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(54) **Title**: PROCESS AND COMPOSITIONS FOR ACHIEVING MAMMALIAN ENERGY BALANCE

(57) **Abstract**: Disclosed is a method of achieving optimal mammalian energy balance using forskolin on a particular physiological and developmental stage of the mammalian cellular system.

# PROCESS AND COMPOSITIONS FOR ACHIEVING MAMMALIAN ENERGY BALANCE

## CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

This application is the PCT filing drawing priority from U.S. non-provisional patent application 14936830 filed on November 10, 2015.

## BACKGROUND OF THE INVENTION

[Para 001] Field of the invention: The invention in general relates to dietary supplements. More specifically, the present invention relates to a method of achieving optimal mammalian energy balance using forskolin on a particular physiological and developmental stage of the mammalian cellular system.

[Para 002] Description of Prior Art: Disruption of mammalian energy balance has been implicated as the cause for worldwide epidemics of metabolic diseases that calls for modifications in life style and food habits and also therapeutic intervention. Current diet regimens, exercise, health care awareness or drug strategies however are often unable to tackle homeostasis of energy in the mammalian body where optimally, a perfect balance between energy accumulation and energy expenditure is sought (Elattar.S and Satyanarayana, "Can Brown Fat Win the Battle against White Fat?", J Cell Physiol. 2015 Mar 11, Zafrir B, "Brown adipose tissue: research milestones of a potential player in human energy balance and obesity", Horm Metab Res. 2013 Oct;45(11):774-85). An impetus to the understanding of critical biological processes controlling brown adipocyte activity and differentiation has been in vogue in view of developing brown adipose tissue (BAT) focussed therapies for energy homeostasis (Giralt M, "White, brown, beige/brite: different adipose cells for different functions? Endocrinology. 2013 Sep; 154(9):2992-3000) where undue energy abundance is effectively countered by optimal energy expenditure. The present invention discusses the potential of forskolin to mediate mammalian energy balance. Accordingly, it is the principle objective of the present invention to disclose,

A. The ability of forskolin to prevent the formation of lipids within adult adipocytes during the differentiation of pre-adipocytes to adipocytes wherein the adipogenesis (fat deposition) inhibition is remarkably enhanced when forskolin is administered (brought into contact) to pre-adipocytes rather than to mature adipocytes;

B. The ability of forskolin to enhance the expression of secreted factors that selectively recruit brown adipose tissue (BAT) like bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF-A) and mitochondrial uncoupling protein (UCP1) wherein said enhanced expression of secreted factors that selectively recruit brown adipose tissue (BAT) is remarkably more enhanced when forskolin is administered (brought into contact) to pre-adipocytes than to mature adipocytes. In other words, forskolin treated pre-adipocytes are selectively able to differentiate into BAT.

[Para 003] The present invention fulfils the aforesaid objectives and provides further related advantages.

## SUMMARY OF THE INVENTION

[Para 004] The present invention discloses,

(A) The ability of forskolin to prevent the formation of lipids within adult adipocytes during the the differentiation of pre-adipocytes to adipocytes wherein the adipogenesis (fat deposition) inhibition is remarkably more enhanced when forskolin is administered (brought into contact) to pre-adipocytes than to mature adipocytes;

(B) The ability of forskolin to enhance the expression of secreted factors that selectively recruit brown adipose tissue (BAT) like bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1) wherein said enhanced expression of secreted factors that selectively recruit brown adipose tissue (BAT) is remarkably enhanced when forskolin is administered (brought into contact) to pre-adipocytes than to mature

adipocytes. In other words, forskolin treated pre-adipocytes are selectively able to differentiate into BAT.

[Para 005] The advantages of the present invention includes the demonstration of a method to achieve mammalian energy balance using forskolin on a particular physiological and developmental stage of the mammalian cellular system wherein forskolin evinces increased potential to (i) inhibit adipogenesis; and (ii) enhance the expression of secreted factors that selectively recruit brown adipose tissue (BAT) like bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1) when brought into contact or administered to pre-adipocytes rather than to mature adipocytes.

