(54) Title: USE OF MTOR INHIBITORS FOR PREVENTION OF AGING-ASSOCIATED DYSFUNCTION OF STEM CELLS

(57) Abstract: Disclosed are methods and compositions for the inhibition of the development of early neoplastic lesions or hyperplastic lesions in the mammary glands or the prevention breast cancer in a patient who has been identified as being at risk for developing breast cancer. The disclosed methods and compositions include rapamycin, a rapamycin analog, or another such inhibitor of the target of rapamycin (TOR).
USE OF MTOR INHIBITORS FOR PREVENTION OF AGING-ASSOCIATED DYSFUNCTION OF STEM CELLS

GOVERNMENTAL RIGHTS

This invention was made with government support under agreement number RO 1 ES022057 awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application serial number 62/058,919 filed October 2, 2014, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

A. Field of the Invention

The invention relates to methods and compositions for inhibiting the development of early neoplastic lesions or hyperplastic lesions in aged mammary glands or preventing breast cancer in a patient who has been identified as being at risk for developing breast cancer. The methods and compositions include rapamycin, rapamycin analogs, or other inhibitors of the mammalian target of rapamycin ("mTOR" or "mTORC1") and stable formulations for efficacious oral administration of such inhibitors.

B. Description of Related Art

Many human diseases, including cancer, increase in frequency during aging (Blagosklonny, 2008; de Magalhaes, 2013). Most (80%) breast cancers are diagnosed in women aged greater than 50 years (Benz, 2008). Instead of targeting each disease separately, it may be more productive to slow aging and minimize all age-related diseases (Fontana et al., 2010). Calorie restriction and rapamycin treatment are dietary and pharmacologic interventions, respectively, that extend life span in many animal species. The mammalian target of rapamycin (mTOR) may be a key regulator, but the exact mechanism underlying the anti-aging effects of calorie restriction and rapamycin are unknown (Johnson et al., 2013).
A primary feature of aging is the decline of tissue function. This may be caused by decreased or altered tissue-specific stem cell function that occurs in various tissues including hematopoietic (Janzen et al., 2006; Liang et al., 2005; Morrison et al., 1996), nervous (Enwere et al., 2004; Kuhn et al., 1996; Maslov et al., 2004; Molofsky et al., 2006), gastrointestinal (Potten et al., 2001), muscle (Conboy et al., 2003; Conboy et al., 2005) and skin tissue (Doles et al., 2012). Therefore, stem cell maintenance may be a mechanism for rapamycin-induced life span extension (Lamming et al., 2013).

SUMMARY OF THE INVENTION

In some aspects, provided are methods for preventing or inhibiting the development of breast cancer, early neoplastic lesions, or hyperplastic lesions in mammary glands of a patient comprising administering an effective amount of a composition comprising rapamycin or an analog thereof to a patient who has been identified as being at risk for developing breast cancer. In some aspects, provided are methods for preventing or inhibiting the development of breast cancer in a patient comprising administering an effective amount of a composition comprising rapamycin or an analog thereof to a patient who has been identified as being at risk for developing breast cancer.

In some aspects, the method is for reducing the number of basal mammary stem-like cells in the mammary glands of a patient comprising administering an effective amount of a composition comprising rapamycin or an analog thereof to the patient. In some embodiments, the patient is one that has been determined to be at risk for breast cancer.

In some aspects, the method is for rejuvenating cell populations of the mammary gland, such as the basal mammary stem-like cells and/or luminal progenitor cells. The cells may be rejuvenated to, phenotypically, resemble a "normal" phenotype (i.e. normal self-renewal, proliferation, tissue function, morphology, passage capability, etc. ...). In some embodiments, the normal phenotype is the phenotype of the same cell type in non-diseased "young" animals, such as adolescent animals.

The term basal mammary stem-like cells refers to cells than have some phenotypes of normal basal mammary stem cells (e.g. CD31-CD45-TER1 19 or Lin-CD24i0-CD49f,i markers), but also have aberrant or dysfunctional phenotypes such as decreased self-renewal, reduced passage capability, and reduced tissue functionality. In this disclosure, the term basal mammary stem cell (MaSC) may refer to the "stem cells" or to the "stem-like cells." Typically, when the cells are discussed in the context of being dysfunction
or from "old" tissues, the cells are stem-like cells, since these cells have undergone changes that contribute to their dysfunction. When the cells are discussed in the context of being from "young" mice or tissues, these are stem cells, since these cells typically exhibit normal phenotypes.

In some embodiments, the basal mammary stem-like cells and/or basal mammary stem cells (normal) are CD31-CD45-TER1 19-cells or Lin-CD24i0CD49fhi cells.

In some embodiments the number of basal mammary stem-like cells is reduced such that the ratio of luminal progenitor cells to basal mammary stem and stem-like cells is significantly increased. In some embodiments, the increase is significant, as compared to un-treated controls, normal controls, and/or the ratio in the patient prior to treatment. In some embodiments, the control is an age-matched patient of the same species. In some embodiments, the ratio is greater than 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, or any derivable range therein. In some embodiments, significant refers to statistical significance. In some embodiments, significant is at least 5, 10, 15, 20, 25, 30, 35, 40, or 50% percent different, or any derivable range therein.

In further aspects, the method is for reducing proliferation of alveologenesis. It is contemplated that the low luminal-to-basal ratio as described herein is derived from increased proliferation of alveologenesis.

In some embodiments, the luminal progenitor cells with CD31-CD45-TER1 19-cells or Lin-CD24siCD49fi0 marks are normalized. In some embodiments, the basal mammary stem and/or stem-like cells with CD31-CD45-TER1 19-cells or Lin-CD24ifiCD49fhi marks are normalized. The term "normalized," as used in this context refers to a reduction or loss of dysfunctional phenotypes such as reduced passage capabilities, reduced tissue functionality, and/or reduced self-renewal capabilities.

In some embodiments, the patient has been identified as being at risk for developing breast cancer. In some embodiments, this risk is identified on the basis of a family history of breast cancer. In some embodiments, a patient is identified as at risk for developing breast cancer because the patient is aged 50 or older. In some embodiments, a patient is identified as at risk for developing breast cancer because the mammary glands of the patient have a luminal-to-basal cell (and cell-like) ratio of less than 1. In some embodiments, the patient is identified as at risk for developing breast cancer because the mammary glands of the patient have a luminal-to-basal cell (and cell-like) ratio that is
significantly less than normal or is a ratio described herein. In some embodiments, the patient is one that has been determined to have a significantly reduced ratio of luminal progenitor cells to basal mammary stem and stem-like cells compared to their normal counterparts. In some embodiments, a patient is identified as at risk for developing breast cancer because the patient is found to have atypical ductal hyperplasia and/or ductal carcinoma in situ in the patient's breasts. In some embodiments, a patient is identified as at risk for developing breast cancer because the patient is found to have mutations of the BRCA1 and/or BRCA2 gene(s).

[0015] In some embodiments, the patient has undergone surgical resection of the breast cancer prior to administration of the composition comprising rapamycin or an analog thereof. In some embodiments, the composition is administered at least one week after the surgical resection. In some embodiments, the composition is administered at least, at most, or exactly 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30 days or 1, 2, 3, 4, 5, or 6 months, or any derivable range therein.

[0016] In some embodiments, the patient is a mammal. In some embodiments, the patient is a human. In some embodiments, the patient is a dog (canine), a cat (feline), a mouse, a rat, or a rabbit.

[0017] In some embodiments, the rapamycin or analog thereof is encapsulated or coated, or the composition comprising the rapamycin or analog thereof is encapsulated or coated. In some embodiments, the encapsulant or coating may be an enteric coating. In some embodiments, the coating comprises cellulose acetate succinate, hydroxypropyl methylcellulose phthalate co-polymer, or a polymethacrylate-based copolymer selected from the group consisting of methyl acrylate-methacrylic acid copolymer, and a methyl methacrylate-methacrylic acid copolymer. In some embodiments, the coating comprises Poly(methacrylic acid-co-ethyl acrylate) in a 1:1 ratio, Poly(methacrylic acid-co-methyl methacrylate) in a 1:1 ratio, Poly(methacrylic acid-co-methyl methacrylate) in a 1:2 ratio, Poly(methyl acrylate-co-methyl methacrylate-co-methacrylic acid) in a 7:3:1 ratio, Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) in a 1:2:0.2 ratio, Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) in a 1:2:0.1 ratio, or Poly(butyl methacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate) in a 1:2:1 ratio, a naturally-derived polymer, or a synthetic polymer, or any combination thereof. In some embodiments, the naturally-derived polymer is selected from the group consisting of
alginites and their various derivatives, chitosans and their various derivatives, carrageenans and their various analogues, celluloses, gums, gelatins, pectins, and gellans. In some embodiments, the naturally-derived polymer is selected from the group consisting of polyethylene glycols (PEGs) and polyethylenoxides (PEOs), acrylic acid homo- and copolymers with acrylates and methacrylates, homopolymers of acrylates and methacrylates, polyvinyl alcohol (PVOH), and polyvinyl pyrrolidone (PVP).

[0018] An effective amount of rapamycin or rapamycin analog or derivative will depend upon the disease to be treated, the length or duration desired and the bioavailability profile of the implant, and the site of administration. In some embodiments, the composition comprises rapamycin or an analog thereof at a concentration of 0.001 mg to 30 mg total per dose. In some embodiments, the composition comprising rapamycin or an analog of rapamycin comprises 0.001% to 60% by weight of rapamycin or an analog of rapamycin. In some embodiments, the average blood level of rapamycin in the subject is greater than 0.5 ng per mL whole blood after administration of the composition.

[0019] The composition can be administered to the subject using any method known to those of ordinary skill in the art. In some embodiments, the composition may be administered intravenously, intracerebrally, intracranially, intraventricularly, intrathecally, into the cortex, thalamus, hypothalamus, hippocampus, basal ganglia, substantia nigra or the region of the substantia nigra, cerebellum, intradermally, intraarterially, intraperitoneally, intralesionally, intratracheally, intranasally, topically, intramuscularly, anally, subcutaneously, orally, locally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in creams, in lipid compositions (e.g., liposomes), or by other method or any combination of the foregoing as would be known to one of ordinary skill in the art. In some embodiments, the composition is administered orally, enterically, colonically, anally, intravenously, or dermally with a patch. In some embodiments, the composition comprising rapamycin or an analog of rapamycin is comprised in a food or food additive.

[0020] The dose can be repeated as needed as determined by those of ordinary skill in the art. In some embodiments, the rapamycin or analog of rapamycin is administered in two or more doses. Where more than one dose is administered to a subject, the time interval between doses can be any time interval as determined by those of ordinary skill in the art. For example, the two doses may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours apart, or any range therein. In some embodiments, the
composition may be administered daily, weekly, monthly, annually, or any range therein. In some embodiments, the interval of time between administration of doses comprising rapamycin or an analog of rapamycin is between 0.5 to 30 days. In some embodiments, the rapamycin or analog thereof may be administered repeatedly for 1, 2, 3, 4, 5, 6, or 7 days, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months, or longer, or any interval therein.

[0021] In some embodiments, the method comprises further administering one or more secondary or additional forms of therapies. In some embodiments, the subject is further administered a composition comprising a second active agent. In some embodiments, the second active agent is surgery with curative intent for localized breast cancer tumors, radiotherapy, chemotherapy and administration of other systemic agents. In some embodiments, the composition comprising rapamycin or an analog of rapamycin is administered at the same time as the composition comprising the second active agent. In some embodiments, the composition comprising rapamycin or an analog of rapamycin is administered before or after the composition comprising the second active agent is administered. In some embodiments, the two treatments may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours apart, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 days apart, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months apart, or one or more years apart or any range therein. In some embodiments, the interval of time between administration of composition comprising rapamycin or an analog of rapamycin and the composition comprising the second active agent is 1 to 30 days.

