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(54) **HORMONE-SECRETING PANCREATIC CELLS MAINTAINED IN LONG-TERM CULTURE**

IN LANGZEITKULTUR GEHALTENE HORMON-ABSONDERNDE PANKREATISCHE ZELLEN

CELLULES PANCREATIQUEES SECRETANT DES HORMONES ET MAINTENUES EN CULTURE DE LONGUE DUREE

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Description

TECHNICAL FIELD

[0001] The invention relates to long-term proliferating in vitro cultures of hormone-secreting pancreatic cells and to methods for establishing, maintaining, and propagating hormone-secreting cells in culture.

BACKGROUND ART

[0002] Hormone-secreting cells are highly differentiated and specialized for the synthesis and secretion of typically one or two specific hormones. Examples of hormone-secreting cells include certain cells of the pituitary gland, the endometrium, the ovary and the pancreas. The pituitary gland contains cells specialized for the synthesis and secretion of glycoprotein hormones known as gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on the gonads. The gonadotrophins secreted by the pituitary enter the blood stream and reach the gonads, where they exert their affects. Within the ovary, upon stimulation with gonadotrophins, granulosa cells surrounding an ovum differentiate within the preovulatory follicle to synthesize and secrete estrogen and progesterone. Specialized cells of the endometrium also synthesize and secrete estrogen and progesterone. Within the pancreas, β-cells of the islets respond to increased blood glucose concentration with an increase in insulin secretion.

[0003] Conventional cell culture technology is sufficient for the propagation of certain cell types in vitro such as fibroblasts taken from normal tissue or from tumors. It has long been a goal of scientists to maintain hormone-secreting cells in vitro, however standard culture conditions do not promote the long-term survival or proliferation of hormone-secreting cells. For practical purposes, it would be desirable to establish in culture cells which both proliferate and perform their specialized functions, i.e. synthesis and secretion of specific hormones.

[0004] For primary tissue culture, normal or tumor cells are removed from an animal or a human cell donor, placed in a liquid chemical medium in laboratory culture dishes, and maintained in an incubator under physical conditions which mimic the cells' environment in vivo. The medium and the incubator environment provide regulated temperature, pH, nutrients, growth factors, protection against pathogens, and in some cases a necessary substrate for cell attachment. Even under optimized culture conditions, however, most types of normal cells have a limited life span in culture. Typically, when cells other than fibroblasts are established in primary tissue culture they do not proliferate; they may or may not continue to perform their differentiated functions over the short-term. When the cells reach the end of their natural life-span they die, thus the cultures are self-limiting. Hormone-secreting cells generally survive in culture for no more than 8 to 12 days, during which time they undergo few or no cycles of cell division. During the life-span of hormone-secreting cells in culture, as they have been maintained using prior known techniques, such cells typically undergo a loss of function as evidenced by a decrease in hormone production.

[0005] In order to increase the life-span of hormone-secreting cells in culture, published techniques have included the use of embryonic cells. The strategy of starting with embryonic cells is based on the fact that embryonic cells are relatively less differentiated than adult cells, and thus can be expected to go through several cycles of cell division before becoming terminally differentiated, i.e. specialized for hormone synthesis. It is an axiom of biology that undifferentiated cells proliferate at a greater rate than differentiated cells. It is generally believed that by the time a cell has developed the necessary intra-cellular machinery for hormone synthesis and secretion, it is no longer able to divide rapidly, if at all.

[0006] Another known strategy for establishing cells in culture is to start with cancer cells, since cancer cells would be expected to have a greater potential for proliferation. However, few cells derived from tumors or other cancerous lesions are able to become established and divide in culture. One cell line was established from a malignant human choriocarcinoma by propagating the tumor cells through 304 serial transplantations to the hamster cheek pouch over a period of 8 years before establishment in vitro (BeWo cell line; ATCC CCL 98; May 1990 supplement to the 1988 American Tissue Culture Collection [ATCC] catalog of cell lines). The BeWo cell line was reported to produce human chorionic gonadotrophin (hCG), polypeptide hormones, human placental lactogen (hPL), estrogens and progestins. A cell line with an abnormal karyotype was established from the malignant ascites of a patient with adenocarcinoma of the ovary (NIH:OVCAR-3; ATCC HTB 161; ref. supra). The OVCAR-3 cell line was reported to possess androgen and estrogen receptors, however no synthesis of hormones by these cells was reported.

[0007] A rat clonal beta-cell line (RIN) was established in culture from a rat insulinoma (Clark, S.A., et al, 1980, Endocrinology 127:2779-2788). RIN cells were reported to secrete insulin in vitro in response to low levels of glucose, with maximal response at 0.6 mM glucose. This response is comparable to that of immature rat beta-cells, and quite different from that of normal mature rat islets which secrete in response to glucose concentrations ranging from 5 mM to 16 mM.

[0008] It is apparent from the forgoing that tumor cells are difficult to establish in vitro. Moreover, tumor cells that do become established in culture often possess abnormal characteristics which diminish their usefulness, such as the

loss or alteration of hormone synthesis or secretogogue responsiveness.

[0009] Using a strategy based on the notion that abnormal cells are more likely to grow in vitro, normal cells have been transformed in culture by various means including the use of UV light, chemical carcinogens, and the introduction of oncogenes. Rat granulosa cells were transformed by co-transfection with the entire SV 40 genome and the activated Ha-ras gene (Baum, G., et al. 1990 Develop Biol 112, 115-128). These cells were reported to retain at least some differentiated characteristics, i.e. they were able to synthesize steroids in response to cAMP.

[0010] Other cell lines established in culture include UMR cells, derived from normal islets of neonatal rats (NG, K. W., et al., 1987, J. Endocrinol. 113:8-10) and HIT cells, derived by simian virus-40 infection of hamster islets (Santerre, R.F., et al., 1981, PNAS 78:4339-4343). The insulin secretory output of these cell lines is low, and response to glucose is lost with passage in culture.

[0011] In order to promote the selection of non-transformed hormone-secreting cells as starting material for culture, a regimen of hormone treatment in vivo was used before removal of cells from the donor (Amsterdam, A., et al. 1989 Endocrinology 124, 1956-1964). Cells were obtained from ovarian follicles removed from women who had received hormonal therapy in preparation for in vitro fertilization. For additional promotion of differentiated function, cells were maintained on extra-cellular matrix and further treated with human chorionic gonadotrophin (hCG). Although the cells had a differentiated appearance and secreted progesterone in culture, the cells were reported to survive in culture for only five days. In a similar study, cells were reported to survive for eight days (Pellicer, A., et al. 1990 Fertility and Sterility 54, 590-596).

[0012] Another strategy for promoting the maintenance of differentiation in culture involved the culturing of the component parts of entire follicles, including the oocyte and cumulus complex (Vanderhyden, B.C., et al. 1990 Develop. Biol. 140, 307-317). In this type of "combination culture", mouse granulosa cells were maintained in a differentiated state for 7 days.

[0013] The above description of the state-of-the-art makes it apparent that there is a need for methods to maintain and propagate hormone-secreting cells in long-term cultures. Such cultures could be developed as biological "factories" for the production of therapeutically useful hormones. Well-established hormone-secreting cell lines would also offer the possibility of in vitro bio-assays based on the cells' responses to drugs such as gonadotrophin preparations. In addition, such cell lines would offer the possibility of in vitro bio-assays for the toxicity of drugs and other chemicals. Established cell lines would also be candidates for implantation to correct diseases due to hormone deficiencies. For instance, diabetics could be stabilized and possibly cured through the implantation of cells which replace the function of insulin-secreting beta-cells of the pancreas.

[0014] There exists a need for methods to produce consistent physiologically correct preparations of gonadotrophin hormones. Human gonadotrophin preparations (hMG), which typically contain both FSH and LH, are administered to women who are undergoing pre-treatment leading to in vitro fertilization. The administered hMG stimulates the woman's ovaries to produce multiple pre-ovulatory follicles, which are subsequently aspirated for in vitro fertilization. hMG is currently derived from the urine of post-menopausal women. Each lot differs according to the age and endocrine status of the urine donors, the differences being in both concentration and types of isoforms present in the final product. There are at least 11 isoforms of human follicle-stimulating hormone (hFSH) and 7 isoforms of human luteinizing hormone (hLH) (Stone, B.A., et al. 1990 Acta Endo (Copenhagen) 123, 161-168). Analysis by high-performance liquid chromatography (HPLC) of various hMG preparations showed between-lot variability in the presence and concentration of isoforms of FSH (Stone, B.A. et al, supra). Different isoforms have different biopotencies (Gharib, S.D., et al 1990 In: Endocrine Reviews, 11, 177-199). Since certain isoforms of FSH are more biopotent than others, there is between-lot variability in biopotency among various hMG preparations. Moreover, the presence of LH isoforms in a preparation affects the biopotency of FSH present in the preparation.

[0015] Scientists are currently attempting to produce genetically engineered FSH of a desired and consistent biopotency. There is a clear need for a cost-effective assay to enable the development of therapeutically useful preparations of genetically engineered gonadotrophins.

[0016] There exist two major forms of chemical assay for gonadotrophins: HPLC and radioimmunoassay (RIA). The HPLC technique is precise but does not identify which chemical properties of hMG preparations relate to biopotency. Moreover, the HPLC technique requires considerable technical expertise, instrumentation, and investment of technical labor. Tests based on immunologic recognition of a gonadotrophin (RIA) are limited by the inherent cross-reactivity of the antibodies with disparate isoforms of the gonadotrophins. For instance, a single RIA numerical value for FSH concentration could include several FSH isoforms of differing biopotency. Thus the current techniques for chemical assay do not provide a means to assess the biopotency of a therapeutic preparation of gonadotrophin.

The need for biopotency assessments of gonadotrophins has been acknowledged by several national agencies, including the U. s. Food and Drug Administration (FDA). The assays currently accepted by the FDA are in vivo assays conducted in rodents. The in vivo assay for FSH is the Steelman-Pohley assay which is based on mouse uterine weight gain. One in vivo assay for LH is the rat Leydig cell assay; the degree of proliferation in the seminal vesicles of the immature male rat is the index for assessing biopotency of LH. Another in vivo bioassay for LH is the rat ovarian

ascorbic acid depletion test. These in vivo assays are disadvantageous because they require the sacrifice of large numbers of laboratory animals. For instance, the sacrifice of 2,000 mice is required to measure the stability factor for one particular batch of hMG. This figure of 2,000 mice does not include the number required to establish the biopotency of the original batch. The need for a more cost-effective bioassay is apparent. Moreover, the results from tests conducted on rodent cells are not necessarily applicable to biopotency in humans.

[0017] The current source for therapeutic gonadotrophins, while convenient, is limited by the inherent biological variability among the human donors. The major source of human gonadotrophin (human menopausal gonadotrophin, hMG) is urine donated by members of a religious order in Switzerland. The post-menopausal women living within the convent pool their urine for sale to a company which derives each lot of its product from a batch of the pooled urine. Since the age and endocrine status of each donor to the urine pool changes from batch to batch, each preparation of gonadotrophin is different in chemical composition and in biopotency. Thus there exists a need for a consistent source of human gonadotrophin.

[0018] There also exists a need for a source of physiologically correct preparations of human sex steroid hormones. Currently, therapeutic estrogen and progesterone compounds, and analogs thereof, are prepared by standardized chemical synthesis. However, the class of compounds designated "estrogens" produced normally in the human female includes several different formulae and isoforms. Similarly, the class of hormones designated "progestins" includes several different compounds and isoforms. The types and amounts of estrogens and progestins produced naturally vary according to the female's age and overall physiological status, i.e. the specific time point in her menstrual cycle, pregnancy, or menopause. The optimal steroid content for any given therapeutic indication has not been determined. Even if the optimal chemical profile of a sex steroid preparation were determined, chemical synthesis would not be a practical route for production of complex steroid mixtures. Therefore, it is desirable to develop methods which inherently provide a physiologically correct mix of human estrogens and progesterones.

[0019] Toxicity testing is another field which scientists have attempted to address through use of in vitro systems (for review see: Nau, H. 1990. in Methods in Developmental Toxicology: Use in Defining Mechanisms and Risk Parameters. Eds. G.L. Kimmel, D. M. Kochhar, CRC Press, pp. 29-43.) To date, in vitro systems based on hormone-secreting cells have been very limited, partly because of the difficulties inherent in maintaining hormone-secreting cells in culture. In theory, the reproductive toxicity of a compound could be assessed by the capacity of the compound to impair hormone-secretion from cells which characteristically secrete a given hormone. A non-human cell line (Chinese hamster ovary, CHO) has been extensively utilized for toxicology analyses, (Tsushimoto, G., et al., 1983 Arch Environ Contam Toxicol, 12, 721). Amphibian oocytes have been proposed as a system for the testing of tumor promoting compounds (U.S. Patent No: 4,983,527; issued January 8, 1991). Xenopus testis explants have been proposed for the testing of mutagenicity and genotoxicity during spermatogenesis (U.S. Patent No: 4,929,542; issued May 29, 1990). Cell lines established from rat embryo fibroblasts have been proposed as systems for screening for protein inhibitors and activators (U.S. Patent No. 4,980,281; issued December 25, 1990). Since it is generally recognized that humans have different toxic susceptibilities compared to amphibians and rodents, the above proposed in vitro testing systems are limited by the non-human origins of the cells.

[0020] Thus, there exists a need for human hormone-secreting cell lines established in long-term culture for the purposes of 1) production of human hormones, 2) bio-assay of therapeutic gonadotrophins, 3) testing of drug efficacy and design, 4) toxicity testing of drugs and chemicals, and 5) implantation to replace deficient hormone secretion.

DISCLOSURE

[0021] This invention provides in a first aspect a method for establishing a cell culture of hormone-secreting human pancreatic cells in vitro comprising the steps of:

(a) selecting cells having insulin secreting potential from a population of human pancreatic non-tumorous similar cells having insulin-secreting potential,
(b) placing said cells in suspension in an establishing medium comprising any of human serum, bovine serum albumin or a serum substitute containing animal proteins and which establishing medium is capable of promoting the viability or proliferation of said cell or cells for at least 13 days in vitro, and
(c) sub-culturing anchorage - independent cells from step (b) such that the cells proliferate and viability is maintained for a period of at least 5.5 months.

[0022] In a further aspect the invention also provides a method for establishing a cell culture of hormone-secreting human pancreatic cells in vitro comprising the steps of :

(a) selecting cells having hormone secreting potential from a population of human pancreatic non-tumorous similar cells having hormone secreting potential,

(b) placing said cells in suspension in an establishing medium comprising any of human serum, bovine serum albumin or a serum substitute containing animal proteins ans which establishing medium is capable of promoting the viability or proliferation of said cell or cells for at least 13 days in vitro, and
(c) sub-culturing anchorrage-independent cells from step (b) in a defined medium having an osmolarity of about 248 mOsm to about 275 mOsm, such that the cells proliferate and produce progeny and viability is maintained for a period of at least 5.5 months.

[0023] In an even further aspect there is provided a method for the long-term maintenance of hormone-secreting human non-tumorous pancreatic cells in vitro obtainable according to the aforementioned method of the invention comprising the steps of:

(a) propagating said cell or cells in step (c) wherein, said defined medium is capable of promoting the viability of at least some of the progeny so that at least some of said progeny are viable in vitro after at least one year from the occurrence of step (a).

[0024] The methods according to the invention advantageously provide for the long-term maintenance and propagation in defined media of hormone-secreting cells in vitro.
[0025] In vitro, the cells secrete hormones characteristic of their tissue of origin in this case. The secreted hormones include insulin and glucagon. Cells may respond to stimulation by secretogogues with increased hormone secretion.
[0026] The methods of the invention also provide for the production of established hormone-secreting pancreatic cell lines which may be cryopreserved and propagated from frozen stock, and which retain a characteristic hormone-secretion profile over several generations in vitro.
[0027] The secreted hormones may be therapeutically useful by propagating hormone-secreting cells in culture and isolating the secreted hormones from the culture medium surrounding the cells. The hormone secreting cells in culture may also be suitable for an in vitro bio-assay for the biopotency of therapeutic hormone preparations.
[0028] An in vitro toxicology assay based on changes in hormone secretion by human pancreatic cells in vitro in response to contact by the chemical agent being tested is also provided. In this aspect the invention provides a method of determining the toxicity of a test compound comprising the steps of:

(a) providing an established human non-tumorous pancreatic cell line produced according to the methods of the invention which cell line comprises having cells which exhibit a characterised response to a known toxin, said response being a known change in the hormone-secretion profile of said cells of said cell line,
(b) contacting said cells with said test compound,
(c) determining the hormone-secretion profile of said cells after step (b), and
(d) comparing the hormone-secretion profile of said cells after step (b) with said known change in hormone-secretion profile to determine the relative toxicity of said test compound.

[0029] The cell cultures obtainable by the methods of the described also form part of the invention, which may also be particularly useful for implantation in a subject to replace the deficient hormones.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Figure 1 is an outline of the general method for establishing and propagating hormone-secreting cells in vitro.
[0031] Figure 2 is a photomicrograph (4900X total magnification) of a typical culture of human ovarian follicular cells after the completion of step 8 in Figure 1.
[0032] Figure 3 provides a second example of the cells depicted in Figure 2.
[0033] Figure 4 provides a third example of the cells depicted in Figure 2.
[0034] Figure 5 is a photomicrograph (approximate final magnification = 560X) of a typical culture of follicular cells after one week in subculture (see Example 2).
[0035] Figure 6 is a photomicrograph of a typical initial sub-culture of follicular cells (SC-1; see Example 2).
[0036] Figure 7 is a photomicrograph (2500X approximate total magnification) of a typical sub-culture of follicular cells after more than 20 rounds of serial sub-culture (see Example 2). The cells were seeded four days previously at a density of 10^6 cells per 15 ml medium in a $25cm^2$ flask.
[0037] Figure 8 is a photomicrograph (1100X approximate total magnification) of a "blastema-like" outgrowth of cells at the edge of a section of original pituitary macroadenoma which had been placed in culture 9 days previously.
[0038] Figure 9 is a photomicrograph (2200X total magnification) of a group of cells which detached from an outgrowing "blastema-like" projection after the original piece of pituitary adenoma had been in culture for 18 days.
[0039] Figure 10 is another photomicrograph taken two days later of the same group of cells as in Figure 9 (20 days

total culture time for the original tumor section). The magnification is the same as in Figure 9, thus illustrating by comparison the rapid proliferation of these cells.

[0040] Figure 11 is a histogram depicting increased insulin secretion by human pancreas cells in response to increased glucose concentration.

BEST MODE OF CARRYING OUT THE INVENTION

[0041] An outline of the general method for establishing cell cultures is provided in Figure 1; steps indicated below refer to Figure 1.

[0042] Briefly, cells with hormone-secreting potential are carefully isolated from a surgical tissue sample according to methods provided herein. Preferably, cells to be established in culture are obtained from a human donor undergoing a medical procedure during which tissue is removed as a part of the procedure (step 2). The cells are gently isolated from the tissues (step 3) and initially established in culture under conditions which sufficiently mimic the in vivo environment so that the viability of the cells is promoted (steps 4-6). After approximately 30 days under establishing culture conditions (step 8), the cells are selected for hormone-secreting potential (step 9). Selected cells are placed into sub-cultures and further maintained and propagated in defined medium (steps 10 and 11). The defined medium is formulated to promote cell proliferation and the continued viability of the sub-cultures. Useful hormones may be isolated from the medium (step 12). After sufficient cells are propagated, the cell cultures are characterized for proliferation rate, secretion of hormones, and responses to secretogogues and toxins (step 13). Aliquots may be cryopreserved (step 14) and tested for retention of cell line characteristics. When a cell line is sufficiently characterized, it may be designated an established cell line and may be used for the production of hormones or for biopotency or toxicity assays.

