(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 February 2006 (23.02.2006)

PCT

(10) International Publication Number
WO 2006/019366 A1

(51) International Patent Classification[7]: C12N 5/08, 5/06, 5/00

(21) International Application Number:
PCT/US2004/009097

(22) International Filing Date: 26 March 2004 (26.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/458,815 28 March 2003 (28.03.2003) US

(71) Applicant: **WISCONSIN ALUMNI RESEARCH FOUNDATION** [US/US]; 614 Walnut Street, P.O. Box 7365, Madison, WI 53707-7365 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LUDWIG, Tenneille, E.** [US/US]; 614 Olin Avenue #1, Madison, WI 53715 (US). **THOMSON, James, A.** [US/US]; 1807 Regent Street, Madison, WI 53705 (US).

(74) Agent: **VINAROV, Sara, D.**; Quarles & Brady LLP, P.O. Box 2113, Madison, WI 53701-2113 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, 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, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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

Published:
— *with international search report*

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.

WO 2006/019366 A1

(54) Title: PHYSIOCHEMICAL CULTURE CONDITIONS FOR EMBRYONIC STEM CELLS

(57) Abstract: Physiochemical parameters to improve the culturing and sub-culturing (here called cloning) of human embryonic stem cells have been investigated. Low levels of oxygen and higher than expected levels of osmolarity in the culture medium have both been found to contribute to the improved culture of human stem cells.

PHYSIOCHEMICAL CULTURE CONDITIONS FOR EMBRYONIC STEM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. provisional patent application Ser. No. 60/458,815 filed March 28, 2003.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with United States government support awarded by the following agencies: NIH RR17721. The United States has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Stem cells are cells which can be maintained in culture *in vitro* and which are capable of differentiation into many, if not all, of the differentiated cell types of a mature body. Stem cells are referred to as pluripotent which means that they are capable of differentiating into many differentiated cell types. One category of pluripotent stem cell of high interest is the human embryonic stem cell, which is a category of stem cell originally created from human embryos. Human embryonic stem cells are capable of indefinite proliferation in culture, are demonstrably pluripotent, and are probably totipotent. One potential use for human embryonic stem cells is to direct differentiation of stem cells into specific differentiation lineages to create differentiated cells or tissues for potential transplantation into human bodies for therapeutic purposes.

[0004] The techniques to create in culture human embryonic stem cells have been described, are replicable and do work, but efforts to refine many of the procedures are still appropriate. Human embryonic stem cells will proliferate in culture indefinitely, but the proliferation in the culture is relatively variable and subject to undesired differentiation in culture. It can take significant amounts of time to proliferate stem cells to make desired quantities for particular scientific experiments or treatments, and so optimizations which increase the culture or proliferation efficiency of the stem cell cultures would be useful. In short, the fully optimized conditions for the culture and proliferation of human embryonic stem cells have not yet been developed. There are several reasons why the development of standard and optimized conditions for the culture and proliferation of human embryonic stem cells would be desirable. One is simply to shorten the time necessary for the proliferation of undifferentiated stem cell cultures so that more stem cells can be created more easily. Another reason is to create well documented and standardized techniques so that different laboratories culturing stem cells can

use common procedures and conditions and thus can obtain similar results. Yet another reason is the potential ultimate therapeutic use of such stem cells. To the extent that ultimate transplantation of cells or tissues derived from stem cells into human beings is an objective, the standardization and characterization of all of the components of the culture system from the beginning to the end of culture is an inherently desirable attribute.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention is summarized in that a culture system has been developed for the culturing of human embryonic stem cells. The culture condition includes culture of the cells in an atmosphere having minimal oxygen, and may include the use of an antioxidant. The culture conditions for a human embryonic stem cell culture may also have an osmolarity in excess of 300 mOsMol.

[0006] It is an object of the present invention to define culture conditions which aid in the optimum growth of human embryonic stem cells.

[0007] It is a feature of the present invention that, surprisingly, the optimal conditions for the culture of human embryonic stem cells differ from the physiological conditions present in the human body.

[0008] Other objects, advantages and features of the present invention will become apparent from the following specification when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0009] Figs. 1 through 4 present graphical representations of data from the experimental work described below.