[0022] Unless otherwise specified, the percent values expressed herein are weight by weight and are in relation to the total composition.

[0023] The term "about" or "approximately" are defined as being close to as understood by one of ordinary skill in the art, and in one non-limiting embodiment the terms are defined to be within 10%, preferably within 5%, more preferably within 1%, and most preferably within 0.5%.

[0024] The terms "inhibiting," "reducing," "treating," or any variation of these terms, includes any measurable decrease or complete inhibition to achieve a desired result. Similarly, the term "effective" means adequate to accomplish a desired, expected, or intended result.
The terms "prevention" or "preventing" includes: (1) inhibiting the onset of a disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease, and/or (2) slowing the onset of the pathology or symptomatology of a disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease.

The terms "rejuvenate" or "rejuvenation" includes: (1) reversing the age-associated phenotypic changes associated with old mammary stem cells whereby rapamycin-treated stem cells functionally resemble young mammary stem cells with respect to an increase in self-renewal and milk production, in combination with a decreased ability to generate early neoplastic lesions; (2) changes in older mammary tissue such that its composition is "younger," more particularly, that treatment with eRapa according to the methods herein described results in changes to the luminal-to-basal cell ratio from less than 1 in rapamycin-treated older patients to a luminal-to-basal cell ratio that is greater than 1, as is typically observed in younger patients; and (3) a decrease in the frequency of mammary stem cells (MaSC) and an increase in the frequency of luminal progenitor cells.

The use of the word "a" or "an" when used in conjunction with the term "comprising" may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

The words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

The compositions and methods for their use can "comprise," "consist essentially of," or "consist of" any of the ingredients or steps disclosed throughout the specification. With respect to the transitional phase "consisting essentially of," in one non-limiting aspect, a basic and novel characteristic of the compositions and methods is the ability of rapamycin to inhibit the development of early neoplastic lesions or hyperplastic lesions in the mammary glands or prevent breast cancer in a patient who has been identified as being at risk for developing breast cancer.
[0030] It is contemplated that any embodiment discussed in this specification can be
implemented with respect to any method or composition of the invention, and *vice versa.*
Furthermore, compositions of the invention can be used to achieve methods of the invention.

[0031] Other objects, features and advantages of the present invention will become
apparent from the following detailed description. It should be understood, however, that the
detailed description and the specific examples, while indicating specific embodiments of the
invention, are given by way of illustration only, since various changes and modifications
within the spirit and scope of the invention will become apparent to those skilled in the art
from this detailed description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0032] The following drawings form part of the present specification and are
included to further demonstrate certain aspects of the present invention. The invention may
be better understood by reference to one or more of these drawings in combination with the
detailed description of specific embodiments presented herein.

[0033] **FIG. 1** provides a cross-sectional graphic illustration of the typical inner
structures of a mammary gland or duct **100**, illustrating various basic features including the
relevant cell types associated with such a mammary gland or duct.

[0034] **FIGS. 2A-2G:** Relation Between Age and Treatment with Enterically-
delivered Rapamycin (eRapa) on Mammary Stem Cell (MaSC) Frequency in C57BL/6 Mice.

(A) Flow cytometry of mammary cells. Cells high in CD49f are MaSC-enriched basal cells,
and cells high in CD24 are luminal progenitor-enriched luminal cells. There was a high
frequency of MaSCs in old control (age, 26 months) mice and a low frequency of MaSCs in
young (age, 4 months) and old eRapa-treated old mice (age, 26 months). Abbreviations:
CD24 PE A, anti-CD24 antibody labeled with phycoerythrin; FITC-A, antibody conjugated
with fluorescein isothiocyanate. (B) Percent basal cells (CD24^hi\text{or}CD49f^hi) (mean ± SD) of
Lin^- mammary cells isolated from young (blue dots), old control (red dots), and old
eRapa-treated (green dots) mice. There was a greater frequency of basal cells in the old
control than young mice. Long-term (2 years) or short-term (5 to 10 days) treatment with
eRapa decreased the frequency of basal cells in old mice. (C) Percent luminal cells
(CD24^hi\text{or}CD49f^hi) (mean ± SD) of Lin^- mammary cells isolated from young, old control, and
old eRapa-treated mice. There was a lower frequency of luminal cells in the old control than
young mice. Long-term (2 years) or short-term (5 to 10 days) treatment with eRapa increased
the frequency of luminal cells in old mice. (D) Luminal-to-basal cell ratio (mean ± SD) of Lin⁺ mammary epithelial cells isolated from young, old control, and old eRapa-treated mice. Most glands from old control mice had luminal-to-basal cell ratio < 1, and most glands from young and eRapa-treated old mice (long- and short-term treatment) had luminal-to-basal cell ratio > 1. (E) Average frequency (mean ± SD) of MaSC expressed as % sphere formation and differentiation initiating cells from basal cells (SFD-ICb) in young, old control, and old eRapa-treated mice. There was a greater frequency of MaSC in the old control than young mice. Long-term (2 years) or short-term (5 to 10 days) treatment with eRapa decreased the frequency of MaSC in old mice. (F, G) Average frequency (mean ± SD) of luminal progenitor cells expressed as % sphere formation and differentiation initiating cells from luminal cells (SFD-IC1) (F) or % colony forming cells (CFC) (G) in young, old control, and old eRapa-treated mice. There was a lower frequency of luminal progenitor cells in the old control than young mice. Long-term (2 years) or short-term (5 to 10 days) treatment with eRapa increased the frequency of luminal progenitor cells in old mice. See also FIGS. 4A-4E.

[0035] FIGS. 3A-3E: Increased Function and Reduced Transformation Potential of Old Mammary Stem Cells (MaSCs) by Treatment with Enterically-delivered Rapamycin (eRapa) in C57BL/6 Mice. (A) Old control MaSCs had decreased in vivo repopulation frequency after the second than first cleared fat pad transplant. Long-term eRapa treatment in old mice significantly increased the engraftment frequency of the second transplant (*difference of engraftment frequency between old control and old eRapa-treated mice: $P = 0.03$, Barnard exact test). (B) Immunohistochemistry staining of casein in regenerated glands (postpartum day 1) from MaSCs isolated from old mice showing greater milk production in old eRapa-treated than old control mice (scale bars for 4x, 500 µm; scale bars for 40x, 100 µm). (C) In both primary (Prim) and MaSC-regenerated glands (Reg), there was greater mean frequency of preneoplastic lesions (expressed as % hyperplasia) and neoplastic lesions (% atypical ductal hyperplasia/ductal carcinoma in situ [ADH/DCIS]) in old control mice (red dots) than long-term or short-term eRapa-treated old mice (green dots), except there was no difference in neoplastic lesions in Reg glands between control old and long-term eRapa-treated old mice. (D) There was greater expression of RNA of pl9ARF and pl6INK4a in old control than young stromal cells. (E) Immunohistochemistry staining of cyclooxygenase 2 of primary glands from young, old control, and old eRapa-treated mice showing increased expression of cyclooxygenase 2 in old control glands but low or no
expression in young and eRapa-treated old glands (scale bars, 100 µm). See also FIGS. 5A-51.

[0036] FIGS. 4A-4E: Mammary Glands from Old Mice Display Altered Cell Lineage in C57BL/6 and Balb/c Mice. (A, B) Solid (A) and hollow (B) structures derived from 3-dimensional gel culture of spheres derived from basal (CD241oCD49fhi) and luminal (CD24hiCD49flo) cells (scale bars, 500 µm). (C) Percent (mean ± SD) basal cells (CD241oCD49fhi), percent luminal cells (CD24hiCD49flo), ratio of luminal-to-basal cells, average frequency of mammary stem cells (MaSC) (% sphere formation and differentiation initiating cell from basal cells [SFD-ICb]), and average frequency of luminal progenitor cells (% SFD-ICI) of Lin− mammary epithelial cells isolated from young (age 2 to 4 months) and old Balb/c mice (age 17 months). (D) The 2 x 2 contingency table showing the number of C57BL/6 mice exhibiting luminal-to-basal cell ratio < 1 or > 1 in old control or old eRapa-treated mice (long-term [2 years] or short-term [5 to 10 days] treatment). Barnard exact test indicates that old control mice are statistically more likely to exhibit luminal-to-basal cell ratio < 1 phenotype and eRapa-treated mice are more likely to exhibit luminal-to-basal cell ratio > 1 phenotype. (E) A 2-dimensional cell colony formed by luminal cells on irradiated NIH3T3 feeder layers (scale bar, 500 µm).

[0037] FIGS. 5A-5I: Decreased Mammary Stem Cell (MaSC) Function and Reversal by Treatment with Enterically-delivered Rapamycin (eRapa), and Elevated Inflammatory Response in Old Mammary Glands from C57BL/6 Mice. (A) Scheme of in vitro 3-dimensional (3D) extracellular matrix culture passage of single 3-dimensional solid structures for 3 consecutive generations (scale bars, 500 µm). Quantitative data showing the number of 3-dimensional solid structures (3Ds) that continued to form 3Ds when they were dissociated and replated in each passage of 15 initial 3Ds from young, old control, and old short-term-eRapa-treated (2 weeks) C57BL/6 GFP mice. (B) Immunohistochemistry staining of casein of regenerated glands (postpartum day 1) from mammary stem cells (MaSCs) isolated from old control or old eRapa-treated C57BL/6 mice (age, 26 months) showing different degrees of casein staining (white, no stain; orange, partial stain; grey, light stain; black, dark stain) (scale bars, 100 µm). (C, D, E) Quantification of different casein staining patterns of regenerated glands from MaSCs isolated from old control or old eRapa-treated C57BL/6 mice that were transplanted into cleared fat pads at the contralateral side of the same young recipient mouse. Regenerated outgrowths were excised on pregnancy day 11 (PI 1), 14 (PI 4), 16 (PI 6), and 18 (PI 8) for the first transplants derived from old MaSCs
isolated from old control or old long-term-eRapa-treated (2 years) C57BL/6 mice (C). Regenerated glands were excised on postpartum day 1 from the second transplants of old MaSCs isolated from old control or old long-term-eRapa-treated (2 years) C57BL/6 mice (D). Regenerated glands were excised on postpartum day 1 from the first transplants derived from old MaSCs isolated from old control or old short-term-eRapa-treated (5 to 10 days) C57BL/6 mice (E). Each pie chart represents percent alveoli with different staining intensities in one section from one regenerated outgrowth and the two pie charts in the same column were from the same recipient mouse with outgrowths generated from old control or old eRapa-treated MaSCs. The Examiner was blind to treatment. (F) Histology showing normal duct, hyperplastic lesion, and atypical ductal hyperplasia/ductal carcinoma in situ (ADH/DCIS) from old mammary glands (hematoxylin-eosin; scale bars, 100 µm). (G) The frequency (mean ± SD) of preneoplastic (expressed as % hyperplasia) and neoplastic (% atypical ductal hyperplasia/ductal carcinoma in situ [ADH/DCIS]) lesions in primary and MaSC-regenerated glands of young (2 to 4 months) and old Balb/c mice (17 months). (H) Venn diagram of differentially expressed genes during aging (by comparing samples from old mice to young controls) in mammary stem cell-enriched (MaSC-enriched) basal mammospheres and stromal cells annotated with the top 10 enriched biological processes from Database for Annotation, Visualization and Integrated Discovery (DAVID) platform (National Institute of Allergy and Infectious Diseases, 2013). (I) Quantification of different phosphorylated S6 kinase (pS6K) staining pattern from young, old control, and old short-term (5 to 10 days) eRapa-treated C57BL/6 mice showing higher expression in old control glands and lower expression in young and eRapa-treated glands. Percentages were mean values of percent ductal structures that had a specific staining intensity (white, no stain; orange, partial stain; black, all cell stained; scale bar, 50 µm) in one tissue section of each mammary gland from four mice in each group.