[0043] Cells obtained from ovarian follicles may be removed from a donor undergoing in vitro fertilization. At the time of follicular extraction, the donor has typically been treated with a combined hormonal regimen to stimulate the development of multiple pre-ovulatory follicles (step 1). The hormonal regimen typically includes leuprolide acetate for mid-luteal suppression combined with human menopausal hormone (hMG) and follicle-stimulating hormone (FSH) for controlled ovarian hyperstimulation.

[0044] Thirty-four hours prior to oocyte retrieval, human chorionic gonadotrophin (hCG) may be administered to promote further growth and differentiation of the follicle. The above described hormonal regimen stimulates the proliferation of the granulosa cells surrounding the ovum. Towards the end of the follicular growth phase, two populations of granulosa cells develop: 1) mural granulosa cells which maintain contact with the basal lamina enclosing the follicle, and 2) the cumulus granulosa cells, also known as zona radiata cells, closest to the ovum, which are coupled by gap junctions to both the oocyte and other surrounding cumulus cells. Gonadotrophin stimulation of granulosa cell differentiation is characterized by changes in cell-cell contacts, cell shape, cytoskeletal organization, and biosynthesis of estrogens, progesterone, progestins, extracellular matrix components, and hormone receptors. FSH acts by a cAMP mediated mechanism on undifferentiated granulosa cells to stimulate the enzymatic activity required for the metabolism of cholesterol to progesterone and for the conversion of androgens to estrogens. As the follicle matures, FSH and estrogen stimulate the production of granulosa cell plasma membrane associated LH receptors. After FSH priming and the synthesis of LH-receptors, granulosa cells become responsive to LH and will then synthesize progesterone in response to added LH.

[0045] The above described hormonal treatment regimen thus favors the development of granulosa cells which exhibit a high level of basal progesterone and estrogen secretion. Such cells are desirable, for instance, for the production of therapeutic human sex steroids or for an in vitro toxicity assay based on reduction of hormone secretion by a toxic agent. When development of the follicles is optimal, the follicles are aspirated in preparation for in vitro fertilization of the ova. In this context "tissue" (step 2) refers to the entire follicle, including the basal lamina, cumulus granulosa cells, mural granulosa cells and ovum. Generally, non-germ line follicular cells accompany the extracted ova (step 2), and these non-germ line cells would usually be discarded during the normal course of the in vitro fertilization method. Cells are isolated for culture (step 3) from the follicles of those patients who donate their non-germ line cells for medical research and development for health care applications.

[0046] In contrast to the above described embodiment, follicular cells which exhibit low basal levels of hormone secretion, but which respond to gonadotrophin with increased hormone secretion, are desirable for in vitro biopotency assays for therapeutic gonadotrophin preparations. Therefore, follicular cells may be obtained from a donor who has undergone only the first part of the above described pretreatment regimen. The donor has received hMG and FSH but not hCG. In this embodiment, relatively undifferentiated follicular cells are obtained from growing primary follicles, rather than from mature pre-ovulatory follicles.

[0047] Follicular cells may also be obtained from a donor who has not undergone any pretreatment regimen. In this embodiment, relatively undifferentiated follicular cells are obtained from primary follicles, rather than from hormone-stimulated pre-ovulatory follicles. Similarly to the above described embodiment, follicular cells obtained by this method exhibit low basal levels of hormone secretion while retaining the ability to respond to gonadatrophin with increased

hormone secretion. Therefore, cells obtained by this embodiment are likewise useful for in vitro gonadotrophin biopotency assays.

[0048] Two important features which distinguish this method from conventional methods are (1) the tissue is not subjected to enzymatic digestion and (2) the cells are not subjected to centrifugation. This contrasts with published methods which require digestion of the tissue matrix by incubation with enzymes such as collagenase, hyaluronidase and trypsin in order to release cells from the tissue matrix. After enzyme treatment, conventional methods typically rely on centrifugation to isolate cells from the resulting debris. Herein, the term "substantially enzyme free" refers to a process in which enzymes are not added to the incubation medium. It is understood that small amounts of enzymes such as proteases may be present in any medium, and the presence thereof is allowed within the definition of the term "substantially enzyme-free".

[0049] The first step after obtaining the follicular cells is to place cells, with or without the ovum, in an establishing medium (EM; step 4). The term "establishing medium" refers to a solution which essentially mimics the critical parameters of the in vivo environment from which the cells were derived. Five specific formulations for establishing media are disclosed herein. Critical parameters of the in vivo environment of follicular cells include an osmolarity which is reduced compared to the osmolarity typically used in previous attempts to culture hormone-secreting cells. The osmolarity of follicular fluid is generally in the range of 270 to 275 mOsm. Thus the final osmolarity of an establishing culture medium of the present invention ranges preferably from about 248 mOsm to about 275 mOsm, most preferably from about 269 mOsm to about 275 mOsm.

[0050] It is preferred to flood the cells in the establishing medium with a medical blood gas mixture composed of 5% CO_2/5% O_2/90% N_2, which mimics the gas mixture in vivo. It is also preferred to supplement the establishing medium with extra glutamine, to a value of about 6.35 mM to about 8.35 mM, most preferably 7.35 mM glutamine.

In one preferred embodiment of the invention, the establishing medium is supplemented with serum obtained from the specific cell donor (establishing medium, homologous serum; EMHS). Preferably, the EMHS contains 0.5% to 15% homologous serum, more preferably 5% to 10% homologous serum, most preferably 7% to 8% homologous serum. The use of homologous serum provides an environment which contains no proteins other than those proteins which are specific to the individual from whom the cells were derived, and thus favors the viability of the donated cells because of the minimization of immunologic reaction. Additionally, donor serum may be favorable for its specific content and concentration of hormones such as progesterones, estrogens, and gonadotrophins.

[0051] In another embodiment of the invention, the establishing medium is supplemented with serum obtained from an individual other than the specific cell donor (establishing medium,non-homologous serum; EMNS). Preferably, the EMNS contains 0.5% to 15% non-homologous serum, more preferably 5% to 10% non-homologous serum, most preferably 7% to 8% non-homologous serum. It will be apparent to those skilled in the art that medium may also be variously supplemented with hormones and growth factors to promote the survival of cells with desired characteristics, such as elevated progesterone production.

[0052] In a further embodiment of the invention, cells are successfully established in a defined medium which does not contain serum, but rather is supplemented with bovine serum albumin (BSA) or a combination of BSA and purchased serum substitute. The term "defined medium" refers to a culture medium which does not contain human serum, and thus contains fewer unknown, unassayed components than does human serum-containing medium. It is understood that medium which contains any animal-derived product, such as BSA, is not as completely defined as a medium which is composed entirely of chemically synthesized components. However, in the art of the present invention, BSA-containing media and serum-substitute containing media are commonly referred to as "defined media". In the present application, the generic term "defined medium" refers to any medium which does not contain human serum. Provided in the experimental examples to follow are formulae for three different defined media: 1) establishing medium-01 (EM-01); 2) defined medium-1 (DM-1); 3) defined medium, serum substitute (DMSS). These three defined media share the characteristic of containing, instead of human serum, BSA and/or a serum substitute containing animal proteins.

[0053] Herein, the term "serum substitute" refers to a combination of proteins and growth factors, preferably added in the amount of about 5% to about 15% of the total volume of the medium. EM-01 was originally devised to promote the viability of fertilized ova from a donor whose serum contained anti-sperm antibody. Fortuitously, the substitution of BSA for donor serum also promoted the viability of non-germ line follicular cells which were cultured at the same time as the ova. Therefore, EM-01 is a preferred establishing medium for follicular cells. The media designated DM-1 and DMSS were originally formulated for use after 30 days in EM containing human serum (EMHS and EMNS). However, it was found that cells may be successfully established in DM-1 or in DMSS without being previously placed in EM containing human serum. EM-01, DM-1 and DMSS share the distinguishing characteristics of EM containing human serum in that they also have a lower osmolarity than do conventional culture media. The osmolarity of EM-01, DM-1 and DMSS is preferably in the range of about 248 mOsm to about 300 mOsm, more preferably in the range of about 260 mOsm to about 280 mOsm, most preferably about 270 mOsm to about 275 mOsm.

[0054] Suitably, the media formulations employed in this method may contain, in addition to glucose, additional energy sources such as lactate and pyruvate. The term "energy source" refers to a chemical which can be used by the cells

to make ATP either through glycolysis or through the tricarboxylic acid cycle.

[0055] As with establishing medium, it is preferred to supplement defined media formulations with added glutamine to the amount of about 6.35 mM to about 8.35 mM, most preferably about 7.35 mM glutamine.

[0056] Suitably, the ovum and surrounding non-germ line follicular cells are placed together in EM (EMHS, EMNS, EM-01, DM-1, DMSS; step 2a). After about 24 hours, sperm may be added to the EM, and the cells may be incubated for an additional 20-24 hours (step 2b). It is generally believed that spermatozoa provide an enzyme known as acrosomal enzyme, which gently releases the non-germ line cells from the matrix surrounding the ovum. It is understood that the amount of spermatozoa-associated acrosomal enzyme is small and is not comparable to the large amounts of enzymes such as collagenase which are employed in traditional methods for digesting tissue matrices to release cells. After incubation in sperm, cells of the corona radiata may be manually stripped from the ovum through use of a hollow needle as described in Example 1 (step 2c). Suitably, follicular cells which do not adhere to the ovum may be simply isolated from the surrounding medium (step 3).

[0057] Viable cells are selected through a dissecting microscope (90X magnification; step 5). The term "viable" refers to cells which typically show monolayer spreading on the bottom of a culture dish. Viability may be confirmed in a dispensable sample of cells by the method of trypan blue dye exclusion as is well known in the art. The method of Fig. 1 is again distinguished in step 5, as well as in step 3 supra, from the methods of Amsterdam, et al (supra) and Pellicer, et al (supra) in that no centrifugation or gradient separation is used in the present technique. Instead, cells are manually and gently selected and isolated for culture, which preserves the membrane integrity of a larger number of cells as compared with cell populations that have undergone centrifugation. The method is again distinguished from techniques which require enzymatic digestion of tissue to isolate cells. Such well known techniques typically involve incubation in trypsin or collagenase, which may be injurious to the cells desired for the practice of this invention.

[0058] Selected cells are placed in fresh EM with the aid of a fine glass micropipette and incubated for a further 24 to 96 hours (step 6). It is preferred to flood the cells with the medical blood gas mixture described supra to best mimic gas conditions in vivo. At this point, cells may be divided into cultures containing 50 to 100 cells in fresh EM (step 7).

[0059] The selected cells are further maintained in the establishing medium for up to about 30 days (step 8). During the first 14 days, culture medium is refreshed only every 6 to 7 days. This produces a slight hypoxia of the cultures and is a physiological selection against fibroblasts, since rapidly dividing cells such as fibroblasts do not survive in a lowered O_2 atmosphere (Aladjem, S. et al 1981 Placenta Suppl 3, 175). The lowered O_2 atmosphere is concomitantly a positive selection for cells of granulosa origin since their normal in vivo milieu is of a similar condition.

[0060] Commonly, in some initial cultures, a high proportion of the cells are able to survive and proliferate without attachment to a substrate. In contrast, a number of the cells spread on the bottom of the culture plate and form attachments to the plastic. Thus a given cell culture may comprise both cell clumps floating in suspension, cell clumps adhering to the plastic dish, and cells spreading in monolayer fashion on the bottom of the dish.

[0061] After about 20 to 30 days in the establishing in vitro environment (step 8), cells are chosen for hormone-secreting potential (step 9) according to morphological criteria as depicted in Figures 2, 3, and 4. The circled clumps of cells are representative of the type of cell clumps that are selected. Preferably, small clumps of 2 to 12 cells are chosen, most preferably clumps of 4 to 5 cells. Selected clumps are combined into groups of 50-150 cells (step 9a) or alternatively into groups of 10 - 15 cells (step 10). These groups are designated first sub-cultures (SC-1). Because of the spatial arrangement of cells within a selected clump, i.e. touching each other in a "string-of-pearls" type arrangement, it is thought that these clumps have a high probability of containing daughter cells arising from the division of 1 or 2 progenitor cells. It is often desired to obtain a clonally selected culture arising from the progeny of a single cell, and thus these clumps have a high probability of providing such a culture when a single clump is used for SC-1 (e.g. step 10 in which a single clump of 10-12 cells forms the starting culture). Figure 6 shows a typical initial sub-culture of follicular cells in SC-1. The dark spots are particularly dense clumps of cells in which individual cells were not photographically resolvable. The lighter colored cell layers in between the dark spots are cells which spread on the bottom of the culture plate and on which the camera was focused. (The white lettering on the photo is merely a record-keeping designation and does not contain information for this patent application.)

[0062] It will be apparent to one skilled in the art that the possibility exists for clonal selection at any point in the subsequent subculturing procedures (step 11).

[0063] Criteria for cells with hormone-secreting potential include an approximately spheroid or ovoid shape, and homogeneity of size and shape within a clump, as illustrated by the circled clumps in Figures 2, 3, and 4. These selection criteria are based on the Applicant's observations of follicular cells, and specifically of granulosa cells, using phase contrast optics and microsurgical manipulations, and on the study of histological preparations and scanning electron micrographs of follicular cells. It will be apparent to one of skill in the art that the above described and depicted selection criteria are to be understood in the context of comparison to other, non-selected cells in the field of view (Figures 2, 3, and 4). Thus the selected cell clumps contain fewer cells than the non-selected clumps, and the individual cells are more homogeneous than cells of the non-selected clumps. Selected cells have a smooth-appearing plasma membrane, in contrast to non-selected cells which typically have plasma membranes with ruffled leading edges. Also, in the case

of follicular cells, the cytoplasm of selected cells typically appears smooth rather than granular.

[0064] Methods for removing cells to subcultures (steps 10 and 11) : In the case of cells which are growing on and attached to the bottom of the culture plate, cells are harvested for sub-culture by gently scraping the cells from the plate with the aid of a fine glass micropipette. This method contrasts with methods which use enzymatic or other harsh methods such as calcium chelation to detach cells from substrate. Of course, in the case of cells which are growing in suspension, no detachment step is necessary.

[0065] Selected cells are transferred to a defined medium (steps 10 and 11, e.g. DM-1, DMSS, described supra). The defined medium formulations described herein allow the cells to proliferate and to maintain hormone secretion capability. The culture flasks or plates containing cells and medium are flooded with the medical blood gas mixture described supra and kept sealed within a 37°C incubator. Preferably, the cells proliferate in culture over a long term of at least about 2 months, more preferably at least 5.5 months, most preferably longer than 15 months. The cell cultures are typically flooded with medical blood gas mixture every other day and subdivided as needed according to the density of the proliferating cell population.

[0066] The defined medium may have a higher initial pH than that typically used in previous attempts to culture follicular cells. The pH values of the media designated DM-1 and DMSS are initially adjusted to 7.65, in contrast to the conventional culture medium pH of 7.4. The rationale for using a higher initial pH is based on the fact that follicular fluid and granulosa cells exist in vivo in a slightly elevated CO_2 environment and mammalian embryos have a higher pH when compared to maternal serum (Nau, H. et al 1986 Nature 323, 276-279; Nau, H. 1990 supra). The higher initial pH of the establishing medium may protect the cells from damage by weak acids by minimizing the production of same. It will be apparent to one skilled in the art of the present invention that there are various alternative techniques which could control the effects of weak acids. Therefore, the initial pH of 7.65 is offered only as an enabling suggestion. Moreover, the establishing media designated EMHS, EMNS, and EM-01 have pH values ranging from 7.2 to 7.45, thus a more conventional pH is sufficient to establish hormone-secreting cells in culture, and may also be sufficient to propagate such cells in culture.

[0067] During the first six days in the first sub-culture (step 10), cell number typically increases about 2-fold, preferably about 3-fold or greater. During the later part of the first sub-culture, and during subsequent sub-cultures, cell proliferation rate increases to a doubling time of preferably about 72 hours, more preferably about 48 to 36 hours or less. Figure 6 shows the typical appearance of an initial sub-culture after 14 days in culture (step 10). In Figure 6 the dark spots are large cell clumps comprising 200 cells or greater; the lighter colored cell areas between the clumps are layers composed of single cells or a few cells spreading on the bottom of the culture dish.

[0068] Figure 7 is a photomicrograph of follicular cells after more than 20 sub-cultures (step 11) which were plated at a density of 10^6 cells/ 15 ml/ 25cm^2 four days previous to the photographic date. In Figure 7 the camera lens is focused on only the lower layer of cells closest to the bottom of the culture dish, and there are many layers of floating clumps of cells between the medium surface and the lowest cell layer. Thus, the typical cell density of a culture similar to that depicted in Figure 7 is preferably about 3 x 10^6 to about 4 x 10^6 cells/15 ml/25 cm^2.

[0069] After a period of proliferation, upon reaching a saturating cell density, an individual culture may exhibit slowed or halted proliferation. Such a "dormant" culture may be useful when differentiated characteristics, such as responses to secretogogues, are preserved. A dormant culture may be useful for the bio-assay of gonadotrophin potency as described below. Alternatively, a proliferating culture may also be used in a gonadotrophin bio-assay.

[0070] By repeated serial sub-culture, a large population of like cells is obtained (step 11). Suitably, portions of the population are frozen in a cryoprotective medium and are stored in liquid nitrogen (step 14). Protocols for freezing cells, as described in Example 15, represent traditional techniques as well as more complex techniques which are currently used for the freezing of embryos. Because of the large size of certain hormone-secreting cells in culture, particularly pituitary cells (Figures 8 - 10; Example 13), it is suitable to employ freezing techniques which are designed for use with embryos, which are of a comparably large size. However, simple and conventional techniques may be employed when a smaller viability percentage is considered acceptable. Upon thawing, the cells may exhibit the characteristic proliferation and hormone-secretion patterns of the population from which they were derived. In a preferred embodiment, a cell population is propagated and cryopreserved to provide an essentially unlimited supply of cells having defined characteristics. Such a characterized and stored population is known as an "established cell line".

[0071] During the time in establishing medium (step 8) and during the time in sub-culture (steps 10 and 11), the cells are capable of secreting at least one human hormone. In a preferred embodiment of the invention, the cells secrete at rates sufficient for the isolation of the hormone(s).

[0072] The cells of a specific population may not secrete a high basal level of steroid hormone. The cells do, however, respond to stimulation by gonadotrophins with detectably increased steroid hormone secretion. Preferably, a maximum dose of FSH, for instance, stimulates a 2-20 fold increase, more preferably a 4-10 fold increase, most preferably a 5-8 fold increase in the amount of steroid hormone secreted into the medium over a period of from 24 to 48 hours. Preferably, the increase in steroid secretion may be correlated with the dose and type of gonadotrophin administered to the cells, thus defining the hormone secretion profile of the population. The term "hormone-secretion profile" refers to (1) the

specific secretogogues to which the cells respond, (2) the type(s) of hormone(s) and (3) the amounts of hormones which are secreted in response to a specified secretogogue. Established cell lines may be employed as bio-assays for gonadotrophin bio-potency.

