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(54) Title: SINGLE BLADDER CELL-DERIVED ORGANOIDS

(57) Abstract: The present invention relates to organoids derived from a single cell, such as a bladder cancer cell, and methods and compositions relating to the production and use thereof, including cell culture medium for producing organoids and methods of personalized treatment for bladder cancer and Interstitial cystitis (IC) or painful bladder syndrome (PBS). The invention further provides a humanized mouse including a bladder organoid derived from a patient's bladder cell.



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SINGLE BLADDER CELL-DERIVED ORGANOID**CROSS-REFERENCE TO RELATED APPLICATION**

[0001] This application claims the benefit of U.S. Provisional Application No. 62/526,078 filed June 28, 2017, the disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The inability to propagate primary tissues represents a major hurdle to understanding the mechanisms of regeneration and the balance of differentiated cells versus stem cells in adult organisms. A need exists to better understand primary human pathological disorders such as injury repair and tumor development. For cancer studies, current cancer models do not adequately represent the molecular and cellular diversity of human cancers. Existing human cancer cell lines lack defined and detailed information regarding the clinical presentation of the cancer and have inherent limitations for deciphering the mechanisms of therapy resistance. For injury repair, there is a lack of understanding of the mechanisms of regeneration and shortage of tissue and organs for transplantation. Therefore, novel methods to maintain primary tissues for cancer, new drug discovery approaches to treat cancer and regenerative medicine indications are needed.

[0003] Maintaining the balance between normal differentiated cells and progenitor or stem cells is complex. Adult stem cells provide regeneration of different tissues, organs, or neoplastic growth through responding to cues regulating the balance between cell proliferation, cell differentiation, and cell survival, with the later including balanced control of cell apoptosis, necrosis, senescence and autophagy. Epigenetic changes, which are independent of the genetic instructions but heritable at each cell division, can be the driving force towards initiation or progression of diseases. Tissue stem cells are heterogeneous in their ability to proliferate, self-renew, and differentiate and they

can reversibly switch between different subtypes under stress conditions. Tissue stem cells house multiple subtypes with propensities towards multi-lineage differentiation. Hematopoietic stem cells (HSCs), for example, can reversibly acquire three proliferative states: a dormant state in which the cells are in the quiescent stage of the cell cycle, a homeostatic state in which the cells are occasionally cycling to maintain tissue differentiation, and an activated state in which the cells are cycling continuously. The growth and regeneration of many adult stem cell pools are tightly controlled by these genetic and/or epigenetic responses to regulatory signals from growth factors and cytokines secreted through niche interactions and stromal feedback signals.

[0004] Bladder cancer is one of the leading causes of cancer-related death. Bladder cancer can be classified into non-muscle invasive bladder cancer, which is more common and has a favorable prognosis, and muscle invasive bladder cancer, which is less prevalent but has a worse prognosis. Notably, bladder cancer is one of the most expensive cancers to treat, primarily due to the considerable costs associated with long-term clinical management of patients with non-muscle invasive bladder cancer, as well as the costs associated with caring for patients after surgical removal of the bladder. Despite its prevalence, bladder cancer has been remarkably understudied relative to other cancers, and remains significantly under-represented by cancer models. Muscle invasive bladder cancers have a relatively poor prognosis, with a 5-year survival of ~50% for localized disease and ~15% for metastatic tumors.

[0005] Studies of bladder tumorigenesis and drug response would greatly benefit from the availability of robust *in vitro* and *in vivo* models that recapitulate the diversity of bladder cancer phenotypes, subtypes, and genomic profiles that characterize the human disease. However, in contrast with many other cancer types, there are very few *in vivo* models of bladder cancer. Most bladder cancer studies have utilized 2-dimensional (2D) cell culture models, however, these

models do not reflect the full genomic heterogeneity of the human disease. As an example, muscle invasive bladder cancer has the highest prevalence of ERBB2 mutations among the most common solid tumors, but currently there is no bladder cancer cell line available for preclinical studies that expresses the S310F ERBB2 mutant, which is the mutant allele found in >90% of ERBB2 mutant bladder tumors. This emphasizes the need for generation of new models, particularly models that represent the full spectrum of genomic alterations that occur in bladder cancer. There is also considerable evidence that chronic urinary track inflammation contributes to the development of bladder cancer.

[0006] Interstitial cystitis (IC) or painful bladder syndrome (PBS) is a chronic urinary condition characterized by bladder pain and urinary frequency and urgency. Its incidence has been estimated at 1.2/100,000 and is growing globally. Despite the tremendous clinical and quality of life implications associated with IC, no clear pathophysiology and etiologies are currently available. The value of cell cultures and particularly cultures of well-characterized cell types specific to disease entity cannot be overstated. The understanding of the molecular determinants as well as development of novel therapeutics has been limited by the lack of three-dimensional (3D) models that accurately characterize IC.

SUMMARY OF THE INVENTION

[0007] In one embodiment, the present invention provides a method of making an organoid from a mammalian bladder tissue *in vitro* comprising: isolating cells from a mammalian bladder tissue to provide isolated cells; culturing the isolated cells in a differentiation medium for a time sufficient to enrich for stem cells and induce differentiation; and amplifying the cells by culturing in an extracellular matrix in an organoid medium for a time sufficient to produce organoids.