[0038] FIG. 6 Experimental flow showing mammary stem cell isolation, sphere and 3-dimensional extracellular matrix (3D-ECM) structure formation, and subsequent in vivo cleared mammary fat pad transplant of single mammosphere and single 3D ECM solid structure derived from FACS -sorted basal cell fraction (CD241oCD49fhi) of the transgenic green fluorescent protein (GFP) mice showing positive outgrowth and subsequent alveolar development upon pregnancy.

[0039] FIGS. 7A-7D MaSCs Frequency Increases during Aging. The left panel shows that MaSC-enriched basal cells increase gradually with age in C57BL/6 mice. The
right panel shows that total cell yield, %lin negative (Lin) cells, sphere formation efficiency, and MaSC frequency increased with age in both C57BL/6 and Idaho mice.

[0040] FIGS. 8A-8D Aging Increases Early Neoplasia Frequency (A) Histology showing normal duct, hyperplastic lesion, and atypical ductal hyperplasia/ductal carcinoma in situ (ADH/DCIS) from old mammary glands (hematoxylin-eosin; scale bars, 100 μm). (B-C) The frequency (mean ± SD) of preneoplastic (expressed as % hyperplasia) and neoplastic (% atypical ductal hyperplasia/ductal carcinoma in situ [ADH/DCIS]) lesions in primary and MaSC-regenerated glands of young (2 to 4 months) and old Balb/c mice (17 months). (D)

[0041] FIG. 9 Decreased Frequency of MaSC and Early Neoplasia after Rapamycin Treatment. Average frequency (mean ± SD) of MaSC expressed as % sphere formation and differentiation initiating cells from basal cells (SFD-ICb) in young, old control, and old eRapa-treated mice. There was a greater frequency of MaSC in the old control than young mice. Long-term (2 years) or short-term (5 to 10 days) treatment with eRapa decreased the frequency of MaSC in old mice. In both primary (P) and MaSC-regenerated glands (R), there was greater mean frequency of preneoplastic lesions (expressed as % hyperplasia) and neoplastic lesions (% atypical ductal hyperplasia/ductal carcinoma in situ [ADH/DCIS]) in old control mice (red dots) than long-term or short-term eRapa-treated old mice (green dots), except there was no difference in neoplastic lesions in the regenerated glands between control old and long-term eRapa-treated old mice.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0042] Many diseases increase in frequency during aging, but the mechanism of age-associated mammary tumorigenesis is unknown. Rapamycin extends life span and improves age-related problems in murine models. In this study, the inventors show that mammary stem cells in older control mice were increased in number and had a functional decline of self-renewal, decreased differentiation, and increased neoplastic transformation potential. Long-term (> 2 years) or short-term (5 to 10 days) treatment with rapamycin reversed phenotypic changes associated with old mammary stem cells. The old rapamycin-treated mammary stem cells were rejuvenated in that they resembled young mammary stem cells in their ability to self-renew and produce milk and had reduced susceptibility to develop neoplastic lesions. Whole transcriptome analysis showed an elevated immune and inflammatory response in old mammary stem cells and stromal cells. Therefore, rapamycin may rejuvenate mammary
tissue by exerting anti-aging and anticancer effects through suppression of aging-associated inflammation and modulation of the function of tissue-specific stem cells.

A. Aging-Associated Dysfunction Of Stem Cells

[0043] Age is the number one risk fact for breast cancer. Recent research implicated that adult mammary stem cells (MaSCs) and progenitors might be responsible for the initiation and progression of certain types of breast cancer such as claudin-low and basal-like breast cancers.

[0044] A preliminary study showed that aging is associated with a significant increase of MaSC frequency, but with a functional decline of self-renewal and differentiation as well as increased neoplastic transformation potential. These findings indicate that aged MaSCs might be the precursors of preneoplastic lesions and serve as the cell of origin for malignant transformation in breast tissue. Therefore, intervention of MaSC aging process could be an effective method for cancer prevention.

[0045] The drug rapamycin has been shown to extend life span and ameliorate age-related pathologies (e.g., cancer) in murine models, and a recent study suggested that rapamycin's anti-aging effect may partially act through enhancing the function of tissue-specific stem cells. In this study, the inventors fed C57BL/6 mice with microencapsulated rapamycin-containing food (14 mg/kg, food designed to deliver -2.24 mg of rapamycin per kg body weight/day to achieve about 4 ng/ml of rapamycin per kg body weight/day) or control diet with empty capsules for 2 years (starting at 2 months old) or 5-10 days (starting at 25.5 months old) and then isolated primary mammary cells at 26 months old for MaSC quantification using an in vitro mammosphere formation and 3D-ECM sphere differentiation assay as well as by the in vivo cleared mammary fat pad transplantation assay. The findings indicate that rapamycin can rejuvenate the function of aged MaSCs as well as reduce their incidence of preneoplastic transformation.

[0046] By using the C57BL/6 mouse model, the study showed that long-term (> 2 years) or short-term (5-10 days) treatment with microencapsulated rapamycin diet (eRapa) reversed phenotypic changes associated with the aged murine mammary stem cell (MaSC), resulting in the rejuvenation of the mammary tissue, which is characterized by rendering these aged MaSCs to resemble young MaSCs in self-renewal, milk production, and the decreased ability of generating early neoplastic lesions. In addition, the inventors discovered significant reduction of early neoplastic lesions in the mammary glands from eRapa-treated
aged mice than those from control old mice. The finding that rapamycin can mitigate age-associated MaSC dysfunction indicates that rapamycin could be one of the first candidates for breast cancer prevention in populations with high risk. The results indicate that aging causes MaSC to increase its pool and to acquire a transforming phenotype, which is likely due to increased chronic immune and inflammatory responses. This transforming phenotype of MaSC can be inhibited by rapamycin treatment.

[0047] Scientists did not link the etiology of breast cancer with mammary stem/progenitor cells until recent years (e.g., Lim et al., 2009, Nature Medicine 15:907-913). The published studies indicate that the luminal progenitor cells and differentiated mammary epithelial cells are the cells of origin for various subtypes of breast cancer in humans. However, the inventors’ study found that it is the stem cell pool that increased with progressive aging. Thus, it is very likely that the stem cells are a putative culprit serving as the cells of origin for breast cancer. No evidence has previously indicated that rapamycin might inhibit the dysfunctional phenotypes of mammary stem cells for the prevention of breast cancer.

[0048] Even further, analysis of the ratio of luminal to basal cells in the mammary epithelia may be valuable. In particular, preventive drug treatment or other preventive methods that result in a change of luminal-to-basal cell ratio greater than 1 indicates that this intervention is effective in rejuvenating mammary tissue resulting in breast cancer prevention.

[0049] By using the C57BL/6 mouse model, the study showed that mammary glands in aged mice (> 25 months old) were characterized with increased basal cells and reduced luminal cells or quantitatively a luminal-to-basal cell ratio of less than 1. In contrast, mammary glands in young mice (e.g., 4-6 months old) were characterized by a luminal-to-basal cell ratio of greater than 1. The inventors have also demonstrated that long-term (> 2 years) or short-term (5-10 days) treatment with microencapsulated rapamycin diet (eRapa) (14 mg/kg food designed to deliver ~2.24 mg of Rapa per kg body weight/day to achieve about 4 ng/ml of Rapa per kg body weight/day) prepared by TestDiet, Inc., Richmond, IN, using Purina 5LG6 as the base, reversed this phenotypic change in aged glands, and resulted in a luminal-to-basal cell ratio of greater than 1. More significantly, this phenotypic reversal of cell lineage associated with rapamycin treatment in old mice can be characterized as the rejuvenation of the mammary tissue which is accompanied by increased mammary stem cell (MaSC) self-renewal, increased milk production and decreased ability of generating early
neoplastic lesions in regenerated glands derived from rapamycin-treated MaSCs. The findings suggest that the luminal-to-basal cell ratio can be used as a reliable indicator for predicting whether a preventive intervention is effective or not. Although the inventors' observations as described in the present disclosure indicate that a threshold value for the luminal-to-basal cell ratio is 1 wherein the efficacy of eRapa administration can be measured, and there is an observable difference in risk of breast cancer between mammary glands characterized by a luminal-to-basal cell ratio less than 1 and those characterized by a luminal-to-basal cell ratio greater than 1, it will be understood by those of ordinary skill in the art that other values above or below 1 may also serve as a luminal-to-basal cell ratio benchmark in other animals such as humans, dogs, cats, rabbits or other mammals.

[0050] For the first time it is recognized that mammary glands in old mice are characterized with increased basal cells and a luminal-to-basal cell ratio of less than 1. This study is also the first to demonstrate that rapamycin treatment can reverse this luminal-to-basal cell ratio from less than 1 to greater than 1 in old mammary glands.

[0051] Current research in breast cancer linked the increased luminal cells (or higher luminal-to-basal ratio) with increased breast cancer risk, which contradicts what was observed in aged mammary glands. Thus, it is counterintuitive to believe that a luminal-to-basal ratio greater than 1 actually indicates effectiveness of breast cancer prevention.

[0052] The inventors initially started exploring the effect of age on murine mammary stem cell function in 2010 with C57BL/6 mice, and the inventors found a significant increase of basal cell fraction in old mammary glands by using flow cytometry - activated cell sorting to fraction out luminal and basal cells from the primary mammary cells. The inventors then found a similar phenomenon in Balb/c mice. These findings led them to develop the ratio of percent luminal to percent basal cell population in lineage-negative (Lin⁻) mammary cells as an index to distinguish young from old mammary glands. The inventors found that the old mammary glands contain early neoplastic lesions, which can also be generated by transplanting MaSCs isolated from old mammary glands into a young mammary fat pad suggesting the reduced luminal-to-basal ratio to lower than 1 is associated with early mammary tumorigenesis. Later on, the inventors found that rapamycin treatment can inhibit early neoplastic transformation in old mammary glands and the transforming phenotype of old MaSCs with a reversal of the luminal-to-basal ratio in old mouse mammary glands back to that as observed in young mouse mammary glands. Thus, the luminal-to-basal ratio can be used as a hallmark to indicate the effectiveness of intervention in breast cancer prevention.
B. mTOR Inhibitors and Rapamycin

[0053] Any inhibitor of mTOR or mTORCl is contemplated for inclusion in the present compositions and methods. In particular embodiments, the inhibitor of mTORCl is rapamycin or an analog of rapamycin. Rapamycin (also known as sirolimus and marketed under the trade name Rapamune) is a known macrolide. The molecular formula of rapamycin is C51H79NO13.

[0054] Rapamycin binds to a member of the FK binding protein (FKBP) family, FKBP 12. The rapamycin-FKBP 12 complex binds to the protein kinase mTOR to block the activity of signal transduction pathways. Because the mTOR signaling network includes multiple tumor suppressor genes, including PTEN, LKB1, TSC1, and TSC2, and multiple proto-oncogenes including P13K, Akt, and eEF4E, mTOR signaling plays a central role in cell survival and proliferation. Binding of the rapamycin-FKBP complex to mTOR causes arrest of the cell cycle in the G1 phase (Janus et al., 2005).

[0055] mTORCl inhibitors also include rapamycin analogs. Many rapamycin analogs are known in the art. Non-limiting examples of analogs of rapamycin include, but are not limited to, everolimus, tacrolimus, CCI-779, ABT-578, AP-23675, AP-23573, AP-23841, 7-epirapamycin, 7-thiomethyl-rapamycin, 7-epi-trimethoxyphenyl- rapamycin, 7-epi-thiomethylrapamycin, 7-demethoxy-rapamycin, 32-demethoxy- rapamycin, 2-desmethyl-rapamycin, and 42-0-(2-hydroxy)ethyl rapamycin.

