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(54) **Title**: MEDIA COMPOSITIONS, METHOD OF INITIATING AND ENRICHING CULTURES OF STEM CELLS AND/OR CANCER STEM-LIKE CELLS

(57) **Abstract**: The present invention provides a culture medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an effective amount wherein the culture medium is essentially serum free. The present invention further provides feeding medium comprising components similar to the culture medium except the feeding medium is devoid of methyl cellulose. Present invention still further provides cell cultures comprising stem cells or cancer stem-like cells and the culture medium of the present invention. Also provides are methods for selectively enriching and maintaining stem cells or cancer stem-like cells using the culture medium of the present invention and kits for the same.

**Title: Media Compositions, method of initiating and enriching cultures of stem cells and/or cancer stem-like cells**

**Field of Invention**

The present invention relates to media compositions and method for initiating, enriching and maintaining culture of stem cells or cancer stem-like cells. The present invention further relates to culture media of the defined composition and cell cultures comprising the same.

**Background of the Invention**

Stem cells are characterized by their ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cells types. Stem cells can be divided into two basic categories i.e. embryonic stem cells that are isolated from embryos and adult stem cells that are found in adult tissues. In embryos, stem cells differentiate to produce the various tissues during growth whereas in adult organisms stem cells and progenitor cells act as repair system for the body replenishing cells and maintain the normal regeneration of regenerative organs for example, oval cells (hepatic stem cells) that produce hepatocytes and bile duct cells, cardiac stem cells that produce cardiomyocytes, "satellite cells" and "myoblasts" produce skeletal muscle cells, neural stem cells that are found in neural tissue such as the brain and spinal cord and produce neurons and glial cells, epidermal stem cells that produce epidermal cells and hair follicle cells, progenitor cells derived from stem cells known as "hematopoietic stem cells", produce blood cells such as erythrocytes, lymphocytes and megakaryocytes.

Stem cells are therefore important in regenerative therapy to cures various diseases and heal wounds. There are protocols available to isolate and grow such stem cells for further studies and research. However, such protocols and media compositions contemplated for culturing stem cells are specific and vary as per the origin of stem

cells. Thus, necessitating the use and investing in unreasonable inventory for different media and protocols for culturing stem cells or cancer stem cells. This makes it challenging and an uneconomical proposition for researchers as well as those in the industrial set up equally causing hindrances in expanding the scope of research or utility of different types of stem cells and/or cancer stem-like cells.

Further, recently, it has been proposed that stem-like cells are found within cancerous tumors, which possesses characteristics of stem cells and are considered to be the cause of tumor formation and recurrence. Such cells are proposed to persist in tumors as a distinct population with the ability to give rise to all cell types found in cancerous tumor as well as replenish them from time to time and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeted at Cancer stem cells or cancer stem-like cells holds hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.

It is considered difficult to separate CSCs from tumors cells. Therefore, it is desirable to develop culture medium, method for obtaining cultures with enriched cancer stem cells or cancer stem-like cells and cultures comprising the same for further use. However, it is challenging to isolate, culture and enrich various types of cancer stem cells or cancer stem-like cells. This may be primarily because cancer stem-like cells differ based on their nature of origin. Cancer stem-like cells derived from breast cancer differs substantially in growth requirements for *in-vitro* culturing as opposed to cancer stem-like cell derived from epithelial tissue or prostrate tissue. Besides, most culturing media available in the market are specific to the different types of cancer cells and require addition of fetal bovine serum (FBS) which is considered as crucial component. Addition of FBS poses additional problems of contamination with pathogens and bacteria.

Thus, problems with existing media are that they are suitable for only selective stem cells proliferation and cannot be used for all cells lines for proliferation of stem cells/ stem like-cells. This creates a unique challenge in devising appropriate culture media and techniques for growth and proliferation of stem cells or cancer stem-like cells.

Thus, there is a need in the art of media composition free of fetal bovine serum (FBS), culturing protocols, which can be used mostly universally without much deviation for initiating, enriching and maintaining cultures of different types of stem cells or cancer stem-like cells as well as cell cultures comprising the same.

**Summary of the Invention**

The present invention in one aspect provides compositions for media for culturing, selectively enriching and maintaining stem cells or cancer stem-like cells which is essentially serum free.

Accordingly, in an embodiment, the present invention provides a composition for a culture medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an effective amount, wherein the composition is essentially serum free.

In another aspect the present invention provides a composition for feeding media for selectively enriching and maintaining stem cells or cancer stem-like cells.

In one of the embodiments in accordance with the above aspect, the present invention provides a composition for a feeding media for selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, and optionally growth factors or other supplementary elements in an effective amount wherein the composition is essentially serum free.

In another aspect the present invention provides a culture media for selectively enriching and maintaining stem cells or cancer stem-like cells.

In one embodiment in accordance with the above aspect, the present invention provides a culture medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an effective amount wherein the culture medium is essentially serum free.

In another aspect the present invention provides a feeding media for selectively enriching and maintaining stem cells or cancer stem-like cells.

In one embodiment in accordance with the above aspect, the present invention provides a feeding medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, and optionally growth factors or other supplementary elements in an effective amount wherein the feeding medium is essentially serum free.

In one additional aspect of the present invention there is provided a cell culture which comprises the stem cells or cancer stem-like cells and any of the culture media of the present invention.

In one embodiment in accordance with the above aspect, the present invention provides a cell culture comprising cancer stem-like cells in essentially serum free culture medium comprised of basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an amount sufficient for maintaining stem cells or cancer stem-like cells.

In one embodiment in accordance with the above aspect, the present invention provides a cell culture comprising stem cells in essentially serum free culture medium comprised of basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an amount sufficient for maintaining stem cells or cancer stem-like cells.

In another aspect the present invention provides a method for isolation and proliferation of stem cells or cancer stem-like cells.

In one embodiment in accordance with the above aspect, the present invention provides a method comprising steps of culturing the single cell suspension of cancer cells or normal cells in the culture medium of the present invention comprising the basic nutrients, heparin, gelatin and methyl cellulose at 37°C in a 5% $CO_2$ atmosphere and adding feeding medium to the growing culture intermittently to obtain spheres of stem cells or cancer stem-like cells.

In another aspect the present invention provides a kit for use in the method of the present invention.

In one embodiment in accordance with the above aspect, the present invention provides a kit comprising the culture medium or components thereof, feeding medium or components thereof, the cell culture system comprising the stem cells or cancer stem-like cells, cell culture containers. The kit may also further comprise written instructions for how to perform the cell culture method.

**Brief Description of the Drawings**

The invention is herein described with reference to the accompanying drawings. With specific reference now to the drawings, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice. The various objects, features, and advantages thereof, may best be understood by reference to the following detailed description when read with the accompanying drawings in which:

Figure 1: Shows photomicrograph of spheroids formed from the cultured MCF7 breast cancer cells as per Example 1.

Figure 2: Shows photomicrograph of spheroids formed from the cultured LNCAP prostate cancer cells as per Example 2.

Figure 3: Shows photomicrograph of spheroids formed from the cultured MCF-10A breast cancer cells as per Example 3.

Figure 4: Shows photomicrograph of spheroids formed from the cultured MDA-MB-231 breast cancer cells as per Example 4.