[0008] In another embodiment, the invention provides an *in vitro* bladder organoid comprising urothelial cells, the organoid exhibiting endogenous three-dimensional organ architecture.

[0009] In one embodiment, the *in vitro* bladder organoid is derived from a single epithelial cell of a bladder tissue, the organoid exhibiting endogenous three-dimensional organ architecture.

[0010] In another embodiment, the invention provides an *in vitro* bladder organoid derived from primary bladder IC tissue, wherein the organoid comprises urothelial cells and exhibits endogenous three-dimensional organ architecture.

[0011] In another embodiment, the invention provides an *in vitro* bladder organoid derived from primary bladder normal tissue, wherein the organoid comprises urothelial cancer cells and exhibits endogenous three-dimensional organ architecture.

[0012] In another embodiment, the invention provides an *in vitro* bladder organoid derived from primary bladder cancer tissue, wherein the organoid comprises urothelial cells and exhibits endogenous three-dimensional organ architecture.

[0013] In another embodiment, the invention provides a cell culture medium supplemented with epidermal growth factor (EGF), bovine pituitary extract (BPE), and Cholera Toxin.

[0014] In another embodiment, the invention provides a cell culture medium supplemented with EGF, basic fibroblast growth factor (bFGF), BPE, and Cholera Toxin.

[0015] In another embodiment, the present invention provides a kit including a cell culture medium supplemented with EGF, BPE and Cholera Toxin, and a cell culture medium supplemented with EGF, bFGF, BPE and Cholera Toxin.

[0016] In another embodiment, the invention provides a method for identifying agents having anticancer activity against bladder cancer cells including selecting at least one test agent, contacting a plurality of patient-specific bladder organoids derived from the patient's bladder

cancer cell with the test agent, determining the number of bladder organoids in the presence of the test agent and the absence of the test agent, and identifying an agent having anticancer activity if the number or the growth of the organoid cells is less in the presence of the agent than in the absence of the agent. In another embodiment, the method provides a step of treating the patient with the agent identified as having anticancer activity against the patient-specific organoids but not against normal organoids. A method for identifying agents having anticancer activity against bladder cancer cells can further include providing a mouse engrafted with bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering the identified agent having anticancer activity to the mouse; and determining if the tumor size is reduced in the presence of the identified agent. In another embodiment, a method for identifying agents having anticancer activity against bladder cancer cells can further include providing a humanized mouse engrafted with components of a patient's immune system and bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering the identified agent to the humanized mouse; and comparing the size of the tumor in the humanized mouse with components of a patient's immune system to the size of the tumor in the mouse in which the identified agent was administered; and determining if the size of the tumor in the humanized mouse with components of a patient's immune system is reduced relative to the size of the tumor in the mouse in which the identified agent was administered. This and other embodiments can further include providing a humanized mouse engrafted with bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering a control agent to the humanized mouse engrafted with bladder cancer cells from the patient; and comparing the size of the tumor in the humanized mouse engrafted with bladder cancer cells from the patient to the size of the tumor in the mouse in which the identified agent was administered;

and determining if the size of the tumor in the mouse in which the identified agent was administered is reduced relative to the size of the tumor in the humanized mouse engrafted with bladder cancer cells from the patient.

[0017] In another embodiment, the invention provides a method for identifying agents having regenerative activity for bladder IC cells including selecting at least one test agent, contacting a plurality of patient-specific bladder organoids derived from the patient's bladder IC cell with the test agent, determining the number of bladder organoids in the presence of the test agent and the absence of the test agent, and identifying an agent having regenerative activity if the number or the growth of the organoid cells is more in the presence of the agent than in the absence of the agent.

[0018] In another embodiment, the method provides a step of treating the patient with the agent identified as having anticancer activity against the patient-specific organoids but not against normal organoids.

[0019] In another embodiment, the present invention provides normal patient-specific bladder organoids, and methods of using such organoids for personalized therapies for bladder cancer, and IC or PBS.

[0020] In another embodiment, the present invention provides immune humanized mice with implanted patient-specific bladder organoids, and methods of using such mice to identify personalized therapies for bladder cancer, IC or PBS, and other bladder disorders.

[0021] In the methods described herein, the organoids exhibit endogenous three-dimensional organ architecture.

DETAILED DESCRIPTION OF THE INVENTION

[0022] In certain embodiments, the present invention provides bladder organoids derived *in vitro* from normal and cancerous tissues, and methods of making and using such organoids, as well as cell culture media and kits. As disclosed in one embodiment herein, certain growth factors in an *in vitro* environment containing extracellular matrix molecules in a 3-dimensional culture device may be used to make the organoids.