[0056] Other analogs of rapamycin include: rapamycin oximes (U.S. Pat. No. 5,446,048); rapamycin aminoesters (U.S. Pat. No. 5,130,307); rapamycin dialdehydes (U.S. Pat. No. 6,680,330); rapamycin 29-enols (U.S. Pat. No. 6,677,357); O-alkylated rapamycin derivatives (U.S. Pat. No. 6,440,990); water soluble rapamycin esters (U.S. Pat. No. 5,955,457); alkylated rapamycin derivatives (U.S. Pat. No. 5,922,730); rapamycin amidino carbamates (U.S. Pat. No. 5,637,590); biotin esters of rapamycin (U.S. Pat. No. 5,504,091); carbamates of rapamycm (U.S. Pat. No. 5,567,709); rapamycin hydroxyesters (U.S. Pat. No. 5,362,718); rapamycin 42-sulfonates and 42-(N-carboxy)sulfamates (U.S. Pat. No. 5,346,893); rapamycin oxepane isomers (U.S. Pat. No. 5,344,833); imidazolidyl rapamycin derivatives (U.S. Pat. No. 5,310,903); rapamycin alkoxysters (U.S. Pat. No. 5,233,036); rapamycin pyrazoles (U.S. Pat. No. 5,164,399); acyl derivatives of rapamycin (U.S. Pat. No. 4,316,885); reduction products of rapamycin (U.S. Pat. Nos. 5,102,876 and 5,138,051); rapamycin amide esters (U.S. Pat. No. 5,118,677); rapamycin fluorinated esters (U.S. Pat. No. 5,164,399); rapamycin amidino carbamates.
No. 5,100,883); rapamycin acetals (U.S. Pat. No. 5,151,413); oxorapamycins (U.S. Pat. No. 6,399,625); and rapamycin silyl ethers (U.S. Pat. No. 5,120,842).

[0057] Other analogs of rapamycin include those described in U.S. Pat. Nos. 6,015,809; 6,004,973; 5,985,890; 5,955,457; 5,922,730; 5,912,253; 5,780,462; 5,665,772; 5,637,590; 5,567,709; 5,563,145; 5,559,122; 5,559,120; 5,559,119; 5,559,112; 5,550,133; 5,541,192; 5,541,191; 5,532,355; 5,530,121; 5,530,007; 5,525,610; 5,521,194; 5,519,031; 5,516,780; 5,508,399; 5,508,290; 5,508,286; 5,508,285; 5,504,291; 5,504,204; 5,491,231; 5,489,680; 5,489,595; 5,488,054; 5,486,524; 5,486,523; 5,486,522; 5,484,791; 5,484,790; 5,480,989; 5,480,988; 5,463,048; 5,446,048; 5,434,260; 5,411,967; 5,391,730; 5,389,639; 5,385,910; 5,385,909; 5,385,908; 5,378,836; 5,378,696; 5,373,014; 5,362,718; 5,358,944; 5,346,893; 5,344,833; 5,302,584; 5,262,424; 5,262,423; 5,260,300; 5,260,299; 5,233,036; 5,221,740; 5,221,670; 5,202,332; 5,194,447; 5,177,203; 5,169,851; 5,164,399; 5,162,333; 5,151,413; 5,138,051; 5,130,307; 5,120,842; 5,120,727; 5,120,726; 5,120,725; 5,118,678; 5,118,677; 5,100,883; 5,023,264; 5,023,263; 5,023,262; all of which are incorporated herein by reference. Additional rapamycin analogs and derivatives can be found in the following U.S. Patent Application Pub. Nos., all of which are herein specifically incorporated by reference: 20080249123; 20080182867; 2008018851; 20080091008; 20080085880; 20080069797; 20070280992; 20070225313; 20070203172; 20070203171; 20070203170; 20070203169; 20070142423; 20060264453; and 20040010002.

[0058] Rapamycin or a rapamycin analog can be obtained from any source known to those of ordinary skill in the art. The source may be a commercial source or natural source. Rapamycin or a rapamycin analog may be chemically synthesized using any technique known to those of ordinary skill in the art. Non-limiting examples of information concerning rapamycin synthesis can be found in Schwecke et al., 1995; Gregory et al., 2004; Gregory et al., 2006; Graziani, 2009.

C. Encapsulated Rapamycin Compositions

[0059] In some aspects, the compositions comprising an inhibitor of mTOR are encapsulated or coated. In some embodiments, the encapsulant or coating may be an enteric coating.

[0060] Many pharmaceutical dosage forms irritate the stomach due to their chemical properties or are degraded by stomach acid through the action of enzymes, thus becoming less effective. The coating may be an enteric coating, a coating that prevents release and
absorption of active ingredients until they reach the intestine. "Enteric" refers to the small intestine, and therefore enteric coatings facilitate delivery of agents to the small intestine. Some enteric coatings facilitate delivery of agents to the colon. In some embodiments, the enteric coating is a EUDRAGIT® coating. Eudragit coatings include Eudragit L 100-55 (for delivery to the duodenum), Poly(methacrylic acid-co-ethyl acrylate) 1:1; Eudragit L 30 D-55 (for delivery to the duodenum), Poly(methacrylic acid-co-ethyl acrylate) 1:1; Eudragit L 100 (for delivery to the jejunum), Poly(methacrylic acid-co-methyl methacrylate) 1:1; Eudragit S 100 (for delivery to the ileum), Poly(methacrylic acid-co-methyl methacrylate) 1:2; Eudragit FS 30D (for colon delivery), Poly(methyl acrylate-co-methyl methacrylate-co-methacrylic acid) 7:3:1; Eudragit RL (for sustained release), Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) 1:2:0.2; Eudragit RS (for sustained release), Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) 1:2:0.1; and Eudragit E (for taste masking), Poly(butyl methacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate) 1:2:1. Other coatings include ethylcellulose and polyvinyl acetate. Benefits include pH-dependent drug release, protection of active agents sensitive to gastric fluid, protection of gastric mucosa from active agents, increase in drug effectiveness, good storage stability, and GI and colon targeting, which minimizes risks associated with negative systemic effects.

Some examples of enteric coating components include cellulose acetate phthalate, methyl acrylate-methacrylic acid copolymers, cellulose acetate succinate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, methyl methacrylate-methacrylic acid copolymers, sodium alginate, and stearic acid. The coating may include suitable hydrophilic gelling polymers including, but not limited to, cellulosic polymers, such as methylcellulose, carboxymethylcellulose, hydroxypropylcellulose, hydroxypropyl methylcellulose, hydroxyethylcellulose, and the like; vinyl polymers, such as polyvinylpyrrolidone, polyvinyl alcohol, and the like; acrylic polymers and copolymers, such as acrylic acid polymer, methacrylic acid copolymers, ethyl acrylate-methyl methacrylate copolymers, natural and synthetic gums, such as guar gum, arabic gum, xanthan gum, gelatin, collagen, proteins, polysaccharides, such as pectin, pectic acid, alginic acid, sodium alginate, polyaminoacids, polyalcohols, polyglycols; and the like; and mixtures thereof. Any other coating agent known to those of ordinary skill in the art is contemplated for inclusion in the coatings of the microcapsules.
The coating may optionally comprise a plasticizer, such as dibutyl sebacate, polyethylene glycol and polypropylene glycol, dibutyl phthalate, diethyl phthalate, triethyl citrate, tributyl citrate, acetylated monoglyceride, acetyl tributyl citrate, triacetin, dimethyl phthalate, benzyl benzoate, butyl and/or glycol esters of fatty acids, refined mineral oils, oleic acid, castor oil, com oil, camphor, glycerol and sorbitol or a combination thereof. The coating may optionally include a gum. Non-limiting examples of gums include homopolysaccharides such as locust bean gum, galactans, mannans, vegetable gums such as alginates, gum karaya, pectin, agar, tragacanth, acacia, carrageenan, chitosan, agar, alginic acid, other polysaccharide gums (e.g., hydrocoUoids), acacia catechu, salai guggal, indian bodeIIum, copaiba gum, asafetida, cambi gum, Enterolobium cyclocarpum, mastic gum, benzoin gum, sandarac, gambier gum, butea frondosa (Flame of Forest Gum), myrrh, Konjac mannan, guar gum, welan gum, gellan gum, tara gum, locust bean gum, carageenan gum, glucomannan, galactan gum, deacetylated xanthan gum, pectin, sodium polypectate, gluten, tamarind gum, ghatti gum, Acaroid Nacca/Red gum, dammar gum, juniper gum, ester gum, ipil-ipil seed gum, gum talha (acacia seyal), and cultured plant cell gums including those of the plants of the genera: acacia, actinidia, aptenia, Carpobrotus, Chickorium, Cucumis, Glycine, Hibiscus, Hordeum, Lactuca, Lycopersicon, Mains, Medicago, Mesembryanthemum, Oryza, Panicum, Phalaris, Phleum, Polianthes, polycarbophil, sida, solanum, trifolium, trigonella, Afzelia africana seed gum, Treculia africana gum, detarium gum, cassia gum, carob gum, Prosopis africana gum, Colocasia esculenta gum, Hakea gibbosa gum, khaya gum, scleroglucan, zea, mixtures of any of the foregoing, and the like.

D. Methods of Using Rapamycin Compositions

"Treatment" and "treating" refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject for the purpose of obtaining a therapeutic benefit for a disease or health-related condition. For example, the rapamycin compositions of the present invention may be administered to a subject for the purpose of inhibiting the development of early neoplastic lesions or hyperplastic lesions in the mammary glands or preventing breast cancer in a patient who has been identified as being at risk for developing breast cancer.

The terms "therapeutic benefit," "therapeutically effective," or "effective amount" refer to the promotion or enhancement of the well-being of a subject. This includes,
but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease.

[0065] "Prevention" and "preventing" are used according to their ordinary and plain meaning. In the context of a particular disease or health-related condition, those terms refer to administration or application of an agent, drug, or remedy to a subject or performance of a procedure or modality on a subject for the purpose of preventing or delaying the onset of a disease or health-related condition. For example, one embodiment includes administering the rapamycin compositions of the present invention to a subject at risk for developing a breast cancer for the purpose of inhibiting the development of early neoplastic lesions or hyperplastic lesions in the mammary glands or preventing breast cancer in a patient who has been identified as being at risk for developing breast cancer.

[0066] Rapamycin compositions, as disclosed herein, may be used to treat any disease or condition for which an inhibitor of mTOR is contemplated as effective for treating or preventing the disease or condition. For example, methods of using rapamycin compositions are disclosed to inhibit the development of early neoplastic lesions or hyperplastic lesions in the mammary glands or prevent breast cancer in a patient who has been identified as being at risk for developing breast cancer.

E. Pharmaceutical Preparations

[0067] Certain methods and compositions set forth herein are directed to administration of an effective amount of a composition comprising the rapamycin compositions of the present invention.

1. Compositions

[0068] A "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, and such like materials and combinations thereof, as would be known to one of ordinary skill in the art (Remington's, 1990). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated. The compositions used in the present invention may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it needs to be sterile for such routes of administration as injection.
[0069] The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions, and these are discussed in greater detail below. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologies standards.

[0070] The formulation of the composition may vary depending upon the route of administration. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure.

[0071] In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g., tablets or other solids for oral administration; liposomal and nanoparticle formulations; enteric coating formulations; time-release capsules; formulations for administration via an implantable drug delivery device; and any other form. One may also use nasal solutions or sprays, aerosols or inhalants in the present invention.

[0072] The capsules may be, for example, hard-shell capsules or soft-shell capsules. The capsules may optionally include one or more additional components that provide for sustained release.

[0073] In certain embodiments, pharmaceutical composition includes at least about 0.1% by weight of the active compound. In other embodiments, the pharmaceutical composition includes about 2% to about 75% of the weight of the composition, or between about 25% to about 60% by weight of the composition, for example, and any range derivable therein.

[0074] The compositions may comprise various antioxidants to retard oxidation of one or more components. Additionally, the prevention of the action of microorganisms can be accomplished by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (e.g., methylparabens and propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof. The composition
should be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[0075] In certain preferred embodiments, an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, and combinations thereof. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both.

