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(54) Title: INDIVIDUALIZED HIGH PURITY COLON CARCINOMA STEM CELLS, METHODS AND USE OF THE SAME

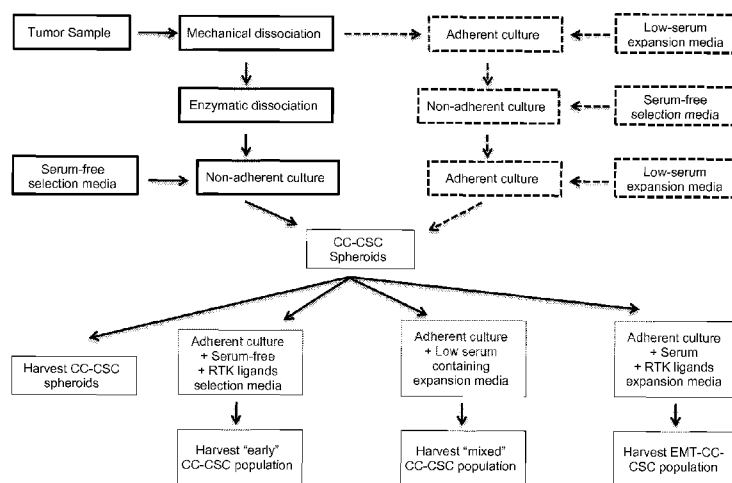


FIG. 1

(57) Abstract: The disclosure provides cancer stem cells, for use in stimulating immune response against a cancer, such as colon carcinoma (CC). Methods for preparing and purifying the cancer stem cells are provided.

INDIVIDUALIZED HIGH PURITY COLON CARCINOMA STEM CELLS, METHODS AND USE OF THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit under 35 U.S.C. §119(e) to United States Provisional Patent Application 61/778,991 filed March 13, 2013. The present application is also a continuation-in-part of International Application PCT/US2013/053850 filed August 6, 2013, which claims the benefit under 35 U.S.C. §119(e) to United States Provisional Patent Applications 61/683,477 filed August 15, 2012 and 61/718,643 filed October 25, 2012. The entire contents of all of which are incorporated by reference herein.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to reagents and methods for stimulating immune response against antigens of colon cancer, and against colon cancer tumors.

BACKGROUND

[0003] The stem cell niche is dormant until proper signaling triggers the re-entry in the proliferation cycle. Re-entry signals can originate from local events such as trauma, cell damage, microorganism aggression (viral, bacterial or fungal), or mediated by local growth factors, cytokines or intercellular communication. The regenerative capacity of any tissue resides in the existence of a subpopulation of progenitor cells that under certain signals can restart the growth cycle and produce cells that are identical to the originating tissue. A systemic determination such as hormonal control can strongly modulate the tissue specific stem cell niche.

[0004] Defects or mutations of the stem cell niche can result in perturbation of the above functions. The cancerous or malignant transformation is considered to be the result of such perturbation, most commonly identified in random mutations that affect the cell cycle control. The mutations are variable from individual to individual and constitute the basis of the variability in cancer.

[0005] Autologous immune therapies use the patient's own tumor tissue to sensitize the immune system, where the result is immune attack against cancer cells. Attack includes killing by way of CD8⁺ T cells, as well as antibody mediated cell cytotoxicity (ADCC), which involves natural killer (NK) cells. The approach seems theoretically ideal, however it is far from being effective due to the low antigenic signal to noise ratio when bulk tumor is used. The majority of the tumor cells are fairly differentiated and mixed with normal cells such as blood vessel constituents, connective tissue, and normal host tissue. The cancer stem cells represent only a small fraction of the bulk, sometimes up to 4% in more aggressive tumors, most commonly less than 1%. Therefore when the bulk tumor is used, the immune response is directed against the more differentiated cells allowing the stem cells to elude the attack and the possibility to relapse or metastases the tumor.

[0006] Colorectal cancer is the third most common cancer diagnosed in North America and the major of patients will undergo surgical resection for treatment. The 5-year overall-survival rates of patients that undergo surgery for colon carcinoma averages around 40 percent. An immunotherapy approach that using the immune system to target those cells responsible for new disease, namely tumorigenic cancer stem cells, could have profound and lasting impact on patient recurrence and survival.

SUMMARY

[0007] The present disclosure provides a cell population obtained from human colon carcinoma tumors that consist of high purity cancer stem cells. These cancer stem cells are specific for the colon progenitors and have the capacity of continuous self-renewal and differentiation to a certain level. The present disclosure also provides a method to produce a purified population of colon carcinoma-derived stem cells that are further used as an antigen source for autologous immune therapy for cancer, as well as the use of the high purity colon carcinoma stem cell population for genetic analysis to identify unique changes that determine the formulation of personalized medicines. The high purity colon carcinoma stem cell population can be used for *in vitro* assays for chemical compounds, drugs, or biologic agents screening.

[0008] Thus, provided herein is an immunogenic composition comprising dendritic cells activated *ex vivo* by tumor antigens derived from the population of purified colon carcinoma (CC) cancer stem cells (CSC). In one embodiment, the tumor antigens comprise cell extracts of the CC-CSC, lysates of the CC-CSC, or intact CC-CSC.

[0009] In another embodiment, the intact CC-CSC are rendered non-proliferative. In another embodiment, the intact CC-CSC are rendered non-proliferative by irradiation. In yet another embodiment, the intact CC-CSC are rendered non-proliferative by exposure of the cells to a protein or nuclear cross-linking agent.

[0010] In another embodiment, the immunogenic composition further comprises a pharmaceutically acceptable carrier and/or excipient. In another embodiment, the immunogenic composition further comprises an adjuvant. In yet another embodiment, the adjuvant is granulocyte macrophage colony stimulating factor.

[0011] In another embodiment, the immunogenic composition comprises activated dendritic cells and CC-CSC, wherein the CC-CSC are in the form of CC-CSC spheroids, early CC-CSC, mixed CC-CSC, or EMT-CC-CSC.

[0012] Also provided herein is a method of treating colon carcinoma in a subject in need thereof, comprising administration of an immunogenic composition disclosed herein to the subject. In one embodiment, the immunogenic composition is administered in a plurality of doses, each dose comprising about $5\text{-}20 \times 10^6$ cells. In another embodiment, the dose comprises about 10×10^6 cells. In yet another embodiment, the dose is administered weekly for 2-5 doses, followed by

monthly for 3-6 doses. In yet another embodiment, the subject receives from 6-10 doses of immunogenic composition.

[0013] Also provided herein is the use of an immunogenic composition disclosed herein in the manufacture of a medicament for the treatment of colon carcinoma.

[0014] Also provided herein in the use of an immunogenic composition disclosed herein for the treatment of colon carcinoma.

[0015] Also provided herein is a method for preparing a population of colon carcinoma (CC) cancer stem cells (CSC), the method comprising: acquiring a sample of CC; dissociating the cells of the sample, and *in vitro* culturing the dissociated cells in a defined medium on a non-adherent substrate, wherein the defined medium is serum free and is supplemented with at least one growth factor that acts through the mitogen activated protein kinase (MAPK) pathway, thereby forming CC-CSC spheroids; wherein at least 80% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, and CD29. In one embodiment, at least 80% of the cells in the CC-CSC spheroid population further express one or more of the biomarkers CK7, CK19, E-cadherin, CD20, ESA, ALDH, CDX1, LGR5, and DCIk1. In another embodiment, at least 90% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, and CD29.

[0016] In another embodiment, the method further comprises culturing the CC-CSC spheroids in a defined medium on an adherent substrate, wherein the defined medium is serum free and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of early CC-CSC, wherein at least 80% of the cells in the early CC-CSC population express two or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133. In another embodiment, at least 80% of the cells in the early CC-CSC population further express one or more of the biomarkers EpCAM, E-cadherin, Sox7, Sox17, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166, and CDCP1. In yet another embodiment, at least 90% of the cells in the early CC-CSC population express two or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133.

[0017] In another embodiment, the method further comprises culturing the CC-CSC spheroids in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of mixed CC-CSC, wherein at least 80% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1. In another embodiment, at least 90% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.

[0018] In another embodiment, the method further comprises culturing the CC-CSC spheroids in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of epithelial to mesenchymal transitioned (EMT)-CC-CSC, wherein at least 80% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117. In another embodiment, at least 80% of the cells in the EMT-CC-CSC population further express one or more of the biomarkers CD44, CD24, γ -synuclein, FMNL2, b-catenin, Nanog, CD147, β 3GhT8, LGR5, CD29, CXCR4, CD133, and DCIk1. In yet another embodiment, at least 90% of the cells in the EMT-CC-CSC population express one or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117

[0019] In another embodiment, the method further comprises culturing CC-CSC spheroids, mixed CC-CSC, or EMT-CC-CSC in a defined medium on an adherent substrate, wherein the defined medium is serum free and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of early CC-CSC, wherein at least 80% of the cells in the early CC-CSC population express two or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133. In another embodiment, at least 80% of the cells in the early CC-CSC population further express one or more of the biomarkers EpCAM, E-cadherin, Sox7, Sox17, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166, and CDCP1. In yet another embodiment, at least 90% of the cells in the early CC-CSC population express one or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133.

[0020] In another embodiment, the method further comprises culturing CC-CSC spheroids, early CC-CSC, or EMT-CC-CSC in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of mixed CC-CSC, wherein at least 80% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1. In another embodiment, at least 90% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.

[0021] In another embodiment, the method further comprises culturing CC-CSC spheroids, early CC-CSC, or mixed CC-CSC in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of EMT-CC-CSC, wherein at least 80% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117. In another embodiment, at least 80% of the cells in the EMT-CC-CSC population further express one or more of the biomarkers CD44, CD24, γ -synuclein, FMNL2, b-catenin, Nanog, CD147, β 3GhT8, LGR5, CD29, CXCR4, CD133, and DCIk1. In yet

another embodiment, at least 90% of the cells in the EMT-CC-CSC population express one or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117.

[0022] In another embodiment, the defined media is any media described in Table 2, a combination of Table 2 and Table 3, a combination of Table 2, Table 3, and Table 4, or a combination of Table 2 and Table 4.

[0023] In another embodiment, the growth factor is one or more of fibroblast growth factor (FGF), epidermal growth factor (EGF), or activin A. In another embodiment, the FGF is basic FGF (bFGF). In yet another embodiment, the defined medium is not supplemented with activin A. In yet another embodiment, the defined medium is supplemented with an agonist of activin A, in an amount effective to prevent spontaneous differentiation of CC-CSC. In another embodiment, the medium further comprises an antagonist of activin A, and the antagonist is follistatin or an antibody that specifically binds to activin A.

[0024] In another embodiment, the medium is not supplemented with an antioxidant such as superoxide dismutase, catalase, glutathione, putrescine, or β -mercaptoethanol. In another embodiment, the medium is supplemented with glutathione.

[0025] In another embodiment, the adherent substrate is configured to adhere to, and to collect, anchorage dependent cells, such as fibroblasts. In another embodiment, the non-adherent substrate is an ultralow adherent polystyrene surface. In another embodiment, the adherent substrate comprises a surface coated with a protein rich in RGD tripeptide motifs.

[0026] Also provided herein is a population of purified CC-CSC prepared by a method disclosed herein, wherein the purified CC-CSC are CC-CSC spheroids, early CC-CSC, mixed CC-CSC, or EMT-CC-CSC.

[0027] Also provided herein is a CC-CSC cell line prepared by a method disclosed herein, wherein the CC-CSC are CC-CSC spheroids, early CC-CSC, mixed CC-CSC, or EMT-CC-CSC.

[0028] Also provided is a method of stimulating an immune response against colon carcinoma in a subject in need thereof, comprising administration of an immunogenic composition disclosed herein, CC-CSC cells disclosed herein, or a CC-CSC cell line disclosed herein to the subject.

[0029] Also provided is the use of CC-CSC cells disclosed herein or a CC-CSC cell line disclosed herein in the manufacture of a medicament for the treatment of colon carcinoma.

[0030] Also provided is the use of CC-CSC cells disclosed herein or a CC-CSC cell line disclosed herein for the treatment of colon carcinoma.

DRAWINGS

[0031] FIG. 1 is a flow chart of the process of isolation, expansion, and harvest of the colon carcinoma (CC) stem cells (CC-CSC) from an excised tumor (solid boxes and arrows) or from a

small sample such as a needle biopsy (dashed boxes and arrows) into spheroids. After generation of spheroids, the pathway of producing CC-CSC subpopulations is a common pathway.

[0032] FIG. 2 depicts spheroid formation in ultralow adherent cell culture flasks from dissociated bulk colorectal tumor (phase contrast x10).

[0033] FIG. 3 depicts CC-CSC colony formation after the dissociation of spheroids and plating on regular tissue culture plastic (phase contrast 10X).

[0034] FIG. 3A-D. FIG. 3A depicts a patient-derived CC-CSC culture staining positive for alpha fetoprotein (AFP) and the epithelial marker CK19, with a nuclear counterstain (bisbenzimidazole) (epifluorescence, 20x). FIG. 3B depicts a red channel image of the cells of FIG. 3A for AFP staining. FIG. 3C depicts a green channel image of the cells of FIG. 3A for CK19 staining. FIG. 3D depicts a blue channel image for bisbenzimidazole.

[0035] FIG. 4A-D. FIG. 4A depicts a patient-derived CC-CSC culture labeled positive for ABCG2 and the epithelial marker CK7. The cells are also stained with a nuclear counterstain, bisbenzimidazole (epifluorescence, 20x). FIG. 4B depicts a red channel image of the cells of FIG. 4A for ABCG2 staining. FIG. 4C depicts a green channel image of the cells of FIG. 4A for CK7. FIG. 4D depicts a blue channel image for bisbenzimidazole.

[0036] FIG. 5A-C. FIG. 5A depicts a patient-derived CC-CSC culture with a high percentage of FoxA2 staining, a marker for a pioneer transcription factor that occurs early in gastrulation, thus demonstrating the early cancer stem cell phenotype. FIG. 5B depicts a red channel image of the cells of FIG. 6A for FoxA2 staining. FIG. 6C depicts a blue channel image for bisbenzimidazole.

[0037] FIG 6A-C. FIG. 6A depicts a patient-derived CC-CSC culture with a high percentage of neural cell adhesion molecule (NCAM) staining, confirming the early cancer progenitor phenotype selection in the established cultures (includes nuclear counterstain, bisbenzimidazole) (epifluorescence, 40x). FIG. 6B depicts a red channel image of the cells of FIG. 6A for NCAM staining. FIG. 6C depicts a blue channel image for bisbenzimidazole.

[0038] FIG. 7A-D. FIG. 7A depicts a patient-derived CC-CSC culture labeled positive for CD44, a marker of invasiveness, specific to cancer stem cells with high metastatic potential and the Sox2 marker specific for early cancer stem cells. FIG. 7B depicts a red channel image of the cells of FIG. 7A for Sox2 staining. FIG. 7C depicts a green channel image of the cells of FIG. 7A for CD44 staining. FIG. 7D depicts a blue channel image for bisbenzimidazole.

[0039] FIG 8A-D. FIG. 8A depicts a patient-derived CC-CSC culture labeled for HLA1 and proliferation marker Ki67. FIG. 8B depicts a red channel image of the cells of FIG. 8A demonstrating intense proliferation (positive for Ki67 marker). FIG. 8C depicts a green channel image of the cells of FIG 8A that shows the up-regulation of HLA1, a marker that is associated with poor immunogenicity and high invasiveness. FIG. 8D depicts a blue channel image for bisbenzimidazole nuclear staining.

[0040] FIG. 9A-D. FIG. 9A depicts epithelial to mesenchymal transition markers, vimentin and Slug/Snail, in a patient-derived CC-CSC culture with a bisbenzimidazole nuclear counterstain. FIG. 9B depicts a red channel image of the cells of FIG. 9A for Slug/Snail staining. FIG. 9C depicts a green channel image of the cells of FIG. 9A for vimentin. FIG. 9D depicts a blue channel image for bisbenzimidazole.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0041] The present disclosure provides a cell population obtained from human colon carcinoma (CC) tumors that consist mainly of high purity cancer stem cells. In embodiments, the purity of the cell population is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% cancer stem cells. These cancer stem cells are colon carcinoma progenitors and have the capacity of continuous self-renewal and differentiation to a certain level. The disclosure also concerns a method to produce a purified population of CC-derived stem cells, for further use as an antigen source for autologous immune therapy of cancer.