[0023] An organoid is a miniature form of a tissue that is generated *in vitro* and exhibits endogenous three-dimensional organ architecture. See, e.g., Cantrell and Kuo (2015) Genome Medicine 7:32-34. The organoids of the present invention can be used, for example, to: a) determine genomic targets within tumors and prediction of response to therapies in preclinical and clinical trials; b) detect the activity of an anti-cancer agent by examining the number of surviving organoids after treatment; c) detect the activity of a proliferative agent by determining the number of proliferating cells within each organoid and determining gene expression profiling of relevant pathways; d) detect the activity of a urothelial regenerative agent by examining the number of organoids after treatment to restore the inner wall of the bladder, similar to the activity of pentosan polysulfate sodium currently used for treatment of IC; e) examine the specificity of agents targeting different cell types within organoids; f) determine the effects of chemotherapy and radiation; g) create mouse models by implantation of the organoid *in vivo*; h) create preclinical models for examining therapy responses and drug discovery both *in vitro* and *in vivo*; and i) determine clonally-targeting anti-cancer therapies.

[0024] Accordingly, in one embodiment, the invention provides a method of making an organoid from a mammalian bladder tissue *in vitro* including: isolating cells from a mammalian bladder

tissue to provide isolated cells; culturing the isolated cells in a differentiation medium for a time sufficient to enrich for stem cells and induce differentiation; and amplifying one or more of the cells by culturing in an extracellular matrix in an organoid medium for a time sufficient to produce organoids. One of ordinary skill in the art can determine a time sufficient to induce differentiation by examining morphological changes associated with differentiation. In one preferred embodiment, the time sufficient to induce differentiation is from about 7 to about 28 days. In another preferred embodiment, the time sufficient to induce differentiation is about 14 days. One of ordinary skill in the art can determine a time sufficient to induce organoid formation by examining morphological changes associated with organoid formation. In one preferred embodiment, the time sufficient to induce organoid formation is from about 7 to about 42 days. In another preferred embodiment, the time sufficient to induce organoid formation is about 21 days. In one embodiment, the isolated cells are epithelial cells and/or mesenchymal cells. In one embodiment, a single bladder epithelial cell or mesenchymal cell is amplified.

[0025] In one preferred embodiment, the differentiation medium comprises keratinocyte serum-free medium (KSFM), EGF, BPE and Cholera Toxin. KSFM is typically used at 1X. The concentration of EGF present in the differentiation medium may range from about 0.1-100 mg/mL (e.g., 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 21 mg/mL, 25 mg/mL, 50 mg/mL, 100 mg/mL, etc.). The concentration of BPE present in the differentiation medium may range from about 1-100 µg/mL (e.g., 1 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, etc.). The concentration of Cholera Toxin present in the differentiation medium may range from about 1-100 ng/mL (e.g., 1 µg/mL, 25 µg/mL, 30 µg/mL, 35 µg/mL, 75 µg/mL, 100 µg/mL, etc.). In a further embodiment, the differentiation medium comprises one or both of Penicillin (500-5000 Units/mL) and Streptomycin (50-500 µg/mL). In a most preferred embodiment, the differentiation medium

comprises the following concentrations: KSFM (ThermoFisher Scientific) (about 1X); EGF (about 20 mg/mL); BPE (about 50 µg/mL); Cholera Toxin (about 30 ng/mL); Penicillin (about 1000 Units/mL); and Streptomycin (about 100 µg/mL). The differentiation medium may further comprise or be substituted with other supplements, growth factors, antibiotics, vitamins metabolites, and hormones, synthetic or natural with similar properties as known in the art.

[0026] In one preferred embodiment, the organoid medium includes KSFM, EGF, bFGF, BPE, and Cholera Toxin. The concentration of EGF present in the organoid medium may range from about 0.1-100 mg/mL (e.g., 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 21 mg/mL, 25 mg/mL, 50 mg/mL, 100 mg/mL, etc.). The concentration of bFGF present in the organoid medium may range from about 0.1-100 mg/mL (e.g., 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 75 mg/mL, 100 mg/mL, etc.). The concentration of BPE present in the organoid medium may range from about 1-100 µg/mL (e.g., 1 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, etc.). The concentration of Cholera Toxin present in the organoid medium may range from about 1-100 ng/mL (e.g., 1 µg/mL, 25 µg/mL, 30 µg/mL, 35 µg/mL, 75 µg/mL, 100 µg/mL, etc.). In a preferred embodiment, the organoid medium further comprises one or both of Penicillin (500-5000 Units/mL) and Streptomycin (50-500 µg/mL). In a most preferred embodiment, the organoid medium includes the following concentrations: KSFM at 1X, approximately 20 mg/mL EGF, approximately 10 mg/mL bFGF, approximately 50 µg/mL BPE, approximately 30 ng/mL Cholera Toxin, approximately 1000 Units/mL Penicillin, and approximately 100 µg/mL Streptomycin. The organoid medium may further include or be substituted with other supplements, growth factors, antibiotics, vitamins metabolites, and hormones, synthetic or natural with similar properties as known in the art.

[0027] In certain embodiments, the cells are from human bladder tissue, IC, and human primary bladder cancer tissue. In certain embodiments, cells that may be used to make an organoid are human bladder stem-like cells. Such cells are known in the art and may be identified and isolated using markers, for example, cytokeratin 5 (CK5), cytokeratin 7 (CK7), cytokeratin 8 (CK8), cytokeratin 14 (CK14), cytokeratin 18 (CK18), cytokeratin 20 (CK20), p63 and uroplakin.