[0076] In particular embodiments, prolonged absorption can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin, or combinations thereof.

2. Routes of Administration

[0077] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

[0078] The composition can be administered to the subject using any method known to those of ordinary skill in the art. For example, a pharmaceutically effective amount of the composition may be administered intravenously, intracerebrally, intracranially, intraventricularly, intrathecally, into the cortex, thalamus, hypothalamus, hippocampus, basal ganglia, substantia nigra or the region of the substantia nigra, cerebellum, intradermally, intraarterially, intraperitoneally, intralesionally, intratraceally, intranasally, topically, intramuscularly, anally, subcutaneously, orally, locally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in creams, in lipid compositions (e.g., liposomes), or by any other method or any combination of the foregoing as would be known to one of ordinary skill in the art (Remington's, 1990).

[0079] In particular embodiments, the composition is administered to a subject using a drug delivery device. Any drug delivery device is contemplated for use in delivering an effective amount of the inhibitor of mTORC1.

3. Dosage

[0080] A pharmaceutically effective amount of an inhibitor of mTORC1 is determined based on the intended goal. The quantity to be administered, both according to
number of treatments and dose, depends on the subject to be treated, the state of the subject, the protection desired, and the route of administration. Precise amounts of the therapeutic agent also depend on the judgment of the practitioner and are peculiar to each individual.

[0081] The amount of rapamycin or rapamycin analog or derivative to be administered will depend upon the disease to be treated, the length of duration desired and the bioavailability profile of the implant, and the site of administration. Generally, the effective amount will be within the discretion and wisdom of the patient's physician. Guidelines for administration include dose ranges of from about 0.01 mg to about 500 mg of rapamycin or rapamycin analog.

[0082] For example, a dose of the inhibitor of mTORCl may be about 0.0001 milligrams to about 1.0 milligrams, or about 0.001 milligrams to about 0.1 milligrams, or about 0.1 milligrams to about 1.0 milligrams, or even about 30 milligrams per dose or so. Multiple doses can also be administered. In some embodiments, a dose is at least about 0.0001 milligrams. In further embodiments, a dose is at least about 0.001 milligrams. In still further embodiments, a dose is at least 0.01 milligrams. In still further embodiments, a dose is at least about 0.1 milligrams. In more particular embodiments, a dose may be at least 1.0 milligrams. In even more particular embodiments, a dose may be at least 30 milligrams. In further embodiments, a dose is at least 100 milligrams or higher. In some embodiments, the dose of the inhibitor of mTORCl may be at least, at most, or exactly about 0.0001, 0.001, 0.01, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 25, or 30 milligrams per dose (or any derivable range therein). In some embodiments, the dose is 1 to 10 mg, 2 to 6 mg, or 4 mg. The dose may be given at least or at most once, twice, or three times per day and may be administered for a time period of at least, at most, or exactly one week, one month, one year, two years, five years, or 10 years (or any derivable range therein). In some embodiments, the rapamycin is administered chronically for the remainder of the patient's life.

[0083] In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 micrograms/kg of body weight, about 10 micrograms/kg of body weight, about 50 micrograms/kg of body weight, about 100 micrograms/kg of body weight, about 200 micrograms/kg of body weight, about 350 micrograms/kg of body weight, about 500 micrograms/kg of body weight, about 1 milligram/kg of body weight, about 5 milligrams/kg of body weight, about 10 milligrams/kg of body weight, about 50 milligrams/kg of body weight, about 100 milligrams/kg of body weight, about 200
milligrams/kg of body weight, about 350 milligrams/kg of body weight, about 500
milligrams/kg of body weight, to about 1000 milligrams/kg of body weight or more per
administration, and any range derivable therein. In non-limiting examples of a derivable
range from the numbers listed herein, a range of about 5 milligrams/kg of body weight to
about 100 milligrams/kg of body weight, about 5 micrograms/kg of body weight to about 500
milligrams/kg of body weight, etc., can be administered, based on the numbers described
above.

[0084] The dose can be repeated as needed as determined by those of ordinary skill
in the art. Thus, in some embodiments of the methods set forth herein, a single dose is
contemplated. In other embodiments, two or more doses are contemplated. In some
embodiments, the two or more doses are the same dosage. In some embodiments, the two or
more doses are different dosages. Where more than one dose is administered to a subject, the
time interval between doses can be any time interval as determined by those of ordinary skill
in the art. For example, the time interval between doses may be about 1 hour to about 2
hours, about 2 hours to about 6 hours, about 6 hours to about 10 hours, about 10 hours to
about 24 hours, about 1 day to about 2 days, about 1 week to about 2 weeks, or longer, or any
time interval derivable within any of these recited ranges. In specific embodiments, the
composition may be administered daily, weekly, monthly, annually, or any range therein.

[0085] In certain embodiments, it may be desirable to provide a continuous supply of
a pharmaceutical composition to the patient. This could be accomplished by catheterization,
followed by continuous administration of the therapeutic agent. The administration could be
intra-operative or post-operative.

4. Secondary and Combination Treatments

[0086] Certain embodiments provide for the administration or application of one or
more secondary or additional forms of therapies or preventative interventions. The type of
therapy is dependent upon the type of disease that is being treated or prevented. The
secondary form of therapy may be administration of one or more secondary pharmacological
agents that can be applied for inhibiting the development of early neoplastic lesions or
hyperplastic lesions in the mammary glands or preventing breast cancer in a patient who has
been identified as being at risk for developing breast cancer.

[0087] If the secondary or additional therapy is a pharmacological agent, it may be
administered prior to, concurrently with, or following administration of the inhibitor of
mTORC1. The interval between administration of the inhibitor of mTORC1 and the secondary or additional therapy may be any interval as determined by those of ordinary skill in the art. For example, the inhibitor of mTORC1 and the secondary or additional therapy may be administered simultaneously, or the interval between treatments may be minutes to weeks. In embodiments where the agents are separately administered, one would generally ensure that a significant period of time did not expire between the times of each delivery, such that each therapeutic agent would still be able to exert an advantageously combined effect on the subject. For example, the interval between therapeutic agents may be about 12 hours to within about 24 hours of each other and, more preferably, within about 6 hours to about 12 hours of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. In some embodiments, the timing of administration of a secondary therapeutic agent is determined based on the response of the subject to the inhibitor of mTORC1.

Examples of secondary treatments useful with methods disclosed herein are: surgery with curative intent for localized breast cancer, radiotherapy, chemotherapy and administration of other local or systemic agents (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008; Granberg 1998; Steinmuller 2008; Plockinger 2004; Kulke 1999; Cooper 2001; Eriksson 2008; Sutcliffe 2004).

Radiotherapy includes radio frequency ablation to stabilize or reduce the size of non-resectable neoplasms or reducing hormone secretion of functional metastatic neoplasms, external beam radiation to alleviate bone pain due to metastases, and targeted radionuclide therapy, such as (131)-I-MIBG, to serve as a palliative option for certain patients with inoperable or metastatic neoplasms (Pasieka 2004; Kwekkeboom 2009).

Chemotherapy and administration of other systemic agents (Kaltsas 2001) include: Somatostatin and related analogues such as octreotide (Sandostatin), lanreotide (Somatuline® Depot), and pasireotide (Kwekkeboom 2009; Kaltsas 2005; Oberg 2004), Proton pump inhibitors to decrease gastric acid to include omeprazole (Prilosec), lansoprazole (Prevacid), rabeprazole (Aciphex), pantoprazole (Protonix), esomeprazole (Nexium), and Zegarid, a rapid release form of omeprazole (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008); Benzothiadiazines, such as DiazaZide, inhibit insulin release which can be used to treat hypoglycemia associated with insulinomas (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008); Interferons, to include interferons such as and similar to interferon-
alpha and pegylated interferon-alpha-2b, may be used to boost the body's natural immune responses (Faiss 2003); Antibodies to VEGF ligand and VEGF complexes to include antibodies such as and similar to Bevacizumab (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008); Inhibitors of tyrosine kinase to include inhibitors such as and similar to Sunitinib (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008); Chemotherapeutics such as Streptozotocin, Temozolomide, fluoropyrimidines such as 5-fluorouracil and xeloda (capecitabine) (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008; Maire 2008; Kouvaraki 2004); leucovorin, platinum derivatives such as Eloxatin (oxaliplatin), picoplatin and cisplatin (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008; Fjallskog 2001); Topoisomerase inhibitors such as Campostar (irinotecan) and etirinotecan pegol; targeted antibodies to epidermal growth factor receptor (EGFR) such as Erbitux and Vectibix (panitumumab), targeted antibodies to VEGF-A such as Avastin (bevacizumab) (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008; Yao 2008); kinase inhibitors such as Stivarga (regorafenib); doxorubicin, dacarbazine, etoposide, everolimus and other rapalogs, (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008; Yao 2008); and all combinations thereof, specifically including the drug combinations that make up the combination therapies referred to as FOLFOX (5-fluorouracil with leucovorin and oxaliplatin), FOLFIRI (5-fluorouracil with leucovorin and irinotecan) and XELOX (oxaliplatin with capecitabine) with and without Avastin, followed by the salvage agents Erbitux, Vectibix or Stivarga (Reidy-Lagunes 2012; Kulke 2011; Matthew 2008).

[0091] Chemopreventives include: non-steroidal anti-inflammatory compounds such as aspirin, sulindac, and cox-2 inhibitors to include sulfonamides such as Celecoxib; efornithine (DFMO - alpha-difluoromethylornithine), elsiglutide, tyrosine kinase inhibitors that act on epidermal growth factor receptors such as erlotinib (Tarceva); inositol, polyunsaturated fatty acids to include omega-3-fatty acids such as eicosapentaenoic acid; biguanides such as metformin; polyethylene glycol, propranolol, etodolac, tinzaparin, bile acids such as ursodeoxycholic acid; curcuminoids such as curcumin, desmethoxycurcumin and bisdesmethoxycurcumin; and estrogen receptor beta agonists such as Eviendep, and combination with any of the aforementioned drugs or compounds.

[0092] Dietary supplements include vitamins C, E and D, calcium, zinc, selenium, curcumin, folate, bioflavonoids, resveratrol, freeze-dried blackberries and green tea extracts, and combination with any of the aforementioned drugs or compounds.
F. Kits

[0093] Kits are also contemplated as being used in certain aspects of the present invention. For instance, a rapamycin composition of the present invention can be included in a kit. A kit can include a container. Containers can include a bottle, a metal tube, a laminate tube, a plastic tube, a dispenser, a pressurized container, a barrier container, a package, a compartment, or other types of containers such as injection or blow-molded plastic containers into which the hydrogels are retained. The kit can include indicia on its surface. The indicia, for example, can be a word, a phrase, an abbreviation, a picture, or a symbol.

[0094] Further, the rapamycin compositions of the present invention may also be sterile, and the kits containing such compositions can be used to preserve the sterility. The compositions may be sterilized via an aseptic manufacturing process or sterilized after packaging by methods known in the art.

EXAMPLES

[0095] The following examples are included to demonstrate certain non-limiting aspects of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[0096] For reference as a backdrop in discussing the examples, FIG. 1 graphically illustrates pertinent cellular structures. Particularly, FIG. 1 provides a cross-sectional graphic illustration of the interior 100 of a mammary gland, which is equally applicable to the interior of a mammary duct. As illustrated, the relevant cell types that are associated with those interior mammary gland structures 100 are shown, as are various other basic features. Overall, FIG. 1 illustrates a typical arrangement of mammary epithelial cells that make up the mammary gland's interior structures 100, including epithelial basal cells 110 and epithelial luminal cells 120. Basal cells 110 are shown along the periphery of the mammary gland's inner structures 100, forming an outer layer of myoepithelial cells within basement membrane 160. The basal cells 110 contain mammary stem cells 140 (MaSCs), which produce an inward layer of the epithelial luminal cells 120. Such luminal cells 120 line the lumen 130 of the mammary gland, interior to the layer of basal cells 110. Basal cells 110 are
characterized as being CD24\textsuperscript{i}, CD49\textsuperscript{f}. Luminal cells \textbf{120} contain progenitor cells \textbf{150} and, in contrast to the basal cells \textbf{110}, the luminal cells \textbf{120} are typically characterized as being CD24\textsuperscript{hi}, CD49\textsuperscript{lo}.