[0042] As used herein, the terms "colon carcinoma" or "colon cancer" include colorectal cancer and colorectal carcinoma.

[0043] Testing and screening embodiments are also encompassed. The present disclosure uses the high purity CC stem cell population for genetic analysis to identify unique changes that drive the formulation of personalized medicines. The present disclosure provides a novel cell line that is modified *in vitro*, where this modification enhances the immune stimulatory characteristics of the CC. The CC cell line is an improvement over similar technologies using crude tumor preparations, as it provides a superior antigenic signal to noise ratio. The cell line lacks contaminant cell populations, such as fibroblasts, that could alter or diminish the *in vitro* applications. The exemplary cell line of the present disclosure is also used for manufacturing of a drug for treating CC.

[0044] As used herein, the term "derived from," in the context of peptides derived from one or more cancer cells, encompasses any method of obtaining the peptides from a cancer cell or a population of cancer cells. The cancer cell can be broken, for example, by a homogenizer or by osmotic bursting, resulting in a crude extract. Peptides, oligopeptides, and polypeptides of the crude extract can be exposed to dendritic cells, followed by processing of the peptides by the dendritic cells. The term "derived from" also encompasses intact cancer cells, where the cancer cells are living, or where the cancer cells have been treated with irradiation but are still metabolically active, or where the cancer cells have been treated with a nucleic acid cross-linking agent but are still metabolically active and therefore still comprise the peptides. "Derived from" also includes mixtures of cancer cell debris, free cancer cell proteins, and irradiated cancer cells, that therefore are derived from the cancer cells.

[0045] "Administration" as it applies to a human, mammal, mammalian subject, animal, veterinary subject, placebo subject, research subject, experimental subject, cell, tissue, organ, or biological fluid, refers without limitation to contact of an exogenous ligand, reagent, placebo, small molecule, pharmaceutical agent, therapeutic agent, diagnostic agent, or composition to the subject, cell, tissue, organ, or biological fluid, and the like. "Administration" can refer, e.g., to therapeutic, pharmacokinetic, diagnostic, research, placebo, and experimental methods. Administration can refer to *in vivo* treatment of a human or animal subject. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" also encompasses *in vitro* and *ex vivo* treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell.

[0046] "Effective amount" encompasses, without limitation, an amount that can ameliorate, reverse, mitigate, prevent, or diagnose at least one symptom or sign of a medical condition or disorder. Unless dictated otherwise, explicitly or by context, an "effective amount" is not limited to a minimal amount sufficient to achieve a desired outcome nor limited to the optimal amount sufficient to achieve the desired outcome.

[0047] The severity of a disease or disorder, as well as the ability of a treatment to prevent, treat, or mitigate, the disease or disorder (achieve the desired outcome) can be measured, without implying any limitation, by a biomarker or by a clinical parameter. Biomarkers include blood counts, metabolite levels in serum, urine, or cerebrospinal fluid, tumor cell counts, cancer stem cell counts, tumor levels. Tumor levels can be determined by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria (Eisenhauer, et al. (2009) Eur. J. Cancer. 45:228-247). Expression markers encompass genetic expression of mRNA or gene amplification, expression of an antigen, and expression of a polypeptide. Clinical parameters include progression-free survival (PFS), 6-month PFS, disease-free survival (DFS), time to progression (TTP), time to distant metastasis (TDM), and overall survival, without implying any limitation.

[0048] A composition that is "labeled" is detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, isotopic, or chemical methods. For example, useful labels include ^{32}P , ^{33}P , ^{35}S , ^{14}C , ^3H , ^{125}I , stable isotopes, epitope tags fluorescent dyes, electron-dense reagents, substrates, or enzymes, e.g., as used in enzyme-linked immunoassays, or fluorettes (disclosed in US 6,747,135 which is incorporated by reference herein for all it discloses regarding fluorettes).

[0049] Therefore, disclosed herein are methods for preparing a population of purified spheroids, or single cells preparations derived from spheroids, of cancer stem cells, the method comprising acquiring a biopsy of CC, dissociating the cells of the biopsy, *in vitro* culturing the dissociated cells in a defined medium on a substrate, wherein the defined medium is supplemented with at least one growth factor that acts through the mitogen activated protein kinase (MAPK) pathway to yield a population of purified CC-CSC spheroids, or single cell

preparations of CC-CSC. At least about 50%, at least about 60%, at least about 70%, or at least about 80% of the cancer stem cells in the population of purified CC-CSC express one or more of the biomarkers ATP-binding cassette sub-family G member 2 (ABCG2; GenBank Accession Number AAG52982.1), alpha-fetoprotein (AFP), CD133, CD44, CD24, CD166, CD29, CD49f, CD9, CD117, CD147, cytokeratin 19 (CK19), cytokeratin 7 (CK7), cytokeratin 20 (CK20), epithelial specific antigen (ESA), aldehyde dehydrogenase (ALDH), homeobox protein CDX1, leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), serine/threonine-protein kinase DCLK1, aldehyde dehydrogenase 1 family, member A1 (ALDH1A1), aldehyde dehydrogenase 1 family, member B1 (ALDH1B1), developmental pluripotency-associated protein 2 (DPPA2), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), polycomb complex protein (BMI-1), CUB domain-containing protein 1 (CDCP1), RNA-binding protein Musashi homolog 1 (MSI1), granulocyte colony stimulating factor (G-CSF), human Piwi homolog (Hiwi), ephrin type-B receptor (EphBR), γ -synuclein (SYNG), formin-like protein 2 (FMNL2), β -catenin, β 3GhT8, CXCR4, c-kit, E-cadherin, epithelial cell adhesion molecule (EpCAM; GenBank Accession Number NP_002345.2), forkhead box A2 (FoxA2), Ki-67, Nanog (GenBank Accession Number NM_024865.2, NP_079141.20), hairy and enhancer of split-1 (Hes1), N-cadherin, neural cell adhesion molecule (NCAM; CD56), Oct3/4 (GenBank Accession Number NP_002692.2; NP_976034.4; NP_001167002.1; NP_068812.10), Slug (SNAI2)/Snail (SNAI1) (Slug/Snail), Sox17, Sox2 (GenBank Accession Number NM_003106.3, NP_003097.1), Sox7, Twist, and vimentin. A flow chart of the formation of the disclosed cell populations is presented in FIG. 1.

[0050] As used herein, the term “spheroids” refers to spherical aggregates of cancer stem cells formed by culture of cancer cells in serum-free medium. The ability to form spheroids is a characteristic of cancer stem cells.

[0051] In certain embodiments, at least about 50%, at least about 60%, at least about 70%, or at least about 80% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, and CD29. In other embodiments, at least 80% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, CD29, CK7, CK19, E-cadherin, CK20, ESA, ALDH, CDX1, LGR5, and DCLK1. In another embodiment, at least 90% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, and CD29.

[0052] The spheroid population can be further expanded into one of three different subpopulations by altering culture conditions such as media composition and substrate. The characteristics of the bulk tumor, spheroid, early, mixed, and EMT populations are presented in Table 1.

Table 1. Summary of the conditions used to produce CC-CSC cell populations from bulk colon carcinoma tumors

Population	Availability	Cell type	Markers (partial list)	Conditions for isolation	Usefulness in therapy
Excised tumor, bulk	immediate	CC cells, normal cells, very few CR-CSC*	Mixed markers from any of the cell populations described below	Lysate and/or enzyme-dissociated	Diluted antigenicity
Spheroids	14 days	CC-CSC	At least two of CD133, Hes1, CD44, CD24, CD166, CD29, Optionally CK7, CK19, E-cadherin, CK20, ESA, ALDH, CDX1, LGR5, DCIk1	Non-adherent culture, bFGF, EGF, Selection media	Proper antigenic signal
Colonies with small cuboid cells, "early" population	21 days or longer	CC-CSC (very early, embryonic-like)	At least two of Nanog, Sox 2, Oct3/4, c-kit, FoxA2, CD133 Optionally EpCAM, E-cadherin, Sox7, Sox17, ALDH1A1, LGR5, Hes1, DPPA2, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166, CDCP1	Adherent culture, Selection media, bFGF, Activin A	Proper antigenic signal
Epithelial monolayer, cells with various sizes, small cuboid to giant cells	21 days or longer	More or less differentiated CC-CSC (mixed)	At least two of Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, DCIk1, ABCG2, NCAM, HLA1, Ki-67, AFP.	Adherent culture, Low calcium expansion media Optional, EGF	Proper antigenic signal
Monolayer with spindle or irregular shaped cells "EMT" population**	28 or and longer	EMT-CC-CSC (mesenchymal-like)	At least two of: N-cadherin, Slug/Snail, Twist vimentin, CD117 Optionally, CD44, CD24, γ -synuclein, FMNL2, b-catenin, Nanog, CD147, β 3GhT8, LGR5, CD29, CXCR4, CD133, DCIk1	Adherent culture, bFGF, Expansion media	Proper antigenic signal

CC = Colon Cancer; ** CSC = cancer stem cell; *** EMT = epithelial to mesenchymal transition

[0053] Furthermore, any of the early CC-CSC, mixed CC-CSC, or EMT-CC-CSC populations can be obtained from CC-CSC spheroids, early CC-CSC, mixed CC-CSC, or EMT-CC-CSC by changing the media and conditions as disclosed in Table 1.

[0054] In one embodiment, the CC spheroids are further cultured on an adherent substrate in the presence of activin A, FGF, and a serum-free media (selection media) to yield colonies with small cells referred to herein as an “early” population of CC-CSC which have characteristics of embryonic stem cells, and at least about 50%, at least about 60%, at least about 70%, or at least about 80% of the cells in the early CC-CSC population express two or more of biomarkers Nanog, Sox 2, Oct3/4, c-kit, FoxA2, and CD133. In another embodiment, at least 80% of the cells in the early CC-CSC population express two or more of biomarkers Nanog, Sox 2, Oct3/4, c-kit, FoxA2, CD133, EpCAM, E-cadherin, Sox7, Sox17, ALDH1A1, LGR5, Hes1, DPPA2, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166, and CDCP1. In another embodiment, at least 90% of the cells in the early CC-CSC population express two or more of biomarkers Nanog, Sox 2, Oct3/4, c-kit, FoxA2, and CD133.

[0055] In another embodiment, the CC spheroids are further cultured on an adherent substrate in the presence of FGF, EGF, and a serum-containing media (expansion media) to yield colonies mixed with a monolayer wherein the cells have heterogeneous morphologies. These cells are referred to herein as a “mixed” population of CC-CSC which have a mixed differentiation profile, and at least about 50%, at least about 60%, at least about 70%, or at least about 80% of the cells in the mixed CC-CSC population express two or more of biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1. In another embodiment, at least 90% of the cells in the early CC-CSC population express two or more of biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.

[0056] In yet another embodiment, the CC spheroids are further cultured on an adherent substrate in the presence of FGF and a serum-containing media (expansion media) to yield a monolayer of spindle- or irregularly-shaped cells referred to herein as mesenchymal-like CC-CSC or “EMT-CC-CSC” (epithelial to mesenchymal transitioned [EMT] cancer stem cells). In this population, the spheroids have undergone a process of EMT characterized by the loss of the expression of at least one epithelial marker. As used herein, loss of the expression of a biomarker refers to undetectable expression or expression in 40% (or less) of the cells, expression in 30% (or less) of the cells, expression in 20% (or less) of the cells, or expression in 10% (or less) of the cells. Additionally, the EMT process is characterized by the increase in the expression of at least one, or all, of the mesenchymal markers Slug/Snail, CD44, Twist, N-cadherin, and vimentin to at

least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the cells in the population expressing the biomarker(s) of interest.

[0057] In one embodiment, at least about 50%, at least about 60%, at least about 70%, or at least about 80% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117. In yet another embodiment, at least 80% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, CD117, CD44, CD24, γ -synuclein, FMNL2, b-catenin, Nanog, CD147, β 3GhT8, LGR5, CD29, CXCR4, CD133, and DCIk1. In yet another embodiment, at least 90% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117.

[0058] In certain embodiments of the cell populations, the cells express one or more of the indicated biomarkers. In other embodiments, the cells express two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more of the indicated biomarkers. In yet other embodiments, the cells express 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 of the indicated biomarkers.

[0059] Expression of biomarkers by a single cell, by a population of cells, or by a population of cells located in a specific structure such as a monolayer or a spheroid, can be determined by measuring expression of the polypeptide form of the biomarker or the mRNA form of the biomarker. Polypeptide expression can be measured using a labeled antibody, while nucleic acid expression can be measured by hybridization techniques, are available to the skilled artisan. Biomarkers that are not polypeptides or nucleic acids, such as oligosaccharides or small molecule metabolites, can also be measured by methods available to the skilled artisan.

[0060] Also disclosed herein are methods to obtain pure populations of isolated CC-CSC from colon tumor samples of various sizes (1 mg to grams). The tumor samples can be fresh or frozen, are dissociated by mechanical and/or enzymatic treatment, or are cultivated directly with minimal mechanical fragmentation.

[0061] Also disclosed herein, a non-adherent substrate is any biocompatible material with anti-biofouling properties or a coating with anti-biofouling properties (reduces accumulation of cells on a wetted surface) applied to a common culture surface. The coating can be applied using coating agents such as amino-silanes. If there is a non-adherent or anti-biofouling substrate, this substrate can be used for about 0-25 days, such as 0-21 days, 5-20 days, 5-10 days, 10-20 days, or any time period between zero and 25 days.

[0062] In another embodiment of the method that uses an adherent substrate, the adherent substrate can be one that is rich in RGD (Arg-Gly-Asp) tripeptide motifs (e.g., collagen, gelatin, MATRIGEL®). An adherent substrate is a surface that is configured to adhere to, and to collect, anchorage dependent cells. Moreover, the substrate can be an adherent substrate that is

configured to adhere to and to collect anchorage dependent cells that are fibroblasts. RGD peptides can also be grafted on polymeric backbones such as polystyrene, hyaluronan, poly-lactic acid, or combinations thereof. The backbone can further carry proteoglycans. The proteoglycans can carry growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), activin A, or follistatin.

[0063] A non-adherent substrate can cause fast and efficient enrichment of the cultures with cancer stem cells. A non-adherent substrate may be used when a large enough sample is provided, for an example surgically excised tumor, so that purification of CC-CSC can begin immediately. If the sample is very small, such as needle aspirate, metastasis, or peritoneal lavage, and non-adherent culture is not feasible, an adherent culture may be used for initial expansion, followed by a purification step on a non-adherent substrate, then followed by another expansion under adherent conditions. The alternative processing method is illustrated in FIG. 1 (dashed lines and boxes) and in detail below.

[0064] In certain culture embodiments, a first period of culture is provided on an adherent substrate, followed by a second period of culture on a non-adherent substrate. Also provided is a first period of culture on a non-adherent substrate, followed by a second period of culture on an adherent substrate. Periods can be, for example, one half day, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, and the like, or any range thereof, such as 2-4 days, or 8-10 days, and so on. Additionally, the cycle can repeat such as an adherent culture followed by a non-adherent culture followed by an adherent culture, etc. In another embodiment, the cycle can repeat such as a non-adherent culture followed by an adherent culture, followed by a non-adherent culture, etc.

[0065] In another embodiment, the defined medium is supplemented with at least one growth factor that acts through the mitogen activated protein kinase (MAPK) pathway. In one embodiment, the growth factor is one or both of FGF and EGF, or analogue thereof. In one embodiment, the FGF is basic fibroblast growth factor (bFGF). In another embodiment, the defined medium is supplemented with activin A. In another embodiment, the defined medium is not supplemented with activin A. Also disclosed is a defined medium supplemented with an agonist of activin A, in amount effective to prevent spontaneous differentiation of CC-CSC. Other ligands to receptor tyrosine kinases (RTK) such as VEGF, HGF, or PDGF may have the same effect on the tumor cell expansion.

[0066] Also provided is a CC-CSC cell line that is unique to each patient obtained from the patient's primary colon tumor, that (a) carries stem cell characteristics of self-renewal and pluripotency and the ability to differentiate; and (b) that carries a unique genomic cancerous signature in the majority of the cells, such as more than 50%.

[0067] The present disclosure encompasses nucleic acids, gene products, polypeptides, and peptide fragments, where identity can be reasonably established by a trivial name alone. Also encompassed, are nucleic acids, gene products, polypeptides, and peptide fragments, based on a particular GenBank Accession No., where the nucleic acid, polypeptide, and the like, has at least 50% sequence identity, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity sequence identity, to that of the GenBank No. where the biochemical function, or physiological function are shared, at least in part, or alternatively, irrespective of function.

