FEEDER INDEPENDENT EXTENDED CULTURE OF EMBRYONIC STEM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. provisional patent application Ser. No. 60/573,545 filed May 21, 2004.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] To be determined.

BACKGROUND OF THE INVENTION

[0003] Stem cells are defined as cells that are capable of a differentiation into many other differentiated cell types. Embryonic stem cells are stem cells from embryos which are capable of differentiation into most, if not all, of the differentiated cell types of a mature body. Stem cells are referred to as pluripotent, which describes this capability of differentiating into many cell types. A category of pluripotent stem cell of high interest to the research community is the human embryonic stem cell, abbreviated here as hES cell, which is an embryonic stem cell derived from a human embryonic source. Human embryonic stem cells are of great scientific interest because they are capable of indefinite proliferation in culture and are thus capable, at least in principle, of supplying cells and tissues for replacement of failing or defective human tissue. The existence in culture of human embryonic stem cells offers the potential of unlimited amounts of human cells and tissues for use in a variety of therapeutic protocols to assist in human health. It is envisioned in the future human embryonic stem cells will be proliferated and directed to differentiate into specific lineages so as to develop differentiated cells or tissues which can be transplanted into human bodies for therapeutic purposes.

[0004] One of most significant features of human embryonic stem cells is the attribute of being capable of self-renewal. By that, it is meant that the hES cells are capable of proliferating into multiple progeny stem cells, each of which seems to have the full potential of its ancestor cell. In other words, the progeny are renewed to have all the developmental and proliferative capacity of the parental cell. This attribute, combined with the pluripotency, are the traits that make hES cells candidates for many potential uses, since, the theory, hES cells can be reproduced indefinitely and in large numbers and then induced to become any cell type in the human body. The attribute of ability to self-renew appears closely linked to

the attribute of being undifferentiated in the sense that at least given present knowledge, only undifferentiated hES cells are capable of indefinite self-renewal and as soon as the cells differentiate, the attribute of self-renewal capability is lost. Since human embryonic stem cells will spontaneously differentiate, care must be taken in culture conditions to maintain the cells in an undifferentiated state.

[0005] Basic techniques to create and culture human embryonic stem cells to maintain the cells in an undifferentiated state have been described. The existing techniques do work, but there are limitations and drawbacks to some of the procedures currently used to culture human embryonic stem cells. One limitation is of particular concern. Most existing human embryonic stem cell lines have been, to one degree or another, exposed directly to mouse cells or to a medium in which mouse cells have been cultured previously. The original techniques for the generation and culture of human embryonic stem cells described the use of mouse embryonic fibroblast (MEF) feeder cells as a feeder layer on which human embryonic stem cells could be cultured. The fibroblast feeder layer acts, through some as yet incompletely understood mechanism, to enable the stem cells to remain in an undifferentiated state. Later, it was discovered that the same phenomenon could be achieved if the stem cells were exposed to "conditioned media." A conditioned medium is nothing more than the stem cell culture medium which had previously been cultured on feeder cells such as MEF. Either the feeder cells impart some factor to the medium or remove some factor from the medium, but the result is that the conditioned medium can be used to culture stem cells without differentiation. Either culture condition, the direct growth on feeder cells, or the use of condition media, raises the concern that one or more agents such as a virus could transmit from the mouse cells to the human ES cells. If one of the objectives of human embryonic stem cell cultures is to create tissues which can ultimately be transplanted into a human body, it is highly desirable that the stem cells never have been exposed to cells of another species or to media which have been used to culture cells of another species. Also, the need for feeder cells of any species represents an unneeded biological variable in the culture of stem cells that is best avoided if possible. Accordingly, defining a culture condition, which will permit the proliferation and culture of human embryonic stem cells without a fibroblast feeder layer and without conditioned medium, is of great interest in the continued development of techniques for the use of human embryonic stem cells.

[0006] Several medium formulations will permit human ES cells to remain undifferentiated for some time, but that state often fails to maintain itself over long term culture. In particular, we define a passage as the growth of human ES cells from an initial

seed culture in a culture plate to growth to cell confluence in the same culture plate as a "passage." We have found several medium formulations that permit the cultivation of human ES cells for one or two passages without severe differentiation, but then the cells differentiate gradually or rapidly upon subsequent passages. We have come to believe that in order for a medium to truly support the indefinite proliferation of human ES cells without differentiation, without feeder cells or feeder conditioned medium, the medium must be demonstrated to support culture of human ES cells in a substantially uniform and undifferentiated state for at least five passages.

BRIEF SUMMARY OF THE INVENTION

[0007] In a first aspect, there is provided a method for culturing human embryonic stem cells in a feeder independent culture, the method comprising the step of culturing the human embryonic stem cells in a medium including salts, vitamins, amino acids, glucose, a fibroblast growth factor and a bone morphogenetic protein antagonist in sufficient amount to maintain the stem cells in an undifferentiated state, wherein the bone morphogenetic protein antagonist is selected from noggin, gremlin, chordin, follistatin, twisted gastrulation, members of the DAN family and antagonist antibodies specific to a bone morphogenetic protein.