[0028] In one embodiment, the cells are positive for at least one marker selected from the group consisting of CK5, CK8, and CK7. In another embodiment, the cells are positive for CK5, CK14, CK18, CK20 and CK7. Such cells may be identified and isolated by methods of cell sorting that are known in the art. For example, in one embodiment, the cells may be isolated by laser capture microdissection, cell sorting, or RNA sorting using methods known in the art, such as molecular beacons and the SmartFlare™ probe protocol (EMD Millipore).

[0029] In one preferred embodiment, the cells are obtained from surgically excised tissues by subjecting the tissues to mechanical dissociation, collagenase treatment, and filtration.

[0030] In certain embodiments the method is performed with a commercially available extracellular matrix such as Matrigel™. Other natural or synthetic extracellular matrices are known in the art for culturing cells. In general, an extracellular matrix comprises laminin, entactin, and collagen. In a preferred embodiment the method is performed using a 3-dimensional culture device (chamber) that mimics an *in vivo* environment for the culturing of the cells, where preferably the extracellular matrix is formed inside a plate that is capable of inducing the proliferation of stem cells under hypoxic conditions. Such 3-dimensional devices are known in the art. An example of such a device is disclosed by Bansal, N., et al. (2014) Prostate 74, 187-200, the disclosure of which is incorporated herein by reference in its entirety. It has been discovered in accordance with the present invention that the use of a 3-dimensional culture device

in a method of making organoids has surprising advantages over other formats, as shown in Table 1.

Table 1. Advantages and disadvantages of tested formats

Format	Consistency of Organoids	Reproducibility	Efficiency
In Matrigel™	+++	+++	++++
On Matrigel™	+	---	++
Hanging Drop plates	---	---	---
Non adherent plate	+	---	+

[0031] In another aspect, the invention provides a bladder organoid. The bladder is comprised of a specialized epithelium known as the urothelium, which is encapsulated by the lamina propria and is surrounded by a thick layer of smooth muscle that forms the bladder wall. The urothelium includes three cell types: (i) basal cells, which are small cuboidal cells that express p63 and high molecular weight cytokeratins, such as CK5 and CK14; (ii) intermediate cells, which express p63, CK5, and CK14 at lower levels than basal cells; and (iii) superficial cells, also called “umbrella cells”, which express uroplakin proteins and low molecular weight cytokeratins such as CK18 and CK20. Studies of the adult mouse urothelium have identified stem cells in the basal layer that can drive the process of tissue repair. The bladder organoids of the present invention resemble the structures of the primary tissue. Upon histological and immunofluorescence analyses, one of skill in the art can determine that the organoids recreate the human urothelium. Bladder tissue origin of organoids can be confirmed by detecting the expression of CK5, CK18, CK7 and uroplakin.

[0032] In another aspect, the invention provides a bladder organoid derived *in vitro* from primary bladder cancer tissue. Tumor heterogeneity can be efficiently modeled using the methods described to make an organoid, by mapping the diagnostic dominant clone and tumor subclones

from each patient biopsy sample, generating organoids derived from each clone and defining the genetic signature of each clone. A bladder organoid derived from primary bladder cancer tissue will generally maintain expression of bladder lineage-specific markers and the functional secretory profile of the original primary tissue. A bladder organoid as described herein can be serially propagated, cryofrozen and regenerated and established as a model for cancer drug discovery and precision therapy.

[0033] In another aspect, the invention provides a bladder organoid derived *in vitro* from surgically excised tissues of tumors identified to express histopathological tissue specific and tumorigenic markers. Single cells from these tissues may be isolated with non-contact laser capture microdissection or by RNA sorting, for example using SmartFlare™ probes to generate single cell organoids with known expression features.

[0034] The organoids described herein exhibit endogenous three-dimensional organ architecture.

[0035] In another embodiment, the invention provides a method for identifying agents having anticancer activity against bladder cancer cells from a patient(s) including selecting at least one test agent, contacting a plurality of patient-specific bladder organoids derived from the patient's bladder cancer cell with the test agent, determining the number of bladder organoids in the presence of the test agent and the absence of the test agent, and identifying an agent having anticancer activity if the number or growth of the organoids is less in the presence of the agent than in the absence of the agent. In another embodiment, the method provides a step of treating the patient with the agent identified as having anticancer activity against the patient-specific organoids. A method for identifying agents having anticancer activity can further include providing a mouse engrafted with bladder cancer cells from the patient and containing a tumor

formed from the bladder cancer cells; administering the identified agent having anticancer activity to the mouse; and determining if the tumor size is reduced in the presence of the identified agent.