DETAILED DESCRIPTION OF THE INVENTION

[00010] This specification is directed to improvements in systems for the optimized culture of human embryonic stem cells. It has heretofore been presumed that human embryonic stem cells would best be cultured in conditions which mimic the conditions found in somatic cells *in vivo* in a human body. This assumption turns out to be incorrect. It is described herein that by reducing the exposure of the human embryonic stem cells to oxygen in general, and oxygen free radicals in particular, and by increasing the osmolarity of the medium in which the stem cells are cultured beyond physiological levels, that the culture of human embryonic stem cells can be facilitated and encouraged. In other words, surprisingly, culture conditions which

mimic the physiochemical conditions of the human body are not the conditions most optimal for the culture of human embryonic stem cells.

[00011] Two parameters which have been found to be important in the culture of stem cells, and two parameters which are different than what might have been expected, are the level of oxygen concentration in the atmosphere in which the cells are cultured and the osmolarity of the culture medium itself in which the stem cells live. Conventionally, mammalian cells in culture are cultured in an atmosphere containing both oxygen and carbon dioxide, generally with oxygen at ambient air concentrations. With regard to the oxygen concentration, it has been found here that stem cells grow better in culture and that the cloning efficiency of human stem cells is increased significantly, both under conditions of low oxygen. Cloning is used here to refer to the process of sub-culturing stem cells, or, in other words, the process by which a cell, or a very few cells, are taken out of one stem cell culture and introduced into a new culture vessel to start a new culture of stem cells. In its ideal practice, the cloning of a stem cell culture should result in a daughter culture of cells all derived from a single parental stem cell. Cloning efficiency refers to the relative degree of success and abundance of undifferentiated cells in the stem cell culture in the new culture vessel. Under poor cloning conditions, daughter culture can either fail to propagate or can propagate as differentiated cells, thereby losing the attribute of being stem cells. It is preferred for stem cell culture in general, and in particular for purposes of creating cultures which can be efficiently cloned, that the oxygen level be held to less than ambient atmospheric levels and preferably to about 5% or less of the content of the atmosphere to which the stem cell culture is exposed. It is also preferred that an antioxidant be added to the culture medium, to further decrease the level of oxygen free radicals in the culture medium. Many compounds having antioxidant effects are known. The addition of an antioxidant will, it is believed, act to lower the overall mutation rate of the stem cells in culture and will thus permit the cloning of undifferentiated stem cells with a lower level of mutation and differentiation than would otherwise be the case in comparable cultures without antioxidants added.

[00012] The osmolarity of the culture is another factor affecting the success and vitality of stem cell cultures. Osmolarity, measured in milli-osmoles, is a measure of the number of dissolved particles in a solution, which is a measure of the osmotic pressure that a solution will generate. Normal human serum has an osmolarity of about 290 milli-osmoles or mOsMol. Media for *in vitro* culture of other mammalian cells vary in osmolarity, but some media have an osmolarity as high as 330 mOsMol. Surprisingly, it has been found here that human embryonic stem cells grow best in an osmolarity of above 330 and preferably about 350 mOsMol. Osmolarity is adjusted in a medium for stem cell culture most simply by adjusting the

concentration of salts, particularly NaCl, in the culture medium to achieve the osmolarity desired. There are any number of other salts that could be added to a medium to increase its osmolarity. The osmolarity of a solution can be measured by suitable instruments and can be calculated by the volume of the solution if one knows the number of molecules of salts which have been added.

[00013] While human embryonic stem cells can be grown in a number of culture media, the most common medium used is referred to as DMEM/DF12. This nomenclature indicates that the medium is a mixture, typically 50% each, of Dulbecco's modified Eagle Medium and Ham's F12 medium. DMEM and Ham's F12 are each media which are commercially available from many sources, separately or combined, and are specific combination of salts, vitamins, glucose, and amino acids. The first human stem cell cultures also included serum in the culture medium, but it has been since found that a serum replacement product may successfully be used to substitute for serum in the culture medium. Serum replacements, which contain purified albumin, vitamins, minerals, antioxidants, insulin, transferrin and lipids, are available commercially or can be formulated originally from these ingredients. A suitable medium for stem cells culture is 80% DMEM/DF12 basal medium and 20% serum replacement, to which is also added glutamine, β-mercaptoethanol, non-essential amino acids, and a fibroblast growth factor. These constituents are all known previously and described in the art and may be used with the alterations discussed in this document.