[0073] Outside the scope of the invention, cells may be derived from human trophoblastic tissue most preferably tissue of non-tumorous origin. The cells are established in culture and sub-cultured as described above and illustrated in Example 12. Trophoblastic cells preferably secrete hCG and other hormones. Populations of cells of non-tumorous trophoblastic origin are suitably employed in bio-assays of potential reproductive toxins. The bio-assays are based on the reduction or alteration of basal sex hormone secretion upon contacting the cells with a potential toxin.

[0074] Outside the scope of the invention, cells may be derived from human endometrium. The endometrial cells are established in culture and sub-cultured according to any of the above described methods. The populations of endometrial cells in culture preferably secrete high levels of progestins and/or estrogens such that they can be used as sources for therapeutically useful hormone preparations. Suitably, the endometrial cell populations are also used in bio-assays for reproductive toxicity.

[0075] Outside the scope of the invention, cells may be isolated from a human pituitary tumor. The cells may be established and propagated in culture by any of the above described methods and as illustrated in Example 13. Suitably, the tumor tissue is initially sectioned into pieces of approximately 1 - 3 mm diameter and individual pieces are placed in establishing medium for 15 to 20 days. During this time, outgrowths of cells develop and separate from the original piece of tumor (Figures 8 - 10). The cell outgrowths are referred to as "blastema-like", an embryological term for a cell group which gives rise to an organ. The separated "blastema" cell groups are then transferred into individual cultures for further propagation in defined medium. Preferably, the pituitary cells secrete at least one human gonadotrophin such as FSH or LH. Suitably, human FSH or LH is isolated from the medium surrounding the cell cultures to form a therapeutically useful gonadotrophin preparation.

[0076] According to the invention, cells are obtained from normal tissue of the pancreas and propagated in vitro according to methods described herein. Suitably, to establish pancreatic cells in culture, a variation of the establishment method is used. The tissue may be placed directly in establishing medium, then the tissue is teased into small chunks through use of a sterile scalpel. Preferably, the chunks contain about 50 to 300 cells. The chunks are aliquoted into several dishes and flooded with the medical blood gas mixture described supra. They are kept in sealed containers for about two weeks, and the gas is refreshed every other day. The cells proliferate during this time. After two weeks, the cell cultures may be subdivided and maintained for approximately 8 additional weeks in establishing medium. The cultures may be subdivided as needed during this time, depending on how quickly the cultures proliferate. The medium of each culture is assayed for hormone content. Suitably, cells derived from pancreas secrete insulin. Cultures containing a desired level of hormone are selected for further culture in defined medium (Figure 1, step 10). Suitably, when suspension culture is desired, cells may be selected which did not adhere to the surfaces of the culture vessels but rather are floating suspended in the medium.

[0077] Pancreas cells are maintained in long-term culture for at least 5.5 months and up to one year or longer. Portions of the pancreas cell cultures are frozen at intervals according to the methods described in Example 17. When these frozen cultures are thawed and placed in defined medium, the cells retain their ability to synthesize and secrete insulin.

[0078] Herein, the term "maintenance level of insulin secretion" refers to the amount of insulin secreted into the defined culture medium. In culturing pancreas cells, it is preferred to use the medium designated herein as "DMSS", however any of the defined media described herein may be used for pancreas cells.

[0079] Preferably, the pancreas cells of the present invention secrete a maintenance level of about 2 uIU to about 1000 uIU insulin/ hour/ 10^5 cells/ milliliter of culture medium. More preferably, the cells secrete a maintenance level of about 20 uIU to about 400 uIU insulin/ hour/ 10^5 cells/ milliliter of culture medium.

[0080] Importantly, the pancreas cells of the present invention, maintained in long-term culture, have the ability to respond to increased glucose and increased amino acid concentrations with increased insulin secretion. The pancreas cells retain these functions for one year or longer in continuous culture.

[0081] In a non-diabetic human, beta-cells within the islets of Langerhans typically are exposed to blood glucose concentrations in the range of about 3 mM to about 8.8 mM. When blood glucose concentration rises above about 5 mM, the normal beta-cells secrete the right amount of insulin to effect the normalization of blood glucose back to 4.4 - 5.3 mM. Another factor influencing the secretion of insulin in the normal subject is the level of amino acids such as alanine, arginine, and leucine in the blood. Elevated amino acid levels can potentiate the secretion of insulin so that secretion is stimulated at lower glucose levels. Thus, normal beta-cells are exquisitely sensitive to glucose and amino acid levels which rise after a meal, and their secretion of insulin is finely tuned to return those levels to normal.

[0082] In contrast, the pancreas cells of human patients who have Type I juvenile onset diabetes are unable to secrete insulin in response to elevated glucose levels. If uncontrolled by exogenous insulin, blood glucose levels in a diabetic may reach 10 mM or greater, at which point glucose is lost through the kidneys leading to dehydration and profound metabolic disturbances. In the clinical management of an adult with Type I diabetes, exogenous insulin is

administered at appropriate times and in appropriate amounts to attempt to maintain blood glucose levels between 3.88 mM to 6.66 mM glucose. In a child with Type I diabetes, it is generally considered safer to maintain blood glucose at a higher level, i.e. 6.1 mM to 9.43 mM, because a child may receive too much insulin and be unable to perceive the symptoms associated with dangerously low blood glucose levels. Clearly, exogenous insulin administration is an imperfect substitute for the function of normal pancreatic beta-cells which respond continuously to fluctuating glucose levels with appropriate insulin secretion.

[0083] The human pancreas cell cultures of the present invention have the capability to respond in dose-response fashion to increased glucose levels in their culture medium, as depicted in Figure 11. Moreover, the in vitro response of these cells in long-term culture is comparable to that expected from normal mature human beta-cells in primary culture. The responsiveness of the cells may be tested by several means.

[0084] To begin the test, the cells may be placed in fresh defined culture medium of the same type in which they are maintained over the long term. The long-term culture medium preferably contains from about 6.5 mM to about 8.0 mM glucose, most preferably 7.4 +/- 0.3 mM glucose. The preferred glucose level in the long-term culture medium is comparable to the high end of the normal range of human blood glucose, so that the cells remain induced for insulin production. The amount of insulin secreted into the defined medium is referrred to as the "maintenance level", as described above.

[0085] Alternatively, to build up stores of insulin within the cells, and to enhance the cells' responsiveness to glucose, the cells may be "glucose-starved" by incubation in "glucose-poor medium". The term "glucose-poor medium" refers to a culture medium which contains less than the normal physiological concentration of glucose, typically from zero to about 2 mM glucose. The cells are incubated for about one to about 16 hours, preferably about 2 hours, in glucose-poor medium prior to the experimental test for response to glucose.

[0086] The cells are then placed in a range of glucose concentrations, typically from about 0.5 mM to about 33 mM glucose. As a control, several cultures are not exposed to increased glucose, but rather are placed in fresh "glucose-poor medium" or the regular defined medium containing a high normal physiological concentration of glucose (approximately 7.4 mM). Samples of medium are removed at various time points for assay of insulin content. The amount of insulin secreted into the control medium without added glucose, whether glucose-poor or defined medium, is referred to as the "basal level" of insulin secretion for a given test.

[0087] Preferably, when exposed to about 1 mM to about 6 mM glucose, the cells secrete insulin at about 1.2 to about 2.5 fold the basal level. Also preferably, when exposed to about 6.1 mM to about 17 mM glucose, the cells secrete insulin at about 3 to about 10 fold the basal level. Generally, the pancreatic cells of the present invention respond maximally to 11 mM to 16.5 mM glucose.

[0088] This glucose response pattern is comparable to that of freshly resected human insulinomas, enclosed in permselective macrocapsules, perfused in vitro (Altman, J.J., et al., 1984, Trans. Am. Soc. Art. Organs 30:382-386). The encapsulated insulinomas were reported to respond maximally to 5.5 mM glucose (220 uIU insulin/ml secreted) and to 16.5 mM glucose (350 uIU insulin/ml secreted).

[0089] The pancreatic cells' response to amino acids is tested similarly. The cells are placed in medium containing various concentrations of glucose. Portions of the cultures are exposed to an amino acid such as alanine or arginine, in concentrations ranging from about 0.5 mM to about 40 mm. After an incubation time of about 0.5 to about 24 hours, preferably after 1.5 hours, samples of the medium are assayed for insulin content.

[0090] Preferably, when incubated in about 1 mM glucose for 1.5 hours, the pancreatic cells secrete an intermediate level of insulin. When 10 mM alanine is added together with 1 mM glucose, insulin secretion is stimulated approximately 1.13 fold over the intermediate level of secretion. When 20 mM arginine is added together with 1 mM glucose, insulin secretion is stimulated approximately 1.4 fold over the intermediate level of secretion. Preferably, similar effects of amino acids are seen in 2 mM glucose. This amino acid response is comparable to that expected from normal pancreas cells, in which insulin secretion is potentiated by amino acids so that more insulin is secreted at lower levels of glucose.

[0091] These tests of human pancreatic cells in long-term culture indicate that the cells of the present invention retain certain characterisitics of normal beta-cells, and therefore they may be useful in therapy for diabetes.

[0092] Human insulin is now commercially produced through the use of genetically engineered bacteria. However, the pancreatic cells of the present invention may prove to be valuable as "bio-factories" for the production of human insulin.

[0093] Importantly, since the cells respond to physiologically relevant changes in glucose and amino acid concentration, they are good candidates for transplantation into diabetic patients to replace the functions of damaged or destroyed beta-cells.

[0094] The cells may be subjected to encapsulation processes, and the resulting capsules may be implanted in the patient. The capsules are porous, to allow glucose from the blood stream to reach the cells, and to allow insulin secreted by the cells to diffuse out of the capsule and into the blood stream. It is expected that the cells will respond to changes in the patient's blood glucose concentration in a similar fashion as they do to glucose concentrations in vitro. Resulting insulin secretion by the cells is expected to normalize the patient's blood glucose level, and the cells will then decrease

their insulin secretion accordingly.

[0095] It will be apparent to one of skill in the art of cell culture that single-cell sub-clone cultures may be established from the cell cultures of the present invention. There may be advantages to single-cell sub-clone cultures in that the cells of a given culture, being progeny of just one cell, are expected to be homogeneous in their characteristics. Numerous sub-clone cultures may then be screened for various desired characteristics such as rate of proliferation and responsiveness to glucose. An optimal culture may be selected for each projected use, such as implantation within capsules.

[0096] It will be apparent to one of skill in the art that the herein provided methods may be applied to many additional cell types, such as mammary cells, which have been traditionally difficult to establish and propagate in culture.

[0097] The experimental examples set forth below illustrate the practice of this invention, whereby examples 1-15 and the part concerning thyroid tissue of example 16 fall outside the scope of the claimed invention.

EXAMPLE 1

[0098] This example sets forth a method for establishing human granulosa cells in culture using donor serum in the establishing medium.

[0099] Cell source: The cells in this example were obtained from follicular cells which accompanied ova extracted from patients undergoing in vitro fertilization.

[0100] Donor serum: Blood was collected from each cell donor 24 hours before ovum retrieval and was allowed to thoroughly clot. The clotted blood was centrifuged at 2700 rpm for 10 min. The clear serum was carefully removed, placed in a sterile Falcon tube and centrifuged again to remove any residual erythrocytes. The serum was used only if there were no signs of hemolysis. The serum was removed, placed in another sterile Falcon tube and heat inactivated at 57°C for 30 minutes. The heat inactivated serum was filtered with a 0.20 micron Nalgene filter and collected into a sterile Falcon test tube before use in making the BDM and EM.

[0101] The formulations for BDM and EM were based on an initial formulation of basal medium designated "IVF Ham's F-10" which was synthesized according to the following protocol:

IVF Ham's F-10: To 1000 ml of Ham's F-10 with L-glutamine (GIBCO) was added 0.9 grams sodium bicarbonate, 0.075 grams penicillin, 0.075 grams streptomycin, and 0.245 grams calcium lactate.

Osmolarity was adjusted to a range between 280 and 285 mOsm with cell culture water (type 1 water, 18 megaohm water, GIBCO or M.A. Bio). The medium was filter sterilized with two, 500 ml, 0.20 micron Nalgene filter units. The pH was 7.7.

[0102] IVF Hams's F-10 was used as the basis for the media described below designated blastocyst development medium (BDM) and establishing medium (EM), and for the media formulations described in Example 2 (DM-1) and in Example 5 (EM-01).

Blastocyst development medium (BDM): 1.5 ml of heat inactivated (37°C, 30 min) donor serum was added to 8.5 ml of IVF Ham's F-10. The pH was 7.35 +/- 0.6. The medium was filter sterilized with two 0.20 um Nalgene filter units.

Establishing medium (EM): 1.5 ml of donor serum was added to 18.5 ml of IVF Ham's F-10. The pH was 7.2 to 7.45. The medium was filter sterilized with two 0.20 um Nalgene filter units.

[0103] Oil plates: Each oil plate was prepared by placing 12 ml of mineral oil equilibrated against IVF Ham's F-10 for about 16 hours in the bottom of a Nunculon culture plate (Nunculon tops were not used). Under the oil overlay was carefully placed a bubble of EM or BDM of approximately 0.4 to 0.5 cc which had been equilibrated overnight with 5% CO_2/5% N_2/90%O_2 at 37°C. Each oil plate had 6 bubbles of EM or BDM. The equilibrated oil overlay provided protection against rapid pH changes in the bubbles.

[0104] Cell donors: All donors were patients who chose in vitro fertilization and who voluntarily donated follicular cells which were aspirated along with ova and which would otherwise have been discarded after the in vitro fertilization procedure. Prior to ovum retrieval, female patients between the ages of 22 and 43 years were treated with a combined hormonal regimen to stimulate the development of multiple follicles. The treatment typically included leuprolide acetate for midluteal suppression combined with human menopausal hormone (hMG) and follicle stimulating hormone (FSH) for controlled ovarian hyperstimulation. Radioimmune assays (RIA) were used to monitor the serum levels of estradiol and progesterone. Ultrasonic scans were used to assess the number of growing follicles and their size. Thirty-four hours (+/- 1 hour) prior to oocyte retrieval, 10,000 IU of human chorionic gonadotropin (hCG) was administered. Follicular contents were aspirated during transvaginal oocyte retrieval, and the follicles were irrigated with 37°C Dulbecco's solution. The follicles in solution were collected in 15cc sterile Falcon disposable test tubes and immediately transferred to the embryology lab. It should be noted that no perfumes were permitted in the embryology lab because fumes from certain perfumes were found to affect the viability of cells.

[0105] Ovum complexes were identified and transferred to Falcon culture well dishes (#3007) containing 5 cc warm

establishing medium (EM). One to 3 ovum complexes were put into each collection dish. An ovum complex includes the ovum, the surrounding zona pellucida, zona radiata, cumulus cells, and attached follicular components. The time limitation for the transfer procedure was 90 seconds in order to minimize pH alterations and temperature fluctuations. The collection dish was immediately placed, with the lid cracked, into a 37°C incubator containing 5% CO_2 (medical grade).

[0106] After about 5 to about 30 minutes, the ovum complexes were transferred into EM bubbles on an oil plate. Generally 1 to 3 ovum complexes were placed within a single EM bubble. During this procedure, the EM plate remained outside the incubator no longer than 7 minutes. The oil plate was then placed into a fail-safe container which was gassed with filtered 5% CO_2/5% O_2/90% N_2 medical mixture, sealed and placed into a 5% CO_2 incubator at 37°C for 4 to 5.5 hours.

[0107] The ovum was then inseminated by the addition of a drop of final sperm suspension to each EM bubble. The amount of sperm added was adjusted to give a final concentration of approximately 50 to 60 million spermatozoa per ml in each EM bubble. The oil plate was incubated overnight as above. The following morning, examination of the EM bubbles showed the presence of two classes of cells: 1) loose cells in the EM, and 2) cells of the zona radiata tightly complexed with the ovum.

[0108] At this time point, about 50 to 52 hours after ovum retrieval, non-germ line cells were selected for further culture.

(a) Selection from loose cells after 50 hours in EM: The ovum was aspirated from the EM and incubated according to conventional IVF methods. Non-germ line cells were selected for culture from cells that showed monolayer spreading on the bottom of the plate. Cells with adherent blood clots were avoided. Selected cells were placed in fresh EM bubbles in a freshly equilibrated oil plate. The transfer was done with gentle scraping to loosen the follicular cells from the culture plate. The borosilicate sterile transfer pipette had been precoated with Ham's F-10. Nunculon culture plates (SECO, Rolling Hills, PA), bottoms only, were used for these cultures. Twenty to 50 selected cells were placed in each EM bubble. (i) These cultures were placed into fail-safe containers and flooded with 5% CO_2/5% O_2/90% N_2; then the containers were sealed and placed into a 5% CO_2 incubator for 3 days. (ii) The cells were then placed in Falcon #3007 well dishes containing 6 ml of EM and overlain with mineral oil equilibrated with IVF Ham's F-10. Each culture well was originally seeded with 50 to 100 cells and maintained under the above conditions for 30 days. During the 30 day establishment period, the cultures were fed every 5 to 6 days with 1 ml of fresh EM (i.e. in a well containing 6 ml, 1 ml was removed and replaced). After 15 days, in certain cultures it was apparent that cells had proliferated to the point that sub-culture was necessary (i.e. the number of cells had increased 3 to 4 fold). The cells were gently scraped from the bottom of the dish and small groups of cells were transferred to new culture dishes containing EM as above.

(b) Selection from cells adhering to ovum after 50 to 52 hours in EM: The ovum plus the complexed zona radiata cells were carefully transferred to blastocyst development medium (BDM) bubbles in a BDM oil plate. Subsequently, the adherent zona radiata cells were manually peeled from the ovum by gently drawing the ovum plus adherent cells into the orifice of a 27½ gauge hypodermic needle and gently expelling the egg. This stripped the adherent zona radiata cells from the smoother membrane, zona pellucida, surrounding the ovum. (The peeled ovum was incubated and prepared for implantation or cryopreservation according to conventional in vitro fertilization methods.) From the stripped zona radiata cells in BDM, cells were selected according to the above criteria and established in culture as described in 1 (a) (i, ii) above.

[0109] After a total of 30 days in EM culture, cells were subcultured as follows in Example 2.

EXAMPLE 2

[0110] This example sets forth a method for maintaining and propagating hormone-secreting cells in long-term culture.

[0111] Upon completion of the initial 30 days of establishing culture (Example 1), the sub-culture selection process was begun. Initial sub-cultures (SC-1) of small groups of cells were manually selected. Cell selection was performed under phase optics according to selection criteria illustrated in Figures 2, 3, and 4. The circled clumps of cells are representative of the type of cell clumps that were chosen as most likely to have hormone-secreting potential. Small clumps of 2 to 12 cells were chosen, most often clumps of 4 to 5 cells. Typically, the cells in the selected clumps were arranged in a semi-linear fashion, i.e. touching each other in a "string-of-pearls" type arrangement. The selected cells were approximately spheroid or ovoid in shape, and were of approximately homogeneous size and shape. The selected cells typically had a smooth appearing membrane, and a smooth-appearing rather than a granular cytoplasm.