[0007a] Disclosed is a method for culturing human embryonic stem cells without the need for feeder cells or conditioned medium of any kind, the method including the step of culturing the human embryonic stem cells in a medium including salts, vitamins, amino acids, glucose, a fibroblast growth factor and a bone morphogenetic protein antagonist in sufficient amount to maintain the stem cells in an undifferentiated state.

[0007b] In a second aspect, there is provided an *in vitro* cell culture for use in a method of maintaining cells in an undifferentiated state comprising in a culture vessel: human embryonic stem cells and a culture medium, the culture medium comprising salts, vitamins, amino acids, glucose, a fibroblast growth factor and a bone morphogenetic protein signaling antagonist in sufficient amount to maintain the stem cells in an undifferentiated feeder independent state, the medium being free of feeder cells and never having been exposed to feeder cells, wherein the bone morphogenetic protein is selected from noggin, gremlin, chordin, follistatin, twisted gastrulation, members of the DAN family and antagonist antibodies specific to a bone morphogenetic protein.

[0008] Also disclosed is an in vitro cell culture of human embryonic stem cells cultured in a medium including a bone morphogenetic protein antagonist so that the stem cells can be cultured indefinitely in an undifferentiated state without the need for fibroblast feeder cells or conditioned medium.

[0008a] In a third aspect, there is provided a medium for culturing embryonic stem cells in a feeder independent culture, the medium comprising salts, vitamins, amino acids, glucose, a fibroblast growth and a bone morphogenetic protein antagonist in sufficient amount to maintain stem cells growth in the medium an undifferentiated state without exposure to feeder cells or medium exposed to feeder cells, wherein the bone morphogenetic protein antagonist is selected from noggin, gremlin, chordin, follistatin, twisted gastrulation, members of the DAN family and antagonist antibodies specific to a bone morphogenetic protein.

[0009] It is an object of the present disclosure to define long term culture conditions for human embryonic stem cells that avoid the use of feeder cells, including both culture on feeder cells and culture in feeder-conditioned medium.

[00010] It is another object of the present disclosure to define culture conditions for human embryonic stem cells that are as defined as possible while being completely independent of feeder cells.

[00011] Other objects, features and advantages of the present disclosure will become apparent from the following specification.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[00012] Not applicable.

DETAILED DESCRIPTION OF THE INVENTION

[00013] The observation that human embryonic stem (ES) cell cultures have previously been maintained in an undifferentiated state only when cultured in the presence of fibroblast feeder cells or in conditioned medium has led to speculation that the fibroblasts release into the medium a factor which acts to inhibit differentiation of the ES cells. This speculation is also based on the parallel observations of murine ES cell lines, which, when cultured with fibroblast feeder cells, respond to leukemia inhibitory factor (LIF) secreted by the fibroblasts to remain undifferentiated. The LIF activates a signal pathway in the murine ES cells that triggers self-renewal. However, human ES cells are unresponsive to LIF and indeed do not seem to possess LIF receptors on their cell surface. Since no single factor has been isolated from conditioned medium that seemed to cause the effect of preventing differentiation in human ES cells, we developed a new hypothesis. We hypothesized that instead the fibroblast cells inactivate differentiation factors present in unconditioned medium.

[00014] Various research groups have investigated factors that initiate differentiation of human ES cells into progeny cell cultures that are enriched in cells of one or more particular lineage. One of these differentiation factors is a category of protein factor known as bone morphogenetic protein (BMP). BMPs are members of the transforming growth factor-β (TGFβ) superfamily of secreted signaling molecules. They play an extensive role in almost all aspects of embryonic development. BMP 4 and other BMP family members, such as BMP2, -5, and –7, bind BMP type II receptor BRII, which recruits type I receptor BR1A (ALK3) or BR1B. Upon ligand activation, the intracellular kinase domain of the type I receptors phosphorylates Smad1, -5, and -8, which are then escorted by a common Smad to enter the nucleus and activate target genes. The relative expression level of BMPs, receptors, and Smads within the cell is an important determinant of BMP-induced responses. Co-stimulation of other signaling pathways also alters the nature of BMP effect. A typical example is the change of BMP action by a co-activated LIF signal in mouse ES cells: BMP signal alone induces non-neural epithelial differentiation, whereas BMP and LIF signals together inhibit differentiation to any lineage. The extracellular BMP antagonists such as noggin, gremlin, chordin, inhibin, follistatin, twisted gastrulation and members of the DAN family, etc. can modify, diminish or totally nullify BMP activities. On the other hand, some signaling pathways can interrupt the BMP signaling intracellularly. For example, the MAPK

signaling activated by fibroblast growth factor (FGF) can inhibit the BMP signaling by preventing the Smads from nuclear translocation via phosphorylation of the linker domain of the Smads. Activation of the transforming growth factor beta (TGFβ), Nodal, or Activin signaling pathways may antagonize the BMP signaling via intracellular cross-talk, such as competition for Smad4 to enter the nucleus. It is anticipated that all of these molecules can be used to antagonize BMP signaling to achieve the effects reported here.