[00014] EXAMPLES

[00015] Effect of gas mixtures on stem cell culture and cloning

[00016] This investigation was to evaluate the effects of O_2 and CO_2 atmosphere on the ability of human embryonic stem cells to proliferate and remain undifferentiated. Cells were cultured in modified DMEM/DF12 plus "Knock-out" (Trademark) Serum Replacement from Gibco. The modified media included an adjustment to the sodium bicarbonate and sodium chloride concentrations to moderate pH and osmolarity. All media were corrected for appropriate pH given the concentration of CO_2, and the osmolarity was adjusted to also remain constant. Ultimate pH and osmolarity were constant across all media tested, despite alteration of atmospheric conditions. All media were conditioned overnight at standard atmospheric conditions (5% CO_2 in air) on mouse embryonic fibroblasts (MEFs) plated at a density of 2.12 x 10^5 cells/ml prior to experimental use. Conditioning of the medium for stem cell culture is done to induce the stem cells to remain undifferentiated without exposing the cells to the MEFs themselves. Conditioning means the medium is used to culture MEFs before the medium is used to culture stem cells. Although the MEFs are removed from the conditioned medium prior to

introduction of the stem cells, the medium is conditioned in some poorly understood manner and supports culture of undifferentiated stem cells in a manner that unconditioned media do not.

[00017] The human stem cells were individualized by treatment with trypsin, counted and plated onto six well plates. The human ES cells had been previously transformed with a green fluorescent protein (GFP) reporter gene under the control of an endogenous Oct4 promoter. Oct4 is known to be a marker of continued undifferentiated status. For the cell sorter analysis, 10K cells per well were plated. For cloning efficiency tests, 4K cells per well were plated. The treatments were all run in triplicate. The cells were fed and the atmosphere changed daily during the growth phase of the assay. FACS and cloning efficiency (CE) data were collected 8 days after plating the cells. The ratios of the total cell number and the geometric mean of GFP fluorescence intensity, compared to a control medium, were determined for each experimental treatment. Multiplication of the cell number ratio and the geometric mean ratio results in a media quality index (MQI) that is assigned to each experimental treatment. An MQI greater than 1 indicated an improvement over control conditions. Through this method, we were able to detect subtle differences due to physiochemical environment and media compositions that might not be apparent by subjective analysis.

[00018] The experimental conditions for atmospheric conditions were:

[00019] 1. 5% CO_2, 5% O_2, balance N_2

[00020] 2. 5% CO_2, 10%O_2, balance N_2

[00021] 3. 5% CO_2, balance air

[00022] 4. 10% CO_2, 5% O_2, balance N_2

[00023] 5. 10% CO_2, 10% O_2, balance N_2

[00024] 6. 10% CO_2 in air

[00025] The results are summarized in the histograms Figs. 1 and 2. In Fig. 1, the three bars for each experimental condition illustrate relative cell numbers, relative geometric mean of fluorescence detected, and relative cloning efficiency. Fig. 2 presents the data on MQI. Note that under any of the analytical metrics measured, the cell cultures that were exposed to less oxygen did better than those exposed to higher levels of oxygen. Thus culture with a lowered oxygen level results in improved stem cell growth in culture and increased cloning efficiency.

[00026] An additional experiment was performed adding a media supplement containing antioxidants. The data from this experiment suggested that addition of antioxidants will increase the attachment of stem cells and decrease the rate of differentiation.

[00027] Effect of osmolarity.

[00028] This investigation was to evaluate the effects of osmolarity on the ability of human embryonic stem cells to proliferate and remain undifferentiated. Human stem cells were again cultured in modified DMEM/DF12 plus "Knock-out" (trademark) Serum Replacement from Gibco. The osmolarity was adjusted to the levels set forth below by adjustment of the amount of sodium chloride in each medium. Media were all tested to assure that no alterations in pH or buffering capacity resulted from the adjustments in osmolarity. All media were again conditioned overnight on MEFs plated at a density of 2.12×10^5 cells/ml prior to experimental use.

