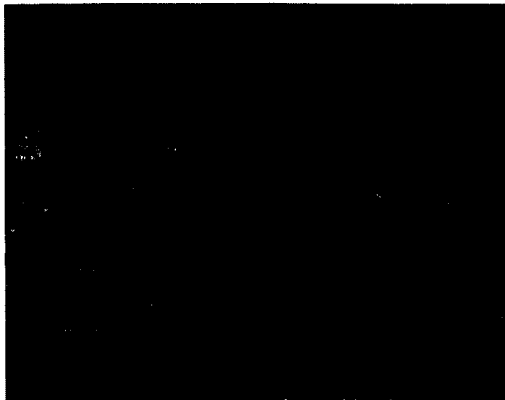


Fig 2(a)



Fig 2(b)

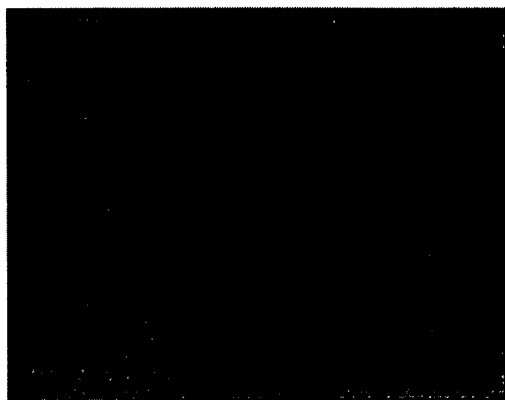


Fig 2(c)

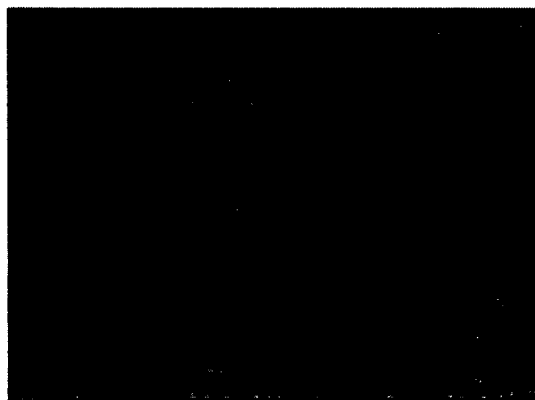


Fig 2(d)



Fig 2(e)



Fig 2(f)



Fig 2(g)

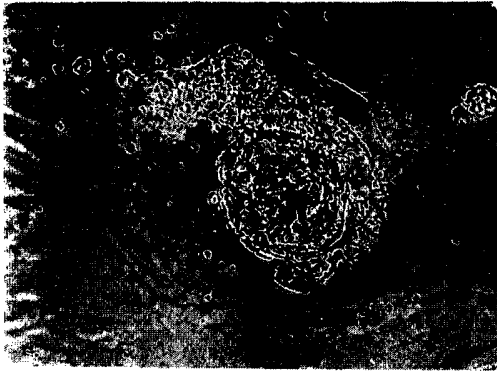


Fig 3(a)

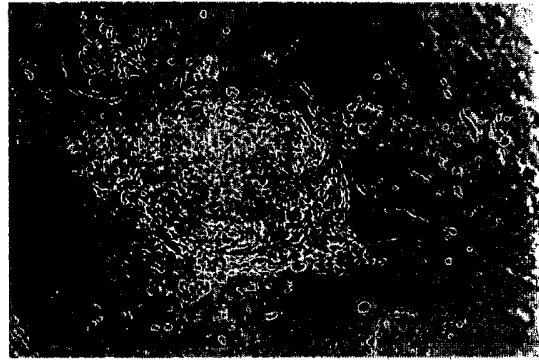


Fig 3(b)

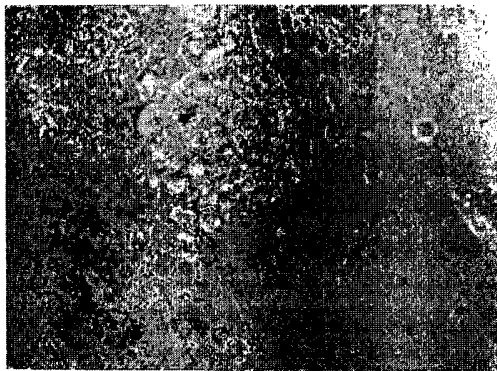


Fig 4



Fig 5

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IN 02/00168

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 C12N13/00 A61B17/435 A61B18/20 B23K26/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61B B23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VEIGA A ET AL: "Laser blastocyst biopsy for preimplantation diagnosis in the human." ZYGOTE, vol. 5, no. 4, November 1997 (1997-11), pages 351-354, XP009002724 ISSN: 0967-1994 page 352, left-hand column, paragraph 3 - paragraph 4 --- -/--	1-17

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

12 December 2002

Date of mailing of the international search report

02/01/2003

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IN 02/00168

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BOADA M ET AL: "Successful use of a laser for human embryo biopsy in preimplantation genetic diagnosis: Report of two cases." JOURNAL OF ASSISTED REPRODUCTION AND GENETICS, vol. 15, no. 5, May 1998 (1998-05), pages 302-307, XP009002723 ISSN: 1058-0468 page 303, right-hand column, paragraph 2 -page 304, left-hand column, paragraph 1; figure 1 page 306, left-hand column, paragraph 3 ---</p>	1-17
A	<p>GERMOND M ET AL: "Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 mu-m diode laser beam." HUMAN REPRODUCTION (OXFORD), vol. 11, no. 5, 1996, pages 1043-1048, XP009002599 ISSN: 0268-1161 cited in the application abstract ---</p>	
A	<p>HOLLIS A ET AL: "ZONA PELLUCIDA MICRODRILLING WITH A 1.48 MUM DIODE LASER" IEEE ENGINEERING IN MEDICINE AND BIOLOGY MAGAZINE, IEEE INC. NEW YORK, US, vol. 16, no. 3, 1 May 1997 (1997-05-01), pages 43-47, XP000700728 ISSN: 0739-5175 -----</p>	