[0112] When it was desired to assay for hormone content within a short time period, the selected clumps were grouped into cultures of 50 to 150 cells each, designated SC-1, in a medium composed of 40 cc IVF Ham's F-10 + 0.25 gm

BSA in Falcon #3037 tissue culture wells with no oil overlay. Each culture was placed in an individual well containing 5 ml of medium, the cultures were flooded with medical blood gas mixture (supra), and placed in a sealed container within a 37°C incubator. The cultures were maintained in this medium for 3 days, after which hormone content of the medium was assayed.

[0113] Alternatively, selected clumps were grouped into smaller starting sub-cultures of 10-15 cells, also designated SC-1. These groups were placed in defined medium (DM-1) formulated as follows.

[0114] Defined medium-1 (DM-1) : 100 ml of IVF Ham's F-10 (see example 1 above) was mixed with 100 ml nutrient Ham's F-12, HEPES and sodium bicarbonate buffered and glutamine supplemented (7.35 mM glutamine, Sigma) plus 30 ml tissue culture water (Sigma), 7.2 grams cell culture tested BSA (Fraction V, Sigma), 1500 IU penicillin-G, and 1.5 mg streptomycin. The medium was equilibrated overnight at 37°C in a 5% CO_2 atmosphere before use. The final osmolarity was 272 +/- 1 mOsm. The pH was adjusted to 7.65. The medium was filter sterilized with one 0.45 um and one 0.20 um Nalgene filter unit.

[0115] These sub-cultures were grown for 14 to 15 days in DM-1; medium was refreshed every 5 to 6 days. During this time, the cell number typically increased about ten to thirty fold. It was found that the SC-1 cultures which had been established from only 10-15 starting cells also secreted hormones which were detectable by radio-immune assay within only a few days.

[0116] After about 15 days, each SC-1 culture was divided into about 4 to 6 second sub-cultures (SC-2).

[0117] Cells in SC-2 were propagated and sub-cultured as above more than 20 times. Medium was collected from each sub-culture at regular intervals and assayed for the presence of secreted estradiol (E_2), progesterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), prolactin, testosterone and the β-chain of human chorionic gonadotrophin (B-hCG). Quantitative radioimmune assays (RIA) were employed as described in Radioassay Systems in Clinical Endocrinology, 1981, Ed. G.E. Abraham, {Basel: Marcel Dekker}, pp. 475-529. Progesterone, testosterone, estradiol, prolactin, thyroid stimulating hormone and luteinizing hormone were assayed by the Coat-a-count procedure (Diagnostic Products Corp., Los Angeles). Follicle-stimulating hormone and B-hCG were assayed by the SERONO MATAclone procedure (Serono, Italy).

[0118] Results from assays of secretion from various cultures after more than 4 months in continuous serial subculture are shown in Table 1.

TABLE 1

			RIA Analyses				
Sub-cultures (after 4+ months) Granulosa Cell Cultures	No. of Cells	Synthesis Days	E_2 pg/ml	Progesterone ng/ml	β-hCG mIU/ml	LH nIU/ml	FSH mIU/ml
100B-4	10^6	5	2576	453.8	0	0	0
100B-5-04-03	10^3	9	<20	14.1	0	<1.8	1.71
100B-5-04-05	103	9	21	51.2	0	0	2.59
100C-2-02-B1	10^3	9	<5	0.3	0	0	3.11
100B-5-OR-04	10^6	10	233	425.8	0	<1.8	3.92
100B-5-OR-01-D	10^3	3	34	59.5	0	0	0
100B-5-OR-01-C	10^3	3	35	48.9	0	0	0
100B-5-OR-02-A	10^6	3	41	65.8	0	0	3.74

TABLE 1 (continued)

			RIA Analyses				
Sub-cultures (after 4+ months) Granulosa Cell Cultures	No. of Cells	Synthesis Days	E_2 pg/ml	Progesterone ng/ml	β-hCG mIU/ml	LH nIU/ml	FSH mIU/ml
100B-5-OR-02	10^6	6	402	202.5	0	0	0

[0119] Conclusions: Using this method, human follicular cells were propagated in culture and transferred to serial subcultures which continued to proliferate and to secrete hormones. Typically, after greater than 4 months in continuous sub-culture, several of the cell cultures secreted moderate to high levels of estrogen, progesterone, and FSH. When 100 mg/ml testosterone (sigma T-5641) was added to provide a required substrate for steroid production, these sub-cultures were found to continue to secrete hormones for as long as 16 months after the cells were first placed in culture.

EXAMPLE 3

[0120] This example demonstrates the level of cell propagation achieved in cultures of hormone-secreting cells.

[0121] Cell counting methods: For seeding original primary cultures, as in Example 1, and first sub-cultures, as in Example 2, cell number was established by direct counting through an inverted phase microscope as the cells were being selected. At various time points after seeding, cell number was established by counting and averaging numbers of cells contained in multiple drops on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). The Makler counting chamber has a grid 0.01 mm^2 X 0.01 mm depth.

[0122] Representative samples of cell concentrations were obtained either directly from suspension cultures or from cell populations that had been detached from substrate.

[0123] Results: During the first six days in the first sub-culture, SC-1, cell number typically increased about 3 fold. Typical SC-1 results are shown below in Table 2.

TABLE 2

Starting cell number		Cell number after 6 days
SC-1a	100	350
SC-1b	50	156
SC-1c	100	294
SC-1d	100	271

[0124] During the later part of SC-1, and during subsequent sub-cultures, cell proliferation rate typically increased to a doubling time of approximately three days.

[0125] As shown in Table 1, Example 2, cells also produced hormones during the days they proliferated.

EXAMPLE 4

[0126] This example describes the establishment of follicular cells in culture using non-homologous human serum.

[0127] Follicular cells from three individual cell donors were established in culture as described in Example 1, with the exception that the cells were placed directly into DM instead of EM, and maintained in DM for 7 days. Subsequently, the cells were transferred into a second medium which was either DM or EM containing, in place of cell donor serum, serum from a different individual. The serum donors were women participating in the IVF program who were being pre-treated with the hormonal regimen as described. The hormonal profile for donor serum "A" was within the normal range for women undergoing this hormonal pre-treatment; the hormonal profile for donor serum "B" was within the "hyper-stimulated" range. Hyperstimulation, which occurs for unknown reasons in certain patients undergoing regular IVF hormonal pretreatment, is marked by high levels of estrogen and an increase in progesterone to about 1.0 ng/ml in the serum. The cells were maintained in either DM or in EM (non-homologous serum) for 2 to 3 weeks, after which

they were subcultured as described in Example 2.

[0128] Results: Follicular cells were successfully established in culture and secreted hormones as shown in Table 3.

TABLE 3

Culture #	Second medium	Prog (ng/ml)	E_2 (pg/ml)
1	DM	59.5	34
1	EM (serum A)	48.9	35
2	DM	86.4	240
2	EM (serum B)	83	585
2	EM (serum A)	82.7	267

[0129] Conclusions: Cells can be established in EM containing non-homologous serum, however the level of hormone secretion is influenced by the level of estrogen and may also be influenced by the progesterone level in the serum employed.

EXAMPLE 5

[0130] This example describes a method for establishing, maintaining, and propagating hormone-secreting cells in a culture medium not containing serum.

[0131] Follicular cells were obtained from a donor as described in Example 1. The serum of this patient contained a significant amount of anti-sperm antibody (greater than 20% of the total IgG was anti-sperm). Therefore, in order to optimize the insemination of the ova from this patient, donor serum was not included in any of the media used during the IVF procedure.

[0132] Follicular cells were obtained, selected, and established in culture as described in Example 1 with the exception that BSA supplemented medium (EM-1) was used in place of donor-serum containing medium (GM and EM). EM-1 consisted of IVF Ham's F-10 (see Example 1) plus BSA (fraction V, Sigma) added to a final concentration of 0.5% to 1.0%. The osmolarity of EM-1 was 273 mOsm; the pH was 7.41.

[0133] Established follicular cells were maintained, sub-cultured, and propagated in culture as described in Example 2.

[0134] Results: Under these conditions, cells proliferated and secreted hormones successfully, as shown in Table 4. Results in Table 4 were obtained during seventh or eighth sub-cultures (SC-7 and SC-8). Control culture 100B-5-OR-04 was established in medium with donor serum (EM); cultures 100B-5-OR-01 and 100B-5-OR-02 were established in medium without serum (DM).

TABLE 4

			Cell Number Determination	
Culture	E_2 (pg/ml) 10^6 cells	Prog (ng/mJ) 10^6 cells	SC start	3days\SC
100B-5-OR-04	233	425.8	100	189
100B-5-OR-01	34	59.5	100	171
100B-5-OR-02	41	65.9	100	163

[0135] None of the cultures secreted a detectable amount of the gonadotrophins FSH, LH or β-hCG.

[0136] Conclusions: Cells which are initially established in DM exhibit a rate of proliferation which is comparable to that of cells which are initially established in EM. Cells established in DM, however, exhibit reduced basal steroid hormone production even after 7 or 8 rounds of serial sub-culture. Therefore, cells established in DM are advantageous for use in assays such as gonadotrophin bioassays, which are favored by low basal steroid secretion.

EXAMPLE 6

[0137] This example describes the maintenance and propagation of cells in defined medium containing a serum substitute (DMSS).

[0138] Follicular cells were established in culture as described in Example 1. The cells were then subcultured as described in Example 2 with the exception that the amount of BSA was reduced as compared to the defined culture

medium (DM-1) and a serum-substitute was added [Seru-Max, Lot No: 107-F-4607, Sigma]. Seru-Max contains, among other components, growth factors such as bovine fibroblast growth factor, murine epidermal growth factor, and bovine insulin, as well as ethanolamine, selenium, transferrin, and hydrocortisone. An analysis of the above lot number of Seru-Max is available from Sigma. This medium formulation, DMSS, represents a more defined medium than DM because a portion of the BSA is replaced by a more defined supplement (Seru-Max). The formulation of DMSS is described below:

[0139] Defined medium, serum substitute (DMSS): To 100 ml Ham's nutrient F-12, HEPES and sodium bicarbonate buffered, was added 5 ml L-glutamine supplement to bring the glutamine level to 7.35 mM total (Sigma), 17 ml cell culture tested distilled water (Sigma), plus 0.25 grams cell culture tested BSA (fraction V, sigma), 2.5 mM Na pyruvate, 1500 IU penicillin-G, 1.5 mg streptomycin, and 10% Seru-Max (Sigma). Seru-Max lot number analysis 107F-4607 is available from Sigma Chemical Co, St. Louis, MO. The final osmolarity was 272 +/- 1 mOsm. The pH was adjusted to 7.65. Final volume, 136 ml, was filter sterilized with two 0.20 um Nalgene filter units.

[0140] Results: Cell proliferation in DMSS proceeded comparably to that of control cell cultures grown in DM-1. Prolactin synthesis increased slightly but not significantly (0.4% in response to the Seru-Max). Progesterone and estradiol synthesis levels were not altered in DMSS as compared to DM-1 controls. In contrast, when 10% or 15% fetal calf serum (FCS) was used in place of Seru-Max, hormone content of the cultures was near zero and cell proliferation was significantly slower (data not shown).

[0141] Conclusions: The use of defined medium with serum supplement is advantageous over the use of FCS supplemented serum for human hormone-secreting cell propagation and maintained hormone secretion potential.

EXAMPLE 7

[0142] This example describes the establishment, maintenance, and propagation in culture of follicular cells which were not exposed to sperm.

[0143] Follicular cells were obtained as described in Example 1 from patients who prior to ovum extraction had elected to have only a specific number of retrieved ova fertilized. This situation allowed for the selection of follicular cells which were treated as described in Example 1 with the exception that they had not been exposed to sperm.

[0144] Cells were selected as in Example 1(a) from loose cells in EM bubbles. (It was impractical to retrieve cells surrounding the ovum as, in Example 1(b), possibly because exposure to sperm is required to loosen the cells of the zona radiata.) Fewer cells were obtained by this method as compared to Example 1, however the cultures were successful. Initial cell counts showed a recovery of only 12 to 27 cells per EM bubble with 3 complexes per bubble. This contrasted to the complexes that had a sperm suspension added (same cell donor) in which the initial cell recovery from each EM bubble for primary culture was 131 to 198 cells. The non-sperm exposed cultures did eventually become established, however an additional 1.5 to 2 weeks were required for satisfactory cell culture establishment. The non-sperm exposed cultures were maintained for approximately 5 weeks, during which time their hormone-secretion profile was comparable to that shown in Table 1 for sperm-exposed cultures.

EXAMPLE 8

[0145] This example describes cells maintained in culture which respond to stimulation by gonadotrophin and by cAMP with increased hormone secretion.

[0146] Ovarian follicular cells, lines 100B-OR-5A, 100B5-OR-B, and 100B5-OR-D, were established in culture as described in Example 1 and maintained and sub-cultured as described in Example 2. From these lines were created sub-cultures designated 1, 2, and 3 respectively, which were employed in the stimulation protocol described below.

(a) Gonadotrophin stimulation: Human chorionic gonadotrophin (hCG; Sigma) was added to the culture medium (DM-1) in the amount of 750 ng/ml. After 70 to 76 hours, progesterone content of the medium was increased approximately two-fold over control. Estradiol synthesis was also stimulated by hCG, leading to an increased estradiol content of 0.4 to 0.5 fold over control within 30 hours. Results are shown in Table 5 below:

TABLE 5

	Prog (ng/ml)		E_2 (pg/ml)	
Culture	No hCG	+ hCG	No hCG	+ hCG
1	5.8	12.1	26	43
2	9.0	19.8	59	76

(b) cAMP stimulation: Cultures were exposed to 1 mM 8-Br-cAMP (Sigma) or to FSH (1IU/ml; Metrodin [urofollitropin], Lot No.: 07321070, Serono, Italy). As shown in Table 6 below, progesterone content in the cultures was increased 5 to 11 fold in response to 24 hours of cAMP stimulation. FSH stimulation for 48 hours led to an increase in progesterone content of 6.5 to 7.7 fold.

TABLE 6

	Prog (ng/10^6 cells)		
Culture	Nothing added	8-Br-cAMP	FSH
1	3.0	24.5	17.2
2	1.4	12.9	10.8
3	2.9	20.8	18.9

[0147] Conclusions: Follicular cells in culture responded to stimulation by gonadotrophin in a manner comparable to their counterparts in vivo, i.e. granulosa cells. This indicates that follicular cells propagated in vitro express a differentiated characteristic of granulosa cells, and are thus potentially useful in bioassays for gonadotrophin potency as well as for chemical toxicity.

EXAMPLE 9

[0148] This example sets forth a method to assay the potency of a preparation of gonadotrophin.

[0149] Follicular cells were obtained from a donor who had been pre-treated with an ovary-stimulating hormonal regimen that did not include hCG. The follicular cells had therefore not been exposed to high levels of gonadotrophin prior to selection for culture, and did not secrete high basal levels of progesterone.

[0150] The cells were established in culture as in Example 1 and propagated in culture as in Example 2 and Example 6. The amount of progesterone secreted by these cells into a culture medium not containing gonadotrophin was typically undetectable, but a significant amount of estradiol was synthesized.

[0151] The gonadotrophin to be bio-assayed (e.g. commercially available FSH preparations) is added to the cell culture; after exposure periods of approximately 24, 48 and 72 hours hormonal content in the culture medium is measured and compared to control. The potency of the gonadotrophin in this in vitro bio-assay is initially related to the FSH values obtained by high-performance liquid chromatography (HPLC; Stone, B. A., et al., 1990, supra). In subsequently employing this bio-assay, the relative potency of a gonadotrophin preparation is obtained by fitting the bio-assay numerical values to standard curves prepared by comparison of bio-assay values and HPLC values.

EXAMPLE 10

[0152] This example describes an in vitro bioassay for the potential toxicity of drugs and other chemical compounds.

[0153] Ovarian follicular cells were established in culture as described in Example 1 and maintained and sub-cultured as described in Example 2. Sub-cultures secreted progesterone and estrogen at levels comparable to those shown in Table 1.

[0154] In order to assess the potential toxicity of a drug, the cells are contacted with the drug to be tested and with a control compound known to be non-toxic. The cells contacted with a non-toxic compound continue to secrete steroid hormones at basal levels. When the experimental drug is toxic, the level of hormone secretion is reduced as compared with control.

EXAMPLE 11

[0155] This example sets forth a method to establish and propagate in culture cells from primary follicles.

[0156] Primary ovarian follicles were obtained from two donors who were undergoing ovariectomy and who had not been pre-treated with an ovary-stimulating hormonal regimen.

[0157] Primary follicles were manually isolated from small pieces of ovarian tissue. The primary follicle complex was placed into culture and maintained in culture for 6 weeks in the medium designated DMSS (see Example 6). After 5 weeks in culture, the cells were found to secrete estradiol plus a detectable amount of progesterone.

TABLE 7

			Primary Follicle				
Sub-cultures (after 4+ months) Primary Follicles (Primary culture - after 9 days of culture)	No. of Cells	Synthesis Days	E_2 pg/ml	Progesterone ng/ml	β-hCG mIU/ml	LH mIU/ml	FSH mIU/ml
	6 follicles total	3	134	3.2	0	0	0
	4 follicles total	3	77	2.3	0	0	0

EXAMPLE 12

[0158] This example sets forth a method to establish and propagate in culture hormone-secreting cells of trophoblastic origin.

[0159] Trophoblastic cells were obtained from a cell donor undergoing surgery for an ectopic pregnancy. A number of the trophoblastic cells were placed directly into EM as described in Example 1 and further cultured in DM as in Example 2. A second group of trophoblastic cells were placed directly into an alternative type of defined medium containing serum substitute (DMSS, formula given in Example 6, supra) and further cultured as in Example 2, with the exception that the growth medium was DMSS.

[0160] Results: The trophoblast cells typically proliferated successfully in culture under both the above described conditions. After 6 days in SC-3 subcultures the medium was assayed for the hormones listed in Table 8 below.

TABLE 8

6 weeks synthesis after 5 weeks of culture	FSH mIU/ml	E_2 pg/ml	β-hCG mIU/ml	Progesterone ng/ml	Testosterone ng/ml	TSH mIU/ml
SREP-1-0	.14	2.83	43.9	7.5	0.1	0.27
SPEP-2-02	0.17	2.63	37.6	5.8	0.13	0.58

[0161] After 5 weeks in culture, the trophoblast cells maintained the secretion of significant levels of β-hCG, (i.e. 43.9 mIU/10^6 cells/10ml/6 days).

EXAMPLE 13

[0162] This example sets forth a method for establishing, maintaining, and propagating gonadotrophin secreting pituitary cells in culture.

[0163] Segments of pituitary macroadenoma were obtained from a male donor undergoing trans-sphenoidal pituitary surgery. Small clumps of cells were teased from the surrounding tissue and manually isolated via dissection using sterile fine glass needles. These small segments (about 0.5 to 1.0 mm diameter) were placed into individual wells containing DMSS (see Example 6). Several individual cultures representing cells selected from disparate tumor regions were thus formed. After 6 hours in primary culture, the medium was changed, and after a further 42 hours, medium samples were taken (48 hours total time in primary culture; 42 hours synthesis time). As shown in Table 9, all the initial cultures secreted high levels of luteinizing hormone and detectable levels of FSH and progesterone. Three of the cultures also secreted detectable levels of β-hCG. Notably, there was no detectable amount of prolactin secretion, indicating the cells had no lactotroph component, and suggesting that they were of pure gonadotroph lineage.