[00029] Again, the human embryonic stem cells were individualized by treatment with trypsin, counted and plated onto six well plates. The human ES cells had been previously transformed with a green fluorescent protein (GFP) reporter gene under the control of an endogenous Oct4 promoter, a marker of continued undifferentiated status. For the cell sorter analysis, 10K cells per well were plated. For cloning efficiency tests, 4K cells per well were plated. The treatments were all run in triplicate. The cells were fed daily during the growth phase of the assay. FACS and cloning efficiency (CE) data were collected 8 days after plating the cells. The same metrics of relative cell number, geometric mean of cell number, and MQI were calculated as were calculated for the experiments already described above.

[00030] The experimental conditions were the following:

[00031] 1. DMEM/F12 270 mOsMol

[00032] 2. DMEM/F12 290 mOsMol

[00033] 3. DMEM/F12 310 mOsMol

[00034] 4. DMEM/F12 330 mOsMol

[00035] 5. DMEM/F12 350 mOsMol

[00036] 6. DMEM/F12 370 mOsMol

[00037] The results are displayed in Figs. 3 and 4. Fig. 3 illustrates the relative cell number and geometric mean results, while Fig. 4 demonstrates the MQI results measured.

[00038] Note that the results, particularly the MQI, demonstrates that an osmolarity in excess of 330 mOsMol, and preferable an osmolarity of about 350 mOsMol is most efficient for stem cell culture. This result is surprising given the physiological conditions (290 mOsMol) of normal human serum.

CLAIM OR CLAIMS

WE CLAIM:

1. A method for culturing human embryonic stem cells comprising culturing the stem cells in a nutrient medium in which the stem cells will remain undifferentiated and in an atmosphere having no more than about 5% oxygen.

2. The method of claim 1 wherein the medium further comprises an antioxidant in the nutrient medium.

3. An improvement in methods for cloning cultures of human embryonic stem cells, the improvement comprising culturing the human embryonic stem cells prior to cloning in a nutrient medium and in an atmosphere having no more than about 5% oxygen.

4. The improvement of claim 3 further comprising adding an antioxidant to the nutrient medium in which the stem cells are cultured.

5. A method for culturing human embryonic stem cells comprising culturing the stem cells in a nutrient medium in which the stem cells can remain undifferentiated and in an atmosphere having no more than about 5% oxygen.

6. An improvement in a medium for the cultivation of human embryonic stem cells, the improvement comprising that the medium is adjusted to have an osmolarity in excess of 330 mOsMol.

7. The improvement as claimed in claim 6 wherein the osmolarity of the medium is about 350 mOsMol.

8. A stem cell culture comprising
a culture plate;
a nutrient medium in the culture plate;
growing human embryonic stem cells in the medium; and
the medium having an osmolarity in excess of 330 mOsMol.

9. A stem cell culture as claimed in claim 8 wherein the osmolarity is about 350 mOsMol.

10. A method for culturing human embryonic stem cells comprising culturing the stem cells in a nutrient medium in which the stem cells will remain undifferentiated, the medium having an osmolarity in excess of 330 mOsMol.