**EXAMPLE 1 - RAPAMYCIN MITIGATES AGING-ASSOCIATED DYSFUNCTION OF MURINE MAMMARY STEM CELLS**

[0097] As described previously above, the inventors have identified that young mice have greater numbers of luminal cells \textbf{120} as compared to basal cells \textbf{110}, wherein the ratio of luminal-to-basal cells is greater than 1. In contrast, as mice age, the number of basal cells \textbf{110} increases as compared to luminal cells \textbf{120}, wherein the same ratio of luminal-to-basal cells is less than 1.

[0098] FIG. 6 shows experimental flow cytometry showing mammary stem cell isolation, sphere and 3-dimensional extracellular matrix (3D-ECM) structure formation, and subsequent in vivo cleared mammary fat pad transplant of single mammosphere and single 3D ECM solid structure derived from FACS -sorted basal cell fraction (CD24\textsuperscript{1}, CD49\textsuperscript{hi}) of the transgenic green fluorescent protein (GFP) mice showing positive outgrowth and subsequent alveolar development upon pregnancy.

[0099] Several different marker combinations (Smalley et al., 2012) have been used to identify basal and luminal cells, and fluorescence-activated cell sorting may enrich for MaSC and luminal progenitor populations. The inventors identified MaSC-enriched basal cells as CD31, CD45, and TER1 19-cells (also known as Lin-CD24\textsuperscript{1}, CD49\textsuperscript{hi} cells), and luminal progenitor-enriched luminal cells (also known as Lin-CD24\textsuperscript{hi}, CD49\textsuperscript{lo} cells) (FIG. 2A).

[00100] The effects of age and rapamycin (microencapsulated or enterically-delivered rapamycin [eRapa]) on murine MaSCs or luminal progenitor cells were evaluated in mammary glands from C57BL/6 mice (age, 2 to 4 months) versus old control mice (age, 25 to 32 months); old control versus old long-term-eRapa-treated mice (eRapa, 2 years; age at termination, 26 months); and old control versus old short-term-eRapa-treated mice (eRapa, 5 to 10 days; age at termination, 26 months). There were significantly more basal cells (FIGS. 2A and 2B), less luminal cells (FIGS. 2A and 2C), and lower mean luminal-to-basal cell ratio (FIG. 2D) in mammary glands from old control than young mice. Long- or short-term treatment with eRapa reversed these aging-associated phenotypes in the older mice (FIGS. 2A-2D). This is further exemplified in FIGS. 7-9. FIGS. 7A-D show that MaSCs frequency
increases during aging, and FIGS. 8A-D shows that aging increases early neoplasia frequency. As shown in FIG. 9, these phenotypes are decreased after eRapa treatment.

[00101] A sphere formation and differentiation assay was used to determine whether an increase in the number of MaSC-enriched basal cells in aged mammary glands may correlate with an increase in MaSC number (Dong et al., 2013). This assay was an in vitro alternative to the in vivo repopulation assay to characterize MaSCs and was based on mammosphere formation in suspension culture from sorted basal or luminal cells and sphere differentiation in 3-dimensional Matrigel culture. Spheres that differentiated into solid 3-dimensional structures (FIG. 4A) originated from MaSCs, and spheres that differentiated into hollow 3-dimensional structures (FIG. 4B) originated from luminal progenitor cells. The MaSC frequency increased 2.75-fold from young (0.08%) to old mice (0.22%), and the luminal progenitor frequency decreased 3.18-fold from young (3.5%) to old mice (1.1%) (FIGS. 2E, 2F). A similar observation was made in Balb/c mice (FIG. 4C).

[00102] The old control mice more frequently had luminal-to-basal cell ratio < 1, and old eRapa-treated mice more frequently had luminal-to-basal cell ratio > 1 (FIGS. 2A and 2D). When the inventors included only mice that had typical old phenotype (luminal-to-basal cell ratio < 1) from the control group and mice that had the typical eRapa-treated profile (luminal-to-basal cell ratio > 1) from the eRapa group, the inventors found a significant decrease in MaSC frequency and an increase in luminal progenitor cell frequency in old eRapa-treated mice, similar to young mice (FIGS. 2E, 2F).

[00103] The luminal progenitor cells also may be quantified by the number of luminal cells capable of forming discrete colonies (FIG. 4E) when plated on an irradiated NIH 3T3 cell feeder layer (Stingl, 2009). Using this assay, the inventors observed similar findings (FIG. 2G). These findings demonstrated that MaSC frequency increased and luminal progenitor frequency decreased with old age, and eRapa treatment (either long- or short-term) reversed the age-related phenotypic changes of MaSC and luminal progenitor cells.

[00104] The inventors evaluated self-renewal and tissue functionality of MaSCs with an in vivo serial transplant assay. After transplanting single 3-dimensional solid structures formed by old MaSCs per cleared fat pad, there was 80% positive outgrowth for the first generation (FIG. 3A), which was similar to previous findings in young animals (70%) (Dong et al., 2013). When the regenerated glands derived from old MaSCs were subjected to secondary transplant, the engraftment was only 50% (FIG. 3A). The MaSCs derived from old
long-term eRapa-treated mice had 100% engraftment in first and second transplants, confirming increased MaSC self-renewal caused by eRapa treatment (FIG. 3A). With short-term eRapa treatment, the inventors observed similar engraftment for the first transplants between old control and old eRapa-treated mice (FIG. 3A). Using in vitro serial passage of single 3-dimensional structures (Dong et al., 2013) as alternative to serial transplantation, the inventors also observed more limited passage capability of these 3-dimensional structures derived from old than young or old eRapa-treated mice (FIG. 5A).

[00105] The inventors mated a subset of MaSC-transplanted recipient mice to measure the ability of the regenerated gland to produce milk. MaSCs from old control and old eRapa-treated mice were transplanted into the cleared fat pads at the contralateral sides of the same recipient mice, and regenerated outgrowths were excised at different times during pregnancy. Immunohistochemistry showed fewer ducts that had positive staining for the milk protein casein and less staining intensity in the outgrowths regenerated from old control MaSCs than old eRapa-treated MaSCs (FIG. 3B); the latter were similar to those from young MaSC (data not shown). This result was observed for all regenerated outgrowths from first and second generation transplants (FIGS. 5B-E), confirming decreased milk production in old control MaSCs.

[00106] The presence of intraductal dysplastic lesions was evaluated with histology (FIG. 5F). Atypical ductal hyperplasia and ductal carcinoma in situ are precursors of carcinoma in rodents and humans and develop into palpable tumors when transplanted into hosts that have intact ovaries (Haslam and Bern, 1977). Mammary ducts of the old MaSC-regenerated glands had a high frequency (60%) of hyperplastic lesions, similar to the ducts of the old primary mammary glands (FIG. 3C), which was much higher than what the inventors normally observed in young C57BL/6 mice (20%). A similar observation was made in Balb/c mice (FIG. 5G). Except for the frequency of atypical ductal hyperplasia and ductal carcinoma in situ in the regenerated glands from the old long-term eRapa-treated group, eRapa significantly reduced the frequency of hyperplasia and atypical ductal hyperplasia/ductal carcinoma in situ in primary glands and MaSC-regenerated glands (FIG. 3C).

[00107] Whole transcriptome sequencing was performed with RNA samples from basal cell derived spheres (highly enriched MaSCs) and stromal cells (MaSC niche), from young and old mice, to understand the causes of increased frequency, decreased function, and increased neoplastic transformation in old MaSCs. The Cdkn2a locus, containing pl9ARF
and pl6INK4a, was most highly upregulated (19-fold) in the old stromal cells (adjusted
P = 0.05), which was confirmed with real time RT-PCR assays (FIG. 3D). Cyclooxygenase
2, which is not expressed under normal conditions in most cells but has elevated levels during
inflammation, was detected at higher frequency in mammary ducts from old control (37%)
than young (8%) or old eRapa-treated mice (17%) (FIG. 3E). Therefore, old stromal cells
had a senescence phenotype (Campisi, 2013; Rodier and Campisi, 2011).

B. Experimental Procedures

[00108] Animals. Animal care and use were conducted according to established
guidelines approved by the Institutional Animal Care and Use Committee of the University of
Texas Health Science Center, San Antonio. Balb/c mice were purchased from the National
Institute of Aging repository at Charles River and housed in the Nathan Shock Center clean
animal facility at the Barshop Institute for Longevity Research. Mice used for long-term
rapamycin treatment were B6.129S2(Cg)-Rbl+/+, and for short-term rapamycin treatment
were C57BL/6 (age, 25.5 months) purchased from the National Institute of Aging repository
at Charles River. Genotyping was done as described previously (Sharp et al, 2003). Other
mice were C57BL/6 mice originally obtained from Jackson Laboratory and raised in the
facility. Mammary glands at different ages were harvested for fluorescence-activated cell
sorting (FACS) analysis.

[00109] Rapamycin treatment. Mice were fed food that contained microencapsulated
or enterically-delivered rapamycin (eRapa). Food (14 mg/kg) (TestDiet, Inc., Richmond, IN;
base, Purina 5LG6, Nestle Purina, St. Louis, MO) delivered eRapa (2.24 mg/kg body
weight/day) to achieve rapamycin (4 ng/ml/kg of body weight/day) (Nadon et al, 2008).
Control diet was the same except the capsules were empty.

[00110] Hematoxylin-eosin staining. Preneoplastic transformation was indicated by
different amounts of ductal hyperplasia. This was assessed from microscopic examination
(hematoxylin-eosin stain) by a pathologist who specialized in breast cancer. Normal ductal
structures were characterized by an outside myoepithelial cell layer and an inside luminal
epithelial cell layer (FIG. 5B). Hyperplastic lesions that had only a few extra layers of
epithelium present were considered mild (FIG. 5B), and those that had dilated ducts that
were completely filled with uniform cells were considered severe and diagnosed as atypical
ductal hyperplasia (ADH). When cytologic atypia and necrosis were present, the diagnosis
was ductal carcinoma in situ (DCIS) (FIG. 5B).
Immunohistochemistry. Mammary glands were fixed (24 to 48 hours) in 10% neutral-buffered formalin, dehydrated in ethanol, and embedded in paraffin wax. Tissue sections (thickness, 4 µm) were treated to remove the paraffin and were rehydrated with graded ethanol solutions. Antigen retrieval was performed by heating in sodium citrate (10 mM; pH 6.0; 95°C) for 10 minutes and cooling at room temperature. Endogenous peroxidase was inhibited by incubating sections with 3% H2O2 for 15 minutes, and nonspecific binding was blocked with 10% normal serum for 30 minutes at room temperature. The sections were incubated with rabbit polyclonal anti-cyclooxygenase 2 (Cell Signaling Technology, Danvers, MA) (dilution, 1:50) or goat polyclonal antiphospho-p70 S6K (Thr 389) (Santa Cruz Biotechnology, Dallas, TX) (dilution, 1:25) overnight at 4°C. Sections were washed with phosphate-buffered saline with 0.025% Triton and incubated with biotin-conjugated secondary antibodies for 1 hour at room temperature. After washing, sections were incubated with streptavidin-horseradish peroxidase for 30 minutes and stained with diaminobenzidine for 15 minutes before dehydration and mounting.