[0068] Provided is a method wherein an immune response to cancer in a subject is stimulated with one of the compositions disclosed herein. The immune response that is stimulated comprises one or more of CD4⁺ T cell response, CD8⁺ T cell response, and B cell response. In certain embodiments, the CD4⁺ T cell response, CD⁺ T cell response, or B cell response, can be measured by ELISPOT assays, by intracellular cytokine staining (ICS) assays, by tetramer assays, or by detecting antigen-specific antibody production, according to assays that are known by persons of ordinary skill in the art. The immune response can comprise a survival time such as a 2-year overall survival (OS), and where the 2-year overall survival is at least 60%. An immune response in a patient can also be assessed by endpoints that are used in oncology clinical trials, including objective response (RECIST criteria), overall survival, progression-free survival (PFS), disease-free survival, time to distant metastasis, 6-month PFS, 12-month PFS, and so on.

[0069] Also disclosed herein dendritic cells stimulated *ex vivo* with the CC stem cells, or antigens derived therefrom, for use in therapy of colon carcinoma. Encompassed herein are immunogenic compositions, such as vaccine compositions, comprising dendritic cells loaded with (exposed to) the CC-CSC *ex vivo*. In certain embodiments, the dendritic cells and tumor cells are from the same human subject although embodiments where the dendritic cells and CC cells are from different subjects are within the scope of the present disclosure.

[0070] Dendritic cells can be loaded with CC tumor cell antigens comprising whole cells, cell lysates, cell extracts, irradiated cells or any protein derivative of a CC tumor cell. Dendritic cell immunogenic compositions can be prepared, and administered to a human subject by one or more routes of administration as are known to persons of ordinary skill in the art.

[0071] In certain embodiments, the CC-CSC cells are irradiated, or otherwise treated to prevent cell division, prior to loading with the dendritic cells. Alternatives to radiation include nucleic acid cross-linking agents that prevent cell division. Also provided is a method that uses of the CC stem cell population, as disclosed above, as a source of antigen for autologous immune therapy, for example, where the CC stem cells are inactivated by a radiant energy (e.g., gamma, UV, X), temperature (e.g., heat or cold), or chemical (e.g., cytostatic, aldehyde, alcohol) methods, or combinations thereof. In other embodiments, the CC stem cells are used as a source of antigen for *ex vivo* activation of dendritic cells.

[0072] The present disclosure provides prepared CC cells, provides DC loaded with the prepared CC cells, and provides immunogenic compositions (or vaccines) comprising dendritic cells loaded the prepared CC cells. Without implying any limitation, an immunogenic composition of the present disclosure can comprise DC loaded with CC spheroids, loaded with a population of cells that comprises spheroids, loaded with a population of cells that was derived from spheroids and that were expanded on an adherent surface prior to loading on DC, loaded with spheroids that were subjected to homogenization or sonication prior to loading on DC, loaded with a population of expanded cells that were subjected to homogenization or sonication prior to loading on DC, and so on. In other embodiments, the DC are loaded with early CC-CSC, mixed CC-CSC, or EMT-CC-CSC. In another embodiment, the DC are loaded with tumor antigens comprising messenger RNA from CC-CSC.

[0073] Also disclosed herein is a population of CC-CSC that are capable of stimulating an effective immune response against a cell expressing at least one CC-specific antigen, wherein the CC-CSC population is contacted with at least one dendritic cell, wherein the CC-CSC population is processed *in vivo* or *ex vivo* by the dendritic cell, and wherein an effective immune response occurs in the subject in response to administration of the at least one dendritic cell to a subject.

[0074] An immune stimulatory amount of the disclosed compositions is, without limitation, an amount that increases ELISPOT assay results by a measurable amount, that increases ICS assay results by a measurable amount, that increases tetramer assay results by a measurable amount, that increases the blood population of antigen-specific CD4⁺ T cells by a measurable amount, that increases the blood population of antigen-specific CD8⁺ T cells by a measurable amount, or where the increase is by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 1.5-fold, 2.0-fold, 3.0-fold, and the like, when compared to a suitable control. A suitable control can be a control composition, where dendritic cells are not loaded with CC cells, or are not loaded with peptide derived from CC cells.

[0075] The disclosure also provides pharmaceuticals, reagents, kits including diagnostic kits, that wherein the pharmaceuticals, reagents, and kits, comprise dendritic cells (DC), antibodies, or antigens. Also provided are methods for administering compositions that comprise at least one dendritic cell and at least one antigen, methods for stimulating antibody formation, methods for stimulating antibody-dependent cytotoxicity (ADCC), methods for stimulating complement-dependent cytotoxicity, and methods and kits for determining patient suitability, for determining patient inclusion/exclusion criteria in the context of a clinical trial or ordinary medical treatment, and for predicting response to the pharmaceutical or reagent. The pharmaceutical compositions, reagents, and related methods, of the disclosure encompass CD83 positive dendritic cells, where CD83 is induced by loading with IFN-gamma-treated, or untreated, cancer cells. In a CD83 aspect of the disclosure, the CD83 is induced by at least 2%, at least 3%, at least 4%, 6%, 7%, 8%, 9%,

10%, and the like. In another aspect, excluded are DC reagents, or DC-related methods, where CD83 on dendritic cells is not detectably induced by loading with IFN-gamma.

[0076] In one embodiment, a kit is provided which includes all of the reagents for generating CC-CSC spheroids, early CC-CSC, mixed CC-CSC, and/or EMT-CC-CSC from tumor samples according to the methods disclosed herein and/or reagents for characterizing the CC-CSC spheroids, early CC-CSC, mixed CC-CSC, and/or EMT-CC-CSC, and instructions for generating and/or characterizing the CC-CSC spheroids, early CC-CSC, mixed CC-CSC, and/or EMT-CC-CSC. In another embodiment, the kit additionally, or alternatively, includes reagents and instructions for isolating dendritic cells, for loading the dendritic cells with CC-CSC, and/or for administering the DC-CC composition to a subject.

[0077] Tumor sample processing

[0078] The colon carcinoma (CC) stem cell population of the present disclosure can originate from fresh or frozen samples of patient tumor. The tumor sample can be a biopsy or a lavage of a tumor-containing tissue. CC stem cells are isolated from needle biopsies and from the lavage fluid.

[0079] The tumor sample may be transported in a generic buffered media with a pH of about 7.4 (+/- 0.6) such as RPMI, DMEM, F12, Williams, or combinations containing a protein source such as animal or human serum in concentrations from 0 to 100% or albumin at concentrations from 0 to 0.5% or macromolecules that ensure a physiological osmotic pressure. Examples of natural or artificial macromolecules are, but not limited to, hyaluronan, dextrans, polyvinyl alcohol. An antibiotic such as penicillin, streptomycin, gentamicin in an optional combination with an antifungal such as amphotericin B, FUNGIZONE® (Life Technologies, Carlsbad, CA), can be used in the media to provide antimicrobial properties and reduce the risk of contamination during transportation.

[0080] The tumor sample can be kept below a metabolic active state by reducing the media temperature to 2 to 30°C, thus allowing the viability maintenance for a limited time (between 0 to 72 hours) before processing. Packaging (e.g., insulated packaging) may be used to ensure the proper temperature control during transportation.

[0081] The solid tumor tissue is then processed by mechanical dissociation using a sharp blade or tissue grinder device into small, less than 1 mm (on any dimension) fragments.

[0082] The solid tissue is optionally further processed by enzymatic dissociation. A variety of enzymes can be used to isolate single cells. Nonspecific proteolytic enzymes such as trypsin and pepsin can be used successfully. Targeting minimal cell membrane damage specific enzymes, including collagenase, dispase, elastase, or combinations thereof, may be used in the disclosed methods. Deoxyribonuclease (DNAse) can be used to degrade the free DNA from cell detritus responsible for unwanted stickiness of the cell preparation. After dissociation, the cells in

suspension are washed from the excess enzyme and debris by straining through a 50-100 μm mesh and repeated centrifugation in a buffered saline (PBS, HBSS) or cell culture media.

[0083] Cell culture conditions and spheroid production

[0084] The single cell suspension described above is transferred in culture conditions that promote isolation, expansion of the stem cells and suppression of the differentiated and/or normal cells. This is accomplished by the congruence of the physical conditions, chemical environment, and manipulations.

[0085] The cell suspension is exposed to a non-adherent (anti-biofouling) substrate that does not allow cell attachment. Mature cells are commonly anchorage dependent and are rapidly eliminated when a proper adherent substrate is not provided. An anti-biofouling substrate can employ commercial products such as ultralow adherent flasks (Corning, Corning, NY), polymers with natural hydrophobic properties (polyvinyl, polyethylene, polypropylene, fluoro-polymers) or coating with natural carbohydrate polymers such as agar-agar, starch, and the like.

[0086] The cancer stem cells will aggregate and/or clonally expand in spheroid formations (FIG. 2) that contain high purity cancer stem cells. The mature cells will remain isolated and non-adherent. A differential gravitational separation can be used to select the larger spheroids from single cells, by simply allowing a timed vertical sedimentation or a short time low force centrifugation (less than 100xG). The selection method described is designed to accomplish the following: (a) eliminate of anchorage dependent cells that are, in general, mature, normal cells; (b) promote the clonal expansion in small clumps or spheroids of the young, stem cells that are anchorage independent; (c) promote the local autocrine activity as a result of clonal expansion of the stem cells; and (d) eliminate the autocrine source of activin A that is secreted by normal fibroblasts or colon cells.

[0087] The ability of cells to form spheres results, in part, from cell-surface proteins called integrins. Homophilic integrins expressed on the cell's surface ensure that cells of the same type "stay together". Spheres are formed directly from enzyme digest which is a single cell suspension at the very beginning of a culture, or can be formed from frozen sample or an existing attached culture at any time. The enzyme digest seeding result in this spherical formations that incorporate the cells with the specific surface properties.

[0088] Fibroblasts, for example, are not incorporated into spheroids and are removed from a culture during gravitational feeding. The media used lacks molecules that promote adhesion in order to prevent the non-specific agglomeration of the cells not having homophilic proprieties and to prevent the adhesion to the culture vessel surfaces. Such cell adhesion molecules (CAMs) are commonly found in the animal or human serum. Therefore a media composition which is serum free is suitable for culture of non-adherent spheroids.

[0089] In the serum free media culture, supplements to the media may include any hormones, nutrients, mineral, and vitamins that are required for supporting growth and maintenance, or other desired aspects of cell physiology and function. In some instance one can stimulate and sustain the stem cell proliferation with the addition or adjustment of amount of growth factors that possess a mitogenic activity, such as the FGF family and EGF.

[0090] Spheres of cells (spheroids), including spheres of cancer stem cells, can be characterized in terms of biomarker expression by way of fixing and staining with labeled antibodies, followed by viewing with confocal microscopy. Biomarkers may also be measured by other immunochemistry methods, e.g., flow cytometry. Spheres can be prepared, for example, from suspensions obtained from fresh tumors, or from cells adapted to grow as adherent cells. The morphology of spheres, for example, large and irregular versus tiny and compact, may be influenced by the choice of medium.

[0091] In another embodiment, a cell population adherent to the anti-biofouling coating can be isolated based on aberrant activation of sonic hedgehog signaling mediated by protein kinase B (AKT) and focal adhesion kinase (FAK) signaling. These phenomena can be enhanced by modifications of the membranes induced by enzymes such as metalloproteases or enzymes used in dissociation (trypsin/collagenase). Such cell population can be associated with rapid proliferative and invasive tumors. FIG. 3 depicts a representative cell population that is attached and expanding to an ultralow adherent surface. Methods for assessing normal or aberrant activation of the sonic hedgehog signaling are available and known to persons of ordinary skill in the art.

[0092] Medium used in cell culture

[0093] The defined media that is used to isolate the CC-CSC promotes cell survival and is specifically formulated for selection. The media is rich in carbohydrates and lipids but has minimal amount of protein (0.1% - 3% albumin or 1%-5% serum). It contains not more than 1.5 mMol total calcium, does not contain inorganic iron compounds; rather, iron is completely bound to a transporter such as transferrin. The media is provided with an excess of essential and non-essential amino acids and essential lipids (alpha-linolenic and linoleic acids) (Table 4). Optionally, the media does not contain activin A and may contain an activin A receptor blocker such as follistatin. Also optionally, the media does not contain antioxidants such as superoxide dismutase (SOD) or catalase, but contains thiolic antioxidants such as glutathione.

[0094] The culture media consists in a basal formulation such as DMEM, F12, Williams, RPMI, Lebovitz supplemented with proteins (in certain formulations), amino acids, antioxidants, energetic substrate (glucose, galactose, L-glutamine), vitamins (B12), hormones (thyroid hormones, insulin) and growth factors (FGF, EGF) as depicted in Table 2.

[0095] The protein can be albumin in concentration of 0.1-0.5%, fetal bovine serum (FBS) 0.5%-20%. The protein can be substituted with macromolecules such as dextrans, hyaluronan,

poly-vinyl alcohol in concentration ranging from 0.1% to 0.5%. The composition of such media is listed in Table 2, Table 3, and Table 4. The supplements are added into the media and mixed for feeding the cell cultures.

[0096] The media can be replaced in a three day a week schedule (e.g., Monday – Wednesday – Friday), or more frequently, e.g., every other day or daily, if the expansion is fast. A continuous feed or a micro-batch feed bioreactor can be used in the expansion phase.

[0097] The media contains growth factors that act through the MAPK pathway such as FGF and EGF. The concentration of these growth factors can vary between 0.1 to 100 ng/mL, commonly around 10 ng/mL.

[0098] In one embodiment, the media is supplemented with FGF at about 0.1 to 100 ng/mL, at about 0.5-50 ng/mL, at about 1-40 ng/mL, at about 2-30 ng/mL, at about 3-20 ng/mL, at about 5-15 ng/mL, at about 6-14 ng/mL, at about 7-13 ng/mL, at about 8-12 ng/mL, at about 9-11 ng/mL, or at about 10 ng/mL. In other embodiments FGF is present in the media at about 5 ng/mL, at about 6 ng/mL, at about 7 ng/mL, at about 8 ng/mL, at about 9 ng/mL, at about 11 ng/mL, at about 12 ng/mL, at about 12 ng/mL, at about 14 ng/mL, or at about 15 ng/mL.

[0099] In another embodiment, the media is supplemented with EGF at about 0.1 to 100 ng/mL, at about 0.5-50 ng/mL, at about 1-40 ng/mL, at about 2-30 ng/mL, at about 3-20 ng/mL, at about 5-15 ng/mL, at about 6-14 ng/mL, at about 7-13 ng/mL, at about 8-12 ng/mL, at about 9-11 ng/mL, or at about 10 ng/mL. In other embodiments EGF is present in the media at about 5 ng/mL, at about 6 ng/mL, at about 7 ng/mL, at about 8 ng/mL, at about 9 ng/mL, at about 11 ng/mL, at about 12 ng/mL, at about 12 ng/mL, at about 14 ng/mL, or at about 15 ng/mL.