TABLE 9

42 hrs. synthesis	E_2 pg/ml	LH mIU/ml	Testosterone ng/ml	FSH mIU/ml	Progesterone ng/ml	β-hCG mIU/ml	Prolactin ng/ml
D/A Macro -01	0	123.2	0	4.09	0.2	2.6	0
D/A Macro -02	0	42.5	0	4.19	0.11	2.7	0
D/A Macro -03	1	71.8	0	5.23	0.1	3.1	0
D/A Macro -04	0	40.6	0	5.16	0.3	0	0
D/A Macro -05	2	71.8	0	5.48	0.1	0	0
D/A Macro -06	0	53.2	0	3.76	0.1	0	0

[0164] After 15 days in culture (10 days synthesis time), the cells continued to secrete hormones. The values for D/A Macro-05, for example, were: LH, 11.3; FSH, 4.04; B-hCG, 5.9 (mIU/ml). After 28 days in culture (8 days synthesis time) the -05 culture contained 3.0 mIU/ml of LH, but the other hormones were undetectable. In contrast, "blastema-like" cell clusters, as illustrated in Figures 9 and 10, were transferred to separate cultures at culture day 20, and were found to secrete relatively large amounts of hormone. For instance, two such "blastema" groups combined in one culture dish secreted 3.0 mIU/ml of LH over a period of 8 days. Given the relatively small number of cells in the culture, this represents a large amount of hormone secretion, and suggests that the "blastema" cells represented the most productive members of the primary cultures.

[0165] A cell line is established which secretes a therapeutically useful form of human gonadotrophin. The gonadotrophin is isolated from the medium surrounding the cell cultures and used in the preparation of a medical composition for the pretreatment of women for the in vitro fertilization procedure.

EXAMPLE 14

[0166] This example describes a method to establish, maintain and propagate human endometrial cells in vitro.

[0167] Endometrial cells were obtained from a woman donor undergoing endometrial biopsy.

[0168] Cells were manually isolated as described in Example 12. Groups of cells were placed into individual wells and subsequently selected for further culture on the basis of their hormone secretory activity, as determined by RIA of the media. Selected cell groups were established in culture as described in Example 1 and propagated in culture as described in Example 2. As shown in Table 10, after greater than 4 months in culture, two of the cell lines continued to secrete very significant amounts of estrogen and progesterone. Thus, these cell lines are useful for the production of human sex steroid hormone for therapeutic use.

TABLE 10

			Endometrium				
Sub-cultures (after 4^+ months) Endometrium (2nd subculture)	No. of Cells	Synthesis Days	E_2 pg/ml	Progesterone ng/ml	β-hCG mIU/ml	LH mIU/mt	FSH mIU/ml
PRUE-02-1	10^6	3	1415	150.8	0	<1.8	2.91
POIE-A	10^3	3	20	0.3	0	0	0
POIE-OR	10^{12}	6	9039	1173.4	0	<1.8	2.46

EXAMPLE 15

[0169] This example describes methods for the cryopreservation of hormone-secreting cells.

[0170] Follicular cells were placed in 3 alternative cryopreservative media:

A) 80% DMSS, 10% dimethylsufoxide (DMSO; cell culture tested, Sigma)), 10% glycerol (Sigma Grade, Sigma)
B) Test-yolk Buffer (Irvine Scientific, CA) plus 15% glycerol
C) 87.5% DMSS (containing 2% BSA and 3.4% sucrose), plus 12.4% 1,2-propanediol.

[0171] All solutions were slowly filtered with a sterile 0.20 um Nalgene filter and equilibrated with 5% CO_2/5% O_2/ 90% N_2 (medical gas mixture) for 16 to 24 hours. The cells in cryopreservatives A or B were frozen at a rate of approximately -1°C per minute to a temperature of -34°C and stored under liquid nitrogen.

[0172] For cryopreservative C, the cells were processed according to the following protocol:

1. 10 ml DMSS + 2% BSA, Fraction V (Cell Culture tested, Sigma); 12 minute incubation of 3 x 10^6 cells. 37°C (36 to 37° C) range.
2. 10 ml [8.75 ml DMSS + 2% BSA + 1.24 ml 1,2-propanediol (Sigma)] 12 min incubation, gentle 1 minute swirling; 3 x 10^6 cells; room temp (=35°C +/-1°)
3. 10 ml [sol.B above + 0.34 grams sucrose (cell culture tested, Sigma)], 12 min incubation, gentle swirling for 1 min; 3 x 10^6 cells; room temp (=35° +/-1°C).
4. Load into 3 cryovials at approximately 10^6 cells per 1.5 ml of solution C.
5. Cool at 4°C for 10 min.

Freezing program for Planer Cell Freezer R204; Liquid/Vapor Phase Nitrogen (PLANER Products Ltd.)
Ramp 1: -2° C/min down to -7°C +/- 0.5°c
Ramp 2: Hold -7°C, 15 min, N_2 vapor.

[0173] Seed (begin crystallization) at the top of miniscus in the freezing vial at the beginning of Ramp 2 by touching the top of the miniscus with forceps that have been prechilled in liquid N_2 vapor.

Ramp 3: -0.3°C/min down to -34°C.
Ramp 4: Hold at -34°C for 30 minutes, then quickly transfer the cryovials to the N_2 vapor storage cryotank.

[0174] Thawing for freezing method using cryopreservative C:
Thawing solutions:

Solution a: DMSS + 2% BSA
Solution b: 1.0 ml of solution A + 0.68 grams sucrose
Solution c: 8.8 ml of Solution B + 1.2 ml 1,2-propanediol.

[0175] All solutions were sterile filtered with a 0.20 um Nalgene filter. The following thawing solutions were prepared in 15 ml Falcon test tubes.

	Solution b	Solution c	
Solution T1	0 ml	+	6 ml
T2	2 ml	+	4 ml
T3	3 ml	+	3 ml
T4	4 ml	+	2 ml
T5	5 ml	+	1 ml
T6	6 ml	+	0 ml

[0176] The thawing solutions were equilibrated in loose/open-top test tubes with medical gas mixture. The frozen cyrovial was quickly thawed in a 30°C water bath. The vial was opened and the cells immediately transferred to 6 ml medium "T1" in a Nunculon petri dish and placed in a gassed (medical gas mixture) sealed glass container for 8 minutes at room temperature.

[0177] The cells were transferred to a second Nunculon (bottom) culture dish with 6 ml of T1 solution, regassed, and incubated for another 8 minutes. This step was repeated for each of the thawing solutions (T2-T6). The thawed, rehydrated cells were transferred to 25cm^2 Falcon tissue culture flasks containing 10 to 15 ml of DMSS plus 10% Hybridoma Enhancing Supplement (H6020 or H8142, Sigma).

[0178] Cells preserved in cryopreservatives A or B were thawed after one month by placing the frozen cryovial in a 36°C water bath. The thawed cells were immediately transferred to 25 cm^2 Falcon tissue culture flasks containing 15 ml DMSS.

[0179] Eighty percent of the thawed cells were found to be viable via the trypan blue dye exclusion test. After one week in culture, the thawed cells typically proliferated at a rate comparable to the cultures from which they originated, and retained the hormone-secretion profiles of their respective parent cultures.

EXAMPLE 16

[0180] This example demonstrates a method to establish hormone secreting cells in culture from a thyroid tumor and a method according to the invention to establish cells cultures from pancreas tissue.

[0181] Sections of thyroid tumor were obtained from a 30 year old female donor. Sections of pancreatic tissue were obtained from the posterior lobe of the pancreas of an elderly female donor who was undergoing surgery because of an injury to the pancreas. Small chunks containing about 50 to 300 cells were teased apart from the tissue and placed in establishing culture medium as described in Example 13. The cultures were flooded with the medical gas mixture described supra every other day. After 2 weeks, the cultures were subdivided and placed in fresh establishing medium. over the course of an additional 8 weeks in culture, the cell cultures were subdivided as needed, depending on the rate of cell proliferation.

[0182] After a total time in primary culture of 8 weeks (10 days synthesis time) the thyroid cells had accumulated thyroxine (T_4) in the medium at a concentration of 7.3 ug/Dl (assayed by a commercial clinical laboratory). This represents a considerable amount of thyroxine secretion when compared to the normal range for adult serum of 4 to 12 ug/Dl, and indicates that the thyroid cells were performing at least one differentiated thyroid function after 8 weeks in culture. The cells secreted no detectable amounts of progesterone or LH, but over 4 weeks of synthesis they accumulated estrogen in the amount of 255 pg/ml. The cells remained in follicle-like clusters throughout the culture period.

[0183] The pancreas cells proliferated in suspension in 15 ml DMSS (25 cm^2 Falcon flasks) during 8 weeks of primary culture. Medium was collected for assay from the time spanning weeks 6 to 8 of culture (2 weeks synthesis time). As would be expected for cells of pancreatic origin, they did not secrete detectable amounts of progesterone, estrogen, or LH. Amylase and insulin concentrations were assayed by Sierra Nevada Labs, Reno, Nevada. Amylase concentration in the medium was very low at 5 U/liter (normal range for human serum = 34-122 U/liter). This indicated that there were very few cells of exocrine pancreas origin in the cultures. In contrast, the medium of most cultures contained greater than 400 IU/ml insulin, (normal range in a fasting individual = 9.1-21.7 IU/ml). This indicated that the cultures contained β-cells of. pancreatic endocrine islet origin, and that the cells actively secreted insulin into the medium. Individual cultures were selected for further propagation according to proliferation rate and amount of insulin secreted.

[0184] The pancreatic cells may be sub-cloned to produce cultures which produce human insulin and which are free of exocrine pancreatic cells.

[0185] Conclusion: The methods of this invention is successful, in establishing cell cultures of pancreatic insulin producing cells for diverse applications.

EXAMPLE 17

[0186] This example demonstrates the maintenance of insulin-secreting cells in long-term culture, and the maintenance of the cells' insulin secretory capacity after freezing and thawing.

[0187] The pancreas cell cultures described in Example 16 were passaged approximately every 3 - 5 days through generation 47. Passaging was done by placing 0.5 - 1.0 ml of cell suspension into 10 ml fresh medium in a flask. Each flask was flooded with medical blood gas mixture (supra), sealed, and maintained in an incubator at 37°C. Typically, each flask was flooded with fresh gas every other day. At passage 47, the cells had been in continuous culture for 9.5 months.

[0188] A portion of the pancreas cells at generation 47 were frozen according to the method described in Example 15 using cryopreservative A. The cells were stored frozen for 1 day, after which they were thawed according to the method described in Example 15.

[0189] The thawed cells and cells from generation 47 which had not been frozen were placed in DMSS medium and centrifuged at 1070 rpm for 5 minutes. The pellets were washed and resuspended in 30 ml of Medium PDM (Dulbecco's salt solution, phosphate buffered, with $MgSO_4$ (no $MgCl_2$), plus 2% BSA Fraction V), final osmolality adjusted to 272 mOsm) at 37°.

[0190] The cells were incubated 30 minutes at 37°, then centrifuged and resuspended as above. Cell count and viability were determined by Trypan blue exclusion.

[0191] The cells were then centrifuged and resuspended in a base medium consisting of 3 parts Medium PDM (supra) and 1 part DMSS, as defined in Example 6 above. The cells were incubated for 1.5 or 3 hours in D(+)glucose at

concentrations ranging from 1 to 21 mM as shown in Table 11 below. RIA analysis demonstrated that the cells responded to glucose by the secretion of insulin as shown in Table 11.

Table 11

Glucose level total	uIU insulin secreted / ml / 10,000 cells	
	pg 47	pg47 frozen/thawed
1 mM	92	99
2 mM	100	105
6 mM	111	114
11 mM	117	128
16 mM	129	125
21 mM	116	124

[0192] Conclusions: The pancreas cells were maintained in long term culture, during which time the cells maintained their capacity to secrete insulin in response to increased glucose concentrations. Moreover, cells which had been frozen and thawed retained their capacity for response to glucose, which response was comparable to that of non-frozen cells.

EXAMPLE 18

[0193] This example shows the time course of the human pancreas cells' response to glucose.

[0194] Human pancreas cell cultures from Example 16 were maintained in continuous culture through 55 generations, at which time they had been in culture for 12 months.

[0195] Two hours prior to the experiment, the cells were centrifuged at 1070 rpm for 5 minutes and then resuspended in glucose-poor medium consisting of 1 part DMSS plus 6 parts PDM. The cells were incubated in glucose-poor medium (glucose-starved) at a concentration of about 1.5 X 10^6 cells/ml in 10 ml culture flasks for two hours at 37°.

[0196] The cells were then centrifuged and resuspended in the experimental medium consisting of one part DMSS and six parts PDM. The glucose concentration in the experimental medium alone was 1.1 mM. To this medium was added glucose in the concentrations shown in Table 12 below, and the cells were incubated for various times. Samples were collected and assayed for insulin content as shown in Table 12.

Table 12

MCC041291 1,500,000 cells/ml 10 ml culture in flask

	1 hr	2 hr	3 hr	5 hr	7 hr	24 hr
mM glucose added				uIU insulin secreted/ 1.5 million cells/ml		
0 mM	29	42	82	99	210	101
1 mM	275	363	451	564	582	812
5.6 mM	3413	982	999	2383	2718	2760
110 mM	3376	1768	2220	1184	3202	1276
16.5 mM	3716	2415	2450	2589	4039	2099
22 mM	2538	1845	2066	1722	1320	989
33 mM	972	345	446	657	1109	1073

[0197] Conclusions: The cells responded in graded dose-response fashion to increasing concentrations of glucose, with a maximal response at 16.5 mM, which is comparable to the response expected from normal human beta-cells in primary culture. The maximal response ranged from approximately 20 fold to 128 fold the basal level of insulin secretion.

EXAMPLE 19

[0198] This example shows that pancreas cells in continuous culture retain their capacity to secrete insulin in response to glucose.

[0199] A portion of the human pancreas cells from Example 16 at passage generation 21 were frozen according to the method described in Example 17, and then thawed 9.5 months later prior to the experiment. Passage generation 60 cells were maintained in continuous culture for 1 year. Both groups of cells were glucose-starved for 2 hours prior to the experiment, as described in Example 18. The cultures were then placed in 24 well plates at a concentration of 10^5 cells/ml/well in an incubation medium of RPMI-1640-Y [100 ml glucose deficient RPMI-1640 (R 1383, Sigma), 1 gm BSA Fraction V, 1.5 ml HEPES solution, 5.5 ml tissue culture water, 1500 IU penicillin-G, 1.5 mg streptomycin, pH 7.4-7.6, final osmolarity adjusted to 272 mosm].

[0200] The cells were incubated for 90 minutes or 5 hours in various concentrations of glucose as shown in Figure 11.

[0201] Results: Cells which were frozen at passage 21 responded maximally to 11 mM and 16.5 mM glucose with an 8 to 9.5 fold increase in insulin secretion compared to control at 5 hours. Cells which had been maintained in continuous culture for 1 year (passage generation 60) responded to 5.6, 11, and 16.5 mM glucose with increases in insulin secretion ranging from 3 to 4.5 fold compared to control (Figure 11).

EXAMPLE 20

[0202] This example demonstrates the response of pancreas cells in long-term culture to amino acids.

[0203] Human pancreas cells at passage generation 47, were prepared for the experiment as described in Example 17 above. The cells were incubated in 6 parts PDM plus 1 part DMSS medium at a concentration of 10^5 cells/ml, at various concentrations of glucose. Alanine (10mM) or arginine(20mM) were added, and the cells were incubated for 90 minutes, at which time samples were collected for assay of insulin content. The results are shown in Table 13.

Table 13

Effect of amino acids on glucose stimulated insulin secretion.			
total mM glucose	control	+10 mM alanine	+20 mM arginine
1 mM	91	103	128
2 mM	108	135	134
6 mM	112	127	105
11 mM	116	117	93
16 mM	128	101	99
22 mM	115	87	94

[0204] Results: At low glucose concentrations (1, 2, and 6 mM), alanine increased insulin secretion beyond the level of glucose stimulation alone. The effect of alanine was most pronounced at 2 mM glucose, where alanine increased insulin secretion 1.25 fold over that stimulated by glucose alone. Arginine had a pronounced effect at 1 mM glucose, where arginine increased insulin secretion 1.4 fold over that stimulated by glucose alone.

EXAMPLE 21

[0205] This example demonstrates that human pancreas cells maintained in long-term culture contain immunoreactive insulin.

[0206] Human pancreas cells from Example 16, passage generation 47, were fixed and permeabilized by -20°C methanol, mounted, and stained by a standard immunochemical technique (Harlow, E. et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories) using as a primary antibody an anti-human insulin antibody raised in guinea pig from Peninsula Laboratories, Belmont, California. The secondary antibody was anti-guinea pig IgG (whole molecule) - TRITC conjugate (Rb) from Sigma (T-7153). As a negative control, in place of the primary

antibody, cells were incubated with the same primary anti-insulin antiserum which had been pre-incubated with synthetic human insulin to adsorb the anti-insulin antibodies. The cells were counterstained with Hoechst dye 33258. The immunostained cells were observed and photographed on a Zeiss IM35 microscope using a Zeiss #15 filter to illuminate the rhodamine dye labeling for insulin. The identical cell fields were observed and photographed using a Zeiss #2 filter to illuminate the Hoechst labeling of DNA in the nuclei of all cells in the field. Photographs of cell nuclei were compared with counterpart photographs of rhodamine labeled cell cytoplasms to determine how many cells in the field contained immunoreactive insulin.

[0207] Results: Controls showed no background staining. Comparison of numbers of labeled nuclei with numbers of cells immunoreactive to insulin revealed that greater than 60% of the cells in the culture contained immunoreactive insulin at different intensities of fluorescent staining.

Claims

1. A method for establishing a cell culture of hormone-secreting human pancreatic cells in vitro comprising the steps of:

(a) selecting cells having insulin secreting potential from a population of human pancreatic non-tumorous similar cells having insulin-secreting potential,
(b) placing said cells in suspension in an establishing medium comprising any of human serum, bovine serum albumin or a serum substitute containing animal proteins and which establishing medium is capable of promoting the viability or proliferation of said cell or cells for at least 13 days in vitro, and
(c) sub-culturing anchorage-independent cells from step (b) such that the cells proliferate and viability is maintained for a period of at least 5.5 months.

2. The method according to claim 1 wherein, said cells are selected to have at least two characteristics selected from the group consisting of:

(a) a smooth outer membrane,
(b) one of an approximately spherical shape and a substantially ovoid shape,
(c) a non-granular cytoplasm, and
(d) being a member of a clump of cells numbering from about 2 to 12 cells and being approximately homogeneous in size and shape.

3. The method according to claim 1 comprising the further step of subdividing the progeny from step (c) into a plurality of cell cultures.

4. The method of claim 2 wherein, said selection is accomplished by visual observation through a microscope, and further comprising the step of aspiration of at least one of an individual cell and cell clumps.

5. The method of claim 1 wherein,
said method is centrifuge-free.

6. The method of claim 5 wherein,
said method is substantially enzyme-free.

7. The method of claim 1 and,
prior to step (a), placing a group of cells including said population of similar cells in a solution having substantially the same chemical composition as the biological fluid which naturally surrounded said group of cells in vivo, the concentration of said group of cells within said solution being less than or equal to the concentration of cells occurring within said biological fluid, and
prior to step (a), separating said population of cells from said group of cells in said solution by selecting cells having Characteristics of viable cells and removing said cells from said solution.