11. A method as claimed in claim 10 wherein the medium has an osmolarity of about 350mOsMol.

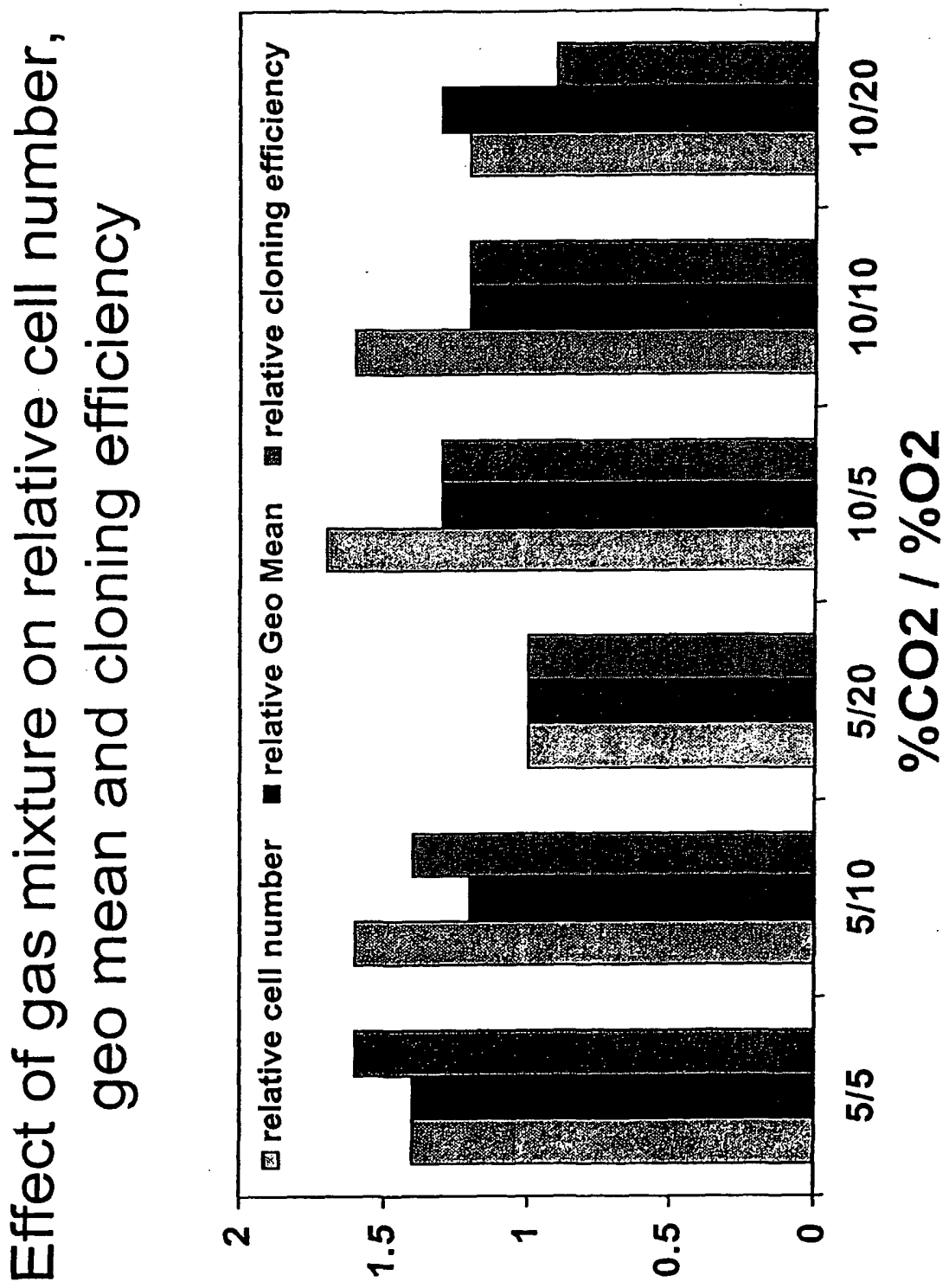
Effect of gas mixture on relative cell number, geo mean and cloning efficiency
relative cell number
relative Geo Mean
relative cloning efficiency
2
1.5
1
0.5
0
5/5
5/10
5/20
10/5
10/10
10/20
%CO2 / %O2