Table 2. Basal media composition options for cancer stem cells:

Components	M.W g/mole	DMEM/F12 (1:1)		William's E		DMEM		RPMI		F12	
		mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM
Amino Acids											
L-Alanine	89.10	4.45	0.05	90	1.010					8.9	0.100
L-Arginine	174.20			50	0.287						
L-Arginine·HCl	210.65	147.5	0.70			84	0.399	200	0.949	211	1.002
L-Asparagine·H ₂ O	150.10	7.50	0.05	20	0.133			50	0.333	15.01	0.100
L-Aspartic Acid	133.10	6.65	0.05	30	0.225			20	0.150	13.3	0.100
L-Cysteine	121.16			40	0.330						
L-Cysteine·HCl·H ₂ O	175.65	17.56	0.10						0.000	35.12	0.200
L-Cystine·2HCl	313.11	31.29	0.10	26.07	0.083	62.57	0.200	65.15	0.208		
L-Glutamic Acid	147.10	7.35	0.05	50	0.340			20	0.136	14.7	0.100
L-Glutamine	146.10	365	2.50	292	1.999	584	3.997	300	2.053	146	0.999
L-Glycine	75.10	18.75	0.25	50	0.666	30	0.399	10	0.133	7.5	0.100
L-Histidine	155.16			15	0.097						
L-Histidine·HCl·H ₂ O	209.65	31.48	0.15			42	0.200	15	0.072	20.96	0.100
L-Hydroxyproline	131.13							20	0.153		
L-Isoleucine	131.20	54.47	0.42	50	0.381	105	0.800	50	0.381	3.94	0.030
L-Leucine	131.20	59.05	0.45	75	0.572	105	0.800	50	0.381	13.1	0.100
L-Lysine·HCl	182.65	91.25	0.50	87.46	0.479	146	0.799	40	0.219	36.5	0.200
L-Methionine	149.20	17.24	0.12	15	0.101	30	0.201	15	0.101	4.48	0.030
L-Phenylalanine	165.20	35.48	0.21	25	0.151	66	0.400	15	0.091	4.96	0.030
L-Proline	115.10	17.25	0.15	30	0.261			20	0.174	34.5	0.300

Components	M.W. g/mole	DMEM/F12 (1:1)		William's E		DMEM		RPMI		F12	
		mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM
L-Serine	105.10	26.25	0.25	10	0.095	42	0.400	30	0.285	10.5	0.100
L-Threonine	119.10	53.45	0.45	40	0.336	95	0.798	20	0.168	11.9	0.100
L-Tryptophan	204.20	9.02	0.04	10	0.049	16	0.078	5	0.024	2.04	0.010
L-Tyrosine·2Na·2H ₂ O	261.20	55.79	0.21	50.65	0.194	103.8	0.397	28.83	0.110	7.8	0.030
L-Valine	117.10	52.85	0.45	50	0.427	94	0.803	20	0.171	11.7	0.100
Sugar											
D-Glucose	180.00	3151	17.51	2000	11.111	4500	25	2000	11.11	1802.00	10.01
Vitamins/Nucleotides/Minute Organics											
Ascorbic acid	176.13			2	1.14E-02						
Vitamin B-12 (cobalamin)	1355	0.6800	5.02E-04	0.2	1.48E-04			0.005	3.69E-06	1.4	
Biotin	244.00	0.0035	1.43E-05	0.5	2.05E-03			0.2	8.20E-04	0.0073	2.99E-05
Choline chloride	140.00	8.98	6.41E-02	1.5	1.07E-02	4	2.86E-02	3	2.14E-02	13.96	0.099714
Ergocalciferol	396.66			0.1	2.52E-04						
Folic acid	441.00	2.65	6.01E-03	1	2.27E-03	4	9.07E-03	1	2.27E-03	1.3	2.95E-03
Inositol	180.00	12.60	7.00E-02	2	1.11E-02	7.2	4.00E-02	35	1.94E-01	18	0.1
Menadione sodium bisulfate				0.01							
Niacinamide	122.00	2.02	1.66E-02	1	8.20E-03	4	3.28E-02	1	8.20E-03	0.037	3.03E-04
D-Calcium pantothenate	477.00	2.21	4.63E-03	1	2.10E-03	4	8.39E-03	0.25	5.24E-04	0.48	1.01E-03
Pyridoxal HCl	204.00	2.00	9.80E-03	1	4.90E-03	4	1.96E-02				
Pyridoxine HCl	206.00	0.03	1.50E-04					1	4.85E-03	0.062	3.01E-04
Riboflavin	376.00	0.22	5.82E-04	0.1	2.66E-04	0.4	1.06E-03	0.2	5.32E-04	0.038	1.01E-04

Components	M.W. g/mole	DMEM/F12 (1:1)		William's E		DMEM		RPMI		F12	
		mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM
Thiamine HCl (Vitamin B1)	337.00	2.17	6.44E-03	1	2.97E-03	4	1.19E-02	1	2.97E-03	0.34	1.01E-03
Thymidine	242.23	0.37	2.07E-03							0.7	
Putrescine 2HCl	88.15	0.08	9.19E-04							0.161	
Sodium pyruvate	110.00	55.00	5.00E-01	25	0.227					110	
α-Tocopherol phosphate				0.01							
Lipoic acid	206.00	0.11	5.10E-04							0.21	
Linoleic acid	280.48	0.04	1.50E-04							0.08	
Para-aminobenzoic acid								1			
Vitamin A acetate				0.1							
Inorganic Bulk Salts, buffers											
Magnesium chloride, anhydrous	95.21	28.64	0.30							57.22	0.601
Magnesium sulfate, anhydrous	120.40	48.84	0.41	97.67	0.81	97.67	0.8112	48.84	0.41		
Potassium chloride	74.55	311.80	4.18	400	5.37	400	5.3655	400	5.37	223.6	3.00
Sodium phosphate, dibasic, anhydrous	142.00	71.02	0.50					800	5.63		
Sodium phosphate dibasic·H ₂ O	160.00					125	0.7813				
Sodium chloride	58.44	6999.50	119.77	6800	116.36	6400	109.5140	6000	102.67	7599	130.03
Sodium phosphate monobasic·H ₂ O	120.00	62.50	0.52	140	1.17						
Calcium chloride, anhydrous	111.00	116.60	1.05	200	1.80	200	1.8018			33.22	0.30

Components	M.W. g/mole	DMEM/F12 (1:1)		William's E		DMEM		RPMI		F12	
		mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM
Calcium nitrate·4H ₂ O	236.00							100	0.42		
Sodium bicarbonate	84.01	2438.0	29.02	2200	26.19	3700	44.0424	2000	23.81	1176	14.00
Hepes buffer (1M)										142.04	
Trace Minerals											
Cupric sulfate·5H ₂ O	249.70	0.0013	5.21E-06	0.0001	4E-07					0.0025	1.00E-05
Ferrous sulfate·7H ₂ O	278.00	0.42	1.50E-03							0.834	0.003
Ferric nitrate·9H ₂ O	101.10	0.05	4.95E-04	0.0001	9.9E-07	0.1	0.0010				
Zinc sulfate	287.50	0.43	1.50E-03								
Zinc sulfate·7H ₂ O				0.0002						0.863	
Manganese chloride·4H ₂ O				0.0001							
Others											
Na hypoxanthine		2.39								4.77	
Phenol red		8.10		10		15		5		1.2	
Glutathione (reduced)				0.05				1			
Methyl linoleate				0.03							

Table 3. Lineage stem cell supplement (50 mL units for reconstitution in 1 L of basal media)

Components	Formulation (per 50 mL supplement or 1 L of final media)	
	value	unit
Water	QS to 50	ml
human serum albumin	2.5	g
Transferrin partially saturated	20	mg
Insulin	20	mg
T3	0.002	mg
Selenite	0.01	mg
Progesterone	0.005	mg
Putrescine	10	mg
Catalase	2.5	mg
Glutathione	1	mg
Carnitine	2	mg
Biotin	0.05	g
L-glutamine	365	mg
Ethanolamine	15	mg
HEPES	1	g
Lipid Mix (see Table 4)	5	ml

Table 4. Lipid mix

Components	Concentration: µg/mL
Linolenic	10
Linoleic	10
Tocopherol acetate	50
Cholesterol	100

The lipid mix is made by o/w emulsions using Pluronic F68, phosphatidyl choline, Tween 80, cyclodextrin, or combinations thereof

[0100] Also provided is a medium which is not supplemented with one or both of superoxide dismutase (SOD) or catalase. The use of antioxidants can have both positive and negative consequences. Cancer stem cells are far more tolerant than normal cells to free radicals and glycolytic metabolism. Therefore in suboptimal cultures such as high density, infrequent media replacement, high concentration of metabolites in the media, it is most likely that the normal sensitive cells to be eliminated first. By not including antioxidants in the media, a population of cells can be selected that is likely to be of a cancerous origin, more resistant than the normal cells. Therefore, in certain embodiments, antioxidants, such as catalase and inhibitors of SOD are

added to the culture medium and in other embodiments, these compounds are omitted from the culture media.

[0101] In an alternative method, the activin/follistatin system can be used to isolate very early cancer stem cells. The addition of activin A can select a subpopulation of activin A-resistant CC stem cells. This subpopulation is associated with more aggressive forms of CC and earlier (less differentiated) cancer stem cells. Follistatin is used to block the activin A receptors and prevent spontaneous differentiation of the CC stem cells, especially when large numbers of cells that endogenously secrete activin A are present, such as fibroblasts and normal cells. The use of follistatin has no effect if the cells are insensitive to activin A or in high purity CC stem cell populations where follistatin can be secreted endogenously.

[0102] Activin A is a protein that is a member of the transforming growth factor-beta (TGF-beta) superfamily. When added or included in culture medium, activin helps maintain stem cell pluripotency and self-renewal. However, activin A promotes maturation and differentiation of young cells and cancer cells that are receptive. Therefore, an initial goal is *in vitro* fast expansion of the tumor that also sustains the proliferation of cancer stem cells by creating a proper autocrine environment in the culture. Although activin A may select a subpopulation of very young cancer stem cells, such conditions applied early in the manufacturing will greatly delay the expansion given the very low concentration of the colon cancer stem cells in the bulk. For example, a "fast expansion" is an expansion that results in the media in the culture vessels having obvious signs of consumption (change of pH for example) and the number of cells is visibly higher every day reflected by increased confluence.

[0103] For fast expansion, activin A is preferably omitted and not added, because it will slow down the culture growth. For some applications the interest is to obtain a very early stem cell population and the use of the activin A will select that cell population. Therefore, in one embodiment, an activin A-containing expansion is initiated and a first composition is administered to a subject comprising the activin A-activated cultured cells, followed by the isolation of the activin A-insensitive cells in an activin-A free culture and administering this second composition comprising the activin A free cultured cells to the subject.

[0104] In one embodiment, the media is supplemented with activin A at about 0.01 to 10 ng/mL, at about 0.05-9 ng/mL, at about 0.1-8 ng/mL, at about 0.5-7 ng/mL, at about 1-6 ng/mL, at about 1-5 ng/mL. In other embodiments, activin A is present in the media at about 0.5 ng/mL, at about 0.7 ng/mL, at about 0.9 ng/mL, at about 1 ng/mL, at about 1.25 ng/mL, at about 1.5 ng/mL, at about 1.75 ng/mL, at about 2 ng/mL, at about 2.25 ng/mL, at about 2.5 ng/mL, at about 2.75 ng/mL, at about 3 ng/mL, at about 3.5 ng/mL, at about 4 ng/mL, at about 4.5 ng/mL, at about 5 ng/mL, at about 6 ng/mL, at about 7 ng/mL, at about 8 ng/mL, at about 9 ng/mL, or at about 10 ng/mL.

[0105] Also disclosed is an embodiment wherein the media is supplemented with an antagonist of activin A, such as, but not limited to, follistatin or an antibody that specifically binds to activin A.

[0106] In another embodiment, the media is supplemented with follistatin at about 0.1 to 100 ng/mL, at about 0.5-50 ng/mL, at about 1-40 ng/mL, at about 2-30 ng/mL, at about 3-20 ng/mL, at about 5-15 ng/mL, at about 6-14 ng/mL, at about 7-13 ng/mL, at about 8-12 ng/mL, at about 9-11 ng/mL, or at about 10 ng/mL. In other embodiments, follistatin is present in the media at about 5 ng/mL, at about 6 ng/mL, at about 7 ng/mL, at about 8 ng/mL, at about 9 ng/mL, at about 11 ng/mL, at about 12 ng/mL, at about 12 ng/mL, at about 14 ng/mL, or at about 15 ng/mL.

[0107] The combination of mitogens (e.g., FGF/EGF), activin A, and adherent substrate may result in an increase in the proliferation of normal cells such as fibroblasts or stellate cells. Thus, conditions are created to promote the expansion of very early CC stem cells or progenitors that are insensitive to activin A in a rich environment or "stroma" constituted by cells with nourishing or encapsulating properties (e.g., fibroblasts, stellate cells). The colonies of CC are progressively observed to develop along and spatially displace the stroma in the course of the next few days to weeks of cell culture. The media used in this method is the combination of the formulation described in Tables 2, 3 and 4.

[0108] There is a relationship between FGF, EGF, and activin A, and "very early" CC-CSC. FGF and EGF cause proliferation of CC-CSC in any differentiation status including the very early ones. Where activin A is in the cell culture medium, the activin A is permissive for (allows) proliferation exclusively of the very early CC-CSC that are insensitive to activin A. If the CC-CSC become sensitive, the proliferation will be stopped or reduced by activin A.

[0109] Insensitivity to FGF and EGF is not common and there are no natural blockers. Insensitivity to activin A can be mediated by follistatin, a natural blocker of the activin receptor. Follistatin can be secreted by the same tumor cell or by cells surrounding the tumor. Activin A is typically secreted by the cells surrounding the tumor, therefore it is possible that the expansion of the tumor is dependent on the surrounding cells (inhibiting) and by the tumor (promoting the expansion). The lack of receptor for activin A, a characteristic of the very early, undifferentiated cancer stem cells can prevent the control of the tumor by the surrounding tissue.

[0110] The *in vitro* cultures will contain embryonic stem cell-like colonies. These colonies may be surrounded by stromal cells, that can be normal fibroblasts, differentiated tumor cells, or mesenchymal transitioned tumor cells.

[0111] The present disclosure provides method for preparing CC-CSC where the total culturing time including time required for manipulations such as changing media, replating, centrifugation, and sedimenting, is less than five months, less than four months, less than three months, less than two months, less than one month, less than 150 days, less than 120 days, less

than 90 days, less than 60 days, less than 30 days, or less than 150 days (+/-20 days), less than 120 days (+/-20 days), less than 90 days (+/-20 days), less than 60 days (+/-20 days), less than 30 days (+/-20 days). In exclusionary embodiments, the present disclosure can exclude any method for preparing cancer stem cells, and any population of cancer stem cells prepared by that method, where time required for manipulation is greater than one of the time-frames disclosed above. Also provided is a time in adherent culture that is indicated by one of the above time-frames. Also provided is a time in non-adherent culture that is one of the above time-frames. Moreover, provided is a combined time in adherent culture and in non-adherent culture that is identified by one of the above time-frames.

[0112] Epithelial to mesenchymal transition (EMT)

[0113] Tumors of epithelial origin are known to regress or trans-differentiate into a mesenchymal state. Epithelial phenotypes are immobile, contribute to volume growth of the tumor limited to the originating tissue and are typically more differentiated. When EMT occurs, the cells gain mobility and produce adjacent tissue infiltration and distant metastases. The transitioned cell also gains a stem cell-like phenotype, with the ability to replicate and differentiate resulting in a new tumor (metastasis) in the host tissue with characteristics of the originating (primary) tumor. By EMT, the tumor cells gain additionally immunosuppressive ability, drug pump and radioresistance.

[0114] The media composition and the physical selection method promote the EMT phenomenon *in vitro*. The advantage of using an EMT transitioned population as an immunogen is in prevention of tumor recurrences. The antigenicity of EMT cancer cells could enable the immune system to recognize and destroy mobile cancer cells that cause metastasis. In the process of metastasis these cells travel in very low number, seed the host tissue, revert to an epithelial phenotype (MTE transition), grow and form a new tumor that has similar characteristics with the primary tumor. The conditions necessary to cause *in vitro* EMT are spheroid formation in serum free media, stimulation with bFGF, then plating on adherent substrate containing RGD (Arg-Gly-Asp) peptide motifs (e.g., collagen, gelatin, etc).

[0115] The EMT-CC-CSC subpopulation is obtained by culturing CC-CSC spheroids, early CC-CSC, or mixed CC-CSC under culture conditions as described in Table 1 and FIG. 1.

[0116] As used herein, the term "CC-CSC" can generally refer to CC-CSC spheroids, early CC-CSC, mixed CC-CSC, or EMT-CC-CSC.

[0117] Obtaining CC-CSC from small sources of tumor (needle biopsy)

[0118] An alternative method for CC-CSC selection is used when the number of sample cells is small. For exemplification, a small number of viable cells obtained from a tumor is less than 10×10^6 viable cells after enzymatic dissociation. For the purposes of this disclosure a small sample refers to a sample obtain for example from a needle biopsy or core biopsy, in contrast to a sample obtained from an excised tumor, which is typically not considered a small sample and weighs at

least 0.5 to 5-10 grams. Core biopsies are done with 18 or 16 or 14 gauge needles, resulting in 5-50 mg samples. A relatively new procedure called a vacuum assisted biopsy is also done with an 11 gauge needle, and a vacuum assisted device (VAD). An 11 gauge probe paired with a vacuum-assisted device typically picks up 94 mg with each core sample. The 14 gauge needle with vacuum assistance typically picks up 37 mg, but only 17 mg when paired with an automated biopsy gun. In this alternative method, depicted in FIG. 1, cells obtained from the tumor sample are transferred, before or after dissociation, to an adherent substrate containing RGD (Arg-Gly-Asp) rich compounds (e.g., collagen, gelatin or MATRIGEL®) and in the presence of a selection (serum-free) culture media described herein. The selection method described is designed to (a) promote initial clonal expansion of the individual cancer stem cells that are present in low number, and (d) promote the local autocrine activity as a result of clonal expansion of the stem cells.