[00015] It was also observed that the levels of bone morphogenetic protein (BMP) stimulated intracellular signal is low is human ES cells grown in conditioned medium, whereas the level of this same signal is high in human ES cells grown out in unconditioned medium (and without fibroblast feeder cells). Perhaps the effect of the conditioning of the medium was due to inhibition of the effects of BMP inducing signals present in the unconditioned medium. We therefore explored the possibility that antagonists of BMP activity could act to enable the cultivation of human ES cells in culture and in an undifferentiated state without the need for feeder cells or conditioned medium. It was discovered, and is reported here, that this possibility was found to be correct. By antagonizing the activity of BMP, it has become possible to culture human ES cells indefinitely, while the cells retain all of the identifying characteristics of embryonic stem cells.

[00016] There are a number of antagonists of BMP that can be used in this invention. The most potent known such antagonist is the protein noggin. Other proteins known to function as antagonists of BMPs include gremlin, chordin, inhibin, follistatin, twisted gastrulation and members of the DAN family. As mentioned above, other proteins include TFGβ and activin and other molecules which activate the signaling pathway for MAPK. It is not required that the antagonist protein be the human form of the protein. It is only required that it be effective in culture to allow unconditioned medium to maintain ES cells without differentiation. It is also possible to use as an antagonist antibodies specific to all BMPs or a specific BMP. The particular protein chosen as the BMP antagonist is less important than that the desired effect is achieved in that BMP signaling activity is inhibited by the molecule added to the medium. The simplest and most straightforward way to accomplish this is to add the BMP antagonist to the medium in which the human ES cells are cultured.

[00017] The most potent BMP inhibitor identified so far, the protein noggin, was originally cloned based on its dorsalizing activity in *Xenopus* embryos. Mouse noggin cDNA encodes a 232 amino acid (aa) residue precursor protein with 19 aa residue putative signal peptide that is cleaved to generate the 213 aa residue mature protein which is secreted as a homodimeric glycoprotein. Noggin is a highly conserved molecule. Mature mouse noggin shares 99% and 83% aa sequence identity with human and *Xenopus* noggin, respectively. Noggin has a complex pattern of expression during embryogenesis. In the adult, noggin is expressed in the central nervous system and in several adult peripheral tissues such as lung, skeletal muscle and skin. Noggin has been shown to be a high-affinity BMP binding protein that antagonizes almost all BMP bioactivities.

[00018] It has also been found that high levels of fibroblast growth factor (FGF) are also useful in the culture of stem cells with or without conditioned medium. While it has been previously reported that the addition of FGF is a useful additive to stem cell culture conditions, as in WO 01/66697, in that work, the FGF in the medium in the work reported was basic FGF (bFGF or FGF2) at concentration of 4 ng/ml. Here it is reported that culture with FGF at levels approximately ten-fold higher produces better results. The preferred concentration of bFGF in the work reported here is 40 ng/ml. While others of the various FGF variants also work for this purpose, the concentrations of other FGFs would need to be adjusted to correspond to the efficacy of this level of bFGF, which has been shown by us to also inhibit the intracellular BMP activity.

[00019] There appears to be a synergistic relationship between the effect caused by a BMP antagonist and that caused by high levels of FGF in the culture medium. In other words, the use of a high level of bFGF, e.g. at 100 ng/ml, will support cultures of hES cells in an undifferentiated state without feeder cells or conditioned medium, but so will a lesser level of bFGF, e.g. 40 ng/ml when combined with the use of a BMP antagonist such as noggin. Either combination makes the culture not just "feeder free", which is a term used for cultures which make use of conditioned medium (conditioned with feeder cells) but completely "feeder independent," meaning entirely independent of the need for feeder cells of any kind at all.

[00020] As the date presented below will demonstrate, this hypothesis has proven to be correct. By adding noggin, or other inhibitor of BMP signaling, and by stimulating the fibroblast growth factor (FGF) signal, human ES cells can be grown indefinitely in an undifferentiated state without either feeder cells or conditioned medium. This permits a

human ES cell culture to be initiated and maintained without exposure to feeder cells or medium exposed to feeder cells, thus enabling animal cell-free proliferation of human ES cell lines in a well defined medium.