Mammary cell preparation. Mammary cells were prepared as described previously (Bandyopadhyay et al., 2012). Dissected inguinal and thoracic mammary glands were digested in dissociation medium (1 part 10X gentle collagenase/hyaluronidase and 9 parts EpiCult-B medium [StemCell Technologies, Vancouver, Canada] supplemented with 5% fetal bovine serum) for 15 hours at 37°C in a 5% carbon dioxide incubator. The organoid pellet was treated sequentially in 0.64% ammonium chloride, 0.25% trypsin and ethylenediaminetetraacetic acid, and 5 mg/mL dispase with 0.1 mg/mL DNase I. The cell suspension was filtered through a 40micron mesh before being labeled with antibodies.

Antibodies. Antibodies included anti-CD24 labeled with phycoerythrin, anti-CD49f labeled with fluorescein isothiocyanate, anti-CD31/CD45/Ter1 19 mixture labeled with biotin (StemCell Technologies), and anti-CD 16/CD32 (BD Biosciences, Franklin Lakes, New Jersey). Allophycocyanin-conjugated streptavidin (Invitrogen, Carlsbad, CA) was used to visualize the antibody mixture that was labeled with biotin.

Cell labeling and flow cytometry. The MaSCs were enriched and isolated from endothelial (CD31) and hematopoietic (CD45 and TER1 19) lineage-depleted (Lin⁻) mammary epithelial cells using cell surface markers CD24 and CD49f (Stingl et al., 2006). Cells were incubated with anti-CD16/CD32 (Fey III/II receptor) for 10 minutes on ice to reduce Fc-receptor-mediated binding. Cells were incubated (15 minutes) on ice with the CD31/CD45/Ter1 19 antibody mixture. After washing, cells were incubated with anti-CD24
labeled with phycoerythrin, anti-CD49f labeled with fluorescein isothiocyanate, and streptavidin-allophycocyanin on ice for 10 minutes. After another wash, cells were sorted (FACSaria-IIIu, BD Biosciences) (FIG. 2A).

Sphere formation and differentiation assay. The sphere formation and differentiation (SFD) assay was performed as described previously (Dong et al, 2013). Sorted cells were cultured in ultralow attachment 96-well plates (Corning, Midland, MI) with mammosphere medium: mouse medium (EpiCult-B, StemCell Technologies) (150 µL per well) that was supplemented with 2% B27 (Invitrogen), 20 ng/mL bovine fibroblast growth factor, 20 ng/mL epidermal growth factor, 10 µg/mL heparin, 10 µg/mL insulin, 1 µg/mL hydrocortisone, and 50 µg/mL gentamicin. After suspension culture (7 days), mammospheres were counted and collected by centrifugation at 400 x g. A total of 30 to 50 individual spheres were resuspended in 60 µL gel (Matrigel, BD Biosciences) for sphere differentiation. The sphere-gel drop was allowed to solidify inside a 37°C incubator for 15 minutes, covered with mammosphere medium supplemented with 5% fetal bovine serum, and incubated at 37°C for 9 days. The solid and hollow three-dimensional structures were counted in ≥ 3 wells (approximately 120 spheres).

Two-Dimensional colony forming cell assay. For colony formation, 1000 sorted cells were plated into each well in 6-well plates that contained mammosphere medium supplemented with 5% fetal bovine serum and irradiated NIH 3T3 cells (104 cells/cm2). After 24 hours, the medium was replaced with serum-free mammosphere medium, and 8 days later the colonies were fixed with 100% cold methanol for 1 minute, stained with 10% Giemsa stain for 30 minutes, and counted. There were 3 replicated wells used for each sample to assess the number of colonies formed per 1000 cells.

Stem and progenitor cell quantification. The MaSC frequency was determined with the SFD assay (Dong et al., 2013). Mammospheres that were derived from MaSC-enriched basal cells and differentiated into solid structures in 3-dimensional gel culture (Matrigel) originated from a single MaSC, and MaSC frequency was determined by the number of SFD initiating cells from the basal cell fraction (% SFD-ICb). In contrast, mammospheres derived from luminal progenitor-enriched luminal cells formed hollow structures in 3-dimensional culture that were representative of luminal progenitor cells (Lim et al, 2009; Shackleton et al, 2006; Stingl et al, 2006), and luminal progenitor frequency was determined by the number of SFD initiating cells from the luminal cell fraction (% SFD-IC1). In addition, the inventors calculated luminal progenitor frequency based on the
frequency of colony forming cells (% colony forming cells) because 2-dimensional colony forming cells were used to determine luminal progenitor cells in other studies (Stingl, 2009). The equations were:

1. Sphere formation efficiency (SFE) = No. spheres per 1000 cells

2. \% SFD-ICb = (SFE/1000 \times \% 3-dimensional [solid]) \times (\% basal cells/\% total epithelial cells) \times 100

3. \% SFD-IC1 = (SFE/1000 \times \% 3-dimensional [hollow]) \times (\% luminal cells/\% total epithelial cells) \times 100

4. \% colony forming cells = 2-dimensional colony forming cells/1000 \times (\% luminal cells/\% total epithelial cells) \times 100.

[00118] The \% 3-dimensional [solid] or \% 3-dimensional [hollow] was defined as the percentage of spheres that formed a solid or hollow structure in gel culture (Matrigel) of the total number of spheres plated. The \% basal or \% luminal cells was the percentage of cells gated as Lin^− CD24_iCD49_f (basal) or Lin^− CD24_hiCD49_flo (luminal), and the \% total epithelial cells was the sum of \% cells gated as basal and luminal.

[00119] Cleared fat pad transplant and analysis. Stem cells in single 3-dimensional solid structures were resuspended in Hank Balanced Salt Solution (Invitrogen) with 0.2% trypan blue (Sigma, St. Louis, MO) and gel medium (50% Matrigel) (5 to 20 μL) and injected with a Hamilton syringe into the inguinal glands of 21-day old virgin female mice cleared of endogenous epithelium. Whole -mount staining was performed on fragments of the removed fat pads to ensure that the cleared fat pads were completely free of endogenous MaSCs; presence of a rudimentary epithelial structure indicated the removal of the endogenous gland. Primary mammary cells isolated from individually regenerated glands were used for secondary transplant.

[00120] Outgrowths, defined as epithelial structures with both ductal and lobular structures, were evaluated after 8 to 10 weeks by whole -mount staining. After excision, the unique ductal growth pattern of the transplants was checked. The regenerated glands from endogenous uncleared mammary ducts usually displayed unidirectional growth similar to primary glands. In contrast, regenerated glands from donor stem cells usually had bidirectional ductal growth (Medina, 1996).
mRNA sequencing: sample preparation and analysis. Stromal cells and mammospheres formed from MaSC-enriched basal cells of mice killed at age 4 months and 26 months were subjected to RNA sequencing analysis. Total RNA was isolated (miRNeasy Mini kit, catalog number 217004, Qiagen, Venlo, Limburg, Netherlands), sequencing libraries were constructed (Illumina TruSeq RNA sample preparation protocol, catalog number RS-122-2002, Illumina, San Diego, CA), and the libraries were sequenced (HiSeq 2000, Illumina) using a single-read 50 bp sequencing protocol. The reads were aligned to the reference mouse genome (University of California Santa Cruz, build mm9) with a spliced read mapper for RNA sequencing (TopHat) (Langmead et al, 2009). Only 2 mismatches were allowed in the alignment. Specialized software was used to count gene expression reads and quantify differentially expressed genes after performing median normalization (Anders, 2010; Anders and Huber, 2010). Differential expressed genes were identified with adjusted P = 0.05 for multiple tests by a method for controlling false discovery rate (Benjamini and Hochberg, 1995) and sequence read abundance per gene > 40% within each sample/library. Software was used to generate a heat map of gene expression levels (Institute for Statistics and Mathematics of Wirtschaftsuniversitat Wien, 2013). Differentially expressed genes were analyzed further for functional enrichment of gene ontology (National Institute of Allergy and Infectious Diseases, 2013).

Statistical analysis. Differences between various treatment groups were evaluated with t test and Barnard exact test. Results were presented as mean ± SD. Statistical significance was defined by P = 0.05.

EXAMPLE 2 - CANINE BREAST CANCER

Breast cancer in canines is similar in ideology and severity as in human breast cancer. However, breast cancer occurs in canines at a rate three times that of humans. Of the dogs that are treated for canine mammary cancer, 50% of the cases are not resolved. Moreover, 50% of those treated require follow-on or secondary treatments. Generally, three primary treatment options are available: chemotherapy, radiation therapy, and surgery. Any of these treatment options may be employed in any particular combination, based on the specific needs of the canine patient. Since most dogs are not tested for the presence of cancer in advance, it is contemplated that in some preferred embodiments, rapamycin would be administered, preferably in an oral form, as a long-term secondary preventative treatment.
for those dogs who have received an initial therapeutic regimen, in other words employing orally administered rapamycin as an adjunct maintenance therapy.

[00124] For instance, if surgery is chosen or deemed necessary as a primary treatment option, it is contemplated that rapamycin would be orally administered to the canine patient subsequent to the surgical resection procedure. It will be understood by those of skill in the art that primary treatments other than surgery, such as chemotherapy or radiation therapy, may also be employed within the framework of the presently described overall treatment regimen. In some preferred embodiments, eRapa can be administered following another primary therapeutic treatment, according to the teachings of the present disclosure, in order to prevent mobilization or relocation of the known cancer to some other organ or area of the patient's body.

[00125] In some preferred embodiments, there would likely be some period of time following the surgery before beginning administration of rapamycin. This time period could be a day, a week, two weeks, or any period of time within such range. It is known in the art that high doses of rapamycin may operate to slow or prevent wound healing subsequent to a surgical procedure. Therefore, delaying treatment with rapamycin following a primary treatment might be necessary to provide an opportunity for the tissue to heal after the surgical procedure. However, in some embodiments, such delay may be further minimized, or alternatively, there may not be a need for any delay at all between the surgery and the administration of rapamycin, particularly if the initial dosage is at such low levels as to preclude any potential wound healing complications. Since rapamycin has a half-life of approximately 24 hours, a dose scheduling regimen may be employed, whereby it is contemplated with some embodiments that a lower dose may begin shortly after a surgical procedure utilizing a comparatively lower dose on an initial dosing schedule of every other day. Thereafter, once any surgical wound has healed, the dosage may be increased as deemed necessary or preferable by the treating physician.

[00126] Rapamycin can be administered after a primary therapeutic treatment, such as surgery, chemotherapy, or radiation, using a low-dose regimen on a regular dosing schedule for as long as would be reasonable as deemed by the treating physician. One regular dosing schedule that is contemplated would be a Monday, Wednesday, Friday treatment schedule. Other dosing schedules could be implemented and still maintain the efficacy of the rapamycin treatment. Such dosing schedule would likely occur for at least two weeks and may be continued for up to six months in duration, or any time period within this range. As
indicated above, the initial dose of rapamycin may need to be low to allow healing of the affected tissue subsequent to surgery; however, the dosage level may be increased if circumstances warrant such an increase after the affected tissue has healed. Post-treatment administration of rapamycin should continue at least until the luminal-to-basal ratio is determined to be greater than 1, which as described above, is a threshold value that is a phenotype consistent with young cells which also tends to indicate a a decreased risk or likelihood of cancer.

[00127] Although, as indicated above, in many instances there is no advance testing for cancer in canines, it is contemplated that rapamycin may be administered following a diagnostic procedure which indicates the presence of cancer in a canine patient or other animal patient. Biopsy of suspected tissue can be performed to confirm whether cancer is present or not. One particular procedure which may be employed in the diagnosis of breast cancer in canines or other animals is needle localization biopsy, whereby a small tissue specimen is removed for identification and diagnostic purposes. In conjunction with an assay, one such assay being flow cytometry, it can be determined whether a patient has cancer. More specifically, the luminal and basal cells are sorted through flow cytometry in order to determine the ratio of luminal cells to basal cells. As described above, a luminal-to-basal cell ratio that is $<1$ indicates a greater risk for development of breast cancer. Conversely, when the luminal-to-basal cell ratio is $>1$, the inventors have found a significant decrease in MaSC frequency and an increase in luminal progenitor cell frequency, such conditions being indicative of a lower risk of breast cancer development. As will be understood by those of skill in the art, other diagnostic techniques are contemplated for use with other embodiments which will also be effective in determining the luminal-to-basal cell ratio in a specimen removed from a patient.