[0119] Adherent substrates are RGD rich proteins such as collagen or gelatin. The substrate can be constructed by attaching the protein or peptide to various materials such as polystyrene polycarbonate, cyclic olefin copolymer or glass. The RGD peptide can be grafted on polymeric backbones such as hyaluronic acid, polylactic acid and combinations. Such polymers can be further enhanced with carrier terminations for growth factors such as proteoglycans (e.g., heparin sulfate, chondroitin sulfate, keratin sulfate, and so on).

[0120] The cell culture surface can be used directly or using coating agents such as aminosilanes. A coating is a compound that has adherent property (substrate) for the cells and is applied on top of the growth vessel's material. It can be a natural compound such as collagen or gelatin and also can be constructed of a more synthetic polymer having the mentioned radicals/terminations. A coating agent (glue, such as silanes) can be used to improve the adherence of the coating to the culture vessel material (for example to glass). Silanes alone can be used if they contain the desired radicals or terminal groups.

[0121] With this method and formulation, a large number of cells can be obtained in relatively short period of time. Starting from a few milligrams, cultures of tissue samples, such as needle biopsies containing 10^3 to 10^6 cells, can be expanded in 3-4 weeks to about 10^8 cells.

[0122] Expansion of CC stem cell cultures and generation of subpopulations

[0123] The CC-CSC can be propagated and expanded indefinitely, as an additional characteristic of stem cells. An expanding culture on an adherent substrate is represented in FIG. 4.

[0124] Furthermore, the CC-CSC can be partial or totally differentiated. If the stem cell expansion conditions are removed, the CC-CSC can slow down or stop the proliferation, and change morphology and phenotype to a more differentiated cell type. The morphology can become flat, epitheloid or stellate having multiple nuclei – a characteristic of the more mature or stellate cells.

[0125] The adherent cultures can be dissociated in single cell suspension and transferred to non-adherent (anti-biofouling) conditions to remove the anchorage dependent differentiated cells. After 2-3 days, the stem cells tend to aggregate and clonally expand in small spheroids that based on differential sedimentation can be separated from the single cells. The spheroids can be re-plated in adherent conditions and further propagated. This method will purify the culture stem cell content if the cultures are overtaken by differentiated cells or normal cells such as fibroblasts, from 1-30% to 90-99% stem cell content. The method can be repeated as many times needed in order to restore stem cell purity.

[0126] Small spheroids generally have the dimensions of between 0.1 mm and 2 mm. The size distribution, in terms of number of cells per small spheroid, is generally between 10 cells and 10,000 cells. The shape of a small spheroid can be spherical or oval, and can also occur as conglomerates of spherical or oval structures.

[0127] A patient-specific CC-CSC cell line can be used to identify the genomic mutation responsible for the neoplastic transformation when compared with normal tissue from the same patient. The genomic mutation may not be expressed in every stage of differentiation. Some regulatory proteins, or transcription factors, are only temporary expressed and may disappear during maturation, resulting in a malformed cell but with normal proteins. Identification of a cell population that is maximally expressing the mutation and exposing this population to the immune system could be a major advantage of using cancer stem cells as an antigen source for immunotherapy.

[0128] By identifying the genomic mutation a personalized formulation can be created for a cancer treatment, for example a small molecule, a DNA sequence, antisense RNA or combinations.

[0129] Such cell lines can be further used to create screening plates (96 wells for example) for drug discovery. Multiple lines from various patients can be combined in a single plate to address variability between individuals.

[0130] Colon carcinoma cancer stem cells may retain some properties of the originating tissue such as secretion of proteins, growth factors and hormones (functional tumors). These properties can be exploited given the immortal characteristics of the cell lines, to produce "self" proteins that can be used for the same patients (for example albumin, transforming growth factor (TGF), insulin, glucagon, DOPA etc). The cells can be introduced in small bioreactors and the secretion product collected, purified and stored for the same patient use. This method is particularly advantageous that the patient will not develop immune resistance such as the more traditional biosimilars.

[0131] Loading dendritic cells

[0132] The individual CC-CSC cell line obtained from the patient can be used to produce an antigen for immune therapy. The advantage of using the purified stem cell line resides in a better signal to noise ratio. The more mature cells from the tumor may have compensatory mechanisms that can mask the antigenicity and could be not identified by the immune system. As an antigen source, the CC-CSC can be used alive, mitotically inactive, nonviable or fragmented. Various methods can be used to modify the cells for optimal antigen exposure: a radiant energy (e.g., gamma, UV, X), temperature (e.g., heat or cold), or chemical (e.g., cytostatic, aldehyde, alcohol) or combinations.

[0133] In exemplary implementations, the present disclosure encompasses reagents and methods for activating dendritic cells (DCs), with one or more immune adjuvants, such as a toll-like receptor (TLR) agonist, e.g., CpG-oligonucleotide (TLR9), imiquimod (TLR7), poly(I:C) (TLR3), glucopyranosyl lipid A (TLR4), murein (TLR2), flagellin (TLR5), as well as an adjuvant such as CD40 agonists, e.g., CD40-ligand, or the cytokine, interferon-gamma, prostaglandin E2, and the like. See, e.g., U.S. Pat. 7,993,659; US 7,993,648; US 7,935,804, each of which is incorporated herein by reference for all it discloses regarding activating DCs. The present disclosure encompasses *ex vivo* treatment of DCs with one or more of the above adjuvant reagents, or in addition, or alternatively, administration of the adjuvant to a human subject, animal subject, or veterinary subject.

[0134] The immune system encompasses cellular immunity, humoral immunity, and complement response. Cellular immunity includes a network of cells and events involving dendritic cells, CD8⁺ T cells (cytotoxic T cells; cytotoxic lymphocytes), and CD4⁺ T cells (helper T cells). Dendritic cells (DCs) acquire polypeptide antigens, where these antigens can be acquired from outside of the DC, or biosynthesized inside of the DC by an infecting organism. The DC processes the polypeptide, resulting in peptides of about ten amino acids in length, transfers the peptides to either MHC class I or MHC class II to form a complex, and shuttles the complex to the surface of the DC. When a DC bearing a MHC class I/peptide complex contacts a CD8⁺ T cell, the result is activation and proliferation of the CD8⁺ T cell. Regarding the role of MHC class II, when a DC bearing a MHC class II/peptide complex contacts a CD4⁺ T cell, the outcome is activation and proliferation of the CD4⁺ T cell. Although dendritic cells presenting antigen to a T cell can "activate" that T cell, the activated T cell might not be capable of mounting an effective immune response. Effective immune response by the CD8⁺ T cell often requires prior stimulation of the DC by one or more of a number of interactions. These interactions include direct contact of a CD4⁺ T cell to the DC (by way of contact the CD4⁺ T cell's CD40 ligand to the DCs CD40 receptor), or direct contact of a TLR agonist to one of the dendritic cell's toll-like receptors (TLRs).

[0135] Humoral immunity refers to B cells and antibodies. B cells become transformed to plasma cells, and the plasma cells express and secrete antibodies. Naïve B cells are distinguished

in that they do not express the marker CD27, while antigen-specific B cells do express CD27. The secreted antibodies can subsequently bind to tumor antigens residing on the surface of tumor cells. The result is that the infected cells or tumor cells become tagged with the antibody. With binding of the antibody to the infected cell or tumor cell, the bound antibody mediates killing of the infected cell or tumor cell, where killing is by NK cells. Although NK cells are not configured to recognize specific target antigens, in the way that T cells are configured to recognize target antigens, the ability of NK cells to bind to the constant region of antibodies, enables NK cells to specifically kill the cells that are tagged with antibodies. The NK cell's recognition of the antibodies is mediated by Fc receptor (of the NK cell) binding to the Fc portion of the antibody. This type of killing is called, antibody-dependent cell cytotoxicity (ADCC). NK cells can also kill cells independent of the mechanism of ADCC, where this killing requires expression of MHC class I to be lost or deficient in the target cell.

[0136] Without wishing to be bound to any particular mechanism, the disclosure encompasses administration of cancer stem cell antigens, or administering dendritic cells loaded with cancer stem cell antigens, where the antigens stimulate the production of antibodies that specifically recognize one or more of the cancer stem cell antigens, and where the antibodies mediate ADCC. The phrase, loaded with antigens, refers to the ability of the dendritic cell to capture live cells, to capture necrotic cells, to capture dead cells, to capture polypeptides, or to capture peptides, and the like.

[0137] Capture by cross-presentation is encompassed by the present disclosure. Also encompassed is the use of antigen-presenting cells that are not dendritic cells, such as macrophages or B cells.

[0138] The technique of "delayed type hypersensitivity response" can be used to distinguish between immune responses that mainly involve cellular immunity or mainly involve humoral immunity. A positive signal from the delayed type hypersensitivity response indicates a cellular response.

[0139] The present disclosure provides compositions and methods, where tumor cells are inactivated, e.g., by radiation, nucleic acid cross-linkers, polypeptide linkers, or combinations of these. Cross-linking is the attachment of two chains of polymers molecules by bridges, composed of either an element, a group, or a compound that join certain carbon atoms of the chains by primary chemical bonds. Cross-linking occurs in nature in substances made up of polypeptide chains that are joined by the disulfide bonds involving two cysteine residues, as in keratins or insulin, trivalent pyridinolone and pyrrole cross-links of mature collagen, and cross-links in blood clots which involve covalent epsilon-(gamma-glutamyl)lysine cross-links between the gamma-carboxy-amine group of a glutamine residue and the epsilon-amino group of a lysine residue.

[0140] Cross-linking can be artificially effected in proteins, either adding a chemical substance (cross-linking agent), or by subjecting the polymer to high-energy radiation. Cross-linking with fixatives and heat-induced aggregation has been shown to enhance immune responses as well as completely inhibit proliferation. Substances that may be used to cross-link proteins on the surface, and therefore prevent proliferation, of CC-CSC include, but are not limited to, 10% neutral-buffer formalin, 4% paraformaldehyde, 1% glutaraldehyde, 0.25-5mM dimethyl suberimidate, ice-cold 100% acetone or 100% methanol. Additionally, combinations of 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer solution may also be used.

[0141] Formaldehyde and glutaraldehyde have both been shown to induce the activation of T helper type 1 and type 2 cells. In particular, heat induced aggregation of antigens was also shown to enhance the *in vivo* priming of cytotoxic T lymphocytes. Cross-linking of antigens by 3,3'-dithiobis(sulfosuccinimidylpropionate) results in increased binding of antigens to dendritic cells and the cross-linked antigens are processed through the proteosomal pathway for antigen presentation. Furthermore, formalin fixed hepatocellular carcinoma tumor cells have been used in clinical trials with no evidence of proliferation.

[0142] In one embodiment, whole CC-CSC are fixed with cross-linking agents, and then used as the antigen source in combination with the dendritic cells.

[0143] In another embodiment, the nucleic acids of the cells are cross-linked. An exemplary nucleic acid alkylator is beta-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. Exemplary cross-linkers, such as psoralens, often in combination with ultraviolet (UVA) irradiation, have the ability to cross-link DNA but to leave proteins unmodified. For instance, the nucleic acid targeting compound can be 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen (S-59). Cells can be inactivated with 150 μ M psoralen S-59 and 3 J/cm² UVA light (FX 1019 irradiation device, Baxter Fenwal, Round Lake, IL). The inactivation with S-59 with UV light is referred to as photochemical treatment, where treatment conditions can be adjusted or titrated to cross-linked DNA to the extent that cell division is completely prevented, but where damage to polypeptides, including polypeptide antigens, is minimized. Cells can be suspended in 5 mL of saline containing 0, 1, 10, 100, and 1000 nM of psoralen S-59. Samples can be UVA irradiated at a dose of approximately 2 J/cm². Each sample can then transferred to a 15 mL tube, centrifuged, and the supernatant removed, and then washed with 5 mL saline, centrifuged and the supernatant removed and the final pellet suspended in 0.5 mL of saline. See U.S. Pat. 7,833,775 and 7,691,393, which are incorporated herein by reference for all they disclose regarding inactivation of cells.

[0144] For any cell preparation that is treated with a cross-linking agent, the ability to divide can be tested by the skilled artisan by incubating or culturing in a standard medium for at least one week, at least two weeks, at least three weeks, at least four weeks, at least five weeks, at least two months, at least three months, at least four months, and so on. Cell division can be assessed by stains that reveal chromosomes, and that reveal that cell division is, or is not, taking place. Cell

division can also be measured by counting cells. Thus, where the number of cells in a culture plate remains stable for a period of two weeks, one month, or two months, and so on, it can reasonably be concluded that the cells cannot divide.

[0145] In one embodiment, the dendritic cell immunogenic composition is administered subcutaneously (SC). In further embodiments, each dose ranges from about 5-20 million loaded DCs, repeated in a series of 6-10 doses. In certain embodiments, the doses are administered every five days, every week, every 10 days, every other week, or every third week for two, three, four, five or six doses, followed by administration of doses every two weeks, every three weeks, every four weeks, every month, every five weeks, or every 6 weeks for two, three, four, five or six doses additional doses for a total of 6-10 doses. In one embodiment, the first four injections are given every week for a month, and then once a month for the next 4 injections. In alternative embodiment, administration is once a week for 3 weeks then once a month for 5 months for a total of 8 administrations.

[0146] Each dose comprises about $5\text{-}20 \times 10^6$ loaded DCs, about $5\text{-}17 \times 10^6$ loaded DCs, about $6\text{-}16 \times 10^6$ loaded DCs, about $7\text{-}15 \times 10^6$ loaded DCs, about $7\text{-}14 \times 10^6$ loaded DCs, about $8\text{-}13 \times 10^6$ loaded DCs, about $8\text{-}12 \times 10^6$ loaded DCs, or about $9\text{-}11 \times 10^6$ loaded DCs. In additional embodiment, each dose comprises about 8×10^6 loaded DCs, about 9×10^6 loaded DCs, about 10×10^6 loaded DCs, about 11×10^6 loaded DCs, or about 12×10^6 loaded DCs. The loaded DCs comprise a mixture of DCs and residual CC-CSCs which have not been taken up by the DCs. The administered dose comprises a mixture of these cells and the dose reflects this mixture.

[0147] In another embodiment, the loaded DCs are administered with a pharmaceutically acceptable carrier or excipients. The pharmaceutically acceptable excipients described herein, for example, vehicles, adjuvants, carriers or diluents, are well-known to those who are skilled in the art and are readily available to the public. It is preferred that the pharmaceutically acceptable carrier or excipient be one which is chemically inert to the loaded DCs and one which has no detrimental side effects or toxicity under the conditions of use.

[0148] The choice of excipient or carrier will be determined in part by the particular therapeutic composition, as well as by the particular method used to administer the composition. The formulations described herein are merely exemplary and are in no way limiting.

[0149] Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include, but are not limited to, saline, solvents, dispersion media, cell culture media, aqueous buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose,

mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

[0150] In some exemplary embodiments, an adjuvant is given simultaneously with every dose. In certain embodiments, the cell dose is suspended in a carrier containing an adjuvant. In alternative exemplary implementations, an adjuvant is administered, but not with every single dose. In other exemplary implementations, there is no adjuvant at all. In one embodiment, the adjuvant is GM-CSF.

[0151] Without limitation, dendritic cells (e.g., autologous or allogeneic dendritic cells) are contacted with cancer stem cell antigens as a cell lysate, acid elution, cell extract, partially purified antigens, purified antigens, isolated antigens, partially purified peptides, purified peptides, isolated peptides, synthetic peptides, or any combination thereof. The dendritic cells are then administered to a subject, for example, a subject having CC, or a control subject not having CC. In exemplary implementations, dendritic cells are contacted with, injected into, or administered, by one or more of a route that is subcutaneous, intraperitoneal, intranodal, intramuscular, intravenous, intranasal, inhaled, oral, by application to intestinal lumen, and the like. Additionally, the immunogenic compositions can be administered directly to the site of a tumor or metastasis.