8. The method of claim 7 wherein,
said step of selecting cells having characteristics of viable cells is accomplished by selecting cells having characteristics selected from the group consisting of:

(a) cells having a smooth plasma membrane,
(b) cells spreading in a monolayer on the bottom of the culture dish, and
(c) cells free from blood clots.

9. The method of one of claims 1, 2, 3, and 8 wherein, said establishing medium of step (b) has an osmolarity of about 248 mOsm to about 275 mOsm.

10. The method of claim 9 wherein,
said establishing medium further comprises:

(i) a basal medium comprising essential minerals, salts, vitamins, amino acids, and lipids,
(ii) a buffering system,
(iii) glutamine in the amount of about 6.35 mM to about 8.35 mM, and
(iv) at least one energy source selected from the group consisting of lactate and pyruvate.

11. The method of claim 9 wherein,
said establishing medium further comprises a serum in the amount of about 0.5% to about 15% of the total volume of the medium.

12. The medium of claim 9 wherein,
said establishing medium further comprises a serum substitute in the amount of about 5% to about 15% of the total volume of the medium.

13. The method of claim 11 wherein,
said serum is human serum.

14. The method of claim 13 wherein,
said serum is obtained from the blood of a donor of said cell population.

15. The method of claim 9 wherein,
said establishing medium further comprises mammalian serum protein in the amount of about 0.5% to about 3.0% (w/v).

16. The method of claim 15 wherein,
said establishing medium comprises bovine serum albumin.

17. A method for establishing a cell culture of hormone-secreting human pancreatic cells in vitro comprising the steps of:

(a) selecting cells having hormone secreting potential from a population of human pancreatic non-tumorous similar cells having hormone secreting potential,
(b) placing said cells in suspension in an establishing medium comprising any of human serum, bovine serum albumin or a serum substitute containing animal proteins and which establishing medium is capable of promoting the viability or proliferation of said cells for at least 13 days in vitro, and
(c) sub-culturing anchorage-independent cells from step (b) in a defined medium having an osmolarity of about 248 mOsm to about 275 mOsm, such that the cells proliferate and produce progeny and viability is maintained for a period of at least 5.5 months.

18. The method of claim 17 wherein,
said step (c) is accomplished by placing cells in said defined medium which will produce progeny having potential for secretion of at least one hormone belonging to the group consisting of glucagon, and insulin.

19. The method of claim 17 wherein,
said step (c) is accomplished by placing said cell in a defined medium further comprising:

(i) a basal medium containing essential minerals, salts, vitamins, amino acids and lipids,
(ii) a buffering system,
(iii) protein,

(iv) at least one energy source selected from the group consisting of lactate and pyruvate and
(v) glutamine in the amount of about 6.35mM to about 8.35mM.

20. The method of claim 19 wherein said defined medium further comprises a serum substitute in an amount of about 5% to about 15% of the total volume of the medium.

21. The method of claim 17 wherein, said step (c) is accomplished by sub-culturing said cells in a defined medium having an osmolarity in the range of about 269 mOsm to about 275 mOsm.

22. A method according to claim 17 comprising the further step of: placing said progeny from step (c) in a glucose-poor defined medium, and thereby causing said cells to secrete a basal level of insulin.

23. A method according to claim 17 further comprising the step of contacting said progeny cells in step (c) with about 0.5 mM to about 22 mM glucose.

24. A method according to claim 17, further comprising the step of contacting said progeny cells in step (c) with about 2 mM to about 9 mM glucose.

25. A method according to claim 17 further comprising the step of, contacting said progeny cells in step (c) with 1 mM to 6 mM glucose, thereby causing said cells to secrete an intermediate level of insulin, and further contacting said cells with an amino acid.

26. A method according to claim 25 wherein said amino acid is at least one of alanine and arginine.

27. A method according to claim 26 wherein said amino acid comprises alanine in a concentration of about 10 mM.

28. A method according to claim 26 wherein said amino acid comprises arginine in a concentration of about 20 mM.

29. The method of claim 17 which comprises the further step of contacting said progeny cells with a secretogogue selected from the group consisting of glucagon-like peptide-1 and glucose.

30. A human pancreatic cell culture obtainable according to the methods of any of claims 1 to 21.

31. A human pancreatic cell culture according to claim 30 and which is capable of proliferation in vitro and of secreting about 2μIU to about 1000μIU insulin/hour/10^5 cells/ml of culture medium

32. A cell culture according to claim 31, wherein, said secretion of insulin is about 20 μIU to about 400μIU insulin/hour per 10^5 cells per millilitre of defined culture medium.

33. A cell culture obtainable according to the method of claim 22 wherein, said basal level of insulin is about 20μIU to about 250 μIU insulin/hour per 1.5 million cells per millilitre of glucose-poor medium.

34. A cell culture obtainable according to the method of claim 23 wherein, said progeny cells are responsive to said glucose contact to produce increased insulin secretion in an amount of about 1.2 fold to about 130 fold a basal level of insulin secretion, said basal level being in the range of about 20 μIU to about 250 μIU insulin per 1.5 million cells per millilitre of medium

35. A cell culture according to claim 34 wherein, said response occurs over a time period comprising about 30 minutes to about 24 hours.

36. A cell culture obtainable according to the method of claim 24, wherein said progeny cells respond to said glucose contact to produce an increase in said insulin secretion in an amount in the range of about 1.5 to about 10 fold said basal level of insulin secretion.

37. A cell culture obtainable according to any of the methods of claims 25-28 wherein, said progeny cells respond to said amino acid contact to produce an increase in insulin secretion in a range of about 1.3 to about 2.0 fold said intermediate level of insulin secretion.

38. A method of determining the toxicity of a test compound comprising the steps of:

(a) providing an established human non-tumorous pancreatic cell line produced according to the methods of claim 1 or 17, which cell line comprises having cells which exhibit a characterised response to a known toxin, said response being a known change in the hormone-secretion profile of said cells of said cell line,
(b) contacting said cells with said test compound,
(c) determining the hormone-secretion profile of said cells after step (b), and
(d) comparing the hormone-secretion profile of said cells after step (b) with said known change in hormone-secretion profile to determine the relative toxicity of said test compound.

39. A cell culture comprising:

hormone-secreting non-tumorous human pancreatic cells according to claim 30 in suspension in an defined medium, said defined medium comprising:

(a) a basal medium having essential minerals, salts, vitamins, amino acids, and lipids,
(b) a buffering system,
(c) an osmolarity of about 248 mOsm to about 275 mOsm, wherein said culture has been gased with a medical gas mixture comprising 5% CO_2, 5% O^2, and 90% N_2 and
(d) glutamine in the amount of about 6.35 mM to about 8.35mM.

40. The cell culture of claim 39 wherein, said medium further comprises at least one energy source selected from the group consisting of lactate and pyruvate.

41. The cell culture of claim 39 or 40 wherein, said medium further comprises serum in the amount of about 0.5% to about 15% of the total volume of the medium.

42. The cell culture of claim 41 wherein, said serum comprises at least one of a human serum and a defined serum supplement.

43. A cell culture as defined in claim 39 wherein said defined medium includes a protein.

44. The cell culture of claim 43 wherein, said medium further comprises at least one energy source selected from the group consisting of lactate and pyruvate.

45. The cell culture of claim 43 wherein, said osmolarity is about 269 mOsm to about 275 mOsm.

46. The cell culture of claim 43 wherein said secreted hormone is selected from the group consisting of glucagon and insulin.

47. A method for the long-term maintenance of hormone-secreting human non-tumorous pancreatic cells in vitro obtainable according to the method of claim 17 comprising the steps of:

(a) propagating said cell or cells in step (c) wherein, said defined medium is capable of promoting the viability of at least some of the progeny so that at least some of said progeny are viable in vitro after at least one year from the occurrence of step (a).

48. A method for determining the toxicity of a test compound comprising the steps of:

(a) providing an established non-tumorous human pancreatic cell line according to claim 30 and wherein said cell line has been propagated in vitro for longer than one year and the cells of which exhibit a characterised response to a known toxin, said response being a known change in the hormone-secretion profile of said cells of said cell line,
(b) contacting said cells with said test compound,
(c) determining the hormone-secretion profile of said cells after step (b), and
(d) comparing the hormone-secretion profile of said cells after step (b) with said known change in hormone-secretion profile to determine the relative toxicity of said test compound.

Patentansprüche

1. Verfahren zur Etablierung einer Zellkultur aus hormonsezernierenden menschlichen Pankreaszellen in vitro, das folgende Stufen umfaßt:

   a) Auswahl von Zellen mit Insulinsekretionspotential aus einer Population menschlicher nichttumoraler Pankreaszellen, die ein Insulinsekretionspotential aufweisen,

   b) Suspendierung dieser Zellen in einem Etablierungsmedium, das eines der Sera der Gruppe Humanserum, Rinderserumalbumin oder Serumersatzstoff umfaßt, das tierische Proteine enthält, wobei das Etablierungsmedium die Wachstumsfähigkeit bzw. Proliferation der Zelle bzw. Zellen während eines Zeitraums von wenigstens 13 Tagen in vitro begünstigt und

   c) Subkultivierung von bindungsunabhängigen Zellen aus Stufe b), so daß die Zellen proliferieren und die Wachstumsfähigkeit während eines Zeitraums von wenigstens 5,5 Monaten aufrechterhalten wird.

2. Verfahren nach Anspruch 1, bei dem die Zellen so ausgewählt werden, daß sie wenigstens zwei Merkmale aufweisen, ausgewählt aus der Gruppe, bestehend aus

   a) glatte Außenmembran,

   b) eine der beiden Formen, ausgewählt unter annähernd sphärischer Form und praktisch ovoider Form,

   c) nichtgranuläres Cytoplasma und

   d) Teil einer Zellmasse mit ca. 2 bis 12 Zellen und annähernd gleicher Größe und Form.

3. Verfahren nach Anspruch 1, das außerdem die Stufe der weiteren Unterteilung der Nachkommen aus Stufe c) in eine Vielzahl von Zellkulturen umfaßt.

4. Verfahren nach Anspruch 2, bei dem die Auswahl durch visuelle, mikroskopische Untersuchung erfolgt und das außerdem noch die Stufe des Aufsaugens wenigstens eines Vertreters der Gruppe individuelle Zelle und Zellmassen umfaßt.

5. Verfahren nach Anspruch 1, bei dem dieses zentrifugenfrei ist.

6. Verfahren nach Anspruch 5, bei dem das Verfahren praktisch enzymfrei ist.

7. Verfahren nach Anspruch 1, bei dem man vor der Stufe a) eine Gruppe von Zellen zugibt, welche die Population ähnlicher Zellen in einer Lösung mit praktisch derselben chemischen Zusammensetzung als die biologische Flüssigkeit, welche diese Gruppe von Zellen unter natürlichen Bedingungen in vivo umgibt, umfaßt, wobei die Konzentration dieser Zellgruppe in der Lösung kleiner ist als die Konzentration der in der biologischen Flüssigkeit enthaltenen Zellen, oder dieser Konzentration entspricht und vor der Stufe a) die Zellpopulation aus der Zellgruppe in der Lösung durch Selektion von Zellen mit Merkmalen wachstumsfähiger Zellen abtrennt und diese Zellen aus der Lösung entfernt.

8. Verfahren nach Anspruch 7, bei dem die Stufe der Selektion der Zellen mit Merkmalen von wachstumsfähigen Zellen durch Selektion von Zellen mit Merkmalen, ausgewählt aus der Gruppe, bestehend aus

   a) Zellen mit glatter Plasmamembran,

   b) Zellen, die in einer Monoschicht auf dem Boden der Kulturschale angeordnet sind und

   c) blutkoagulatfreie Zellen,

   durchgeführt wird.

9. Verfahren nach einem der Ansprüche 1, 2, 3 und 8, bei dem das Etablierungsmedium der Stufe b) eine Osmolarität

von ca. 248 mOsm bis ca. 275 mOsm aufweist.

10. Verfahren nach Anspruch 9, bei dem das Einstellungsmedium außerdem noch folgendes umfaßt:

i) ein Basismedium, das essentielle Mineralstoffe, Salze, Vitamine, Aminosäure und Lipide,

ii) ein Puffersystem,

iii) Glutamin in einer Menge von ca. 6,35 mM bis ca. 8,35 mM und

iv) wenigstens eine Energiequelle, ausgewählt aus der Gruppe, bestehend aus Lactat und Pyruvat,

umfaßt.

11. Verfahren nach Anspruch 9, bei dem das Etablierungsmedium außerdem noch ein Serum in einer Menge von ca. 0,5 bis ca. 15 %, bezogen auf das Gesamtvolumen des Mediums, umfaßt.

12. Medium nach Anspruch 9, bei dem das Etablierungsmedium außerdem noch einen Serumersatzstoff in einer Menge von ca. 5 bis ca. 15 %, bezogen auf das Gesamtvolumen des Mediums, umfaßt.

13. Verfahren nach Anspruch 11, bei dem das Serum Humanserum ist.

14. Verfahren nach Anspruch 13, bei dem das Serum aus dem Blut eines Spenders der Zellpopulation erhalten wird.

15. Verfahren nach Anspruch 9, bei dem das Etablierungsmedim außerdem noch Säugerserumprotein in einer Menge von ca. 0,5 bis ca. 3,0 % (G/V) umfaßt.

16. Verfahren nach Anspruch 15, bei dem das Etablierungsmedium Rinderserumalbumin umfaßt.

17. Verfahren zur Etablierung einer Zellkultur aus hormonsezernierenden menschlichen Pankreaszellen in vitro, das folgende Stufen umfaßt:

a) Auswahl von Zellen mit Insulinsekretionspotential aus einer Population menschlicher nichttumoraler Pankreaszellen, die ein Insulinsekretionspotential aufweisen,

b) Suspendierung dieser Zellen in einem Etablierungsmedium, das eines der Sera der Gruppe Humanserum, Rinderserumalbumin oder Serumersatzstoff umfaßt, das tierische Proteine enthält, wobei das Etablierungsmedium die Wachstumsfähigkeit bzw. Proliferation der Zelle bzw. Zellen während eines Zeitraums von wenigstens 13 Tagen in vitro begünstigt und

c) Subkultivierung von bindungsunabhängigen Zellen aus Stufe b), so daß die Zellen proliferieren und die Wachstumsfähigkeit während eines Zeitraums von wenigstens 5,5 Monaten aufrechterhalten wird.

18. Verfahren nach Anspruch 17, bei dem die Stufe c) so durchgeführt wird, daß die Zellen in das definierte Medium gegeben werden, das Nachkommen mit einem Potential für die Sekretion wenigstens eines Hormons der Gruppe, bestehend aus Glucagon und Insulin, produziert.

19. Verfahren nach Anspruch 17, bei dem die Stufe c) so durchgeführt wird, daß die Zellen in das definierte Medium außerdem folgendes umfaßt:

i) ein Basismedium, das essentielle Mineralstoffe, Salze, Vitamine, Aminosäuren und Lipide,

ii) ein Puffersystem,

iii) Protein,

iv) wenigstens eine Energiequelle, ausgewählt aus der Gruppe, bestehend aus Lactat und Pyruvat, und

v) Glutamin in einer Menge von ca. 6,35 bis ca. 8,35 mM

umfaßt.

20. Verfahren nach Anspruch 19, bei dem das definierte Medium außerdem noch einen Serumersatzstoff in einer Menge von ca. 5 bis ca. 15 %, bezogen auf das Gesamtvolumen des Mediums, umfaßt.

21. Verfahren nach Anspruch 17, bei dem die Stufe c) durch Subkultivierung der Zellen in einem definierten Medium mit einer Osmolarität im Bereich von ca. 269 mOsm bis ca. 275 mOsm erfolgt.

22. Verfahren nach Anspruch 17, das außerdem noch die Stufe der Übertragung der Nachkommen aus Stufe c) in ein glucosearmes definiertes Medium umfaßt, wodurch die Zellen dazu gebracht werden, einen Basalspiegel an Insulin zu sezernieren, umfaßt.

23. Verfahren nach Anspruch 17, das außerdem noch die Stufe der Kontaktierung der Nachkommenzellen von Stufe c) mit ca. 0,5 bis ca. 22 mM Glucose umfaßt.

24. Verfahren nach Anspruch 17, das außerdem noch die Stufe der Kontaktierung der Nachkommenzellen von Stufe c) mit ca. 2 bis ca. 9 mM Glucose umfaßt.

25. Verfahren nach Anspruch 17, das außerdem noch die Stufe der Kontaktierung der Nachkommenzellen von Stufe c) mit 1 bis 6 mM Glucose umfaßt, wodurch die Zellen dazu gebracht werden, einen mittleren Insulinspiegel zu sezernieren, sowie der Kontaktierung der Zellen mit einer Aminosäure umfaßt.

26. Verfahren nach Anspruch 25, bei dem die Aminosäure wenigstens eine Aminosäure, ausgewählt unter Alanin und Arginin ist.

27. Verfahren nach Anspruch 26, bei dem die Aminosäure Alanin in einer Konzentration von ca. 10 mM umfaßt.

28. Verfahren nach Anspruch 26, bei dem die Aminosäure Arginin in einer Konzentration von ca. 20 mM umfaßt.

29. Verfahren nach Anspruch 17, das außerdem eine Stufe der Kontaktierung der Nachkommenzellen mit einem Sekretogog, ausgewählt aus der Gruppe, bestehend aus Glucagon-ähnlichem Peptid-1 und Glucose, umfaßt.

30. Menschliche Pankreaszellkultur, die nach einem der Verfahren gemäß Anspruch 1 bis 21 erhalten werden kann.

31. Menschliche Pankreaszellkultur nach Anspruch 30, die zur Proliferation in vitro befähigt ist und zur Sekretion von ca. 2 bis ca. 1000 μIU Insulin/h pro 10^5 Zellen pro ml Kulturmedium, befähigt ist.

32. Zellkultur nach Anspruch 31, bei der die Sekretion von Insulin ca. 20 bis ca. 400 μIU Insulin/h/105 Zellen pro ml definiertes Kulturmedium beträgt.

33. Zellkultur, herstellbar nach dem Verfahren nach Anspruch 22, bei dem der Basalspiegel an Insulin ca. 20 bis ca. 250 μIU Insulin/h pro 1,5 Millionen Zellen pro glucosearmes Medium beträgt.

34. Zellkultur, herstellbar nach dem Verfahren nach Anspruch 23, bei dem die Nachkommenzellen auf den Kontakt mit Glucose reagieren, indem sie zu einer erhöhten Insulinsekretion in einer Menge führen, die ca. das 1,2- bis ca. das 130fache des Basalspiegels der Insulinsekretion ausmacht, wobei der Basalspiegel in einem Bereich von ca. 20 bis a. 250 μIU Insulin pro 1,5 Millionen Zellen pro ml des Mediums liegt.

35. Zellkultur nach Anspurch 34, bei der die Reaktion über eine Zeitdauer von ca. 30 Minuten bis ca. 24 Stunden erfolgt.

36. Zellkultur, herstellbar nach dem Verfahren nach Anspruch 24, bei dem die Nachkommenzellen auf den Kontakt mit Glucose reagieren, indem sie zu einer erhöhten Insulinsekretion in einer Menge führen, die ca. das 1,5- bis ca. das 10fache des Basalspiegels der Insulinsekretion ausmacht.