FIG 1

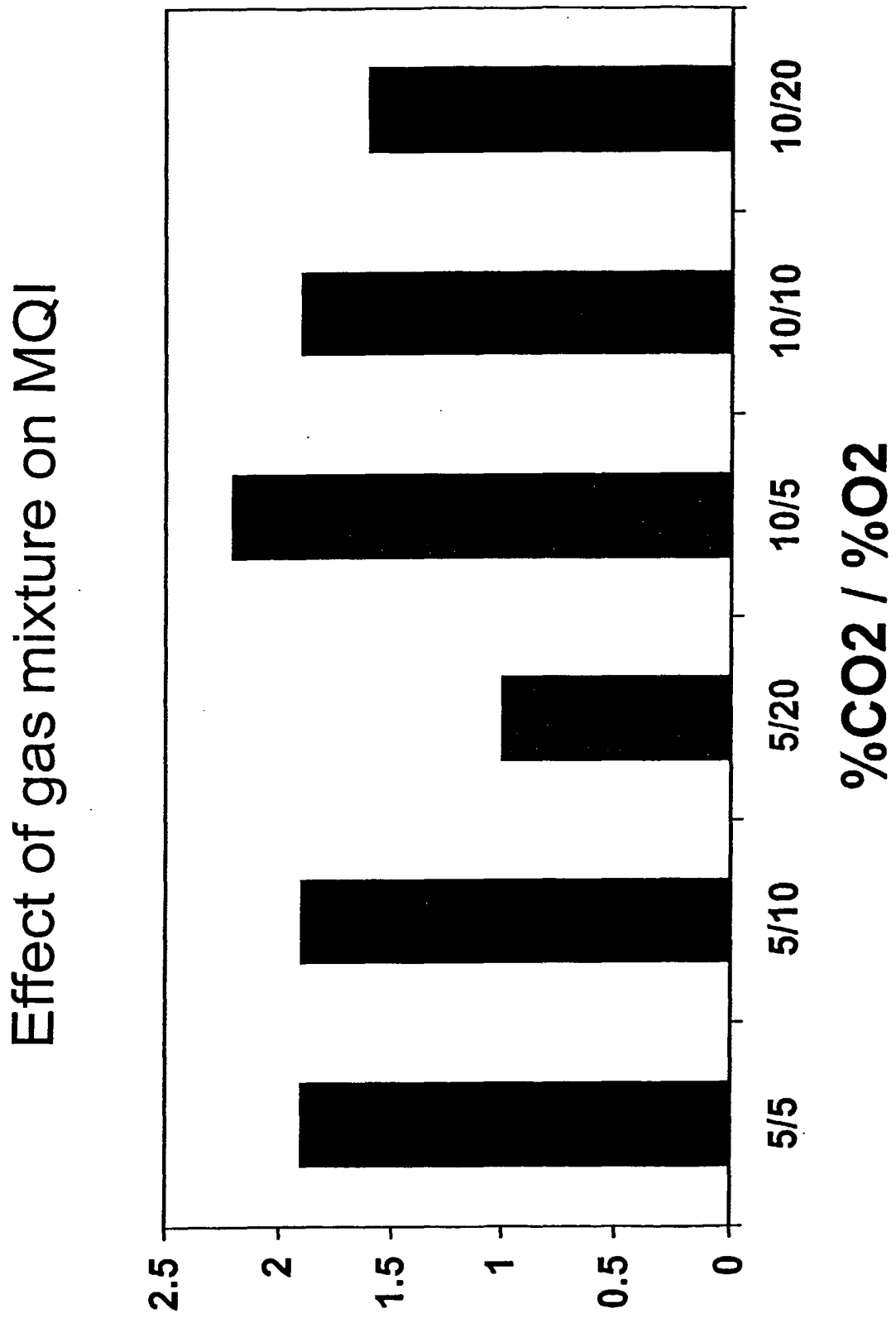
Effect of gas mixture on MQI
2.5
2
1.5
1
0.5
0
5/5
5/10
5/20
10/5
10/10
10/20
%CO2 / %O2