[00021] A related concern in the culture of human ES cells is to remove, to the extent possible, undefined constituents and constituents of animal origin from ES cell culture conditions. This is done for two reasons. One reason is to standardize culture conditions so as to minimize the normal variations in biological materials to the extent possible. The other objective is to avoid the use of materials, cells, exudates or constituents of animal origin so as to avoid any possible cross-species viral transmission through the culture system. Thus it is an objective to define a culture condition that avoids the use of products of animal origin.

[00022] So a defined medium for human ES cells begins with a basal medium containing salts, vitamins, glucose and amino acids. The basal medium can be any of a number of commercially available media. We prefer combination of Dulbecco's Modified Eagle Medium and Hams F12 medium, sold as a combination (DMEM/F12). To that basal medium is added glutamine, β-mercaptoethanol, and non-essential amino acids. Other possible additives include antioxidants and lipids. A protein constituent of the medium is a serum substitute product. Albumin or purified albumin products, like the commercial product AlbuMax™, will work, but we prefer a defined protein product made up of albumin, insulin and transferrin. Human proteins are preferred but not essential so long as uncharacterized animal products are excluded.

[00023] It is worthy of mention that the observations here indicate a fundamental difference between the mechanisms of self-renewal among stem cells of mammalian species. The conditions which support the maintenance of murine stem cells, including the addition of LIF and BMP to the medium, are insufficient to maintain hES cells in an undifferentiated state. We have been unable to maintain undifferentiated hES cells in serum-free medium supplemented with LIF and BMP4. BMPs cause human ES cells to differentiate into trophoblast cells. In fact, the data presented here teach that BMP signaling must be antagonized to foster human stem cell self-renewal. Thus it is clear that, at a minimum, the cellular mechanisms for self-renewal are very different between the mouse and human cell systems.

EXAMPLES

[00024] Methods and materials

[00025] Media and cell culture. Unconditioned medium (UM) contained 80% DMEM/F12 and 20% KNOCKOUT serum replacement, and was supplemented with 1 mM L-glutamine, 1% Nonessential Amino Acids (all from Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma). Conditioned medium (CM) is prepared by incubating unconditioned medium with mouse embryonic fibroblasts overnight and collecting the medium afterwards, which is then supplemented with 4 ng/ml bFGF and refrigerated to be used within 2 weeks. hESCs were cultured on plates coated with Matrigel (BD Scientific) in CM or UM with or without either 0.5 μg/ml mouse noggin (R&D Systems), or 40 ng/ml human bFGF (Invitrogen), or both, and propagated by using 2 mg/ml Dispase (Invitrogen) to loosen the cell colonies. For evaluation of $Oct4^+$ cell number, suspended colonies containing 35,000 cells were added to each medium in multiple wells and cultured for 7 days. Cells were harvested and counted on days 1 and 7, and $Oct4^+$ cells on day 7 were detected by fluorescence-activated cell sorting (FACS, see below). Embryoid bodies (Ebs) were formed by suspending hESCs that had been cultured in CM or UM/bFGF/noggin (UMFN), as cell clumps in UM on a non-coated plate, and culturing them on a rocker for 7 days. The EB cells were then re-plated in DMEM medium supplemented with 10% fetal bovine serum on gelatin-coated plate and cultured for 5 days followed by harvesting and reverse transcription-PCR (RT-PCR) analysis. Experiments were repeated multiple times and ANOVA was used for statistic analysis throughout the studies.

[00026] Immunoprecipitation and western blotting. 15 ml of DMEM/F12 medium was conditioned on 2.12 x 10^5/ml irradiated mouse embryonic fibroblast cells in a T75 flask overnight. The medium was collected and concentrated to about 0.7 ml with a 5 kD molecular weight cut-off filter (Millipore) and immunoprecipiated with goat anti-mouse noggin and gremlin antibodies (R&D Systems) (5 μg each) or 10 μg goat IgG as a negative control. The precipitated proteins or cell lysates (Fig. 2A) were electrophoresized on a 4%–20% linear gradient Polyacrylamide Tris-HCl Precast Gel (BioRad) for western blotting. The antibodies against mouse noggin and gremlin were used for the immunoprecipitated proteins, and antibodies against human Smad1/5/8, phosphorylated Smad1/5/8 (Cell Signaling Technology), BMP2/4 (R&D Systems), and β-Actin (Abcam) were used for the cell lysates.

The blots were treated with the ECL substitute solutions 1 and 2 (Amersham Biosciences) and exposed in a Fuji Imager for chemiluminescence.

[00027] **BMP/Smad-Luciferase Reporter Assay.** hESCs cultured in CM were transfected with a BMP/Smad-responsive firefly luciferase reporter plasmid, pID120-Lux, together with trace amount of pRL-tk plasmid (Promega) to express Renilla luciferase as an internal control. One day post-transfection, the cells were treated variously for 24 h. Cell lysates were extracted and both the firefly and Renilla luciferase activities tested by using the Dual-Luciferase Reporter Assay System (Promega) on a 3010 Luminometer (BD Biosciences). Results were recorded as the firefly luciferase activity normalized by the Renilla luciferase activity.