EXAMPLES

Example 1. Isolation and expansion of CC cancer stem cells

[0152] Colorectal carcinoma (CC) tumor samples are histologically heterogeneous, consisting in more or less differentiated cancer cells, along with normal parenchymal, stromal and vascular cells. The purpose of the methods presented here are to isolate and expand a population of cancer stem cells derived from CC samples. Further, these cells are used to prepare an autologous therapy for the treatment and recurrence prevention of the CC.

[0153] The procedures and reagents were designed to sustain typical stem cells, and do not sustain the persistence/proliferation *in vitro* of differentiated epithelia, vascular endothelial cells, smooth muscle and fibroblasts.

[0154] Needle aspiration biopsies and tissue fragments were obtained from consented patients diagnosed with colorectal cancer tumors from the macroscopically identified pathological areas at the primary lesion or from colon metastases. The biopsies were transferred immediately in a closed container in transport media and delivered to the tissue processing facility at controlled temperature (4-8°C). The biopsies were then dissociated in a solution of collagenase IV (4 mg/mL) for 30 minutes.

[0155] Depending on the sample size, the larger biopsies were transferred after dissociation in ultralow adherent cell culture flasks (Corning) in serum free media consisting of DMEM:F12 and supplemented with lineage supplement (as described in Tables 2, 3 and 4), and containing 10

ng/mL bFGF and 10 ng/mL of EGF, while the smaller samples such as needle biopsies were first expanded first on adherent substrate.

[0156] For adherent substrate culture, polystyrene culture flasks were coated with 0.1% gelatin for 10-30 minutes, into which the cells resulted from enzymatic and mechanical dissociation of the needle biopsy samples were transferred and allowed to expand for about 1-2 weeks. Every 2nd day (or on a Monday-Wednesday-Friday schedule) cultures were fed with fresh media consisting of a basal formulation (DMEM:F12, Table 2) and supplemented with lineage supplement (Tables 3 and 4), 5% FBS, 10 ng/mL bFGF, and 10 ng/mL EGF.

[0157] After robust culture establishment and reaching a 60-100% confluence, the adherent cultures were dissociated with a proteolytic enzyme (TrypLE, Life Sciences) and suspended in ultra-low adherent conditions.

[0158] The cultures in ultralow adherent flasks initiated immediately after dissociation of the larger tumors samples, or after the establishment and expansion on adherent substrate of the smaller samples, were seeded at the initial concentration of 100,000 cells/cm². Feeding was performed by gravitational separation every second day (Monday-Wednesday-Friday) with a serum free media consisting of a basal media from Table 2 (DMEM:F12) supplemented with lineage supplement (Table 3), bFGF 10ng/mL and EGF 10 ng/mL. Spheroids formation was observed over 14 days, initially in smaller, irregular clumps, then progressively developing larger, regular spheres (FIG. 2).

[0159] After 2 weeks of growth in the ultralow adherent conditions, the spheres were gently dissociated with exposure to collagenase IV and mechanical pipetting, the enzyme removed by centrifugation, the cells resuspended in fresh media and transferred in regular, gas plasma treated tissue culture flasks.

[0160] The cultures started to grow slowly in the first, after about 7-14 days colony reassembling structures (FIG. 3) were identified that progressively reached confluence. Enzymatic dissociation and passaging these colonies resulted of monolayers of intense proliferating cultures. The dissociation during passaging with collagenase IV allowed expansion rates of 1:2 to 1:6 over the next 4 weeks.

[0161] After 2 to 6 passages, the cell lines were immunocytochemically analyzed for cytokeratins CK19 (FIG. 3C) and CK7 (FIG. 4C), tumor specific markers alpha fetoprotein (FIG. 3B) and ABCG2 (FIG. 4B), adhesion molecules EpCAM and NCAM (FIG. 6A-C), CD44 (FIG. 7C), proliferation marker Ki67 (FIG. 8B), and epithelial to mesenchymal transition (EMT) markers Slug/Snail (FIG. 9B) and Twist.

Table 5. ICC marker expression by the expanded lines from CC lines derived from patient biopsies

Cell Line	CK19	AFP	EpCAM	NCAM	CK7	HLA1	Ki67	Notes
CC-004 p3	4+ / 3	0 / 0	4+ / 2	2+ / 2	1+ / 3	4+ / 3	1+ / 3	Colonies; CK19 most abundant
CC-004 p5	1+ / 3	2+ / 1	1+ / 2	0 / 0	1+ / 3	4+ / 2	1+ / 2	Some clumping, small round undefined cells
CC-006 p2	1+ / 3	0 / 0	1+ / 2	1+ / 2	1+ / 3	4+ / 2	2+ / 2	Colonies, abundant stroma
CC-006 p3	4+ / 2	1+ / 1	4+ / 2	3+ / 2	2+ / 3	4+ / 3	2+ / 3	Colonies; most intense and abundant CK7
CC-011 p1	3+ / 3	0 / 0	1+ / 3	3+ / 1	1+ / 3	4+ / 1	3+ / 3	Colonies, and stroma; good cell density;
CC-011 p3	1+ / 3	0 / 0	1+ / 3	2+ / 1	1+ / 1	4+ / 2	2+ / 3	Good cell morphology; good cell density;

% Positive scale: 0=Negative; 1+ = 1-25%; 2+ = 26-50%; 3+ = 51-75%; 4+ = 76-100%

Staining Intensity Scale: 1 = light; 2 = medium; 3 = high

[0162] The results show that the cultures are mostly of epithelial nature demonstrated by the cytokeratins (CK7 and CK19) with invasive properties by the high proliferative activity (Ki67) and by the increased expression of HLA class I (FIG. 8A-D).

[0163] In a separate culture, the cells were expanded in a serum-free media as previously described in the presence of ligands for receptor tyrosine kinase (bFGF 10 ng/mL and EGF 10 ng/mL) and optional activin A (5 ng/mL), resulting in compact colonies with small, cuboid cells with large nuclei reassembling embryonic stem cell cultures. The ICC profile of these cultures identified the presence of the FoxA2 marker that is a pioneer transcription factor involved in early endoderm specification during gastrulation of the embryo (FIG. 5A-C). In the same cultures, the presence of the Sox2 transcription factor (FIG. 7B) was seen, that is maintaining the non-differentiated, pluripotent state of the cells.

[0164] In these cultures the presence of the very early cancer stem cells resembling embryonic stem cell cultures were seen, expressing transcription factors that are associated with pluripotency or very early embryonic development.

[0165] The culture conditions also enriched for cell types that are associated with aggressive colorectal cancers such as the presence of HLA class I, NCAM, and EMT morphology (FIG. 6A-C and FIG 9A-D). The surface antigen CD44, hyaluronan receptor, has a known association with mobility and metastatic properties of the tumor cells. In the analyzed samples the majority of the cells (more than 90-100%) were positive for CD44 (FIG. 7C).

[0166] This data shows that the presented serum-free media formulation, the substrate and propagation method, caused selection and/or transition of the cancer stem cell progenitors from the original mixed tumor population obtained from a small needle biopsy. Expansion and propagation in a serum containing media (0.5-5%) can accelerate the expansion phase after 1-2 passages, however in combination with a collagen based substrate (gelatin, RGD peptides) and growth factors such as FGF, EGF may cause unwanted proliferation of fibroblasts or myoblasts that may persisted during selection phase.

Example 2. Production of Loaded Dendritic Cell Compositions

[0167] The antigen source is autologous tumor cells from continuously proliferating, self-renewing cells derived from the patient's fresh tumor tissue. These cells have the characteristics of tumor stem cells. At all times in the surgical and pathology setting, biopsies are handled with strict adherence to sterility protocols to ensure that samples are sterile.

[0168] The pathologist obtains fresh tissue from biopsy of the patient's tumor. Using sterile scalpels and forceps, the specimen is cut into 10 mm slices and transferred to the transport tubes containing transport media, working quickly to avoid specimen drying. Specimens are shipped by overnight courier to the manufacturing facility within 48 hours of surgical resection.

[0169] At the manufacturing facility, samples are dissociated into single cell suspensions in a clean room and placed in cell culture conditions designed to enrich for and proliferate the CC-CSC. During the processing of the tumor specimen, normal cells such as lymphocytes, stromal cells and connective tissue are eliminated. Upon completion of the expansion and purification steps, the enriched proliferating CC-CSC (tumor cells, TC) are inactivated by irradiation (apoptosis, which facilitates antigen exposure to antigen presenting cells) and placed in vapor phase liquid nitrogen storage. This process can take up to eight weeks, depending on the quantity and quality of the tumor specimen.

[0170] Once the tumor cell product has cleared quality assurance, the patient is notified to undergo a procedure called leukapheresis (usually a six liter procedure). This process entails the filtering of blood to collect peripheral blood mononuclear cells (PBMCs). The collected PBMC is shipped to the manufacturing facility by overnight courier for further purification by counter flow density centrifugation called elutriation. Elutriation is a process by which monocytes are purified from other lymphocytes in order to enrich for cells that can be turned into antigen presenting cells

or dendritic cells. To generate the dendritic cells, the elutriated monocytes are incubated with the cytokines GM-CSF and interleukin-4 (IL-4) for six days.

[0171] On Day 6, the purified tumor cell product is removed from cryostorage, thawed and combined with the dendritic cells for 18-24 hours. This process results in "antigen loading" of the DC. The final product is either entirely DC or may contain some residual irradiated TC (which is considered permissible), and is referred to as DC-TC. The combined dendritic cell/tumor cell mixture is collected, cryopreserved to retain viability of the dendritic cells and stored in vapor phase liquid nitrogen.

[0172] Upon completion of the quality controls assays and release of the autologous cell therapy product, the batch is shipped to the treatment facility under vapor phase liquid nitrogen conditions. After arrival, the cell therapy product is stored under vapor phase liquid nitrogen conditions until prepared for administration.

[0173] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." As used herein the terms "about" and "approximately" means within 10 to 15%, preferably within 5 to 10%. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0174] The terms "a," "an," "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No

language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0175] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0176] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0177] Specific embodiments disclosed herein may be further limited in the claims using consisting of or consisting essentially of language. When used in the claims, whether as filed or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the invention so claimed are inherently or expressly described and enabled herein.

[0178] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above-cited references and printed publications are individually incorporated herein by reference in their entirety.

[0179] In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

[0180] Thus, while there have shown and described and pointed out fundamental novel features of the disclosure as applied to an exemplary implementation and/or aspects thereof, it will be understood that various omissions, reconfigurations and substitutions and changes in the form

and details of the exemplary implementations, disclosure and aspects thereof may be made by those skilled in the art without departing from the spirit of the disclosure and/or claims. For example, it is expressly intended that all combinations of those elements and/or method steps which perform substantially the same function in substantially the same way to achieve the same results are within the scope of the disclosure. Moreover, it should be recognized that structures and/or elements and/or method steps shown and/or described in connection with any disclosed form or implementation may be incorporated in any other disclosed or described or suggested form or implementation as a general matter of design choice. It is the intention, therefore, to not limit the scope of the disclosure. All such modifications are intended to be within the scope of the claims appended hereto.

[0181] All publications, patents, patent applications, references, and sequence listings, cited in this specification are herein incorporated by this reference as if fully set forth herein.

[0182] The Abstract is provided to comply with 37 CFR §1.72(b) to allow the reader to quickly ascertain the nature and gist of the technical disclosure. The Abstract is submitted with the understanding that it will not be used to interpret or limit the scope or meaning of the claims.

Claims:

1. An immunogenic composition comprising dendritic cells activated *ex vivo* by tumor antigens derived from the population of purified colon carcinoma (CC) cancer stem cells (CSC).
2. The immunogenic composition of claim 1, wherein the tumor antigens comprise cell extracts of the CC-CSC.
3. The immunogenic composition of claim 1, wherein the tumor antigens comprise lysates of the CC-CSC.
4. The immunogenic composition of claim 1, wherein the tumor antigens comprise intact CC-CSC.
5. The immunogenic composition of claim 4, wherein the intact CC-CSC are rendered non-proliferative.
6. The immunogenic composition of claim 5 wherein the intact CC-CSC are rendered non-proliferative by irradiation.
7. The immunogenic composition of claim 4, wherein the intact CC-CSC are rendered non-proliferative by exposure of the cells to a nuclear cross-linking agent.
8. The immunogenic composition of any one of claims 1-7, further comprising a pharmaceutically acceptable carrier and/or excipient.
9. The immunogenic composition of any one of claims 1-8, further comprising an adjuvant.
10. The immunogenic composition of claim 9, wherein the adjuvant is granulocyte macrophage colony stimulating factor.
11. The immunogenic composition of any one of claims 1-10, wherein the composition comprises activated dendritic cells and CC-CSC.
12. The immunogenic composition of any one of claims 1-11, wherein the CC-CSC are in the form of CC-CSC spheroids.
13. The immunogenic composition of any one of claims 1-11, wherein the CC-CSC are in the form of early CC-CSC.
14. The immunogenic composition of any one of claims 1-11, wherein the CC-CSC are in the form of mixed CC-CSC.
15. The immunogenic composition of any one of claims 1-11, wherein the CC-CSC are in the form of EMT-CC-CSC.
16. A method of treating colon carcinoma in a subject in need thereof, comprising administration of the immunogenic composition of any one of claims 1-15 to the subject.

17. The method of claim 16, wherein the immunogenic composition is administered in a plurality of doses, each dose comprising about $5\text{-}20 \times 10^6$ cells.

18. The method of claim 17, wherein the dose comprises about 10×10^6 cells.

19. The method of any one of claims 16-18, wherein the dose is administered weekly for 2-5 doses, followed by monthly for 3-6 doses.

20. The method of any one of claims 16-19, wherein the subject receives from 6-10 doses of immunogenic composition.

22. Use of the immunogenic composition of any one of claims 1-15 in the manufacture of a medicament for the treatment of colon carcinoma.

23. Use of the immunogenic composition of any one of claims 1-15 for the treatment of colon carcinoma.

24. A method for preparing a population of colon carcinoma (CC) cancer stem cells (CSC), the method comprising:

acquiring a sample of CC;

dissociating the cells of the sample, and

in vitro culturing the dissociated cells in a defined medium on a non-adherent substrate, wherein the defined medium is serum free and is supplemented with at least one growth factor that acts through the mitogen activated protein kinase (MAPK) pathway, thereby forming CC-CSC spheroids;

wherein the at least 80% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, and CD29.

25. The method of claim 24, wherein at least 80% of the cells in the CC-CSC spheroid population further express one or more of the biomarkers CK7, CK19, E-cadherin, CD20, ESA, ALDH, CDX1, LGR5, and DCIk1..

26. The method of claim 24, wherein at least 90% of the cells in the CC-CSC spheroid population express two or more of the biomarkers CD133, Hes1, CD44, CD24, CD166, and CD29.

27. The method of claim 24, further comprising:

culturing the CC-CSC spheroids in a defined medium on an adherent substrate, wherein the defined medium is serum free and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of early CC-CSC, wherein at least 80% of the cells in the early CC-CSC population express two or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133.

28. The method of claim 27, wherein at least 80% of the cells in the early CC-CSC population further express one or more of the biomarkers EpCAM, E-cadherin, Sox7, Sox17, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166, and CDCP1.

29. The method of claim 27, wherein at least 90% of the cells in the early CC-CSC population express two or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133.

30. The method of claim 24, further comprising:
culturing the CC-CSC spheroids in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of mixed CC-CSC, wherein at least 80% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.

31. The method of claim 30, wherein at least 90% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.

32. The method of claim 24, further comprising:
culturing the CC-CSC spheroids in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of epithelial to mesenchymal transitioned (EMT)-CC-CSC, wherein at least 80% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117.

33. The method of claim 32, wherein at least 80% of the cells in the EMT-CC-CSC population further express one or more of the biomarkers CD44, CD24, γ -synuclein, FMNL2, b-catenin, Nanog, CD147, β 3GhT8, LGR5, CD29, CXCR4, CD133, and DCIk1.

34. The method of claim 32, wherein at least 90% of the cells in the EMT-CC-CSC population express one or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117

35. The method of any one of claims 24, 30, or 32, further comprising:
culturing the CC-CSC spheroids, the mixed CC-CSC, or EMT-CC-CSC in a defined medium on an adherent substrate, wherein the defined medium is serum free and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of early CC-CSC, wherein at least 80% of the cells in the early CC-CSC population express two or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133.