Figure 5: Shows photomicrograph of spheroids formed from the cultured L 929 mouse connective tissue cells as per Example 5.

Figures 6A – 6C: shows RT-PCR - gene expression analysis of spheroids of cells obtained in Example 3-5.

FIG 6A:

Upper Gel: Lane 1: Marker 1- 100bp, Lane 2: Marker 2- 1Kb, Lane 3-4: primer quantification of primers 1 & 2 FOXC2 I, Lane 5-6: Lane(3-6) primer quantification of primers 3 & 4 GADPH 1.

Lower Gel : Lane 1: Marker 1 100bp, Lane 2: Marker 2 - 1Kb, Lane 3: cDNA : negative control : 1 ul :no band, Lane 4: Enzyme negative control: no band, Lane 5: Enzyme + water negative control :no band, Lane 6: cDNA :MCF 10 A - faint band seen near the periphery of well, Lane 7: cDNA : L929 - faint band seen near the periphery of well, Lane 8: cDNA: MDA MB 231 : faint band seen near the periphery of well.

FIG 6B:

Upper Gel: Lane 1: Ladder – indicating 494bps, Lane 2: Control 1: good prominent band, Lane 3: Control 2: good prominent band, Lane 4: Negative control : enzyme negative, Lane 5: MCF 10 A: good prominent band, Lane 6: L929 : good prominent

band, Lane 7: MDA MB 231: good prominent band

Lower Gel: Lane 1: Ladder – indicating 494bps, Lane 2: Blank Lane, Lane 3: Control : good prominent band, Lane 4: Negative control: enzyme negative, Lane 5: MCF 10 A: good prominent band, Lane 6: L929 : good prominent band, Lane 7: MDA MB 231: good prominent band, Lane 8: Negative: negative .

FIG 6C:

Upper gel: Lane 1: Ladder – indicating 494bps, Lane 2: Control 2: good prominent band, Lane 3: Negative control : enzyme negative: no band seen, Lane 4: MCF 10 A: with P1/P2 2ul cDNA :Faint band, Lane 5: L929 : with P1/P2 : 2ul cDNA No band, Lane 6: MDA MB 231: with P1/P2: 2ul cDNA faint band, Lane 7: Control 2: 5ul cDNA good prominent band, Lane 8: Negative control : enzyme negative: no band seen, Lane 9: MCF 10 A: with P1/P2 5ul cDNA:Faint band, Lane 10: L929 : with P1/P2 5ul cDNA: No band, Lane 11: MDA MB 231: with P1/P2 5ul cDNA: faint band.

Lower Gel: Lane 1: Ladder – indicating 494bps, Lane 2: Control 2: good prominent band, Lane 3: Negative control : enzyme negative: no band seen, Lane 4: MCF 10 A: with P3/P4 2ul cDNA : prominent band, Lane 5: L929 : with P3/P4 : 2ul cDNA prominent band, Lane 6: MDA MB 231: with P3/P4: 2ul cDNA : prominent band, Lane 7: Control 2: 5ul cDNA good prominent band, Lane 8: Negative control : enzyme negative: no band seen, Lane 9: MCF 10 A: with P3/P4 5ul cDNA: prominent band, Lane 10: L929 : with P3/P4 5ul cDNA:Faint band, Lane 11: MDA MB 231: with P3/P4 5ul cDNA: prominent band.

**Detailed Description of the Invention**

In the following detailed description, various specific details are set forth in order to provide a better understanding of the invention. However, it will be understood by those skilled in the art that the present invention may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present

invention.

The present invention is directed towards obviating the problems associated with initiation, proliferation and maintenance of cultures of stem cells or cancer stem-like cells. More particularly the present invention is directed to selectively enriching and maintaining the population of stem cells or cancer stem-like cells and to make them accessible for further research, regenerative therapies, clinical investigations or drug screening for anticancer drug therapeutics.

Accordingly, the present invention is directed towards providing compositions for media for example culture medium and feeding medium, for culturing, selectively enriching and maintaining stem cells or cancer stem-like cells.

In an embodiment, the present invention provides a composition of a medium for selectively enriching and maintaining stem cells or cancer stem-like cells originating from cancerous and non-cancerous tissue comprising basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an effective amount wherein the composition is essentially serum free.

As used herein the phrase "serum free" refers to being devoid of a human or an animal serum.

According to some embodiments of the invention, the serum free media compositions do not comprise of serum or portions thereof.

The basic nutrients refers to a mixture comprising of salts, amino acids, vitamins that provide cells with water and certain bulk inorganic ions essential for normal cell metabolism, maintain intra- and extra-cellular osmotic balance, provide a carbohydrate as an energy source, and provide a buffering system to maintain the medium within the physiological pH range. The basic nutrients may be formulated into a base media by mixing the above mentioned nutrient components. Alternately,

basic nutrients may be incorporated based on the known basal media as such, or after modification. Non-limiting examples of basic nutrients to be incorporated based on the known basal media include Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F-12, or KO-DMEM, Minimal Essential Medium (MEM), Basal Medium Eagle (BME), BGJb Medium, RPM1 1640, Ham's F-10, Ham's F-12, α-Minimal Essential Medium (αMEM), Brinster's BMOC-3 Medium, CO2-Independent Medium, CMRL Medium, Glasgow's Minimal Essential Medium (G-MEM), Iscove's Modified Dulbecco's Medium, Waymouth's MB 752/1 Media, Williams Media E, Medium NCTC-109, neuroplasma medium, Leibovitz's L-15 Media, McCoy's 5A Media (modified), MCDB 131 Mediumor the likes and/or mixtures thereof.

According to some embodiments of the invention, gelatin is present typically from about 0.001% to about 0.01% w/v, for example from about 0.001% to about 0.5% w/v, for example from about 0.001% to about 0.05% w/v, or for example from about 0.001% to about 0.01% w/v. Preferably gelatin is incorporated in an amount from about 0.001% to about 0.01% w/v.

According to some embodiments of the invention, methyl cellulose is present typically from about 0.01% to about 10% w/v, for example from about 0.01% to about 5% w/v, for example from about 0.05 % to about 2.5% w/v, or for example from about 0.05 % to about 1.5% w/v.

According to some embodiments of the invention, the composition further comprises heparin. According to some embodiments the concentration of said heparin is from about 0 ng/ml to about 100 ng/ml, for example from about 2 ng/ml to about 50 ng/ml, for example from about 2.5 ng/ml to about 25 ng/ml, for example from about 5 ng/ml to about 15 ng/ml, for example from about 4 ng/ml to about 10 ng/ml, for example from about 4 ng/ml to about 8 ng/ml.

The composition of a medium for selectively enriching and maintaining stem cells or cancer stem-like cells of the present invention further comprises growth factors selected from the group consisting of but not limited to, members of the epidermal growth factor family (EGFs), members of the fibroblast growth factor family (FGFs), members of the hydrocortisone growth factor family, B27 supplement, insulin and/or other suitable growth factors.

Preferably, one or more growth factors and insulin to be incorporated in the composition are recombinantly produced molecules. Alternately, they may be isolated from natural sources. With regard to the growth factors and insulin proteins, the invention also contemplates the use of homologs, or proteins having sequence identity of at least about 70% and the receptor activating activity of the respective naturally occurring protein that is growth factors or insulin, artificial analogs, polypeptide fragments that activate the respective growth factors or insulin receptor and/or downstream signaling, and other molecules that activate one or more growth factors or insulin receptors and/or their downstream signaling.