FIG 2

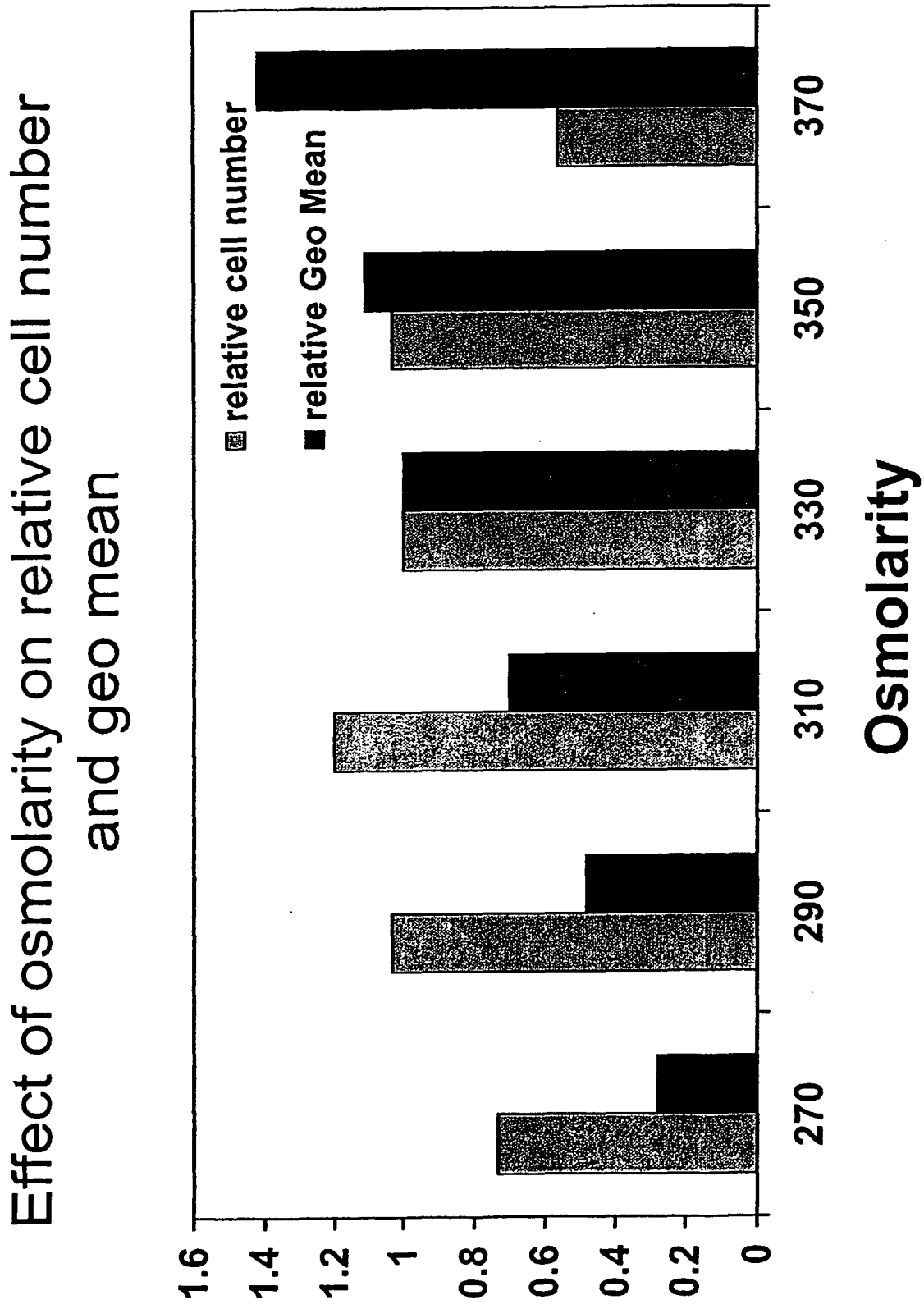
Effect of osmolarity on relative cell number and geo mean
relative cell number
relative Geo Mean
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
270
290
310
330
350
370
Osmolarity