[00128] For particular subjects where cancer is found to be present, it is anticipated that some embodiments will involve administering a preferred rapamycin prior to beginning another treatment regimen for the cancer. As one example, nanoparticle formulations of eRapa may be administered at an effective dosage as a pretreatment, prior to initiation of radiation therapy or chemotherapy for treatment of a cancerous tumor. Pretreatment with such formulations in this regime would preferably occur on a daily or periodic schedule for a duration continuing over a period of from one to six weeks prior to initiation of the primary therapy - whether in that primary therapy is either therapeutic radiation therapy or chemotherapy. The pretreatment will preferably be undertaken for from two to four weeks,
although other durations may also be used as alternatives, particularly if a basis is validated for varying the duration outside of that range. Such pretreatment administration of rapamycin, in part, can serve as a sensitizer to enhance the beneficial effects that the anticipated radiation or chemotherapy will have on the cancer cells. Thus, the present invention contemplates that pretreatment administration of rapamycin may result in a more effective treatment with radiation, chemotherapy or the like.

[00129] It will be understood by those of skill in the art that although the description herein relates most specifically to embodiments used for treatment of breast cancer in canines, the treatments described may also be implemented with respect to other animals including humans. It is also contemplated that in other embodiments, eRapa may be administered as a primary therapeutic treatment option. As one non-limiting example, it is known that people who have a harmful mutation in the BRCA1 or BRCA2 gene have a greater lifetime risk of developing breast cancer among other types of cancer. Genetic testing is commercially available which can identify such harmful mutations in the BRCA1 or BRCA2 gene. It is contemplated as being within the scope of the present invention and the embodiments described herein that if a person is identified as a carrier of a harmful BRCA1 or BRCA2 gene mutation, eRapa may be used as a primary therapeutic treatment option.
REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U.S. Patent Application Serial No. 14/717,844
U.S. Patent Publication No. 2012/0064143
Sharp & Richardson, Target Onco 16, 41-51, 2011.
What is claimed is:

1. A method of preventing or inhibiting the development of breast cancer, early neoplastic lesions, or hyperplastic lesions in mammary glands of a patient comprising administering an effective amount of a composition comprising rapamycin or an analog thereof to a patient who has been identified as having or being at risk for developing breast cancer.

2. A method of reducing the number of basal mammary stem-like cells in the mammary glands of a patient comprising administering an effective amount of a composition comprising rapamycin or an analog thereof to the patient.

3. The method of claim 1, wherein the rapamycin or analog thereof is encased in a coating that comprises a cellulose acetate succinate or hydroxypropyl methylcellulose phthalate copolymer, or a polymethacrylate-based copolymer to include: methylacrylate-methacrylic acid copolymer, or a methyl methacrylate-methacrylic acid copolymer.

4. The method of claim 1, wherein the coating comprises Poly(methacrylic acid-co-ethyl acrylate) in a 1:1 ratio, Poly(methacrylic acid-co-methyl methacrylate) in a 1:1 ratio, Poly(methacrylic acid-co-methyl methacrylate) in a 1:2 ratio, Poly(methyl acrylate-co-methyl methacrylate-co-methacrylic acid) in a 7:3:1 ratio, Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) in a 1:2:0.2 ratio, Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) in a 1:2:0.1 ratio, or Poly(butyl methacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate) in a 1:2:1 ratio, a naturally-derived polymer, or a synthetic polymer, or any combination thereof.

5. The method of claim 4, wherein the naturally derived polymer is selected from the group consisting of alginates and their various derivatives, chitosans and their various derivatives, carrageenans and their various analogues, celluloses, gums, gelatins, pectins, and gellans.

6. The method of claim 4, wherein the naturally derived polymer is selected from the group consisting of polyethyleneglycols (PEGs) and polyethyleneoxides (PEOs), acrylic acid homo and copolymers with acrylates and methacrylates, homopolymers of acrylates and methacrylates, polyvinyl alcohol (PVOH), and polyvinyl pyrrolidone (PVP).
7. The method of claim 2, wherein the basal mammary stem-like cells are CD31-CD45-TER1 19-cells or Lin-CD24LOCD49fl cells.

8. The method of claim 2, wherein the patient is one that has been determined to be at risk for breast cancer.

9. The method of claim 2, wherein the number of basal mammary stem-like cells is reduced such that the ratio of luminal progenitor cells to basal mammary stem and stem-like cells is significantly increased.

10. The method of claim 9, wherein the luminal progenitor cells with CD31-CD45-TER1 19-cells or Lin-CD24LOCD49fl marks are normalized.

11. The method of claim 10, wherein the basal mammary stem and/or stem-like cells with CD31-CD45-TER1 19-cells or Lin-CD24LOCD49fl marks are normalized.

12. The method of claim 1 or 2, wherein the patient is at least 50 years old.

13. The method of claim 1 or 2, wherein the patient has been diagnosed with a breast cancer.

14. The method of claim 13, wherein the patient has been diagnosed with atypical ductal carcinoma and/or ductal carcinoma in situ.

15. The method of claim 13, wherein the patient has undergone surgical resection of the breast cancer prior to administration of the composition comprising rapamycin or an analog thereof.

16. The method of claim 15, wherein the composition is administered at least one week after the surgical resection.

17. The method of claim 1 or 2, wherein the patient has a family history of breast cancer.

18. The method of claim 1 or 2, wherein the patient has been diagnosed as carrying a mutation in the BRCA1/BRCA2 genes.

19. The method of claim 1 or 2, wherein the composition comprises rapamycin or an analog thereof at a concentration of 0.001 mg to 30 mg total per dose.
20. The method of claim 1 or 2, wherein the composition comprising rapamycin or an analog of rapamycin comprises 0.001% to 60% by weight of rapamycin or an analog of rapamycin.

21. The method of claim 1 or 2, wherein the composition is administered orally or enterically.

22. The method of claim 1 or 2, wherein the rapamycin or analog of rapamycin is administered in two or more doses.

23. The method of claim 22, wherein the interval of time between administration of doses comprising rapamycin or an analog of rapamycin is 0.5 to 30 days.

24. The method of claim 1 or 2, wherein the subject is further administered a composition comprising a second active agent that is a chemopreventive or chemotherapeutic agent, radiotherapy, other systemic agent, or surgery.

25. The method of claim 24, wherein the composition comprising rapamycin or an analog of rapamycin is administered at the same time as the composition comprising the second active agent.

26. The method of claim 24, wherein the composition comprising rapamycin or an analog of rapamycin is administered before or after the composition comprising the second active agent is administered.

27. The method of claim 26, wherein the interval of time between administration of the composition comprising rapamycin or an analog of rapamycin and the composition comprising the second active agent is 1 to 30 days.

28. The method of any of claim 1 or 2, wherein the composition comprising rapamycin or an analog of rapamycin is comprised in a food or food additive.

29. The method of claim 1 or 2, wherein the composition comprising rapamycin or an analog thereof is administered daily for a period of at least one year.

30. The method of claim 1 or 2, wherein the patient is a human, feline, or canine.
31. The method of claim 1 or 2, wherein the patient is one that has been determined to have a significantly reduced ratio of luminal progenitor cells to basal mammary stem and stem-like cells compared to their normal counterparts.
FIG. 2A
FIG. 2B

FIG. 2C

SUBSTITUTE SHEET (RULE 26)
FIG. 2D

FIG. 2E

SUBSTITUTE SHEET (RULE 26)
**FIG. 2F**

- **LP frequency (% SFD-IC)** vs. **Age**
- **Old control** vs. **Old eRapa**
- **P = 0.0077**
- **Long-term** vs. **Short-term**
- **P = 0.0318**
- **P = 0.0120**

**FIG. 2G**

- **LP frequency (% CFC)** vs. **Age**
- **Old control** vs. **Old eRapa**
- **P = 0.0022**
- **P = 0.0035**
- **P = 0.0111**

SUBSTITUTE SHEET (RULE 26)
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Positive / total cleared fat pads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Old control</td>
</tr>
<tr>
<td>Long-term 1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>12/15 (80%)</td>
</tr>
<tr>
<td>Long-term 2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Short-term 1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>11/15 (73%)</td>
</tr>
</tbody>
</table>

**FIG. 3A**

![Control (4x) and (40x)](image)

![eRapa (4x) and (40x)](image)

**FIG. 3B**

SUBSTITUTE SHEET (RULE 26)
FIG. 3C

SUBSTITUTE SHEET (RULE 26)
FIG. 3D

Relative expression level

$P = 0.0162$

$P = 0.0063$

$p19^{ARF}$ vs $p16^{INK4a}$

FIG. 3E

Young

Old control

Old eRapa

Cox2

SUBSTITUTE SHEET (RULE 26)
### 2 x 2 contingency tables for Barnard exact test
(Luminal-to-basal ratio, L/B)

<table>
<thead>
<tr>
<th></th>
<th>Long-term</th>
<th></th>
<th>Short-term</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>eRapa</td>
<td>Control</td>
<td>eRapa</td>
</tr>
<tr>
<td>L/B &lt; 1</td>
<td>11</td>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>L/B &gt; 1</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>P-value</td>
<td>$P = 0.041$</td>
<td></td>
<td>$P = 0.038$</td>
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</tbody>
</table>

**FIG. 4D**

![Image of a cell culture](image)

**FIG. 4E**
No growth

No. of initial solid 3Ds capable of continuing forming 3Ds in each passage

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old control</th>
<th>Old eRapa</th>
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</thead>
<tbody>
<tr>
<td>1st</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>2nd</td>
<td>7</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>3rd</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

N = 15

5000 cells

5000 cells

All cells

Single 3D

FIG. 5A

FIG. 5B

Dark stain

Light stain

Partial stain

No stain

β-casein
FIG. 51

p-S6K

Young
- 5.6%
- 30.2%
- 64.2%

Old control
- 25.8%
- 31.9%
- 42.2%

Old eRapa
- 25.9%
- 29.0%
- 45.1%
FIG. 7
FIG. 9
(Cont'd)
**INTERNATIONAL SEARCH REPORT**

**International application No.**
PCT/US20 15/053725

**A. CLASSIFICATION OF SUBJECT MATTER**
- **IPC(8)**: A61K 31/4353 (2015.01)
- **CPC**: A61K 31/4353 (2015.12)

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)
- **IPC(8)**: A61K 31/00, 31/33, 31/395, 31/435, 31/4353 (2015.01)
- **CPC**: A61K 31/00, 31/33, 31/395, 31/435, 31/4353 (2015.12)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
- **US Classes**: 514/1, 183, 277, 279, 290
- **CPC**: A61K 31/00, 31/33, 31/395, 31/435, 31/4353 (Keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
- Orbit, Google Patents, PubMed, Google

**Search terms used**: breast cancer rapamycin sirolimus polymer coating stem-like cells cd31-cd45-ter1

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>X</td>
<td>WO 2009/108361 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 03 September 2009 (03.09.2009) entire document</td>
<td>1, 4-6, 12-30</td>
</tr>
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<td>Y</td>
<td>PETERSEN et al. &quot;Stem Cells in the Human Breast,&quot; Cold Spring Harbor Perspectives in Biology, 01 May 2010 (01.05.2010), Vol. 2, Iss. 5, Pgs. 1-15. entire document</td>
<td>2, 7-1 1</td>
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</table>

- 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: 10 December 2015
Date of mailing of the international search report: 19 JAN 2016

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Form PCT/ISA/210 (second sheet) (January 2015)