According to some embodiments of the invention, the composition comprises Basic fibroblast growth factor (also known as bFGF, FGF2 or FGF-β) a member of the fibroblast growth factor family. In some embodiments the concentration of said bFGF is from about 5 ng/ml to about 100 ng/ml, for example from about 10 ng/ml to about 50 ng/ml, for example from about 10 ng/ml to about 25 ng/ml, for example from about 15 ng/ml to about 25 ng/ml.

According to some embodiments of the invention, the composition comprises Epidermal growth factor (also known as EGF). In some embodiments the concentration of said EGF is from about 1 ng/ml to about 100 ng/ml, for example from about 2.5 ng/ml to about 50 ng/ml, for example from about 2.5 ng/ml to about 25 ng/ml, for example from about 5 ng/ml to about 15 ng/ml.

According to some embodiments of the invention, the composition comprises insulin. In some embodiments the concentration of said insulin is from about 1 µg/ml to about 100 µg/ml, for example from about 2.5 µg/ml to about 50 µg/ml, for example from about 2.5 µg/ml to about 25 µg/ml, for example from about 5 µg/ml to about 15 µg/ml.

According to some embodiments of the invention, the composition comprises hydrocortisone. In some embodiments the concentration of said hydrocortisone is at least about 0.1 µg/ml, for example at least about 2.5 µg/ml, for example at least about 5 µg/ml, for example at least about 7.5 µg/ml, for example at least about 1 µg/ml.

According to some embodiments of the invention, the composition comprises B27 supplement. One of the commercially available B27 supplement is without vitamin A which is available from Gibco-Invitrogen, Corporation, Grand Island, NY USA, Catalogue No. 12587-010. The B27 supplement is a serum-free formulation which includes d-biotin, fatty acid free fraction V bovine serum albumin (BSA), catalase, L-carnitine HC1, corticosterone, ethanolamine HC1, D-galactose (Anhyd.), glutathione (reduced), recombinant human insulin, linoleic acid, linolenic acid, progesterone, putrescine-2-HCl, sodium selenite, superoxide dismutase, T- 3/albumin complex, DL alpha-tocopherol and DL alpha tocopherol acetate. However, the use of B27 supplement is limited since it includes albumin from an animal source. When incorporated, it may be included at the concentration of 1X to 5X.

The composition may further comprise of supplementary nutrients for example L-glutamine or stable glutamine. The composition of a medium according to the invention can also be supplemented with any compound(s) that will not interfere with, and preferably supports the enrichment and/or maintenance of stem cells or cancer stem-like cells over time. Preferred examples of such compounds include

non-essential amino acids, anti-oxidants, reducing agents, vitamins, organic compounds, inorganic salts, transferring, and albumins.

Preferably, all ingredients included in the composition of the present invention are substantially pure, with a tissue culture grade.

In one embodiment, the present invention provides a culture medium. The culture medium for culturing, selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an effective amount wherein the culture medium is essentially serum free.

As used herein the phrase "culture medium" refers to a solid or a liquid substance used to support the growth of stem cells and selectively enriching and maintaining stem cells or cancer stem-like cells. Preferably, the phrase "culture medium" as used herein refers to a liquid substance.

In one of the preferred embodiments the present invention provides a medium for initiating, selectively enriching and maintaining stem cells or cancer stem-like cells originating from cancerous and non-cancerous cells comprising gelatin, heparin, the basic nutrients comprising of an approximately 50:50 mixture of DMEM and Ham's F12 nutrients, L- glutamine or stable glutamine, basic Fibroblast Growth Factor- FGF, Epidermal Growth Factor –EGF, recombinant growth factor Insulin, B27 supplement, and hydrocortisone. The medium when used as a culture medium is supplemented with methyl cellulose prior to use. The medium may be used as such, as a feeding medium.

In another preferred embodiments the present invention provides a medium for initiating, selectively enriching and maintaining stem cells or cancer stem-like cells originating from cancerous and non-cancerous cells comprising gelatin present at concentration from about 0.001% to about 0.01% w/v, methyl cellulose present at

concentration from about 0.05 % to about 1% w/v, heparin present at the concentration from about 4 ng/ml to about 8 ng/ml, the basic nutrients comprising of an approximately 50:50 mixture of DMEM and Ham's F12 nutrients, basic Fibroblast Growth Factor- bFGF present at concentration from about 15 ng/ml to about 25 ng/ml, Epidermal Growth Factor –EGF present at concentration from about 5 ng/ml to about 15 ng/ml, Insulin present at concentration from about 5 µg/ml to about 15 µg/ml, B27 supplement 1X to 5X, and hydrocortisone present at concentration of at least about 1 µg/ml. The medium when used as a culture medium is supplemented with prior to use. The medium may be used as such, as a feeding medium.

The normal cells as well as cancer cells of various origins may be used in the present invention as a source of stem cells or cancer stem-like cells. In one of the embodiments, stem cells or cancer stem-like cells of the present invention are derived from a cell line.

In another embodiment, stem cells or cancer stem-like cells of the present invention are derived from a primary cell culture.

In another embodiment, stem cells of the present invention are derived using a well known protocol from blood, or from tissues or from various organs comprising the stem cells such tissues or organs may be selected from but not limiting to liver, bile duct, cardiac tissue, skeletal muscles, neural tissue from brain and spinal cord, epidermal tissue, skin, hair follicle or any other organ or tissue comprising the stem cells.

In another embodiment, the primary cell culture comprising cancer stem-like cells is derived from a tumor or cell metastasis.

In another embodiment, tumors and cell metastasis are derived from but not limited to: prostate cancer, breast cancer, carcinoid tumor, carcinoma, cervical cancer,

colon cancer, endometrial cancer, esophageal cancer, extrahepatic bile duct cancer, ewings family of tumors (pnet), extracranial germ cell tumor, eye cancer, intraocular melanoma, gallbladder cancer, gastric cancer, germ cell tumor, extragonadal gestational trophoblastic tumor, head and neck cancer, hypopharyngeal cancer, islet cell carcinoma, laryngeal cancer, adrenocortical carcinoma, anal cancer, bladder cancer, brain tumor, brain stem glioma, brain tumor, cerebellar astrocytoma, cerebral astrocytoma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal, pineal tumors, hypothalamic glioma, leukemia, acute lymphoblastic, leukemia, oral cavity cancer, liver cancer, lung cancer, small cell, lymphoma, AJDS- related, lymphoma, central nervous system (primary), lymphoma, cutaneous T-cell, lymphoma, hodgkin's disease, non-hodgkin's disease, malignant mesothelioma, melanoma, merkel cell carcinoma, metasatic squamous carcinoma, multiple myeloma, plasma cell neoplasms, mycosis fungoides, myelodysplastic syndrome, myeloproliferative disorders, nasopharyngeal cancer, neuroblastoma, oropharyngeal cancer, osteosarcoma, ovarian epithelial cancer, ovarian germ cell tumor, ovarian low malignant potential tumor, pancreatic cancer, exocrine, pancreatic cancer, islet cell carcinoma, paranasal sinus and nasal cavity cancer, parathyroid cancer, penile cancer, pheochromocytoma cancer, pituitary cancer, plasma cell neoplasm, rhabdomyosarcoma, rectal cancer, renal cell cancer, salivary gland cancer, sezary syndrome, skin cancer, cutaneous T-cell lymphoma, skin cancer, kaposi's sarcoma, skin cancer, melanoma, small intestine cancer, soft tissue sarcoma, soft tissue sarcoma, testicular cancer, thymoma, malignant, thyroid cancer, urethral cancer, uterine cancer, sarcoma, unusual cancer of childhood, vaginal cancer, vulvar cancer, or wilms' tumor.