[0036] A method for identifying agents having anticancer activity can further include providing a humanized mouse engrafted with components of a patient's immune system and bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering the identified agent to the humanized mouse; and comparing the size of the tumor in the humanized mouse with components of a patient's immune system to the size of the tumor in the mouse in which the identified agent was administered; and determining if the size of the tumor in the humanized mouse with components of a patient's immune system is reduced relative to the size of the tumor in the mouse in which the identified agent was administered. In this embodiment, the humanized mice with the patient's immune system can be used to compare the effects of the identified agent (e.g., candidate therapeutic) on tumors in the presence or absence of immune cells to examine a potential role for combination with immunotherapy. These methods can further include providing a humanized mouse (an immune-deficient control mouse) engrafted with bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering a control agent to the humanized mouse engrafted with bladder cancer cells from the patient; and comparing the size of the tumor in the humanized mouse engrafted with bladder cancer cells from the patient to the size of the tumor in the mouse in which the identified agent was administered; and determining if the size of the tumor in the mouse in which the identified agent was administered is reduced relative to the size of the tumor in the humanized mouse engrafted with bladder cancer cells from the patient. In this method, if the size of the tumor in the mouse in which the identified agent was administered is reduced relative to the size of the tumor

in the humanized mouse engrafted with bladder cancer cells from the patient, the identified agent can be confirmed as a successful treatment for cancer in the patient.

[0037] In another embodiment, the invention provides a method for identifying agents, growth factors or peptides having urothelial regenerative activity against bladder IC cells including selecting at least one test agent, contacting a plurality of patient-specific bladder organoids derived from the patient's bladder IC cell with the test agent, determining the number of bladder organoids in the presence of the test agent and the absence of the test agent, and identifying an agent having regenerative activity if the number or growth of the organoids is more in the presence of the agent than in the absence of the agent. In another embodiment, the method provides a step of treating the patient with the agent identified as having regenerative activity against the patient-specific organoids.

[0038] In another embodiment, the invention provides a method of selecting a personalized treatment for bladder cancer in a subject including: selecting at least one form of treatment, contacting a plurality of bladder organoids with the form of treatment, wherein the organoids are derived from bladder cancer cells from the subject, determining the number of bladder organoids in the presence of the treatment and the absence of the treatment, and selecting the treatment if the number or growth of the bladder organoids is less in the presence of the treatment than in the absence of the treatment. Various types of therapy can then be examined using the organoids to determine therapy resistance before initiation, to tailor the therapy for each individual patient based on oncogenic driver expression in the organoids, as well as further study induced clonal selection processes that are the frequent causes of relapse. Various forms, combinations, and types of treatment are known in the art, such as radiation, hormone, chemotherapy, biologic, and bisphosphonate therapy. The term "subject" refers to any animal (e.g., a mammal), including, but

not limited to humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject. Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition.

[0039] The foregoing methods may be facilitated by comparing therapeutic effects in organoids derived from cancer cells and normal cells from the same patient. For example, normal organoids and cancer organoids derived from cells of the same patient can be assessed to determine genetic and epigenetic mutations and gene expression profiles that are cancer-specific, thereby allowing the determination of gene-drug associations and optimization of treatment. Such comparisons also allow one to predict a therapeutic response and to personalize treatment in a specific patient.

[0040] In another aspect of this method, clonally targeted therapies can be determined by testing the effect of a therapeutic agent on multiple organoids derived from subsequently determined dominant clones of bladder cancer cells identified in the tumor tissue from a patient, and comparing to the effect of the therapeutic agent on organoids derived from normal cells of the same patient.

[0041] In another aspect, the invention provides a cell culture (e.g., organoid) medium supplemented with EGF, bFGF, and BPE. In another embodiment, the invention provides a cell culture (e.g., organoid) medium supplemented with EGF, bFGF, BPE and Cholera Toxin. In another embodiment, the invention provides a cell culture (e.g., organoid) medium supplemented with EGF, bFGF, BPE, Cholera Toxin, Penicillin and Streptomycin. In a preferred embodiment, the medium is a commercially available cell growth medium such as KSFM (Thermo Fisher Scientific).

[0042] In another aspect, the invention provides kits to make an organoid from a single cell. In an embodiment, a kit contains containers for a differentiation medium and an organoid medium as previously described. The containers may also contain the necessary supplements (growth factors, antibiotics, hormones, vitamins, amino acids, and combinations thereof) for a differentiation medium and an organoid medium. The kit may further include the necessary components for a 3-dimensional culture device, for example, plates, and/or materials for an extracellular matrix, e.g. Matrigel™. The kit may further contain a set of instructions to perform the methods of making an organoid from a single cell as previously described.

[0043] In another embodiment, the present invention provides a mouse with an implanted patient-specific bladder organoid. In one embodiment, the mouse is a humanized mouse. In another embodiment, the mouse is a human immune system (HIS) –reconstituted mouse. In another embodiment, the mouse is non-obese diabetic (NOD)-Rag (-)- γ chain (-) (NRG) mouse. In another embodiment, the mouse is an NSG immune-deficient PDX mouse.

[0044] Methods of making HIS-reconstituted mice are known in the art and disclosed for example by Drake et al. (2012) Cell Mol Immunol 9:215-24 and Harris et al. (2013) Clinical and Experimental Immunology 174:402-413. In accordance with one aspect of the present invention, human stem cells from patient, for example from a diagnostic bone marrow or blood sample or HLA-matched, are transplanted into neonatal NRG mice to engraft components of the patient's immune system. Methods of making NSG immune-deficient PDX mice are also known in the art and disclosed for example by Ciamporcero et al. (2016) Oncogene 12:1541-1553. The mice are later subjected to grafting with bladder organoids derived from bladder cells of the same patient subcutaneously, under the renal capsule or orthotopically in the mouse bladder. The mice are

useful for identifying new treatments, urothelial regeneration agents, assessing responses to therapy, and evaluating combination therapies.