[00028] **Quantitative-PCR and RT-PCR.** Total cellular RNA was extracted by RNeasy kit (Qiagen), and treated with RNase-free DNase according to the manufacturer's instructions. One μg RNA was reverse transcribed to cDNA with Improm-II Reverse Transcription System (Promega). Quantitative-PCR was performed by using the SYBR green Q-PCR Mastermix (Stratagene) on the AB 7500 Real Time PCR System (Applied Biosystems) under the following conditions: 10 min at 95°C, 40 cycles of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C, and 3 min extension at 72°C. *GAPDH* transcript was tested as an endogenous reference to calculate the relative expression levels of target genes according to Applied Biosystems' instructions. For RT-PCR, following conditions were used: 3 min at 94°C, various cycles (see below) of 20 sec at 94 °C, 30 sec at 55 °C, and 1 min at 72 °C. The PCR reactions were separated on gel by electrophoresis and the DNA bands were visualized under ultraviolet light for photography. The primer sequences and PCR cycle numbers are listed below.

[00029] Table 1. Primers and cycle numbers for Q-PCR and RT-PCR

Gene Name	Test	Forward Primer/Reverse Primer	SEQ ID NO:	PCR Cycle #
Id1	Q-PCR	Forward Primer 5'-GGT GCG CTG TCT GTC TGA G Reverse Primer 5'-CTG ATC TCG CCG TTG AGG	(SEQ ID NO: 1) (SEQ ID NO: 2)	40
Id2	Q-PCR	Forward Primer 5'-GCA GCA CCT CAT CGA CTA CA Reverse Primer 5'-AAT TCA GAA GCC TGC AAG GA	(SEQ ID NO: 3) (SEQ ID NO: 4)	40
Id3	Q-PCR	Forward Primer 5'-CTG GAC GAC ATG AAC CAC TG Reverse Primer 5'-GTA GTC GAT GAC GCG CTG TA	(SEQ ID NO: 5) (SEQ ID NO: 6)	40
Id4	Q-PCR	Forward Primer 5'-ATG AAG GCG GTG AGC CCG GTG CGC C Reverse Primer 5'-TGT GGC CGT GCT CGG CCA GGC AGC G	(SEQ ID NO: 7) (SEQ ID NO: 8)	40
GAPDH	Q-PCR	Forward Primer 5'-GAG TCC ACT GGC GTC TTC AC Reverse Primer 5'-CTC AGT GTA GCC CAG GAT GC	(SEQ ID NO: 9) (SEQ ID NO: 10)	40
Oct4	RT-PCR	Forward Primer 5'-GGG AAG GTA TTC AGC CAA ACG Reverse Primer 5'-GGT TCG CTT TCT CTT TCG GG	(SEQ ID NO: 11) (SEQ ID NO: 12)	35
Nanog	RT-PCR	Forward Primer 5'-AAT ACC TCA GCC TCC AGC AGA TG Reverse Primer 5'-CAA AGC AGC CTC CAA GTC ACT G	(SEQ ID NO: 13) (SEQ ID NO: 14)	35
Rex1	RT-PCR	Forward Primer 5'-CCT GGA GGA ATA CCT GGC ATT G Reverse Primer 5'-TCT GAG GAC AAG CGA TTG CG	(SEQ ID NO: 15) (SEQ ID NO: 16)	35
CGβ	RT-PCR	Forward Primer 5'-TGA GAT CAC TTC ACC GTG GTC TCC Reverse Primer 5'-TTT ATA CCT CGG GGT TGT GGG G	(SEQ ID NO: 17) (SEQ ID NO: 18)	30
Pax6	RT-PCR	Forward Primer 5'-CGT CCA TCT TTG CTT GGG AAA TC Reverse Primer 5'-GAG CCT CAT CTG AAT CTT CTC CG	(SEQ ID NO: 19) (SEQ ID NO: 20)	30

NeuroD1	RT-PCR	Forward Primer 5'-AAG CCA TGA ACG CAG AGG AGG ACT (SEQ ID NO: 21) Reverse Primer 5'-AGC TGT CCA TGG TAC CGT AA (SEQ ID NO: 22)	30
Brachyury	RT-PCR	Forward Primer 5'-AAC CCA ACT GTG GAG ATG ATG CAG (SEQ ID NO: 23) Reverse Primer 5'-AGG GGC TTC ACT AAT AAC TGG ACG (SEQ ID NO: 24)	35
HNF3α	RT-PCR	Forward Primer 5'-CCA AGC CGC CTT ACT CCT ACA (SEQ ID NO: 25) Reverse Primer 5'-CGC AGA TGA AGA CGC TGG AGA (SEQ ID NO: 26)	30
B-Actin	RT-PCR	Forward Primer 5'-TGG CAC CAC ACC TTC TAC AAT GAG C (SEQ ID NO: 27) Reverse Primer 5'-GCA CAG CTT CTC CTT AAT GTC ACG C (SEQ ID NO: 28)	25