In one additional aspect of the present invention there is provided a cell culture which comprises the stem cell or cancer stem-like cells and any of the culture media described hereinabove.

The stem cell or cancer stem-like cells or cell cultures comprising the same are capable of keeping their self-renewal potential during 1-100 passages of in-vitro cultivation. In another embodiment, stem cell or cancer stem-like cells are capable of keeping their self-renewal potential during 1-90 passages of in-vitro cultivation. In another embodiment, stem cell or cancer stem-like cells are capable of keeping their self-renewal potential during 20-60 passages of in-vitro cultivation.

In another embodiment of the present invention, a method is provided for isolation and proliferation of stem cells or cancer stem-like cells, in which, a method comprises steps of culturing the single cell suspension of cancer cells or normal cells in culture medium as per the present invention comprising the basic nutrients, heparin, gelatin and methyl cellulose at 37°C in a 5% $CO_2$ atmosphere and adding feeding medium to the growing culture intermittently to obtain spheres of stem cells or cancer stem-like cells. The propagation time for generation of spheroids of stem cells or cancer stem-like cells by the culture method and using the culture medium with methyl cellulose as well as feeding medium of the present invention is shorter compared to the other known protocols. Spheres or spheroids of stem cells or cancer stem-like cells may be obtained by the method and media of the present invention in 5-10 days depending upon the type of cells / cell line and cell density plated. Unless stated otherwise the terms "spheroid" and "spheres" are used interchangeably herein and refer to a certain "ball-shaped" globular structure, consisting of more than a single cell that has initially developed from a single or from multiple cells.

The single cell suspension of the normal cells or cancer cells is cultured as per the present invention method at an appropriate cell density. The cell density may range from about $1X10^4$ cells /ml to $1X10^6$ cells/ml. For obtaining the single cell suspension of normal cells or cancer cells for culturing as per the method of the present invention, normal cells or cancer cells are expanded by incubating them in a complete minimal essential medium supplemented with antibiotic, vitamins and

serum for period of 24-48 hours at about 37 degree centigrade in a 5% $CO_2$ atmosphere. On attaining the 90% confluency, cells are washed twice with DPBS and trypsinised using 0.25% trypsin-EDTA at $37^0$C in a 5% $CO_2$ atmosphere for 2-5 minutes and cells are dissociated into a single cell suspension, which are initially cultured in a 96 well non-treated plate with the medium as per the present invention.

It will be appreciated that although single-cell suspensions of stein cells are usually seeded, small clusters may also be used. While using the small clusters, enzymatic digestion (such as with type IV collagenase) utilized for cluster disruption is terminated before stem cells become completely dispersed and the cells are triturated with a pipette such that clumps (i.e., 10-200 cells) are formed. However, measures are taken to avoid large clusters which may cause cell differentiation.

For further enriching the stem cells or cancer stem-like cells, the spheres or spheroids comprising stem cells or cancer stem-like cells are isolated from the primary growing sphere cultures and passaged in the culture medium of the present invention, further supplemented periodically with the feeding medium to obtain large spheres comprising of stem cells or cancer stem-like cells. In one of the embodiments, the methods of the present invention provide that stem cells or cancer stem-like cells can be passaged without loosing their respective phenotype for at least 5 passages. In other embodiments, the methods of the present invention provide that stem cells or cancer stem-like cells can be passaged without loosing their respective phenotype for at least 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 passages.

According to some embodiments of the invention, when cultured according to the teachings of the present invention, the growth of the stem cells or cancer stem-like cells is monitored. Monitoring the formation of spheres or spheroids comprising stem cells or cancer stem-like cells is within the capabilities of those skilled in the art

and can be effected by morphological evaluations and determination of expression of differentiation-specific markers [e.g., using immunological techniques or RNA-based analysis (e.g., RT-PCR, cDNA microarray)].

In some embodiments, the growth of the stem cells or cancer stem-like cells is monitored to determine their differentiation state. The differentiation state can be determined using various approaches including, for example, morphological evaluation (e.g., as shown in Figures 1-5) and/or detection of the expression pattern of typical markers of the undifferentiated state using immunological techniques such as flow cytometry for membrane-bound markers, immunohistochemistry or immunofluorescence for extracellular and intracellular markers and enzymatic immunoassay, for secreted molecular markers. For example, the level of transcripts of specific markers e.g., FOXC2 or undifferentiation markers e.g., Oct 4, Nanog, Sox2, Rexl, Cx43, FGF4 or differentiation markers e.g., albumin, glucagons, -cardiac actin, β-globulin, Flkl, AC133 and neurofilament can be detected using RNA-based techniques such as RT-PCR analysis and/or cDNA microarray analysis.

In one additional embodiment, the present invention provides a kit comprising the culture medium or components thereof, feeding medium or components thereof, the cell culture system comprising the normal cells or stem cells or cancer cells or cancer stem-like cells, cell culture containers. Optionally, the kit also comprises written instructions for how to perform the cell culture method.

Compositions of the culture and feeding media, the use of the culture and feeding media of the present invention and the method of culturing as per the present invention allows selectively enriching and maintaining stem cells or cancer stem-like cells in liquid suspension as spheroids and thereby provides ease in separating said stem cells or cancer stem-like cells from differentiated cells.

In particular, the present invention employs passaging suspended cells in liquid serum-free medium to enrich the cells with non-differentiated cells. Additionally, the system is an ideal *in vivo* mimicking study model for stem cells or cancer stem-like cells.

The compositions of media and methods of culturing of the present invention provides advantages of a well-defined culture system for obtaining spheroids of stem cells or cancer stem-like cells of different origin; shorter culture cycle; reduced exposure to pathogens and ability for the isolation and proliferation of stem cells or cancer stem-like cells. Due to the serum-free nature of the media as per the present invention toxic effects of serum are avoided. Further, sensitive proteins are not degraded by serum proteases. Also, downstream processing of products from such cultures of stem cells or stem-like cells is easier.

The selectively enriched stem cells or cancer stem-like cells or cell cultures comprising the same as obtained by the present invention can provide a means of exploring basic mechanisms in cancer cell biology and disease, the same can be used advantageously in cancer stem cell research, clinical research and drug screening of anticancer therapeutics. The selective enrichment and maintains of stem cells or cancer stem-like cells from a particular tumor or metastatic lesion is useful, for example, in diagnosing a pathology and/or developing a rational therapeutic treatment that targets a developing pathology. In some instances, enrichment and maintains of stem cells or cancer stem-like cells is desirable for further in-vitro studies exploring physiological and molecular mechanisms, wherein in other instances, these cells can be used to inoculate a test animal for further studies of cancer progression or therapy.Effects of the test compound on cancer stem cells can also be measured for example by determining the number of cancer stem cells that persist in culture or in the tumors in vivo after treatment with the test compound. In addition to determining the number of cancer stem cells, the effects of the test compound on cancer stem cells, cell cycle status and marker expression can also be

determined by flow-cytometry.