[0045] The following non-limiting examples serve to further illustrate the invention.

EXAMPLE 1

[0046] 3D organoid technology for generating a large repository of organoid cell lines from normal, IC and bladder cancer was developed. To establish bladder cancer organoids, a novel protocol for the dissociation and 3D culture of fresh normal, IC and bladder cancer tissue was developed. Using these organoid culture conditions, fresh bladder cells obtained by transurethral resection (TUR) were dissociated and cultured. Organoid lines were propagated for at least three passages, and were successfully cryopreserved, allowing their long-term storage and retrieval. To determine whether the histological phenotypes of the patient-derived organoids (PDOs) resembled their corresponding original tissues, hematoxylin-eosin (H&E) staining of paraffin sections was performed. Light microscopic examination of the H&E-stained slides showed that the histopathological features of the PDOs were identical to those of their corresponding original tissues. This analysis indicates the presence of strong phenotypic concordance between the parental tissues and corresponding organoids. Table 2 below includes the media and culture conditions in a typical embodiment of producing bladder tissue organoids.

Table 2. Bladder Organoid Media

Primary Tissue	Collection Media	Process Time	2D Culture (phase I)	3D Culture (Phase II in Matrigel)	Days to organoids
Bladder	DMEM medium +10%FBS Penicillin (3,000 Units/mL) + Streptomycin	Stripping buffer (PBS, HEPES, 0.1% EDTA) 4°C O/N, then	KSFM medium + EGF 20mg/mL + BPE 50µg/mL	KSFM medium + EGF 20mg/mL + bFGF 10mg/mL + BPE 50µg/mL	Phase I: 14 Phase II: 21

	(300 µg/mL)	RPMI + Collagenase 45 min	+ Cholera Toxin (30ng/mL)+ Penicillin (1,000 Units/mL) + Streptomycin (100 µg/mL)	+ Cholera Toxin (30ng/mL)+ Penicillin (1,000 Units/mL) + Streptomycin (100 µg/mL)	
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EXAMPLE 2

[0047] Cystoscopic bladder biopsies from patients all meeting the NIDDK diagnostic criteria for IC/PBS (age range, 30-60 years) were obtained. All the specimens were de-identified at collection in the Urology operating room and were numerically identified. Cystoscopy was performed under general anesthesia employing normal saline as a bladder irrigant. A rigid cold cup biopsy forceps was used to obtain the bladder biopsy specimen measuring 4 cubic mm and was transported in epithelial specific medium in a sterile sealed container at room temperature for immediate processing. Biopsy specimens were minced into 0.5 cubic mm pieces to increase the surface area and placed in uncoated plastic tissue culture dishes containing complete epithelial cell medium supplemented with growth factors and cytokines that support epithelial and mesenchymal cell growth, as developed for normal urothelial cells. Epithelial cells were isolated by dispase enzyme treatment and single cells are subjected to 3D cell cultures with different mixtures of growth factors to determine the optimum growth conditions for normal, IC and cancer organoids.

[0048] Cystoscopic bladder biopsies from IC patients and cystectomy derived normal adjacent tissue (NAT) and bladder cells were utilized to determine best conditions for generating organoids.

A two-step methodology was employed comprising a first phase of adult bladder stem cell enrichment, conducted in a 2D setting (stage I), followed by a second phase of organoid 3D growth obtained in pure matrigel chambers (stage II). For 2D stage I culture, the optimum media to use was identified: KSFM medium with EGF (20 mg/mL), BPE (50 µg/mL), cholera toxin (30 ng/mL) and penicillin streptomycin that allowed enrichment of bladder stem cells within 14 days to allow the generation of organoids in the following phase II culture. The growth of organoids in stage II from single cells occurred within 21 days. Stage II media included using serum-free conditions with KSFM medium with EGF (20 mg/mL), bFGF (10 mg/mL), BPE (50 µg/mL), cholera toxin (30 ng/mL) and penicillin streptomycin. Organoid cellular growth and molecular markers (detailed above) are identified, and measured using Q-PCR, IF (immunofluorescence) and immunohistochemistry (IHC) techniques. The molecular markers of different cell types in bladder are identified and quantified, and the conditions that produce organoids with expression of molecular markers of epithelial and mesenchymal features are determined. In addition, the organoids are studied morphologically and by imaging. Identification of the molecular signature of IC/PBS, identification of candidate genes (by microarray and whole genome sequencing), and identification of urothelial-muscle cell interaction are pursued.

[0049] Analyses of marker expression in PDOs by immunofluorescence are performed. For these analyses, immunostaining for the basal epithelial marker cytokeratin 5 (CK5), the luminal marker cytokeratin 8 (CK8), and CK7, which is strongly expressed by all urothelial cells, is performed. Organoids are expected to display strong widespread expression of the luminal marker CK8, as well as the urothelial marker CK7, consistent with the phenotype of their corresponding original tissues. The percentage of cells in organoids with expression of the basal marker CK5 is examined, which is compared to the corresponding original tissues. This finding determines the

degree of phenotypic heterogeneity in the parental tissues and the extent of it being retained in the corresponding organoids.