[00030] **FACS and immunocytochemistry.** hESCs cultured in various media were processed for FACS analysis to detect $Oct4^+$ cells. Mouse anti-human Oct4 antibody (Santa Cruz Biotechnology) at 2 μg/ml and fluorescent isothiocyanate-labeled rabbit anti-mouse secondary antibody (Molecular Probes) at 1:1000 dilution were used. Statistic analysis was performed on Arcsine numbers converted from the percentages of $Oct4^+$ cells. For immunocytochemistry, the mouse anti-Oct4 antibody (at 0.2 μg/ml) was used and followed by Alexa Fluor 488-labeled anti-mouse IgG secondary antibody (Molecular Probes) at 1:1000 dilution.

[00031] **Immunoassay of HCG in the culture medium.** hESCs cultured in UMFN (unconditioned medium with bFGF and noggin) for multiple passages were subsequently cultured in CM plus 100 ng/ml BMP4 up to 7 days with daily refreshment of the medium and BMP4. The spent media were collected on days 3, 5, and 7, and assayed for HCG as described.

[00032] **G-banding and fluorescence *in situ* hybridization.** hESCs cultured in UMFN for various passages were processed for G-banding and fluorescence *in situ* hybridization. From all the dispersed and fixed cells, 20 cells at metaphase were analyzed for G-banding, and 100-200 nuclei were assayed for fluorescence *in situ* hybridization using probes to detect marker genes in chromosomes of interest. Representative images captured by the CytoVysion® digital imaging system (Applied Imaging) were reviewed.

[00033] **Results**

[00034] **UM contains BMP-like differentiation-inducing activity.** UM contained 20% KNOCKOUT ™ serum replacement (Invitrogen), which includes a proprietary lipid-rich bovine albumin component, ALBUMAX ™. UM was conditioned on fibroblasts overnight and then supplemented with 4 ng/ml human bFGF to obtain CM. We cultured hESCs (H1) in CM, UM, a 1:1 mixture of CM with UM, or a 1:1 mixture of CM with DMEM/F12. The cells in CM or the 1:1 CM-DMEM/F12 mixture remained undifferentiated, and were characterized by typical hESC morphology. However, the cells in UM or the 1:1 CM-UM mixture both rapidly differentiated within 48 h. We next substituted purified fetal bovine serum albumin (16.6 g/L, Fisher Scientific) for the serum replacement to determine whether albumin caused the differentiation. This medium allowed hESCs to maintain an

undifferentiated morphology for about 7 days; however, the cells had a reduced proliferation rate and eventually differentiated into a mixed population of cells. These results suggest that components other than albumin contained in the serum replacement are responsible for the rapid differentiation of UM-cultured cells. CM reduces this differentiation-inducing activity, but also provides positive factors to sustain hESC self-renewal. In addition to albumin, serum replacement also contains other components that are required for hESC culture, so serum replacement rather than albumin was used in all subsequent studies.

[00035] To examine whether the differentiation-inducing activity in UM stimulates BMP signaling in hESCs, we assessed by western blotting the level of phosphorylated Smad1, an immediate effector downstream of BMP receptors. Smad1 phosphorylation (the antibody used here could also detect phosphorylation of other BMP effectors Smad5 and –8) was low in H1 cells cultured in CM, but was high in cells cultured for 24 h in UM, or in CM + BMP4. The addition of noggin to UM reduced the level of Smad1 phosphorylation, but the addition of 40 ng/ml bFGF to UM left the level of Smad1 phosphorylation unchanged. BMP signaling can induce expression of BMP ligands, forming a positive feedback loop in cells from various species, including hESCs. BMP2/4 proteins were, indeed, detected at an increased level in UM-cultured hESCs compared to cells cultured in CM or in UM plus noggin. It is at present unclear whether there are BMPs in UM that directly stimulate BMP signaling in hESCs, or other differentiation-inducing molecules that indirectly stimulate BMP signaling by inducing BMP secretion. Noggin and another BMP antagonist gremlin were both detected in medium conditioned by the fibroblast. These data demonstrate that an elevated, but repressible, BMP signaling activity is present in UM-cultured hESCs, and that both BMP agonists and antagonists are present in fibroblast-supported culture of hESCs.