The invention will be further described by way of examples providing specific details thereof. It should be noted that the examples are only for the purpose of illustration and do not in any way limit the scope of the invention. The various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above description illustrate the invention in a non limiting fashion. Many changes and modifications may be made within the scope of the embodiments and examples disclosed herein without departing from the true spirit of the invention. The invention herein includes all such modifications.

Example 1:

Cells from breast cancer cell line MCF 7 were expanded in Minimal essential Medium MEM containing 1X antibiotic, L - glutamine and 10 % FBS in a cell culture plate and incubated at 37°C in a 5% CO2 atmosphere. After attaining 90% confluency the cells were washed twice with PBS and trypsinised with 0.25% trypsin -EDTA. Later the cells were dissociated into single cells using a trypsin solution and 100ul of cell suspension containing a pre-defined number of (1000) cells were seeded into 96 well low attachment plates in a culture medium comprising of basic nutrients of DMEM:F12 medium emulsified with 1% methyl cellulose and further supplemented with gelatin 0.001%, heparin 8ng/ml, and other growth factors including stable glutamine 1 %, Fibroblast Growth Factor- FGF 20ng/ml, Epidermal Growth Factor -EGF 10ng/ml, recombinant Insulin 10ug/ml, growth factor B27- 1X and hydrocortisone 1ug/ml, and incubated at 37°C in a 5% $CO_2$ atmosphere. Feeding

medium comprising of all the constituents of the culture medium except methyl cellulose was added at three days intervals.

The cell culture split up into adherent cells and non-adherent cells during growth and enrichment, wherein the non-adherent cells developed close packed and hollow acinaric spheres. After spheres formation was visibly detected, they were isolated, passaged and newly seeded into the culture medium initially and further supplemented with feeding medium periodically. Wherein, the composition of the culture medium and the feeding medium was the same as disclosed above for culturing and feeding. It was observed that after such passaging, again new spheres were formed. The obtained spheres were dissociated enzymatically with 0.25% trypsin-EDTA and seeded onto a 96 well plate to a density of an average of 1000 cells per well and incubated at 37°C in a 5% CO2 atmosphere. This gave large spheroids comprising of breast cancer stem cells or cancer stem-like cells as seen in FIG. 1.

Example 2:

Cells form LNCaP prostate cancer cell line were firstly expanded in Rose Parker Memorial Institute 1640 (RPMI 1640) containing 2mM sodium Pyruvate, 0.075% sodium bicarbonate, 1X antibiotic, 2 mM L-glutamine and 10 % FBS in a cell culture plate 100mm and incubated for 24-48 hrs at 37°C in a 5% CO2 atmosphere. After attaining 90% confluency, the cells were washed twice with PBS and trypsinised with 0.25% trypsin - EDTA. Later the cells were dissected into single cells using a trypsin solution and 100ul of cell suspension containing $2.5x10^4$ /ml cells were seeded into 96 well low attachment plates with the medium as per the present invention comprising of basic nutrients of DMEM:F12 medium emulsified with 1% methyl cellulose and further supplemented with gelatin 0.001%, heparin 4 ng/ml, and other growth factors including stable glutamine 1 %, Fibroblast Growth Factor- FGF 15 ng/ml, Epidermal Growth Factor -EGF 12 ng/ml, recombinant Insulin 15 ug/ml,

growth factor B27- 0.5X and hydrocortisone 2 ug/ml, and incubated at 37°C in a 5% $CO_2$ atmosphere. Feeding medium comprising of all the constituents of the culture medium except methyl cellulose was added at three days intervals.

At around 5-10 days formation of spheres was detectable. Spheres were extracted, passaged three times and newly seeded into the culture medium initially and further supplemented with feeding medium periodically, wherein the composition of culture medium and feeding medium was the same as disclosed above for culturing and feeding. It was observed that after such passaging again new spheres were formed. The obtained spheres were dissociated enzymatically with 0.25% trypsin-EDTA and seeded onto a 96 well plate to a density of an average of 1000 cells per well and incubated at 37°C in a 5% CO2 atmosphere. This gave large spheroids comprising of prostate cancer/ stem cells or cancer stem-like cells as seen in FIG. 2.

Example 3:

Cells from breast cancer cell line MCF-10A were expanded in DMEM/F12 500 ml, supplemented with FBS 10%, 100µl EGF (100 mg/ml), 250µl Hydrocortizone (1mg/ml), 50µl CholeraToxin (1mg/ml), 500µl Insulin (10mg/ml and 5.0ml Pen/Strep in a cell culture plate and incubated at 37°C in a 5% CO2 atmosphere. After attaining 90% confluency the cells were washed twice with PBS and trypsinised with 0.25% trypsin -EDTA. Later the cells were dissociated into single cells using a trypsin solution and 100ul of cell suspension containing a pre-defined number of (1000) cells were seeded into 96 well low attachment plates in a culture medium comprising of basic nutrients of DMEM:F12 medium emulsified with 1% methyl cellulose and further supplemented with gelatin 0.001%, heparin 3 ng/ml, and other growth factors including stable glutamine 1 %, Fibroblast Growth Factor- FGF 16 ng/ml, Epidermal Growth Factor -EGF 8 ng/ml, recombinant Insulin 12 g/ml, growth factor B27- 1X and hydrocortisone 1ug/ml, and incubated at 37°C in a 5% CO2

atmosphere.

The cell culture split up into adherent cells and non-adherent cells during growth and enrichment, wherein the non-adherent cells developed close packed and hollow acinaric spheres. At around 5-10 days formation of spheres was detectable. Spheres were extracted, isolated, passaged and newly seeded into the culture medium initially and further supplemented with feeding medium periodically. Wherein, the composition of the culture medium and the feeding medium was the same as disclosed above for culturing and feeding. It was observed that after such passaging, again new spheres were formed. The obtained spheres were dissociated enzymatically with 0.25% trypsin-EDTA and seeded onto a 96 well plate to a density of an average of 1000 cells per well and incubated at 37°C in a 5% CO2 atmosphere. This gave large spheroids comprising of breast cancer stem cells or cancer stem-like cells as seen in FIG. 3.

Example 4:

Cells from breast cancer cell line MDA-MB-231 were expanded in DMEM/F12 500 ml, supplemented with containing 1X antibiotic, L - glutamine and 10 % FBS in a cell culture plate and incubated at 37°C in a 5% CO2 atmosphere. After attaining 90% confluency the cells were washed twice with PBS and trypsinised with 0.25% trypsin -EDTA. Later the cells were dissociated into single cells using a trypsin solution and 100ul of cell suspension containing a pre-defined number of (1000) cells were seeded into 96 well low attachment plates in a culture medium comprising of basic nutrients of DMEM:F12 medium emulsified with 1% methyl cellulose and further supplemented with gelatin 0.001%, heparin 5 ng/ml, and other growth factors including stable glutamine 1 %, Fibroblast Growth Factor- FGF 20 ng/ml, Epidermal Growth Factor -EGF 10 ng/ml, recombinant Insulin 14 ug/ml, growth factor B27- 1X and hydrocortisone 1ug/ml, and incubated at 37oC in a 5% CO2 atmosphere.