[0050] The optimal conditions for *in vitro* cell cultures of IC/PBS by organoid cell cultures was determined in the presence of growth factors that support urogenital mesenchyme growth (see above and Table 2 for culture conditions). With the primary biopsy tissue and organoid cell cultures, molecular profiling based on genomic and RNA seq analyses is performed and measurement of listed molecular biomarkers for IC/PBS can be achieved, e.g., anti-proliferative factor (APF), uroplakins and bladder nerve growth factor (NGF). This study is the first to develop and molecularly characterize 3D organoid cell cultures in patients with IC/PBS. Moreover, since many of the listed molecular biomarkers of IC/PBS could be modulated with currently available targeted therapies, these therapies are evaluated in the IC/PBS organoid cultures once established to identify potential molecular subtypes and novel targeted therapies for IC/PBS. Increased levels of these bladder biomarkers not only could serve a diagnostic purpose, but also allows one to quantify the level of response to therapy by monitoring the progress during the treatment period. Lastly, targeted therapies including pharmacological interventions (e.g. intravesical administration of peptides or liposomes) aimed at targeting urothelial receptor/ion channel expression may provide new strategies for the clinical management of IC and PBS.

[0051] The foregoing examples and description of the preferred embodiments should be taken as illustrating, rather than as limiting the present invention as defined by the claims. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the scope of the invention, and all such variations are intended

to be included within the scope of the following claims. All references cited herein are incorporated herein by reference in their entireties.

WE CLAIM:

1. A method of making an organoid from a mammalian bladder tissue *in vitro* comprising: isolating cells from a mammalian bladder tissue to provide isolated cells; culturing the isolated cells in a differentiation medium for a time sufficient to enrich for stem cells and induce differentiation; and amplifying one or more of the cells by culturing in an extracellular matrix in an organoid medium for a time sufficient to produce organoids that exhibit endogenous three-dimensional organ architecture.
2. The method of Claim 1 wherein the differentiation medium comprises epidermal growth factor (EGF), bovine pituitary extract (BPE) and Cholera Toxin.
3. The method of Claim 1 wherein the organoid medium comprises EGF, basic fibroblast growth factor (bFGF), BPE and Cholera Toxin.
4. The method of Claim 3 wherein the organoid medium further comprises one or more of Penicillin and Streptomycin.
5. The method of Claim 1 wherein the mammalian tissue is a human tissue.
6. The method of Claim 5 wherein the human tissue is human bladder tissue.
7. The method of Claim 6 wherein the human bladder tissue is primary human normal bladder tissue, or primary human bladder cancer tissue.
8. The method of Claim 1 wherein the organoids comprise at least one of: epithelial cells and mesenchymal cells.
9. The method of Claim 1 wherein the time sufficient to produce organoids is about twenty-one days.
10. The method of Claim 3 wherein the EGF is present at a concentration of about 1-50 mg/mL, the bFGF is present at a concentration of about 1-50 mg/mL, the BPE is present at a

concentration of about 1-100 $\mu\text{g/mL}$, and the Cholera Toxin is present at a concentration of about 1-100 ng/mL .

11. The method of Claim 3 wherein the EGF is present at a concentration of about 20 mg/mL , the bFGF is present at a concentration of about 10 mg/mL , the BPE is present at a concentration of about 50 $\mu\text{g/mL}$, and the Cholera Toxin is present at a concentration of about 30 ng/mL .

12. The method of Claim 4 wherein the EGF is present at a concentration of about 20 mg/mL , the bFGF is present at a concentration of about 10 mg/mL , the BPE is present at a concentration of about 50 $\mu\text{g/mL}$, and the Cholera Toxin is present at a concentration of about 30 ng/mL , and the medium comprises Penicillin at a concentration of about 1000 Units/ mL and Streptomycin at a concentration of about 100 $\mu\text{g/mL}$.

13. The method of Claim 1 wherein the isolated cells are sorted for the presence of at least one marker selected from the group consisting of: cytokeratin 5 (CK5), cytokeratin 18 (CK18), and cytokeratin 7 (CK7).

14. A bladder organoid comprising at least one of epithelial cells and mesenchymal cells, the organoid exhibiting endogenous three-dimensional organ architecture.

15. A bladder organoid derived *in vitro* from primary bladder normal tissue, wherein the organoid comprises at least one of epithelial cells and mesenchymal cells and exhibits endogenous three-dimensional organ architecture.

16. A bladder organoid derived *in vitro* from primary bladder cancer tissue, wherein the organoid comprises at least one of epithelial cells and mesenchymal cells and exhibits endogenous three-dimensional organ architecture.