[00036] We further assessed BMP signaling in hESCs (H14) cultured in various media in the presence or absence of protein factors, by using a luciferase reporter plasmid specifically responsive to BMP/Smads. The reporter activity increased with an increasing concentration of the serum replacement or BMP4, and decreased with an increasing concentration of noggin or bFGF. 500 ng/ml Noggin and 40 ng/ml bFGF had synergistic effect in reducing the reporter activity to the level similar to that achieved by CM. Somewhat surprisingly, even higher levels of bFGF (100 ng/ml) reduced BMP signaling to a level comparable to that found in CM without the addition of noggin. These results suggest that serum replacement indeed contains BMP-like activity, which can be reduced by noggin and/or bFGF.

[00037] The *Id1* promoter contains BMP responsive elements, and *Id1* was previously shown to be a target of BMP4 signaling in both human and mouse ESCs. We therefore examined the expression of *Id* genes as a second indicator of BMP signaling activity in hESCs cultured in various media. *Id1-4* transcripts were higher in hESCs (H9) cultured for 24 h in UM or CM + BMP4 than in cells cultured in CM, and addition of noggin to UM reduced expression of the *Id* genes.

[00038] **UM/bFGF/noggin sustains undifferentiated proliferation of hESCs.** UM supplemented with 0.5 μg/ml noggin and 40 ng/ml bFGF sustained undifferentiated proliferation of hESCs. H1 cells were plated at an equal number and cultured for 7 days in CM, UM, UM plus bFGF, UM plus noggin, or UM plus bFGF and noggin. $Oct4^+$ cell numbers were significantly higher after 7 days in CM and UM/bFGF/noggin than in UM, UM/bFGF, or UM/noggin. Intermediate $Oct4^+$ cell numbers were detected in UM/bFGF and UM/noggin, suggesting a synergistic effect between noggin and bFGF. hESCs cultured in UM/bFGF or UM/noggin could be propagated for multiple passages, but differentiated cells accumulated in either the middle (in UM/bFGF) or edge (in UM/noggin) of the hESC colonies. Increased differentiation also occurred in cells cultured in UM/bFGF/noggin if the noggin concentration was reduced to 0.1 μg/ml and the bFGF concentration was reduced to 10 ng/ml. The noggin in UM/bFGF/noggin could be substituted by gremlin (5 μg/ml) or a soluble BMP receptor IA (0.5 μg/ml) (data not shown), supporting that noggin's effects are indeed through the interruption of BMP receptor activation by BMPs.

[00039] Three different hESC lines (H1, H9, and H14) that had been expanded in UM/bFGF/noggin for more than 40 days (7, 6, and 6 passages, respectively) remained positive for Oct4, but subsequently differentiated if switched to UM lacking bFGF and noggin. UM/bFGF/noggin-cultured hESCs continued to express other ES cell markers, including *Nanog* and *Rex1*, and the cell surface markers SSEA4 and TRA-1-60 (data not shown). Even in the best cultures, hESCs are mixed with a small percentage of spontaneously differentiated cells. For example, low levels of the trophoblast marker chorionic gonadotropin β-subunit (*CGβ*) can be detected in CM-cultured ES cells, indicating the existence of small populations of trophoblast. This marker, however, was not detectable in UM/bFGF/noggin-cultured cells. The neural progenitor markers *Pax6* and *NeuroD1*, the mesodermal marker *brachyury*, and the endodermal marker *HNF3α* were all negative in CM- and UM/bFGF/noggin-cultured hESCs. Thus, ES cells propagated in UM/bFGF/noggin maintained characteristic ES cell markers following extended culture.

[00040] We further examined hESCs after long-term culture in UM/bFGF/noggin. H9 cells were continuously cultured in UM/bFGF/noggin for 32 passages. H1 and H14 cells cultured in UM/bFGF/noggin were frozen after passages 20 and 16, respectively. H14 cells were subsequently thawed directly into UM/bFGF/noggin and cultured to passage 18. The population doubling time and percentage of $Oct4^+$ cells of both H9 and H14 cells cultured in UM/bFGF/noggin for 27 and 18 passages, respectively, were similar to those for CM-cultured control hESCs.

[00041] **UM/bFGF/noggin maintains the developmental potential of hESCs.** When treated with BMP4 in CM for 3-7 days, hESCs that had been previously cultured in UM/bFGF/noggin for 10 passages differentiated into a flattened epithelium and secreted human chorionic gonadotropin (HCG) into the medium, indicating trophoblast differentiation. Embryoid bodies (EBs) derived from H1 cells cultured in UM/bFGF/noggin for 5 passages, and from control CM-cultured cells, expressed the trophoblast marker *CGβ* and markers of the three germ layers, including *Pax6*, *NeuroD1*, *brachyury*, and *HNF3α*. EB cells also had reduced expression of the ES cell markers *Oct4*, *Nanog*, and *Rex1*. H1 and H9 cells cultured in UM/bFGF/noggin for 7 and 6 passages, respectively, were injected into SCID-beige mice. Teratomas exhibiting complex differentiation developed in the mice 5-6 weeks post-inoculation.