The cell culture split up into adherent cells and non-adherent cells during growth and enrichment, wherein the non-adherent cells developed close packed and hollow acinaric spheres. At around 5-10 days formation of spheres was detectable. Spheres were extracted, isolated, passaged and newly seeded into the culture medium initially and further supplemented with feeding medium periodically. Wherein, the composition of the culture medium and the feeding medium was the same as disclosed above for culturing and feeding. It was observed that after such passaging, again new spheres were formed. The obtained spheres were dissociated enzymatically with 0.25% trypsin-EDTA and seeded onto a 96 well plate to a density of an average of 1000 cells per well and incubated at 37°C in a 5% CO2 atmosphere. This gave large spheroids comprising of breast cancer stem cells or cancer stem-like cells as seen in FIG. 4.

Example 5:

Cells from mouse connective tissue cell line L929 were expanded in DMEM supplemented with 1% Penicillin Streptomycin / L-glutamine and 10% heat inactivated Fetal Bovine Serum at 56°C for 30 min. and 7.5% CO2 atmosphere. After attaining 90% confluency the cells were washed twice with PBS and trypsinised with 0.25% trypsin -EDTA. Later the cells were dissociated into single cells using a trypsin solution and 100ul of cell suspension containing a pre-defined number of (1000) cells were seeded into 96 well low attachment plates in a culture medium comprising of basic nutrients of DMEM:F12 medium emulsified with 1% methyl cellulose and further supplemented with gelatin 0.001%, heparin 6 g/ml, and other growth factors including stable glutamine 1 %, Fibroblast Growth Factor- FGF 18 ng/ml, Epidermal Growth Factor - EGF 8ng/ml, recombinant Insulin 13 ug/ml, growth factor B27- 1X and hydrocortisone 1ug/ml, and incubated at 37°C in a 5% CO2 atmosphere. $CO_2$ atmosphere. Feeding medium comprising of all the

constituents of the culture medium except methyl cellulose was added at three days intervals.

The cell culture split up into adherent cells and non-adherent cells during growth and enrichment, wherein the non-adherent cells developed close packed and hollow acinaric spheres. At around 5-10 days formation of spheres was detectable. Spheres were extracted, isolated, passaged and newly seeded into the culture medium initially and further supplemented with feeding medium periodically. Wherein, the composition of the culture medium and the feeding medium was the same as disclosed above for culturing and feeding. It was observed that after such passaging, again new spheres were formed. The obtained spheres were dissociated enzymatically with 0.25% trypsin-EDTA and seeded onto a 96 well plate to a density of an average of 1000 cells per well and incubated at 37°C in a 5% CO2 atmosphere. This gave large spheroids comprising of breast cancer stem cells or cancer stem-like cells as seen in FIG. 5.

Example 6:

RT-PCR Analysis:

10-14 day-old spheres were harvested for RNA isolation and cDNA synthesis.

RT-PCR of cells cultured on 3-D culture systems - Total RNA was isolated from 10-14 day-old spheres on three-dimensional culture systems in the culture media having composition as mentioned above in examples 3, 4 and 5 respectively for respective cell types MCF10A, MDA MB 231 and L929. RNA was isolated by following protocol provided in instruction manual pages 25 – 30 of Qiagen RneasyMini Kit. cDNA was synthesized as per the protocol provided in instruction manual of PureExtreme® by Fermentas Life Science.

Primer sets used were:

Primer 1 :hFOXC2 (Forward) : 100mM stock- GCCTAAGGACCTGGTGAAGC

Primer 2 :hFOXC2 (Reverse) : 100mM stock-TTGACGAAGCACTCGTTGAG

Primer 3 :hGADPH (Forward): 100mM stock- ACCCAGAAGACTGTGGATGG

Primer 4 :hGADPH( (Reverse) : 100mM stock-TCTAGACGGCAGGTCAGGTC

The primers were quantified upon re-constitution and appropriate enzyme and positive and negative controls were used in the PCR assay.. RNA was extracted from breast and prostrate cancer stem cells harvested in the existing medium, using the RNeasy Mini Kit with an on-column DNase I digest (Qiagen). First-strand cDNA was synthesized from 1 µg total RNA, which was synthesized in a 20 µl reaction by reverse transcription using SuperScript II (Fermentas). The following conditions for cDNA synthesis were applied: 37°C for 60 minutes and cooling for 1 minute at 4°C, then 42°C for 50 minute and 72°C for 15 minutes. In a 50 µl PCR reaction mixture, 2 µl cDNA (total 100 ng RNA) were amplified in eppendorf master cycler with incubation at 94°C for 3 minutes, followed by 30 to 40 cycles of a three temperature program of 30sec at 94°C, 30 seconds at 58°C, and 45 seconds at 72°C. The PCR reaction was terminated after a 10 minute extension at 70°C and stored at 4°C until analysis.. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control (Table 2). All inhouse primers were synthesized by SIGMA. PCR products were size- fractionated using 1 % agarose gel electrophoresis using ethidium bromide and 10X orange G dye. DNA markers were used to confirm the size of the resultant fragments. For quantitative PCR (Q-PCR), densitometry of tested genes was normalized to GAPDH. Three repeats were conducted for each tested line.

Results :

As seen in Gel picture (FIG. 6A) primer quantification shows that intact primers were used for PCR.

As seen in gel picture (FIG. 6B) GADPH primer P3 & P4 (Fermentas) as well as P1 & P2 designed in-house helped in normalization of mRNA used in assay/ served as good house keeping genes for all the cell lines.

As seen in gel picture (FIG. 6C) FOXC2 results show that FoxC2 expression which is prominent in cancer stem-like cells is highly expressed in human breast cell line MDA MB 231 and non transformed breast cell line MCF 10 A cultured using the media and the method of the present invention.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. Thus, it is understood that any dependent claim among the appended claims merely represents particular embodiments within the scope of the subject matter bounded by the claim(s) from which the claim depends, and the inventors reserve the right to pursue subject matter that is within the scope of a more broad claim but is not specifically recited in an appended claim.

**Claims :**

1. A culture medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising basic nutrients, gelatin, methyl cellulose and optionally growth factors or other supplementary elements in an effective amount wherein the culture medium is essentially serum free.
2. The culture medium as claimed in claim 1, wherein said gelatin is present from about 0.001% to about 0.01% w/v
3. The culture medium as claimed in claim 1, wherein said methyl cellulose is present from about 0.01% to about 10% w/v,
4. The culture medium as claimed in claim 1, wherein said medium further comprises heparin at concentration from about 0 ng/ml to about 100 ng/ml.
5. The culture medium as claimed in claim 1, wherein said medium further comprises growth factors selected from the group consisting of but not limited to, members of the epidermal growth factor family, members of the fibroblast growth factor family (FGFs), members of the hydrocortisone growth factor family, B27 supplement and insulin.
6. The culture medium as claimed in claim 5, wherein said fibroblast growth factor family member is bFGF present at concentration from about 5 ng/ml to about 100 ng/ml.
7. The culture medium as claimed in claim 5, wherein said epidermal growth factor family member is epidermal growth factor (EGF) present at concentration from about 1 ng/ml to about 100 ng/ml.
8. The culture medium as claimed in claim 5, wherein said insulin is present at concentration from about 1 µg/ml to about 100 µg/ml.
9. The culture medium as claimed in claim 5, wherein said hydrocortisone growth factor family member is hydrocortisone present at concentration of least about 0.1 µg/ml.