FIG 3

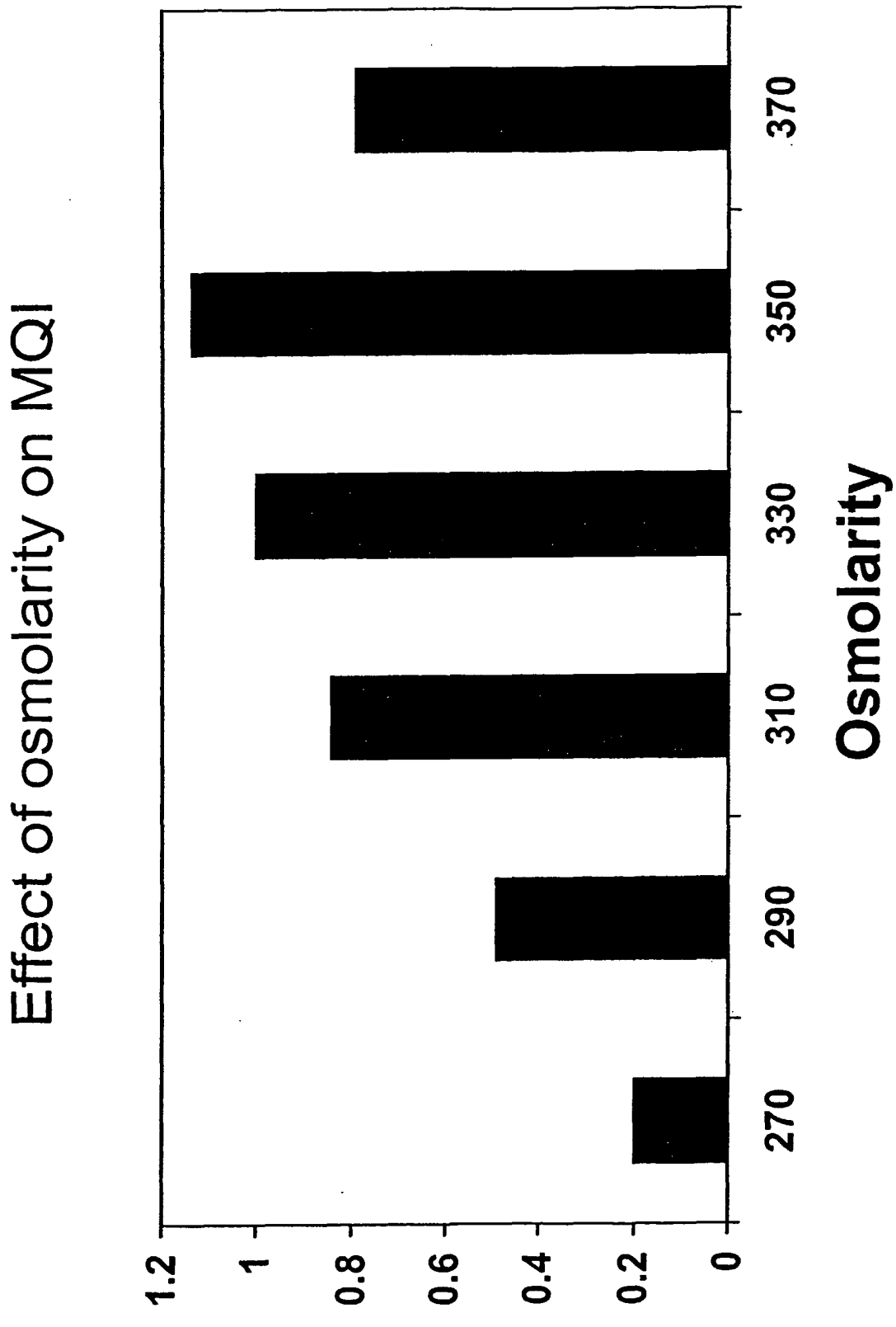
Effect of osmolarity on MQI
1.2
1
0.8
0.6
0.4
0.2
0
270
290
310
330
350
370
Osmolarity

FIG 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/09097

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 5/08, 5/06, 5/00
US CL : 435/366, 374, 375, 377
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)
U.S. : 435/366, 374, 375, 377

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 practicable, search terms used)
Please See Continuation Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,184,035 A (CSETE et al) 6 February 2001 (06.02.2001), entire document including abstract; column 3, lines 10-25; column 5, lines 40-45; column 6, lines 28-35.	1-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family 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 application or patent 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
24 October 2005 (24.10.2005)

Date of mailing of the international search report
06 DEC 2005

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703) 305-3230

Authorized officer
Vera Afremova

Telephone No. (571) 272-1600

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/09097

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-5

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/09097

BOX III. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5, drawn to a first method for culturing and/or cloning human embryonic stem cell in an atmosphere having no more than 5% oxygen.

Group II, claim(s) 6 and 7, drawn to a first composition that is a medium having osmolarity in excess of 330 mOsm.

Group III, claim(s) 8 and 9, drawn to a second composition that is a stem cell culture comprising support plate, embryonic stem cells and a medium with osmolarity in excess of 330 mOsm.

Group IV, claim(s) 10 and 11, drawn to a second method for culturing human embryonic stem cell in a medium having osmolarity in excess of 330 mOsm.
The inventions listed as Groups I-IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
This application contains claims drawn to more than one of permissible combinations of invention categories such as more than one product and more than one process of using the product. Furthermore, the corresponding special technical features are known in the prior art. For example: US 4,724,206 teaches a medium with osmolarity in excess 350 mOsm for protein production (abstract). For example: US 6,184,035 teaches the use of low oxygen conditions including less than 5% for culturing embryonic stem cells (abstract; col. 5, lines 41-45).

Continuation of B. FIELDS SEARCHED Item 3:
WEST: USPT, PGPUB; STN: BIOSIS, WPIDS
search terms: embryonic stem cells, human embryonic stem cells, low oxygen conditions