36. The method of claim 35, wherein at least 80% of the cells in the early CC-CSC population further express one or more of the biomarkers EpCAM, E-cadherin, Sox7, Sox17, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166, and CDCP1.

37. The method of claim 35, wherein at least 90% of the cells in the early CC-CSC population express one or more of the biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, and CD133.

38. The method of any one of claims 24, 27, or 32, further comprising:
culturing the CC-CSC spheroids, the early CC-CSC, or EMT-CC-CSC in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of mixed CC-CSC, wherein at least 80% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.
39. The method of claim 38, wherein at least 90% of the cells in the mixed CC-CSC population express two or more of the biomarkers Hes1, MSI1, ALDH1B1, ALDH1A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP, and DCIk1.
40. The method of any one of claims 24, 27, or 30, further comprising:
culturing the CC-CSC spheroids, the early CC-CSC, or mixed CC-CSC in a defined medium on an adherent substrate, wherein the defined medium contains serum and is supplemented with at least one growth factor that acts through the MAPK pathway, thereby forming a population of EMT-CC-CSC, wherein at least 80% of the cells in the EMT-CC-CSC population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117.
41. The method of claim 40, wherein at least 80% of the cells in the EMT-CC-CSC population further express one or more of the biomarkers CD44, CD24, γ -synuclein, FMNL2, b-catenin, Nanog, CD147, β 3GhT8, LGR5, CD29, CXCR4, CD133, and DCIk1.
42. The method of claim 40, wherein at least 90% of the cells in the EMT-CC-CSC population express one or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist, and CD117.
43. The method of any one of claims 24-42, wherein the defined media is any media described in Table 2.
44. The method of any one of claims 24-42, wherein the defined media is any media from a combination of Table 2 and Table 3.
45. The method of any one of claims 24-42, wherein the defined media is any media from a combination of Table 2, Table 3, and Table 4.
46. The method of any one of claims 24-42, wherein the defined media is any media from a combination of Table 2 and Table 4.
47. The method of any one of claims 24-46, wherein the growth factor is one or more of fibroblast growth factor (FGF), epidermal growth factor (EGF), or activin A.
48. The method of claim 47, wherein the FGF is basic FGF (bFGF).

49. The method of any one of claims 24-48, wherein the defined medium is not supplemented with activin A.

50. The method of any one of claims 24-49, wherein the defined medium is supplemented with an agonist of activin A, in an amount effective to prevent spontaneous differentiation of CC stem cells.

51. The method of any one of claims 24-49, wherein the medium further comprises an antagonist of activin A, and the antagonist is follistatin or an antibody that specifically binds to activin A.

52. The method of any one of claims 24-51, wherein the medium is not supplemented with an antioxidant.

53. The method of claim 52, wherein the antioxidant is superoxide dismutase, catalase, glutathione, putrescine, or β -mercaptoethanol.

54. The method of any one of claims 24-51, wherein the medium is supplemented with glutathione.

55. The method of any one of claim 24-54, wherein the adherent substrate is configured to adhere to, and to collect, anchorage dependent cells.

56. The method of claim 55, wherein the anchorage dependent cells are fibroblasts.

57. The method of any one of claim 24-54, wherein the non-adherent substrate is an ultralow adherent polystyrene surface.

58. The method of claim 57, wherein the adherent substrate comprises a surface coated with a protein rich in RGD tripeptide motifs.

59. A population of purified CC-CSC cells prepared by the method of any one of claims 24-58.

60. The population of claim 59, wherein the purified CC-CSC cells are CC-CSC spheroids.

61. The population of claim 59, wherein the purified CC-CSC cells are early CC-CSC.

62. The population of claim 59, wherein the purified CC-CSC cells are mixed CC-CSC.

63. The population of claim 59, wherein the purified CC-CSC cells are EMT-CC-CSC.

64. A CC-CSC cell line prepared by the method of any one of claims 1-29.

65. The CC-CSC cell line of claim 64, wherein the CC-CSC are CC-CSC spheroids.

66. The CC-CSC cell line of claim 64, wherein the CC-CSC are early CC-CSC.

67. The CC-CSC cell line of claim 64, wherein the CC-CSC are mixed CC-CSC.

68. The CC-CSC cell line of claim 64, wherein the CC-CSC are EMT-CC-CSC.

69. A method of stimulating an immune response against colon carcinoma in a subject in need thereof, comprising administration of the immunogenic composition of any one of claims 1-15, the CC-CSC cells of any one of claims 59-63, or the CC-CSC cell line of any one of claims 64-68 to the subject.

70. Use of the CC-CSC cells of any one of claims 59-63 or the CC-CSC cell line of any one of claims 64-68 in the manufacture of a medicament for the treatment of colon carcinoma.

71. Use of the CC-CSC cells of any one of claims 59-63 or the CC-CSC cell line of any one of claims 64-68 for the treatment of colon carcinoma.

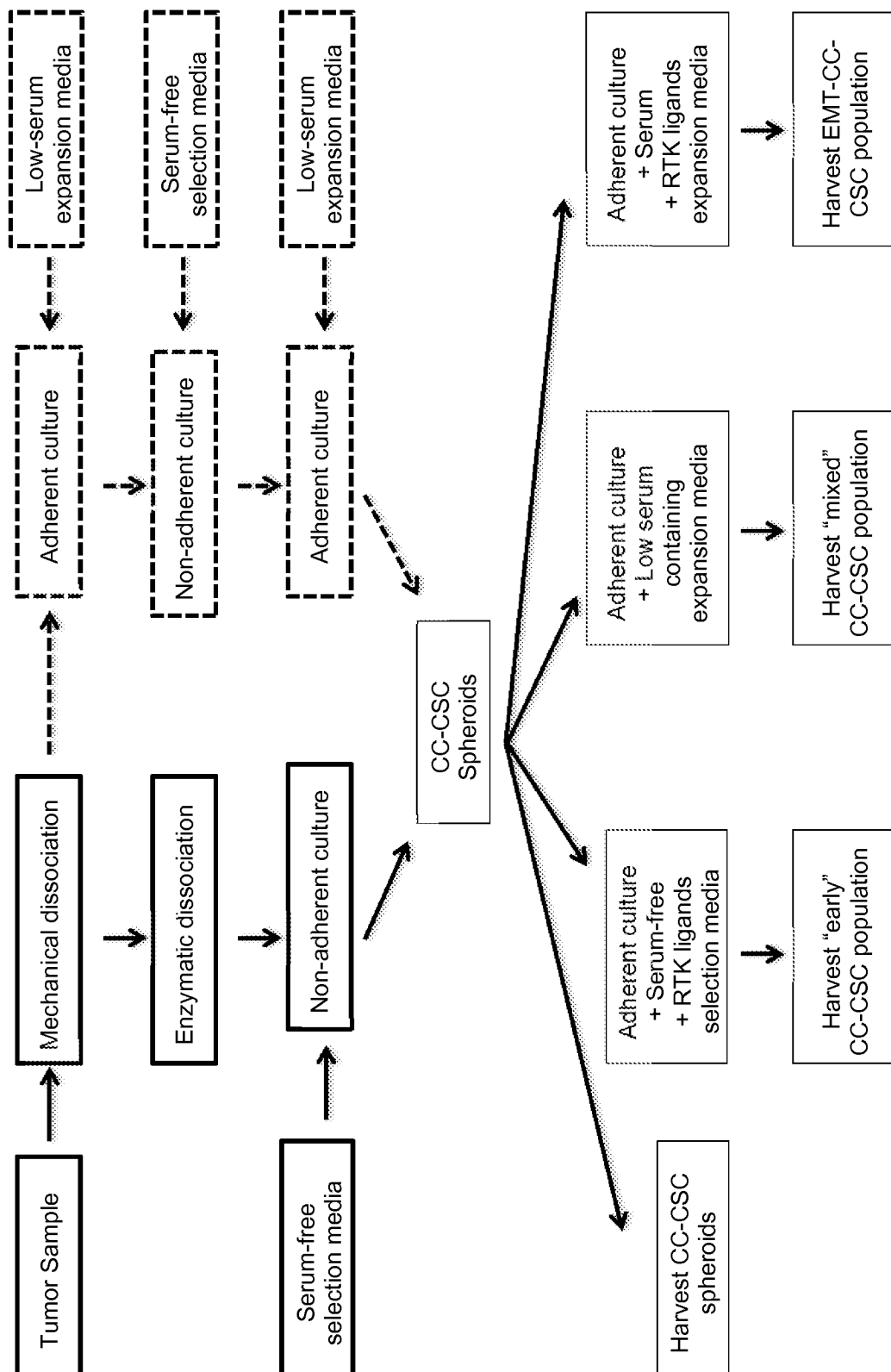


FIG. 1

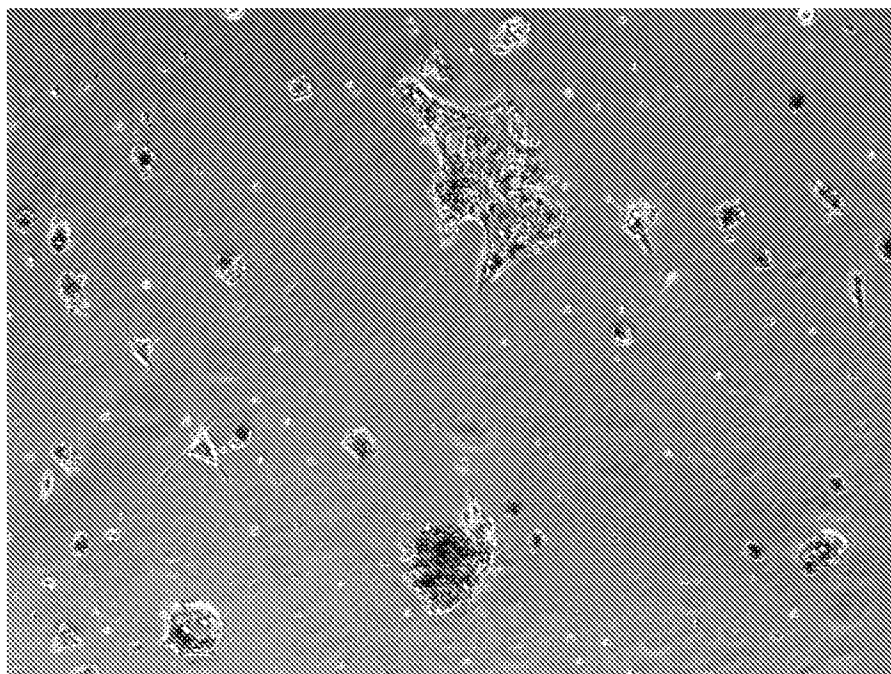


FIG. 2.

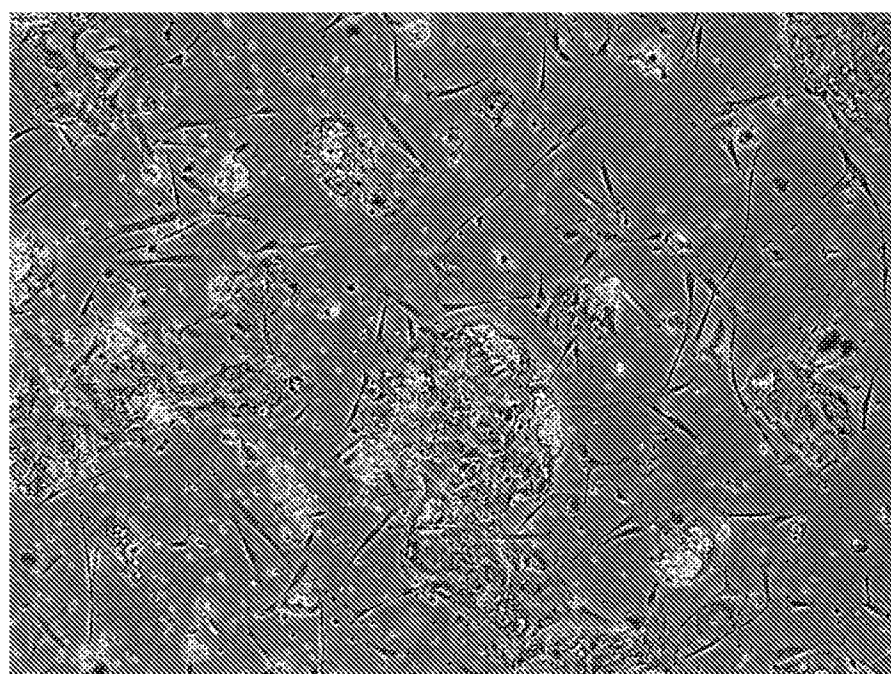


FIG. 3.