10. The culture medium as claimed in claim 1, wherein said basic nutrients are in the form of basal medium selected from but not limiting to Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F-12, or KO-DMEM, Minimal Essential Medium (MEM), Basal Medium Eagle (BME), BGJb Medium, RPM1 1640, Ham's F-10, Ham's F-12, α-Minimal Essential Medium (αMEM), Brinster's BMOC-3 Medium, CO2-Independent Medium, CMRL Medium, Glasgow's Minimal Essential Medium (G-MEM), Iscove's Modified Dulbecco's Medium, Waymouth's MB 752/1 Media, Williams Media E, Medium NCTC-109, neuroplasma medium, Leibovitz's L-15 Media, McCoy's 5A Media (modified), MCDB 131 Mediumor and/or combination thereof.

11. A culture medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising gelatin present at concentration from about 0.001% to about 0.01% w/v, methyl cellulose present at concentration from about 0.05% to about 1.5% w/v, heparin present at the concentration from about 4 ng/ml to about 8 ng/ml, the basic nutrients comprising of an approximately 50:50 mixture of DMEM and Ham's F12 nutrients, basic Fibroblast Growth Factor- bFGF present at concentration from about 15 ng/ml to about 25 ng/ml, Epidermal Growth Factor –EGF present at concentration from about 5 ng/ml to about 15 ng/ml, Insulin present at concentration from about 5 µg/ml to about 15 µg/ml, B27 supplement 1X to 5X, and hydrocortisone present at concentration of at least about 1 µg/ml.

12. The culture medium as claimed in claim 1 or 11, wherein said culture medium does not contain human or an animal serum or portion thereof.

13. A feeding medium for selectively enriching and maintaining stem cells or cancer stem-like cells comprising components as claimed in claim 1 or 11 except methylcellulose.

14. A cell culture comprising stem cells or cancer stem-like cells and the culture medium of claim 1 or 11.

15. A method for selectively enriching and maintaining stem cells or cancer stem-like cells comprising steps of culturing the single cell suspension of cancer cells or normal cells in the culture medium of claims 1 or 11 at 37°C in a 5% $CO_2$ atmosphere and adding feeding medium of claim 12 to the growing culture intermittently to obtain spheres of stem cells or cancer stem-like cells.

16. The method as claimed in claim 16, wherein, said single cell suspension of the normal cells or cancer cells is cultured at the cell density ranging from about $1X10^4$ cells /ml to $1X10^6$ /ml.

17. The method as claimed in claim 16, wherein, said spheres or spheroids of stem cells or cancer stem-like cells are obtained in around 5-10 days.

18. A kit comprising the culture medium as claimed in claim 1or 11 or components thereof, feeding medium as claimed in claim 13 or components thereof, the cell culture system comprising the normal cells or stem cells or cancer cells or cancer stem-like cells, cell culture containers and optionally the written instructions for how to perform the cell culture method of claim 15.

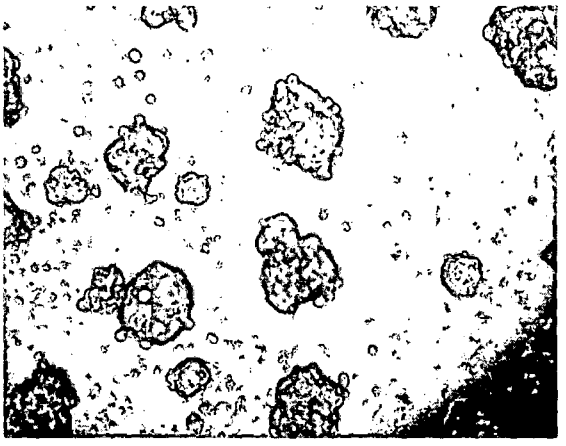

FIG 1

FIG 2

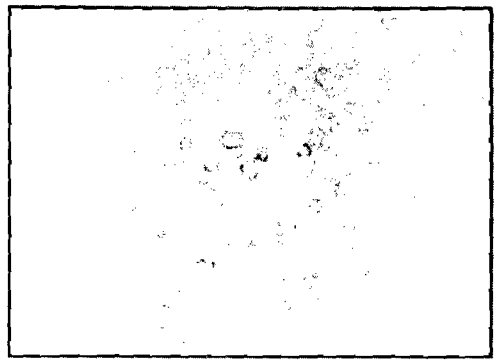

FIG 3

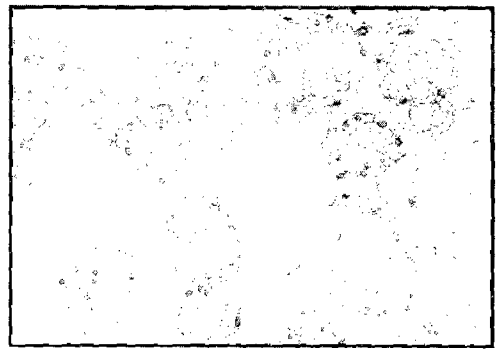

FIG 4

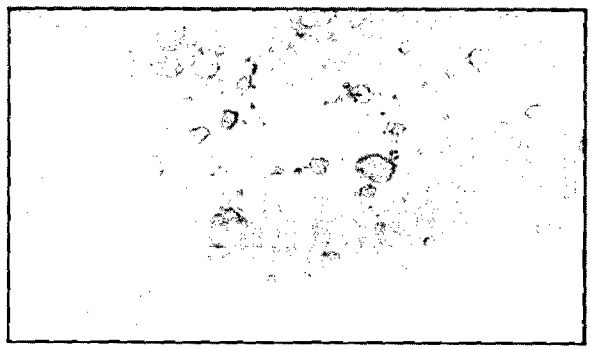

FIG 5 (L929)