[00042] **UM/bFGF/noggin-cultured ES cells are karyotypically normal.** H1 cells cultured in UM/bFGF/noggin for 5 passages, H9 for 33 passages, and H14 for 19 passages were karyotyped by standard G-banding, and chromosomes 12 and 17 were examined by fluorescence *in situ* hybridization. The cells retained normal karyotypes.

[00043] **ES cells cultured in defined and humanized system remain undifferentiated.** Although replacement of the CM with UMFN has eliminated the need for mouse-derived feeder cells, the UM still contained fetal bovine serum-derived albumin extract – an incompletely defined component, and the plate-coating material Matrigel is a solubilized basement membrane matrix extracted from a mouse tumor. Thus, further removing these animal materials was thought to be appropriate to define a humanized culture system for human ES cells. We first searched for a defined and humanized serum replacement to substitute for the KNOCKOUT SR product previously used, and Sigma's 50x Seral Replacement 3 (SR3) which is composed of three human proteins: albumin, insulin, and transferrin was considered. It has been shown that laminin can substitute for Matrigel to coat plates for human ES cell culture in the CM. We then established a system where human ES

cells were cultured on laminin-coated plates and in a UM containing 5 X SR3 instead of KNOCKOUT SR, plus 40 ng/ml FGF2 and 0.5 μg/ml noggin. ES cells in this system also retained ES cell identity after multiple weekly passages. Therefore, this combination makes up a defined and humanized culture system suitable for human ES cells.

[00044] These sets of data, taken together, demonstrate that feeder cells and conditioned medium can be avoided by the use of culture conditions including a bone morphogenetic protein antagonist, like noggin, together with an FGF. The other constituents of the culture medium can then be selected to avoid animal products. The result is a highly defined medium that permits the long term culture and proliferation of human embryonic stem cells while retaining all of the potential of those cells,

CLAIMS:

1. A method for culturing human embryonic stem cells in a feeder independent culture, the method comprising the step of

culturing the human embryonic stem cells in a medium including salts, vitamins, amino acids, glucose, a fibroblast growth factor and a bone morphogenetic protein antagonist in sufficient amount to maintain the stem cells in an undifferentiated state,

wherein the bone morphogenetic protein antagonist is selected from noggin, gremlin, chordin, follistatin, twisted gastrulation, members of the DAN family and antagonist antibodies specific to a bone morphogenetic protein.

2. The method of claim 1 wherein the medium includes the fibroblast growth factor in a concentration of at least 4 ng/ml.

3. The method of claim 1 wherein the bone morphogenetic protein antagonist is noggin.

4. The method of claim 1 wherein the fibroblast growth factor is bFGF at a concentration of 40ng/ml.

5. An *in vitro* cell culture for use in a method of maintaining cells in an undifferentiated state comprising in a culture vessel:

human embryonic stem cells and

a culture medium, the culture medium comprising salts, vitamins, amino acids, glucose, a fibroblast growth factor and a bone morphogenetic protein signaling antagonist in sufficient amount to maintain the stem cells in an undifferentiated feeder independent state, the medium being free of feeder cells and never having been exposed to feeder cells,

wherein the bone morphogenetic protein is selected from noggin, gremlin, chordin, follistatin, twisted gastrulation, members of the DAN family and antagonist antibodies specific to a bone morphogenetic protein.

6. A cell culture of claim 5 wherein the bone morphogenetic protein signaling antagonist is noggin.

7. A cell culture as claimed in claim 5 or 6 wherein the medium includes the fibroblast growth factor in a concentration of at least 4ng/ml of bFGF.

8. A cell culture as claimed in 5, 6 or 7 wherein the medium further comprises proteins selected from the group consisting of albumin, insulin and transferin.

9. A cell culture as claimed in claim 8 wherein the proteins are human.

10. A cell culture as claimed in claim 8 wherein the proteins are recombinant proteins.

11. A medium for culturing embryonic stem cells in a feeder independent culture, the medium comprising salts, vitamins, amino acids, glucose, a fibroblast growth and a bone morphogenetic protein antagonist in sufficient amount to maintain stem cells growth in the medium an undifferentiated state without exposure to feeder cells or medium exposed to feeder cells,

wherein the bone morphogenetic protein antagonist is selected from noggin, gremlin, chordin, follistatin, twisted gastrulation, members of the DAN family and antagonist antibodies specific to a bone morphogenetic protein.

12. The medium of claim 11 wherein the bone morphogenetic protein antagonist is noggin.

13. The medium of claim 11 or 12 wherein the medium further comprises proteins selected from the group consisting of albumin, insulin and transferin.

14. The medium of claim 11, 12 or 13 wherein the fibroblast growth factor is bFGF present in the medium at a concentration of at least 4 ng/ml.

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