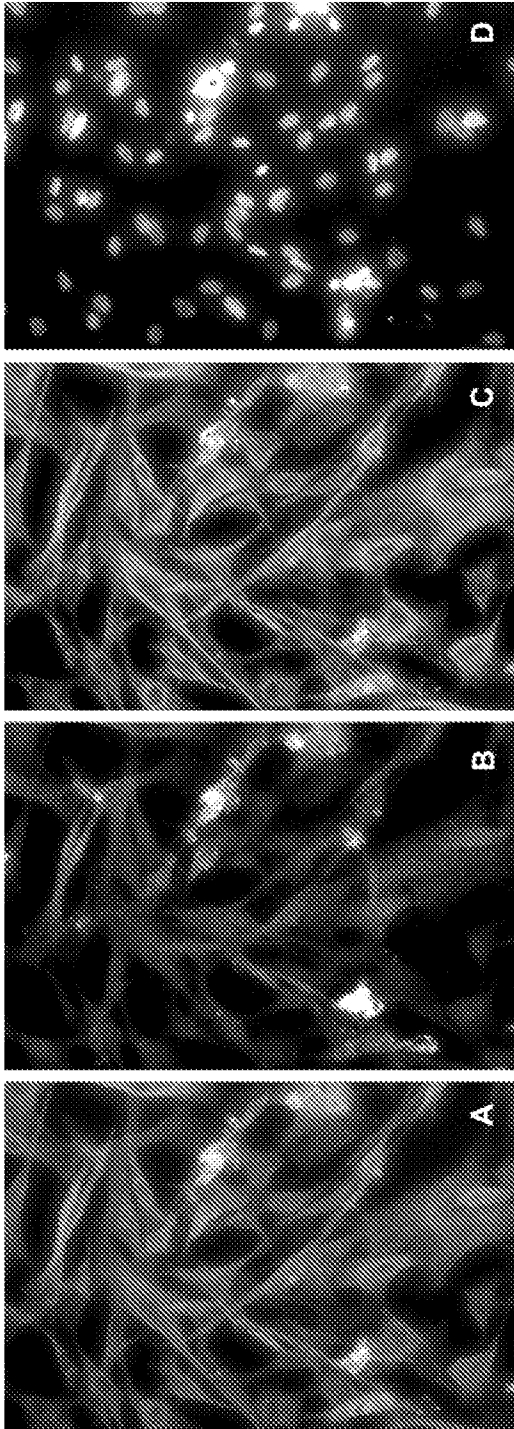


FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

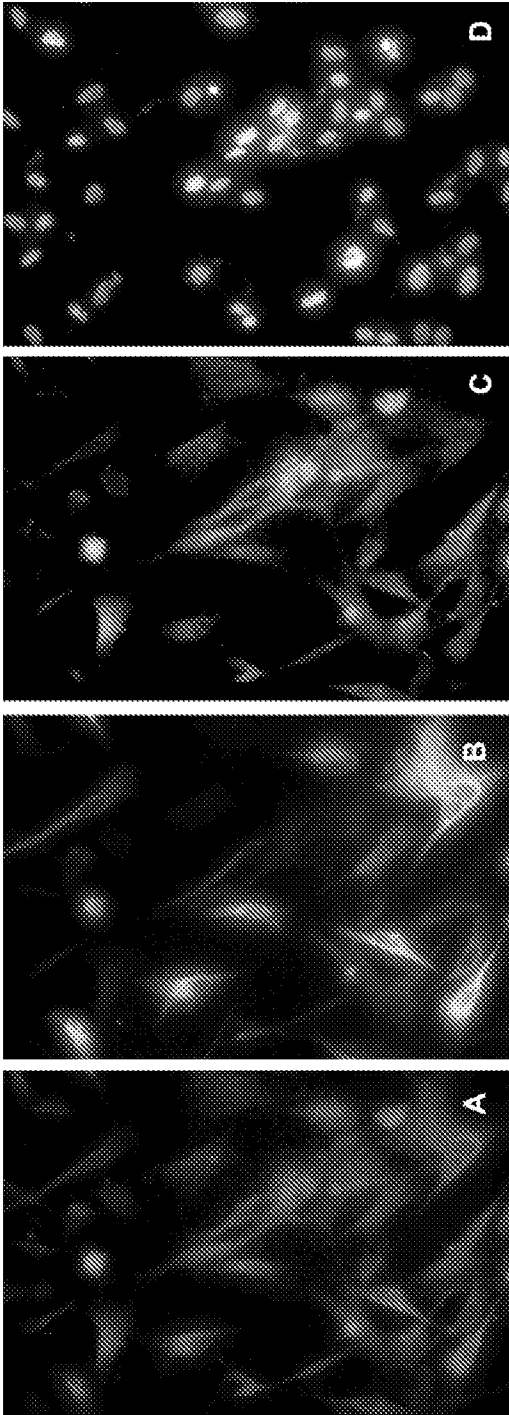


FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

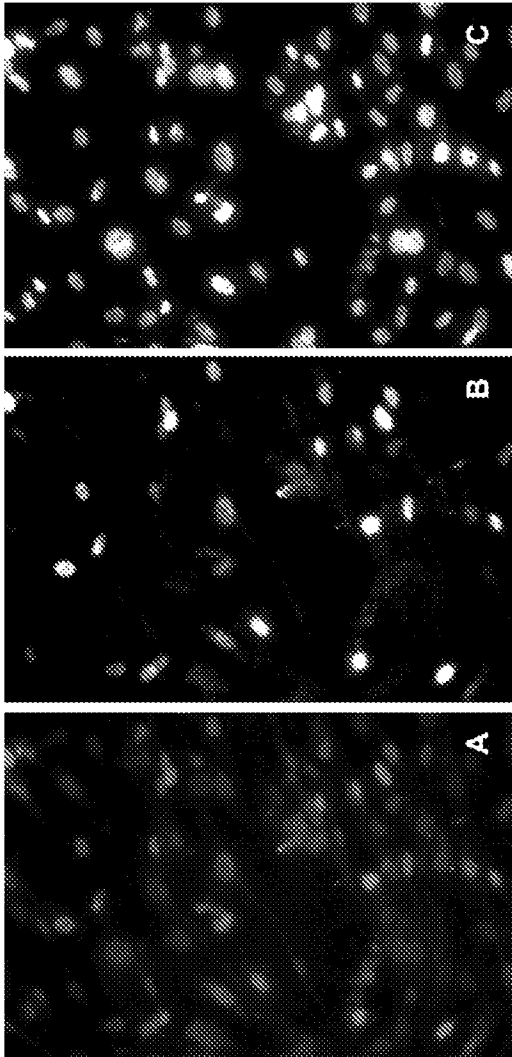


FIG. 5A FIG. 5B FIG. 6C

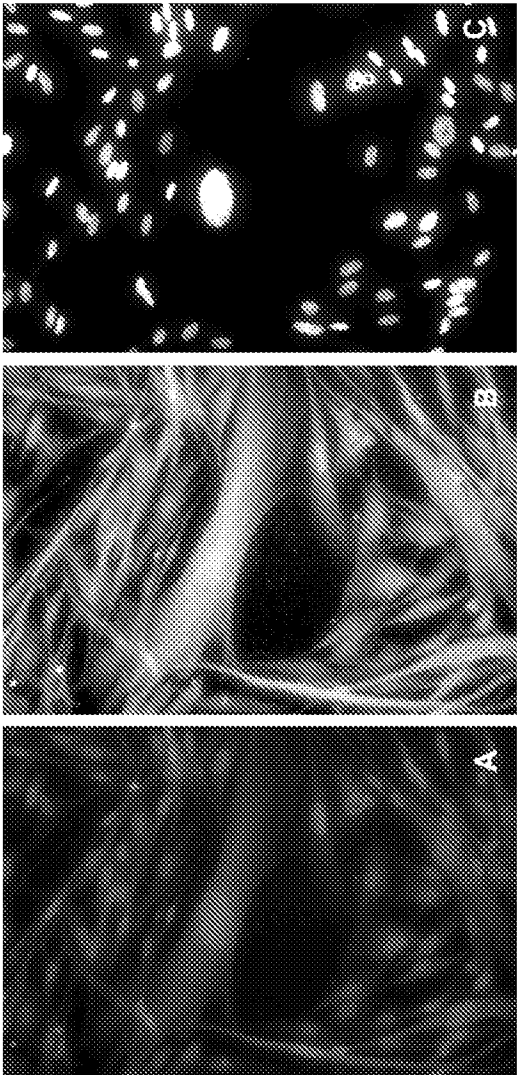


FIG. 6A FIG. 6B FIG. 6C

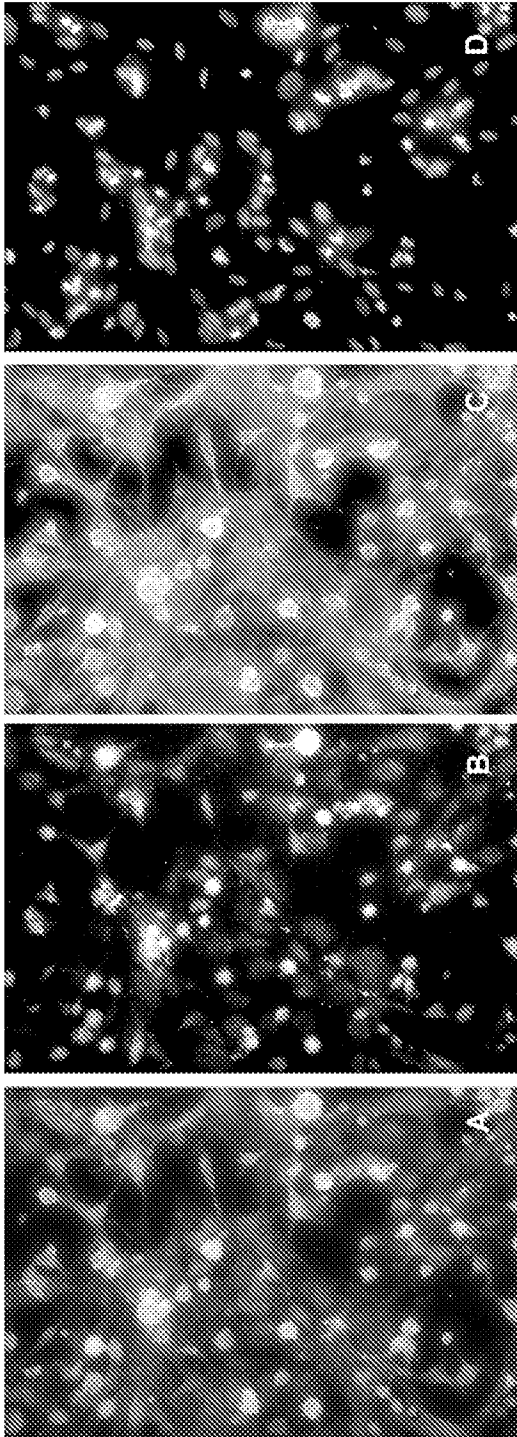


FIG. 7A

FIG. 7B

FIG. 7C

FIG. 7D

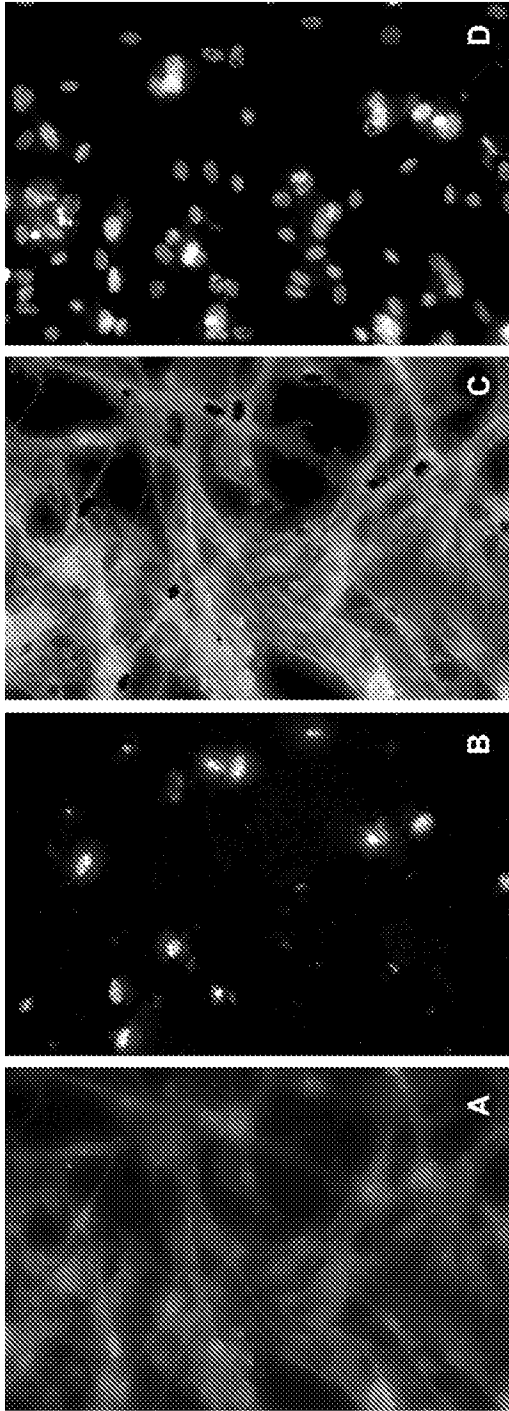


FIG. 8A

FIG. 8B

FIG. 8C

FIG. 8D

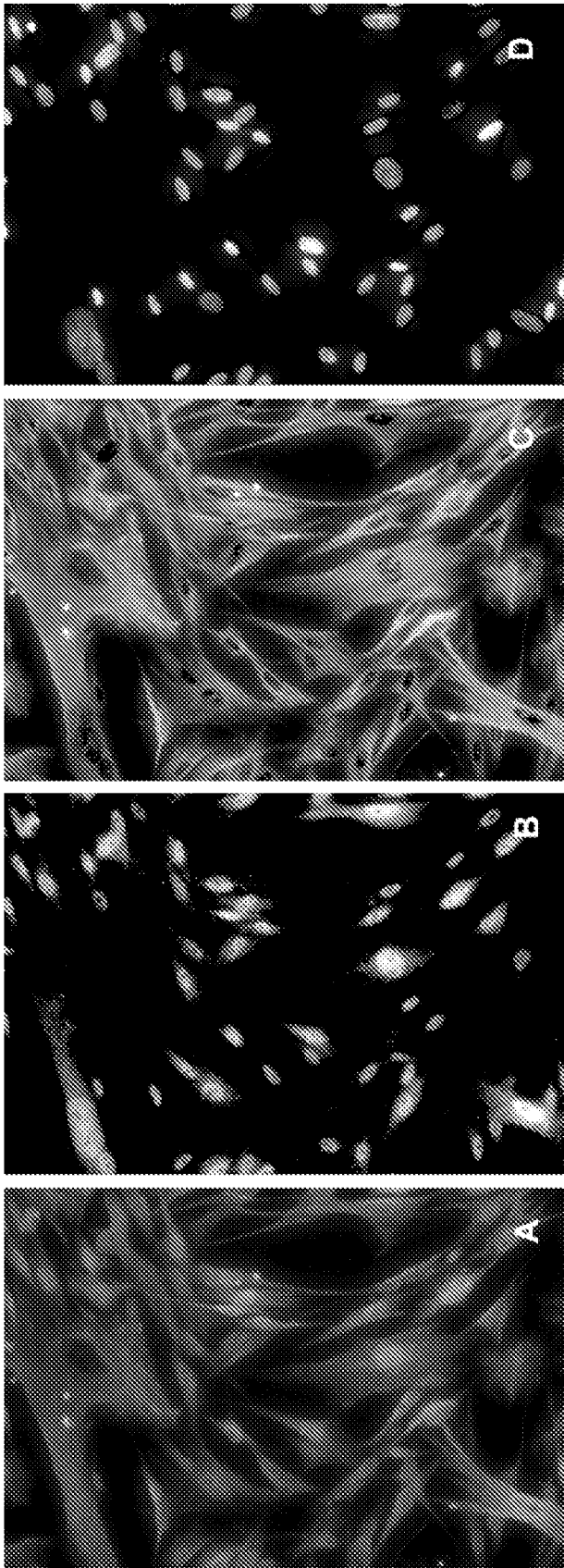


FIG. 9

FIG. 9B

FIG. 9C

FIG. 9D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/024382**A. CLASSIFICATION OF SUBJECT MATTER****A61K 35/12(2006.01)i, A61K 39/39(2006.01)i, A61K 39/395(2006.01)i, A61P 35/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/12; C12Q 1/02; C12N 5/00; A61N 5/00; A61P 37/00; A61K 39/00; A61P 35/00; A61P 37/04; A61K 39/39; A61K 39/395

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: colon carcinoma stem cells, dendritic cell activation, sphere formation, biomarkers, epithelial-to-mesenchymal transition

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0313229 A1 (SUGAYA, K. et al.) 22 December 2011 See abstract, pages 4-6, claims 11, 15, 20.	1-8
Y		24-42
Y	KONG, D. et al. "Cancer Stem Cells and Epithelial to Mesenchymal Transition (EMT)-Phenotypic Cells: Are They Cousins or Twins?" Cancers. 2011. Vol. 3, pp. 716-729 See abstract, pages 718, 719.	24-42
A	BELOV, L. et al. "Cell Surface Markers in Colorectal Cancer Prognosis." Int. J. Mol. Sci. 2011. Vol. 12, pp. 78-113 See the whole document.	1-8, 24-42
A	BURGDORF, S. K. et al. "Dendritic cell vaccination of patients with metastatic colorectal cancer." Dan. Med. Bull. 2010. Vol. 57: B4171, pp. 1-17 See the whole document.	1-8, 24-42
A	JUN, D. et al. "Latest Therapeutic Approaches Based on Cancer Stem Cells." Cancer Stem Cells - The Cutting Edge, Prof. Stanley Shostak (Ed.). 2011. pp. 389-406 See the whole document.	1-8, 24-42

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 July 2014 (31.07.2014)

Date of mailing of the international search report

31 July 2014 (31.07.2014)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/024382**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. ☒ Claims Nos.: 16-20, 69
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 16-20, 69 pertain to methods for treatment of the human body by surgery or therapy, as well as diagnostic methods, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☒ Claims Nos.: 10, 17, 18, 21, 48, 53, 56, 58, 60-63, 65-68
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 10, 17, 18, 48, 53, 56, 58, 60-63, 65-68 are unclear, as they refer to the multiple dependent claim which is not drafted in accordance with the third sentence of Rule 6.4(a), and claim 21 is missing in this application.
3. ☒ Claims Nos.: 9, 11-16, 19, 20, 22, 23, 43-47, 49-52, 54, 55, 57, 59, 64, 69-71
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/024382

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012-0189664 A1 (YU, J. S.) 26 July 2012 See the whole document.	1-8, 24-42
A	US 2008-0175870 A1 (MATHER, J. P. et al.) 24 July 2008 See the whole document.	1-8, 24-42
A	WO 2012-046797 A1 (PHARMACOLOGICALS RESEARCH PTE. LTD) 12 April 2012 See the whole document.	1-8, 24-42

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/024382

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0313229 A1	22/12/2011	CA 2731091 A1 EP 2318040 A1 EP 2318040 A4 JP 2011-529080 A WO 2010-011893 A1	28/01/2010 11/05/2011 01/05/2013 01/12/2011 28/01/2010
US 2012-0189664 A1	26/07/2012	CA 2700573 A1 EP 2084267 A2 EP 2084267 A4 US 2008-206286 A1 US 8129184 B2 WO 2008-039874 A2 WO 2008-039874 A3	03/04/2008 05/08/2009 02/03/2011 28/08/2008 06/03/2012 03/04/2008 10/07/2008
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WO 2012-046797 A1	12/04/2012	CN 103328626 A EP 2626414 A1 EP 2626414 A4 SG 189302 A1 US 2013-0288248 A1	25/09/2013 14/08/2013 23/04/2014 31/05/2013 31/10/2013