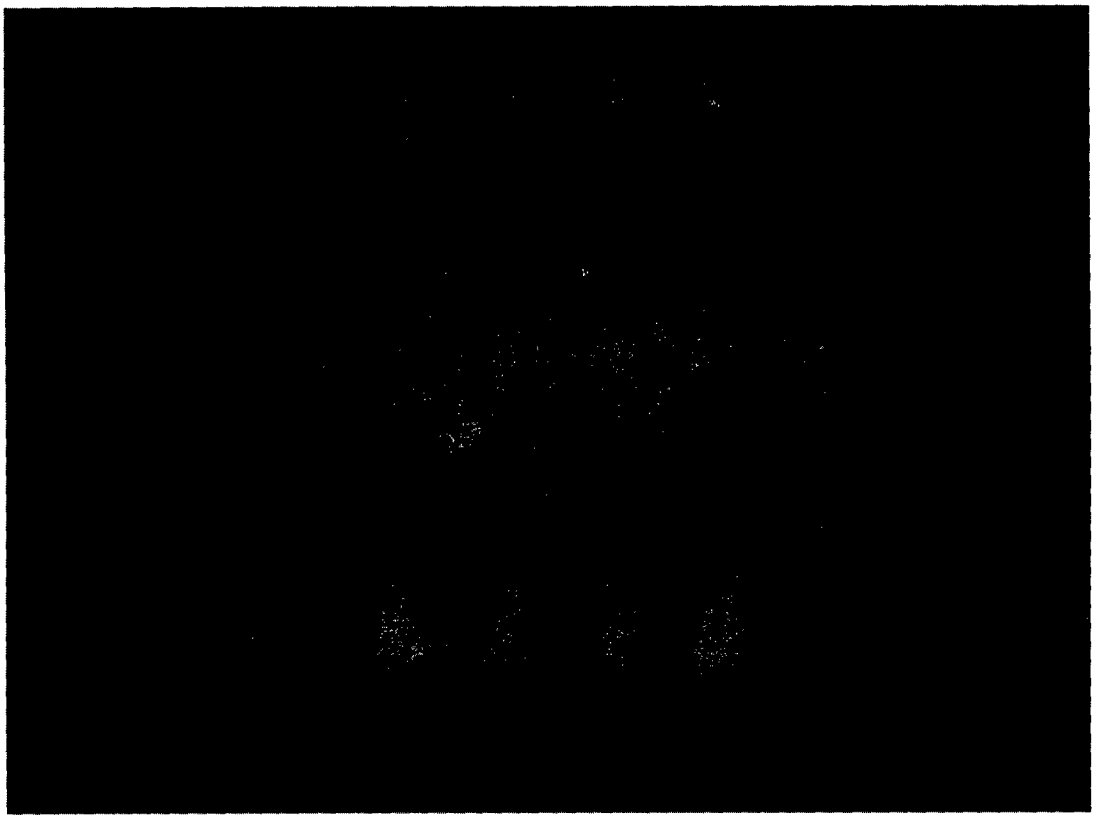

FIG 6A

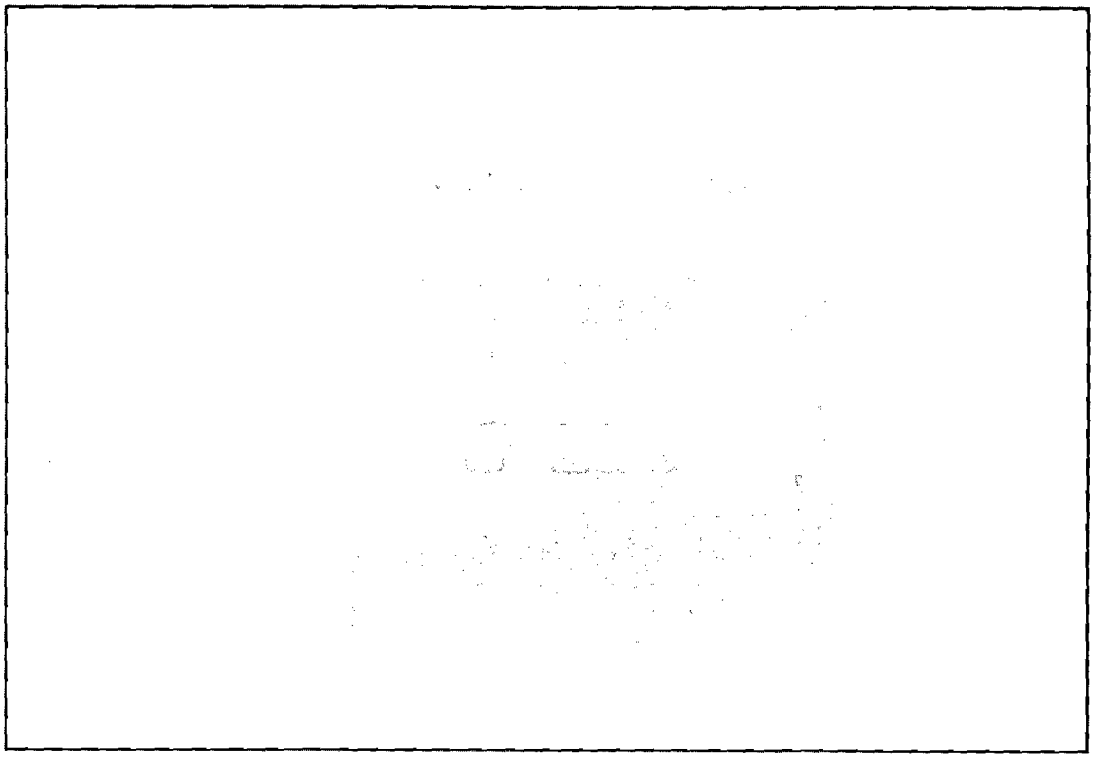

FIG 6B

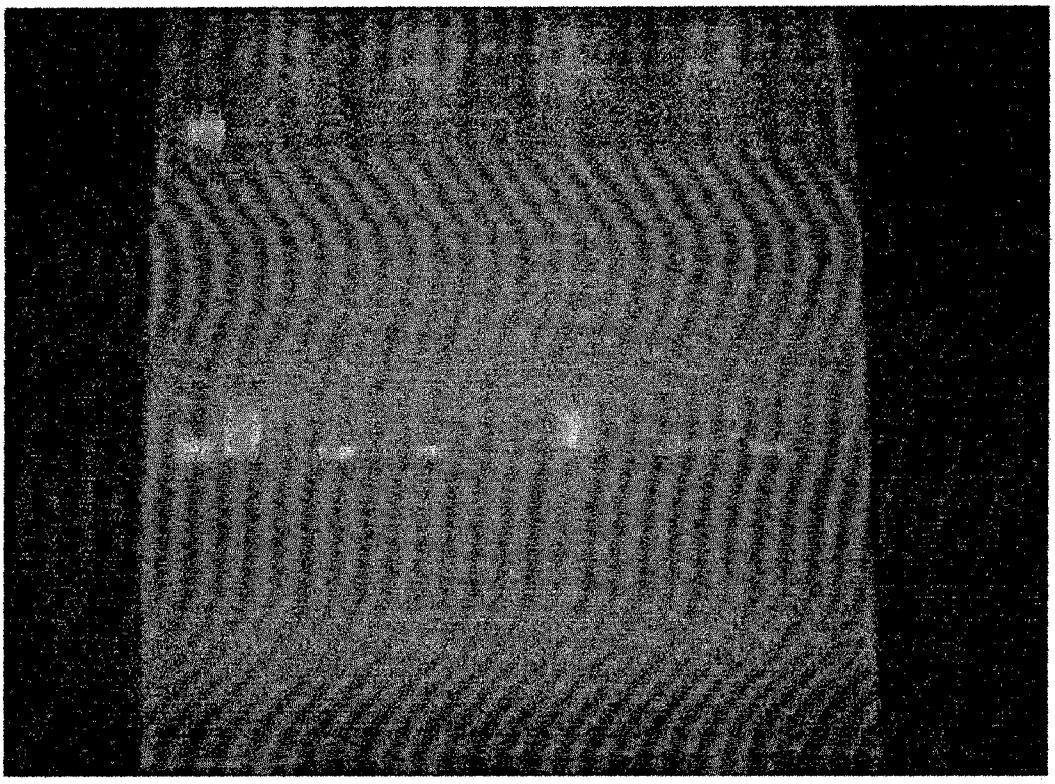

FIG 6C