37. Zellkultur, herstellbar nach dem Verfahren nach einem der Ansprüche 25 bis 28, bei dem die Nachkommenzellen auf den Kontakt mit Aminosäure reagieren, indem sie zu einem Spiegel der Insulinsekretion in einer Menge führen,

die das 1,3- bis 2,0fache der mittleren Insulinsekretion ausmacht.

38. Verfahren zur Ermittlung der Toxizität einer Testverbindung, das folgende Stufen umfaßt:

a) Bereitstellung einer etablierten menschlichen nichttumoralen Pankreaszellinie, hergestellt nach einem der Verfahren gemäß Anspruch 1 oder 17, wobei die Zellinie Zellen umfaßt, die eine kennzeichnende Reaktion auf ein bekanntes Toxin zeigen, und diese eine bekannte Veränderung im Hormonsekretionsprofil der Zellen der Zelllinie darstellt,

b) Kontaktierung der Zellen mit der Testverbindung,

c) Ermittlung des Hormonsekretionsprofils der Zellen nach der Stufe b) und

d) Vergleich des Hormonsekretionsprofils der Zellen nach der Stufe b) mit der bekannten Änderung im Hormonsekretionsprofil zur Ermittlung der relativen Toxizität der Testverbindung.

39. Zellkultur, die menschliche hormonsezernierende nichttumorale Pankreaszellen nach Anspruch 30 in Suspension in einem definierten Medium umfaßt, das seinerseits folgendes umfaßt:

a) Ein Basismedium mit essentiellen Mineralstoffen, Salzen, Vitaminen, Aminosäuren und Lipiden,

b) ein Puffersystem,

c) eine Osmolarität von ca. 248 bis ca. 275 mOsm, wobei die Kultur mit einem medizinischen Gasgemisch aus 5 % CO_2, 5 % O^2 und 90 % N_2 begast wurde, und

d) Glutamin in einer Menge von ca. 6,35 bis ca. 8,35 mM.

40. Zellkultur nach Anspruch 39, bei der das Medium außerdem noch wenigstens eine Energiequelle, ausgewählt aus der Gruppe, bestehend aus Lactat und Pyruvat, umfaßt.

41. Zellkultur nach Anspruch 39 oder 40, bei der das Medium außerdem noch ein Serum in einer Menge von ca. 0,5 bis ca. 15 %, bezogen auf das Gesamtvolumen des Mediums, umfaßt.

42. Zellkultur nach Anspruch 41, bei dem das Serum wenigstens ein Serum, ausgewählt aus der Gruppe, bestehend aus Humanserum und definiertem Serumsupplement, umfaßt.

43. Zellkultur nach Anspruch 39, bei der das definierte Medium ein Protein umfaßt.

44. Zellkultur nach Anspruch 43, bei der das Medium außerdem noch wenigstens eine Energiequelle, ausgewählt aus der Gruppe, bestehend aus Lactat und Pyruvat, umfaßt.

45. Zellkultur nach Anspruch 43, bei der die Osmolarität ca. 269 bis ca. 275 mOsm beträgt.

46. Zellkultur nach Anspruch 43, bei der das sezernierte Hormon ausgewählt wird aus der Gruppe, bestehend aus Glucagon und Insulin.

47. Verfahren zur Langzeithaltung von menschlichen hormonsezernierenden, nichttumoralen Pankreaszellen in vitro, die nach dem Verfahren nach Anspruch 17 erhalten werden können, wobei das Verfahren folgende Stufen umfaßt:

a) Vermehrung der Zelle bzw. Zellen auf Stufe c), wobei das definierte Medium geeignet ist, die Wachstumsfähigkeit wenigstens eines Teils der Nachkommen zu begünstigen, so daß wenigstens ein Teil der Nachkommen in vitro nach wenigstens einem Jahr nach Ablauf der Stufe a) noch wachstumsfähig ist.

48. Verfahren zur Ermittlung der Toxizität einer Testverbindung, das folgende Stufen umfaßt:

a) Bereitstellung einer etablierten menschlichen nichttumoralen Pankreaszelllinie gemäß Anspruch 30, wobei die Zellinie in länger als ein Jahr in vitro vermehrt wird und die Zellen eine kennzeichnende Reaktion auf ein

bekanntes Toxin zeigen und diese eine bekannte Veränderung im Hormonsekretionsprofil der Zellen der Zellinie darstellt,

b) Kontaktierung der Zellen mit der Testverbindung,

c) Ermittlung des Hormonsekretionsprofils der Zellen nach der Stufe b) und

d) Vergleich des Hormonsekretionsprofils der Zellen nach der Stufe b) mit der bekannten Änderung im Hormonsekretionsprofil zur Ermittlung der relativen Toxizität der Testverbindung.

Revendications

1. Procédé pour l'établissement d'une culture cellulaire de cellules pancréatiques humaines sécrétrices d'hormone *in vitro*, comprenant les étapes consistant :

(a) à sélectionner les cellules ayant un potentiel de sécrétion d'insuline à partir d'une population de cellules pancréatiques humaines similaires non tumorales présentant un potentiel de sécrétion d'insuline,
(b) à mettre lesdites cellules en suspension dans un milieu d'établissement comprenant l'un quelconque des agents consistant en sérum humain, sérum-albumine bovine et un agent de substitution du sérum contenant des protéines animales, milieu d'établissement qui est capable d'améliorer la viabilité ou la prolifération de ladite ou desdites cellules pendant au moins 13 jours *in vitro,* et
(c) à soumettre les cellules à une sous-culture, indépendantes de l'ancrage, de l'étape (b) de telle sorte que les cellules prolifèrent et que la viabilité soit maintenue pendant une période de temps d'au moins 5,5 mois.

2. Procédé suivant la revendication 1, dans lequel lesdites cellules sont choisies de manière à présenter au moins deux caractéristiques choisies dans le groupe consistant en :

(a) une membrane extérieure lisse,
(b) une des formes consistant en une forme approximativement sphérique et une forme pratiquement ovoïde,
(c) un cytoplasme non granulaire, et
(d) un membre d'un amas de cellules comprenant un nombre d'environ 2 à 12 cellules, ayant des dimensions et une forme approximativement homogènes.

3. Procédé suivant la revendication 1, comprenant l'étape supplémentaire de subdivision de la descendance de l'étape (c) en une pluralité de cultures cellulaires.

4. Procédé suivant la revendication 2, dans lequel la sélection est effectuée par observation visuelle au microscope, et ledit procédé comprenant en outre l'étape d'aspiration d'au moins un des éléments consistant en une cellule distincte et un amas cellulaire distinct.

5. Procédé suivant la revendication 1, qui est mis en oeuvre sans centrifugation.

6. Procédé suivant la revendication 5, qui est mis en oeuvre pratiquement sans enzyme.

7. Procédé suivant la revendication 1, comprenant en outre les étapes consistant
avant l'étape (a), à placer un groupe de cellules comprenant ladite population de cellules similaires dans une solution ayant une composition chimique pratiquement identique à celle du fluide biologique qui entourait naturellement ledit groupe de cellules *in vivo,* la concentration dudit groupe de cellules dans ladite solution étant inférieure ou égale à la concentration de cellules existant dans ledit fluide biologique, et
avant l'étape (a), à séparer ladite population de cellules dudit groupe de cellules dans ladite solution en sélectionnant les cellules ayant des caractéristiques de cellules viables et en séparant lesdites cellules de ladite solution.

8. Procédé suivant la revendication 7, dans lequel
ladite étape de sélection des cellules ayant les caractéristiques de cellules viables est effectuée en sélectionnant les cellules ayant des caractéristiques choisies dans le groupe consistant en :

(a) des cellules ayant une membrane plasmatique lisse,
(b) des cellules s'étalant sous forme d'une couche monocellulaire sur le fond de la boîte de culture, et
(c) des cellules dépourvues de caillots sanguins.

9. Procédé suivant l'une des revendications 1, 2, 3 et 8, dans lequel le milieu d'établissement de l'étape (b) a une osmolarité comprise dans l'intervalle d'environ 248 mOsm à environ 275 mOsm.

10. Procédé suivant la revendication 9, dans lequel
le milieu d'établissement comprend en outre :

(i) un milieu basal comprenant des substances minérales essentielles, des sels, des vitamines, des amino-acides et des lipides,
(ii) un système tampon,
(iii) de la glutamine en une quantité d'environ 6,35 mM à environ 8,35 mM, et
(iv) au moins une source d'énergie choisie dans le groupe consistant en lactate et pyruvate.

11. Procédé suivant la revendication 9, dans lequel
le milieu d'établissement comprend en outre un sérum en une quantité d'environ 0,5 % à environ 15 % du volume total du milieu.

12. Procédé suivant la revendication 9, dans lequel
le milieu d'établissement comprend en outre un agent de substitution du sérum en une quantité d'environ 5 % à environ 15 % du volume total du milieu.

13. Procédé suivant la revendication 11, dans lequel
le sérum consiste en sérum humain.

14. Procédé suivant la revendication 13, dans lequel
le sérum est obtenu à partir du sang d'un donneur de ladite population cellulaire.

15. Procédé suivant la revendication 9, dans lequel
le milieu d'établissement comprend en outre une protéine sérique de mammifère en une quantité d'environ 0,5 % à environ 3,0 % (en poids/volume).

16. Procédé suivant la revendication 15, dans lequel
le milieu d'établissement comprend de la sérum-albumine bovine.

17. Procédé pour l'établissement d'une culture cellulaire de cellules pancréatiques humaines sécrétrices d'hormone, *in vitro,* comprenant les étapes consistant :

(a) à sélectionner des cellules ayant un potentiel de sécrétion d'hormone à partir d'une population de cellules pancréatiques humaines non tumorales similaires présentant un potentiel de sécrétion d'hormone,
(b) à mettre lesdites cellules en suspension dans un milieu d'établissement comprenant l'un quelconque des constituants consistant en sérum humain, sérum-albumine bovine ou un agent de substitution du sérum contenant des protéines animales, le milieu d'établissement étant capable d'améliorer la viabilité ou la prolifération desdites cellules pendant au moins 13 jours *in vitro,* et
(c) à soumettre à une sous-culture des cellules, indépendantes de l'ancrage, de l'étape (b) dans un milieu défini ayant une osmolarité d'environ 248 mOsm à environ 275 mOsm, de telle sorte que les cellules prolifèrent et produisent une descendance et la viabilité soit maintenue pendant un temps d'au moins 5,5 mois.

18. Procédé suivant la revendication 17, dans lequel
l'étape (c) est mise en oeuvre en plaçant dans le milieu défini des cellules qui produiront une descendance ayant un potentiel de sécrétion d'au moins une hormone appartenant au groupe consistant en le glucagon et l'insuline.

19. Procédé suivant la revendication 17, dans lequel
l'étape (c) est mise en oeuvre en plaçant les cellules dans un milieu défini comprenant en outre :

(i) un milieu basal contenant des substances minérales essentielles, des sels, des vitamines, des amino-acides et des lipides,
(ii) un système tampon,
(iii) une protéine,
(iv) au moins une source d'énergie choisie dans le groupe consistant en lactate et pyruvate, et
(v) de la glutamine en une quantité d'environ 6,35 mM à environ 8,35 mM.

20. Procédé suivant la revendication 19, dans lequel le milieu défini comprend en outre un agent de substitution du sérum en une quantité d'environ 5 % à environ 15 % du volume total du milieu.

21. Procédé suivant la revendication 17, dans lequel l'étape (c) est mise en oeuvre en soumettant à une sous-culture les cellules dans un milieu défini ayant une osmolarité comprise dans l'intervalle d'environ 269 mOsm à environ 275 mOsm.

22. Procédé suivant la revendication 17, comprenant l'étape supplémentaire consistant : à placer la descendance de l'étape (c) dans un milieu défini pauvre en glucose, et à provoquer ainsi la sécrétion par lesdites cellules d'un taux basal d'insuline.

23. Procédé suivant la revendication 17, comprenant en outre l'étape de mise en contact des cellules de la descendance dans l'étape (c) avec environ 0,5 mM à environ 22 mM de glucose.

24. Procédé suivant la revendication 17, comprenant en outre l'étape de mise en contact des cellules de la descendance dans l'étape (c) avec environ 2 mM à environ 9 mM de glucose.

25. Procédé suivant la revendication 17, comprenant en outre l'étape de mise en contact des cellules de la descendance dans l'étape (c) avec 1 mM à 6 mM de glucose, ce qui provoque la sécrétion par lesdites cellules d'un taux intermédiaire d'insuline, et en outre la mise en contact desdites cellules avec un amino-acide.

26. Procédé suivant la revendication 25, dans lequel l'amino-acide est au moins un des amino-acides consistant en l'alanine et l'arginine.

27. Procédé suivant la revendication 26, dans lequel l'amino-acide comprend l'alanine à une concentration d'environ 10 mM;

28. Procédé suivant la revendication 26, dans lequel l'amino-acide comprend l'arginine à une concentration d'environ 20 mM.

29. Procédé suivant la revendication 17, qui comprend l'étape supplémentaire de mise en contact des cellules de la descendance avec un sécrétagogue choisi dans le groupe consistant en le peptide 1 analogue au glucagon et le glucose.

30. Culture de cellules pancréatiques humaines pouvant être obtenue par les procédés suivant l'une quelconque des revendications 1 à 21.

31. Culture de cellules pancréatiques humaines suivant la revendication 30, et qui est capable de proliférer in *vitro* et de sécréter environ 2 μUI à environ 1000 μUI d'insuline/heure/105 cellules/ml de milieu de culture.

32. Culture cellulaire suivant la revendication 31, dans laquelle la quantité sécrétée d'insuline est comprise dans l'intervalle d'environ 20 μUI à environ 400 μUI d'insuline/heure pour 10^5 cellules par millilitre de milieu de culture défini.

33. Culture cellulaire pouvant être obtenue par le procédé suivant la revendication 22, dans laquelle le taux basal d'insuline est compris dans l'intervalle d'environ 20 μUI à environ 250 μUI d'insuline/heure pour 1,5 million de cellules par millilitre de milieu pauvre en glucose.

34. Culture cellulaire pouvant être obtenue par le procédé suivant la revendication 23, dans laquelle les cellules de la descendance sont sensibles au contact avec le glucose pour parvenir à une sécrétion accrue d'insuline en une quantité d'environ 1,2 fois à environ 130 fois le taux basal de sécrétion d'insuline, ledit taux basal étant compris dans l'intervalle d'environ 20 μUI à environ 250 μUI d'insuline pour 1,5 million de cellules par millilitre de milieu.

35. Culture cellulaire suivant la revendication 34, dans laquelle la réponse se produit en une période de temps comprise dans l'intervalle d'environ 30 minutes à environ 24 heures.

36. Culture cellulaire pouvant être obtenue par le procédé suivant la revendication 24, dans laquelle les cellules de la descendance répondent au contact avec le glucose en présentant une augmentation de la sécrétion d'insuline en une quantité comprise dans l'intervalle d'environ 1,5 à environ 10 fois le taux basal de sécrétion d'insuline.

37. Culture cellulaire pouvant être obtenue suivant l'un quelconque des procédés des revendications 25 à 28, dans laquelle les cellules de la descendance répondent au contact avec l'amino-acide en présentant une augmentation de la sécrétion d'insuline dans l'intervalle d'environ 1,3 à environ 2,0 fois le taux intermédiaire de sécrétion d'insuline.

38. Procédé pour déterminer la toxicité d'un composé d'essai, comprenant les étapes consistant :

(a) à prendre une lignée de cellules pancréatiques humaines non tumorales établie produite par les procédés suivant la revendication 1 ou 17, lignée cellulaire qui comprend des cellules qui présentent une réponse caractéristique à une toxine connue, ladite réponse consistant en une variation connue du profil de sécrétion d'hormone desdites cellules de ladite lignée cellulaire,
(b) à mettre en contact lesdites cellules avec ledit composé d'essai,
(c) à déterminer le profil de sécrétion d'hormone desdites cellules après l'étape (b), et
(d) à comparer le profil de sécrétion d'hormone desdites cellules après l'étape (b) à ladite variation connue de profil de sécrétion d'hormone pour déterminer la toxicité relative dudit composé d'essai.

39. Culture cellulaire comprenant :

des cellules pancréatiques humaines non tumorales sécrétrices d'hormone suivant la revendication 30 en suspension dans un milieu défini, ledit milieu défini comprenant :

(a) un milieu basal comprenant des substances minérales essentielles, des sels, des vitamines, des amino-acides et des lipides,
(b) un système tampon,
(c) une osmolarité d'environ 248 mOsm à environ 275 mOsm, ladite culture ayant été additionnée d'un gaz consistant en un mélange gazeux médical comprenant 5 % de CO_2, 5 % de O_2 et 90 % de N_2, et
(d) de la glutamine en une quantité d'environ 6,35 mM à environ 8,35 mM.

40. Culture cellulaire suivant la revendication 39, dans laquelle le milieu comprend en outre au moins une source d'énergie choisie dans le groupe consistant en lactate et pyruvate.

41. Culture cellulaire suivant la revendication 39 ou 40, dans laquelle le milieu comprend en outre du sérum en une quantité d'environ 0,5 % à environ 15 % du volume total du milieu.

42. Culture cellulaire suivant la revendication 41, dans laquelle le sérum comprend au moins un des agents consistant en un sérum humain et un supplément de sérum défini.

43. Culture cellulaire répondant à la définition suivant la revendication 39, dans laquelle le milieu défini comprend une protéine.

44. Culture cellulaire suivant la revendication 43, dans laquelle le milieu comprend en outre au moins une source d'énergie choisie dans le groupe consistant en lactate et pyruvate.

45. Culture cellulaire suivant la revendication 43, dans laquelle l'osmolarité est comprise dans l'intervalle d'environ 269 mOsm à environ 275 mOsm.

46. Culture cellulaire suivant la revendication 43, dans laquelle l'hormone sécrétée est choisie dans le groupe consistant en le glucagon et l'insuline.

47. Procédé pour le maintien à long terme de cellules pancréatiques humaines non tumorales sécrétrices d'hormone, *in vitro,* pouvant être obtenues par le procédé suivant la revendication 17, comprenant les étapes consistant :

(a) à multiplier ladite ou lesdites cellules dans l'étape (c) dans laquelle le milieu défini est capable d'améliorer la viabilité d'au moins une partie de la descendance de telle sorte qu'au moins une partie de ladite descendance soit viable *in vitro* après au moins un an depuis la mise en oeuvre de l'étape (a).

48. Procédé pour déterminer la toxicité d'un composé d'essai, comprenant les étapes consistant :

(a) à prendre une lignée de cellules pancréatiques humaines non tumorales établie suivant la revendication 30, ladite lignée cellulaire ayant été multipliée *in vitro* pendant un temps supérieur à un an et ses cellules présentant une réponse caractérisée à une toxine connue, ladite réponse consistant en une variation connue du profil de sécrétion d'hormone desdites cellules de ladite lignée cellulaire,
(b) à mettre en contact lesdites cellules avec ledit composé d'essai,
(c) à déterminer le profil de sécrétion d'hormone desdites cellules après l'étape (b), et
(d) à comparer le profil de sécrétion d'hormone desdites cellules après l'étape (b) à ladite variation connue de profil de sécrétion d'hormone pour déterminer la toxicité relative dudit composé d'essai.