17. A cell culture medium supplemented with EGF, BPE and Cholera Toxin.

18. A cell culture medium supplemented with EGF, bFGF, BPE and Cholera Toxin.
19. The cell culture medium of Claim 17 or Claim 18 further comprising Penicillin and Streptomycin.
20. A kit comprising the cell culture medium of claim 19.
21. A method for identifying an agent having anticancer activity against bladder cancer cells from a patient comprising selecting at least one test agent, contacting a plurality of bladder organoids derived from bladder cancer cells from the patient with the test agent, determining the number of bladder organoids in the presence of the test agent and the absence of the test agent, and identifying an agent having anticancer activity if the number or growth of the organoids derived from bladder cancer cells from the patient is less in the presence of the agent than in the absence of the agent.
22. A method for identifying an agent having regenerative activity for bladder Interstitial cystitis (IC) cells comprising selecting at least one test agent, contacting a plurality of bladder organoids derived from bladder IC cells with the test agent, determining the number of bladder organoids in the presence of the test agent and the absence of the test agent, and identifying an agent having regenerative activity if the number or growth of the organoids derived from bladder IC cells is more in the presence of the agent than in the absence of the agent.
23. A method of personalized treatment for bladder cancer in a subject comprising: selecting at least one form of treatment, contacting a plurality of bladder organoids comprising with the form of treatment, wherein the organoids are derived from bladder cancer cells from the subject, determining the number of bladder organoids in the presence of the treatment and the absence of the treatment, and selecting the treatment if the number or growth of the bladder organoids is less in the presence of the treatment than in the absence of the treatment.

24. The method of Claim 22 further comprising treating the subject with the selected treatment.

25. A method of personalized treatment for bladder disorders in a subject comprising: selecting normal bladder cells to generate organoids, wherein the organoids are derived from bladder normal cells from the subject, or HLA-matched donors, generating normal patient-specific or HLA-matched bladder organoids, and using such organoids for personalized therapies for bladder cancer and Interstitial cystitis (IC) or painful bladder syndrome (PBS).

26. A humanized mouse engrafted with components of a patient's immune system and comprising a bladder organoid derived from the patient's bladder cell grafted into the mouse.

27. The method of Claim 6 wherein the human bladder tissue is bladder cancer tissue.

28. The method of claim 21, further comprising providing a mouse engrafted with bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering the identified agent having anticancer activity to the mouse; and determining if the tumor size is reduced in the presence of the identified agent.

29. The method of claim 21, further comprising providing a humanized mouse engrafted with components of a patient's immune system and bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering the identified agent to the humanized mouse; and comparing the size of the tumor in the humanized mouse with components of a patient's immune system to the size of the tumor in the mouse in which the identified agent was administered; and determining if the size of the tumor in the humanized mouse with components of a patient's immune system is reduced relative to the size of the tumor in the mouse in which the identified agent was administered.

30. The method of claim 21 or 29, further comprising providing a humanized mouse engrafted with bladder cancer cells from the patient and containing a tumor formed from the bladder cancer cells; administering a control agent to the humanized mouse engrafted with bladder cancer cells from the patient; and comparing the size of the tumor in the humanized mouse engrafted with bladder cancer cells from the patient to the size of the tumor in the mouse in which the identified agent was administered; and determining if the size of the tumor in the mouse in which the identified agent was administered is reduced relative to the size of the tumor in the humanized mouse engrafted with bladder cancer cells from the patient.

31. The method of any one of claims 21-25 and 27-30, wherein the organoids exhibit endogenous three-dimensional organ architecture.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/040041

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

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

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

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

3. ☒ Claims Nos.: 31
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 additional fees, this Authority did not invite payment of 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/US2018/040041

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/071; C12N 5/0789; C12N 5/095; G01N 33/15; G01N 33/50 (2018.01)

CPC - A01K 2267/03; A01K 2267/0331; C12N 5/0685; C12N 5/0688; C12N 5/0689; C12N 5/0693; C12N 5/0695; C12N 2500/25; C12N 2501/01; C12N 2501/11; C12N 2501/115; C12N 2501/39; C12N 2503/02; G01N 33/5011; G01N 33/5044; G01N 33/5082; G01N 33/5088 (2018.08)

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)

See Search History document

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

USPC - 435/4; 435/325; 435/366; 435/404 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2015/156929 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK et al) 15 October 2015 (15.10.2015) entire document	1, 5-9, 13-16, 21, 27, 28 ----- 2-4, 10-12, 23, 25, 29, 30
X -- Y	US 2007/0003541 A1 (FAUDOA et al) 04 January 2007 (04.01.2007) entire document	17-20 ----- 2-4, 10-12
X	US 2014/0302491 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 09 October 2014 (09.10.2014) entire document	22, 24
X -- Y	WO 2016/209865 A1 (THE JACKSON LABORATORY) 29 December 2016 (29.12.2016) entire document	26 ----- 29, 30
Y	US 2013/0274226 A1 (CHENG et al) 17 October 2013 (17.10.2013) entire document	12
Y	WO 2016/083613 A2 (KONINKLIJKE NEDERLANDSE AKADEMIE VAN WETENSCHAPPEN) 02 June 2016 (02.06.2016) entire document	23, 25
A	WO 2016/015158 A1 (UNIVERSITY HEALTH NETWORK) 04 February 2016 (04.02.2016) entire document	1-30



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

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"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

21 September 2018

Date of mailing of the international search report

02 OCT 2018

Name and mailing address of the ISA/US

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