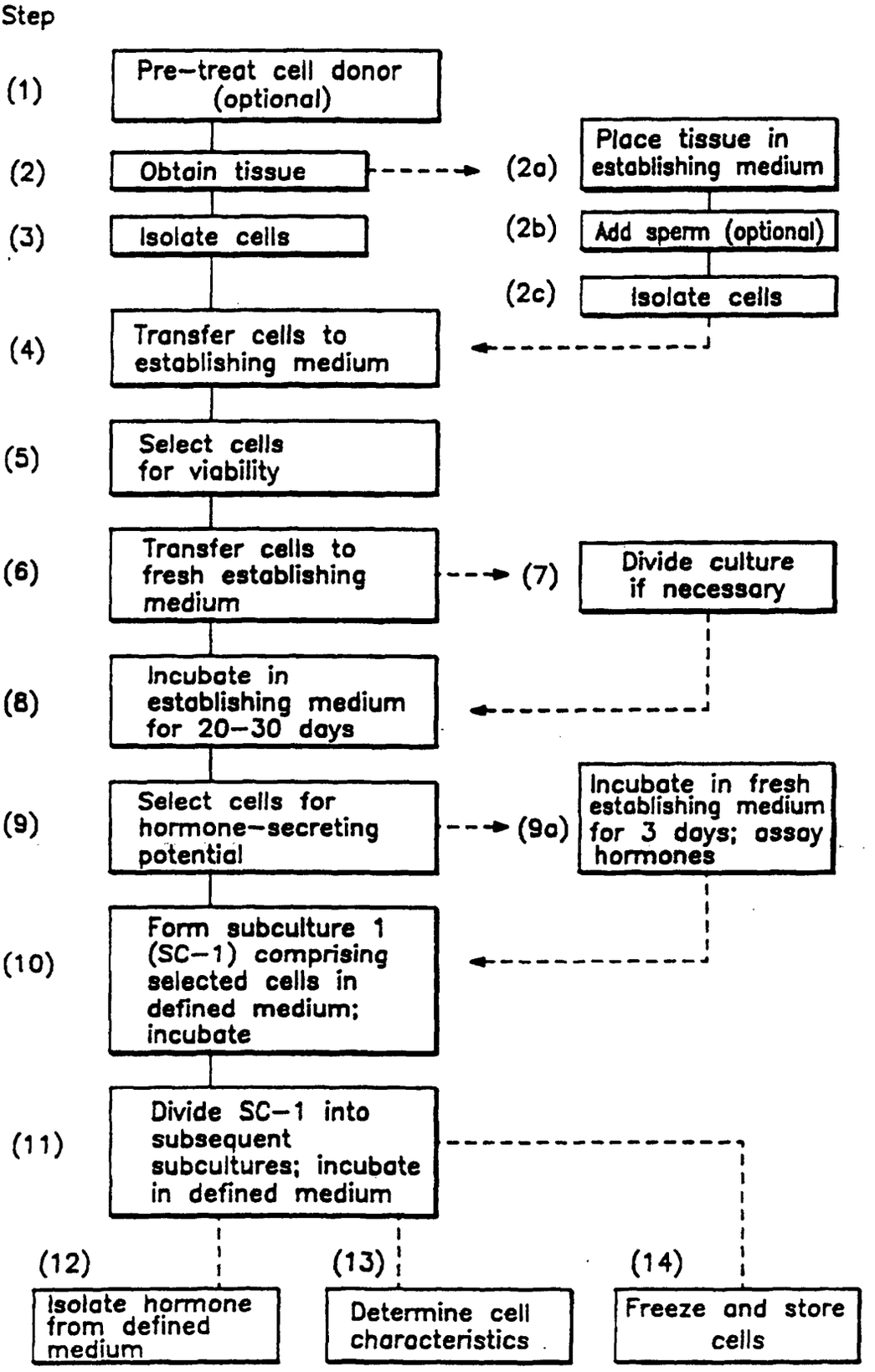
Step
(1)
Pre-treat cell donor (optional)
(2)
Obtain tissue
(2a)
Place tissue in establishing medium
(2b)
Add sperm (optional)
(2c)
Isolate cells
(3)
Isolate cells
(4)
Transfer cells to establishing medium
(5)
Select cells for viability
(6)
Transfer cells to fresh establishing medium
(7)
Divide culture if necessary
(8)
Incubate in establishing medium for 20-30 days
(9)
Select cells for hormone-secreting potential
(9a)
Incubate in fresh establishing medium for 3 days; assay hormones
(10)
Form subculture 1 (SC-1) comprising selected cells in defined medium; incubate
(11)
Divide SC-1 into subsequent subcultures; incubate in defined medium
(12)
Isolate hormone from defined medium
(13)
Determine cell characteristics
(14)
Freeze and store cells

FIG.—1

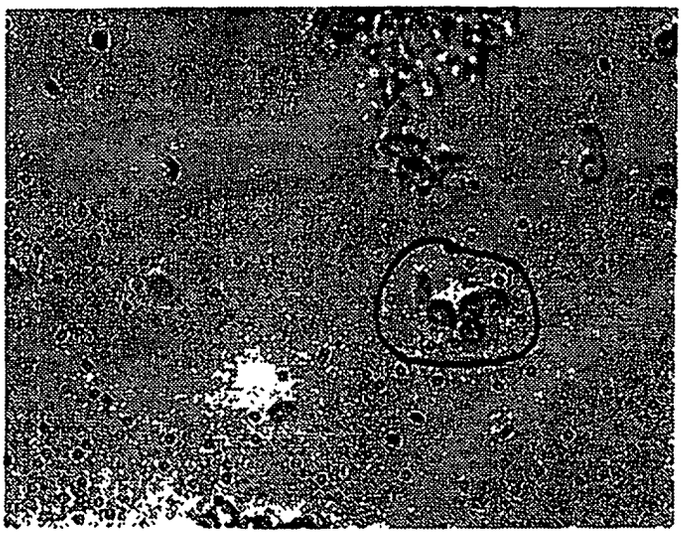

FIG.—2

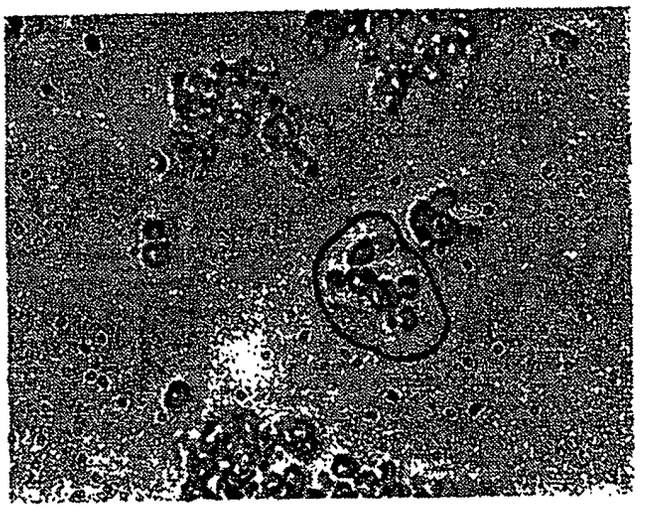

FIG.—3

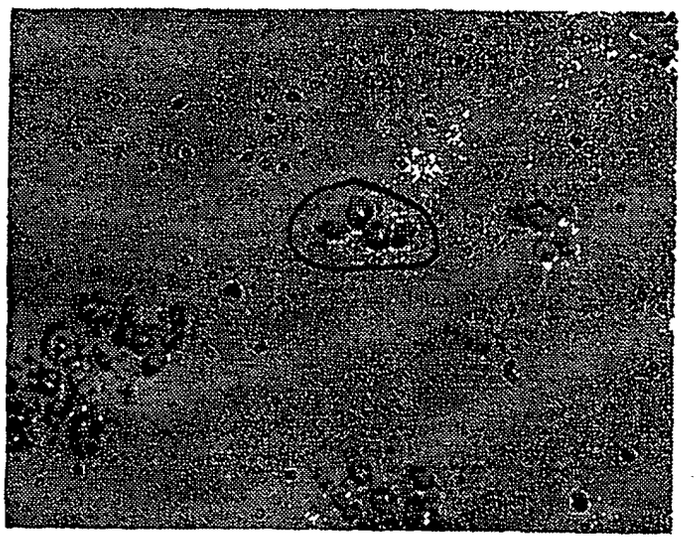

FIG.—4

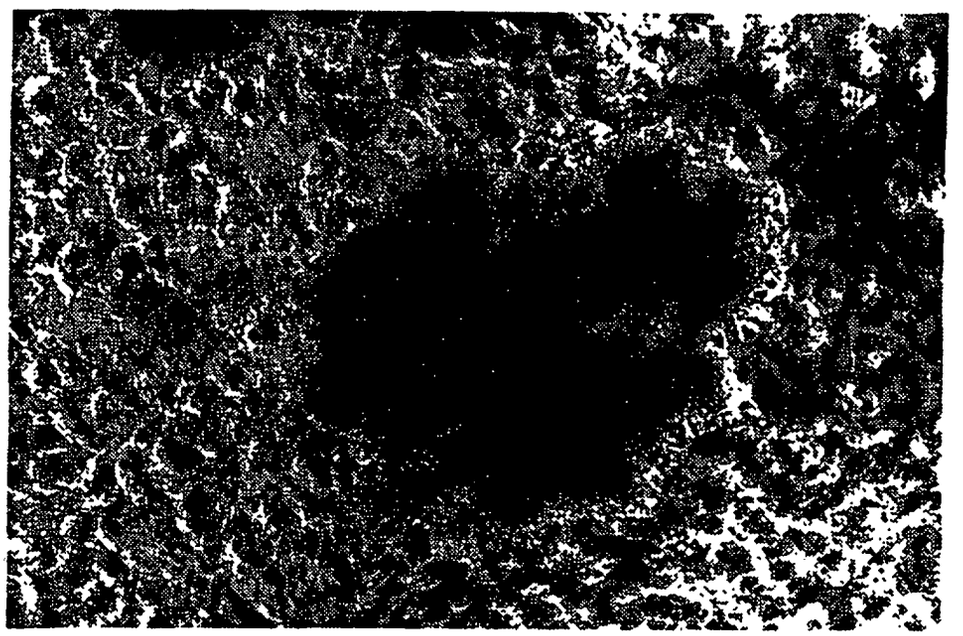

FIG.—5

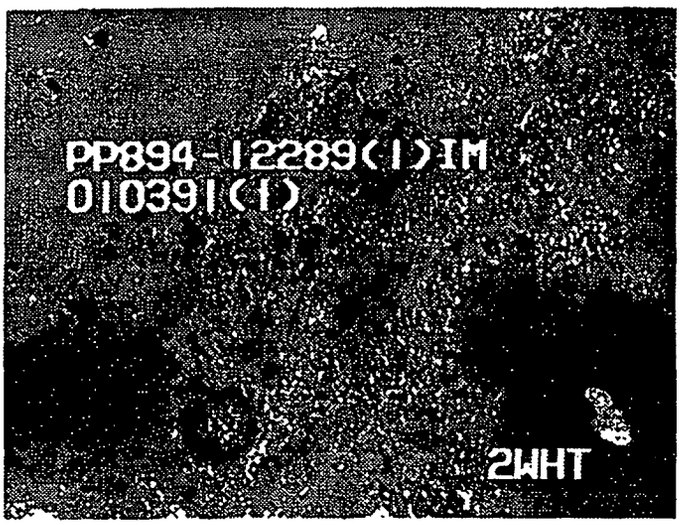

FIG.—6

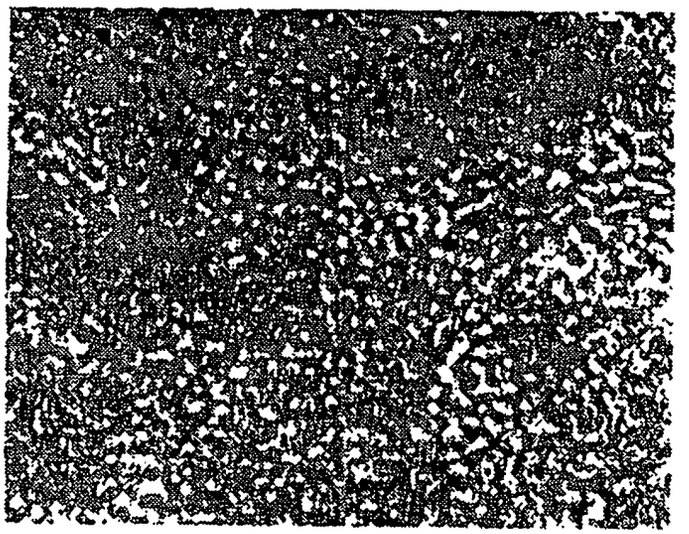

FIG.—7

FIG.—8

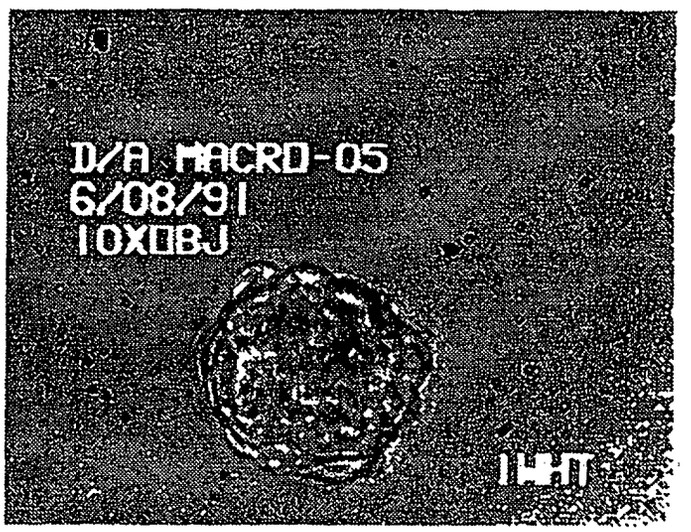

FIG.—9

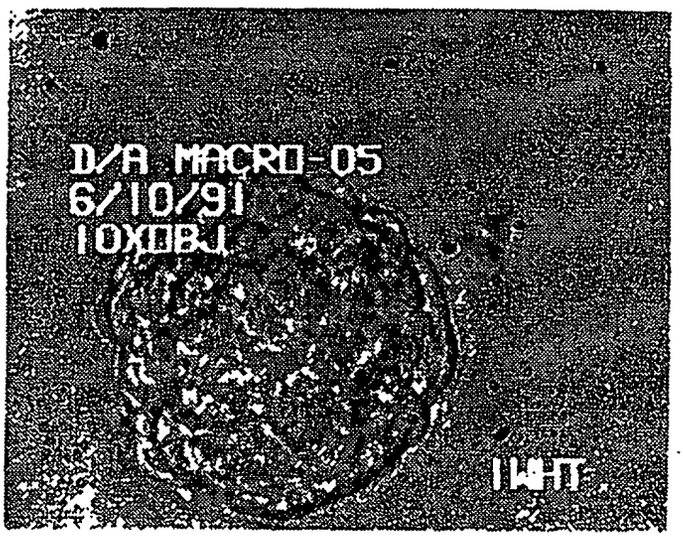

FIG.—10

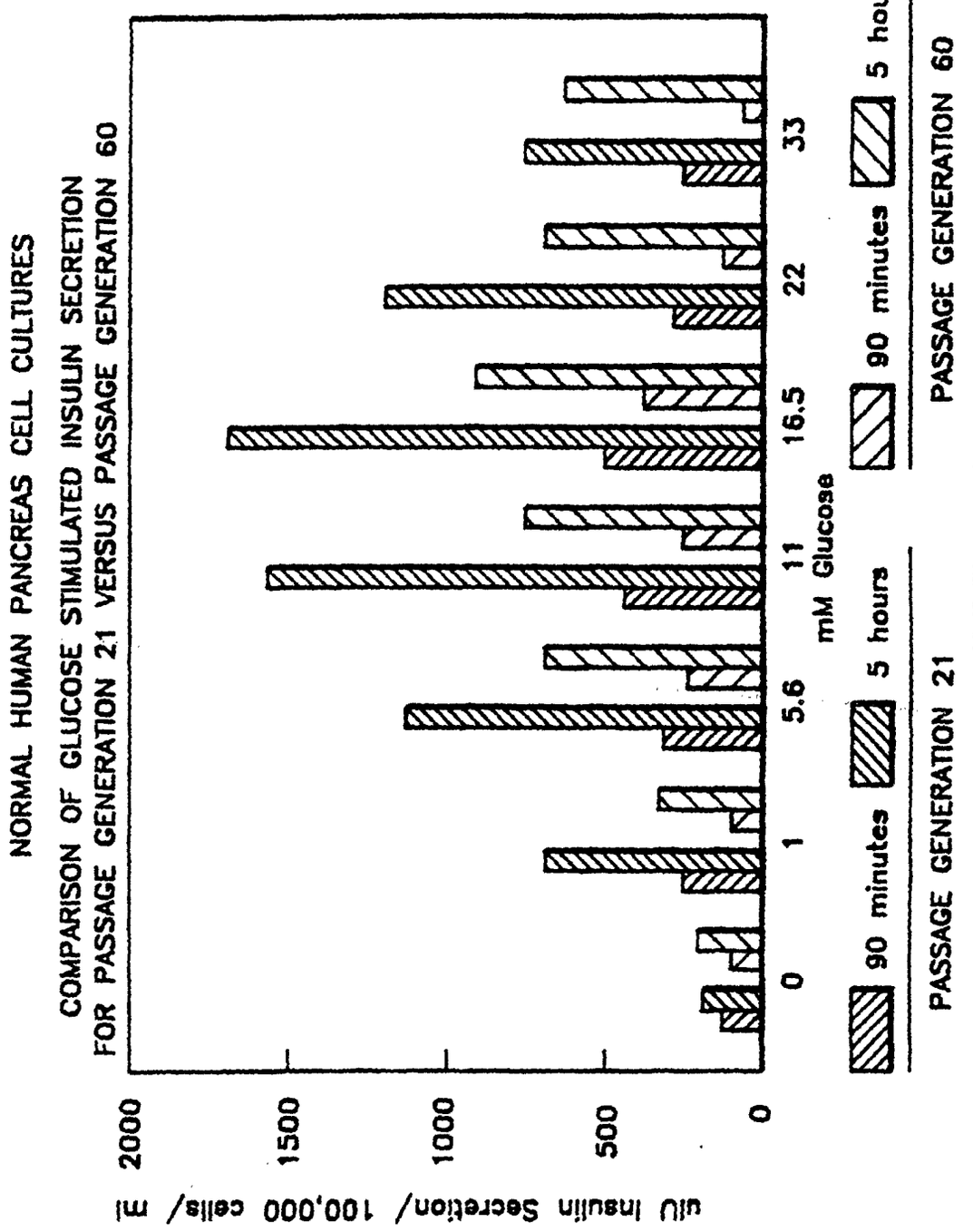
NORMAL HUMAN PANCREAS CELL CULTURES
COMPARISON OF GLUCOSE STIMULATED INSULIN SECRETION
FOR PASSAGE GENERATION 21 VERSUS PASSAGE GENERATION 60
uIU Insulin Secretion/ 100,000 cells/ ml
2000
1500
1000
500
0
0
1
5.6
11
16.5
22
33
mM Glucose
90 minutes
5 hours
PASSAGE GENERATION 21
90 minutes
5 hours
PASSAGE GENERATION 60

FIG. — 11