[Para 006] Other features and advantages of the present invention will become apparent from the following more detailed description, taken in conjunction with the accompanying images, which illustrate, by way of example, the principle of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

[Para 007] **Fig.1** shows the graphical representation of BMP-7 in cell culture supernatant of cultured 3T3-L1 adipocytes when forskolin (50µg/ml and 100µg/ml) are respectively added before the differentiation of pre-adipocytes to adipocytes and after the differentiation of pre-adipocytes to adipocytes.

[Para 008] **Fig.2** shows the graphical representation of BMP-4 in cell culture supernatant of cultured 3T3-L1 adipocytes when forskolin (50µg/ml and 100µg/ml) are respectively added before the differentiation of pre-adipocytes to adipocytes and after the differentiation of pre-adipocytes to adipocytes.

[Para 009] **Fig.3** shows the graphical representation of VEGF-A in cell culture supernatant of cultured 3T3-L1 adipocytes when forskolin (50µg/ml and 100µg/ml) are respectively added before the differentiation of pre-adipocytes to adipocytes and after the differentiation of pre-adipocytes to adipocytes.

[Para 0010] **Fig.4** shows the graphical representation of UCP1 in cell culture supernatant of cultured 3T3-L1 adipocytes when forskolin (50µg/ml and 100µg/ml) are respectively added before the differentiation of pre-adipocytes to adipocytes and after the differentiation of pre-adipocytes to adipocytes.

## DETAILED DESCRIPTION OF THE MOST PREFERRED EMBODIMENTS

### (Figs. 1, 2, 3 and 4)

[Para 0011] In the most preferred embodiment, the present invention relates to a method of achieving mammalian energy balance using forskolin in a process of adipogenesis inhibition wherein forskolin is added seperately to pre-adipocytes before differentiation and also to mature adipocytes to comparatively evaluate adipogenesis inhibition potential of, said process comprising steps of:

a) Seeding mammalian adipocyte precursor cells (pre-adipocytes) in wells of microplates wherein approximately $60\times10^4$ cells are seeded for 48-72 hours to get 70-80% confluence;
b) Adding forskolin at concentrations of 50µg/ml and 100µg/ml in the pre-seeded microplates of step a consisting of undifferentiated pre-adipocytes;
c) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells;
d) Adding 200 µl of freshly prepared Adipogenesis progression medium after 72 hours of incubation with the Adipogenesis induction medium in step c;
e) Incubating the cells treated with forskolin (step b), adipogenesis induction medium (step c) and adipogenesis progression medium (step d) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air;
f) Fixing the cells of step e by adding 100 µl of 10% formalin and staining using the Oil Red O technique;
g) Reading the optical density of cells of step f at 492 nm in a microplate reader and expressing the results as inhibitory concentration (IC50) values using the graph pad prism software;
h) Calculating the percentage inhibition of adipogenesis in the cells of steps f and g using the formula, C-T/T×100, wherein C is the absorbance of Oil Red O in

differentiating/undifferentiated cells and T is the absorbance of Oil Red O in sample treated differentiating/undifferentiated cells.

i) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells of step a.

j) Adding 200 µl of freshly prepared Adipogenesis progression medium comprising graded concentrations of forskolin(50µg/ml and 100µg/ml respectively) to the wells of step i after 72 hours of incubation with the Adipogenesis induction medium;

k) Incubating the cells treated with forskolin (step j), adipogenesis induction medium (step i) and adipogenesis progression medium (step j) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air;

l) Fixing the cells of step l by adding 100 µl of 10% formalin and staining using the Oil Red O technique;

m) Reading the optical density of cells of step m at 492 nm in a microplate reader and expressing the results as inhibitory concentration (IC50) values using the graph pad prism software;

n) Calculating the percentage inhibition of adipogenesis in the cells of steps m and n using the formula, C-T/T×100, wherein C is the absorbance of Oil Red O in differentiating/undifferentiated cells and T is the absorbance of Oil Red O in sample treated differentiating/undifferentiated cells; and

o) Comparing percentage inhibition of adipogenesis in the cells of steps h and o.

[Para 0012] In another most preferred embodiment, the present invention also relates to a method of mammalian energy balance using forskolin in a process of promoting the expression of secreted factors that selectively recruit brown adipose tissue (BAT) like bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1) wherein said expression of secreted factors that selectively recruit brown adipose tissue (BAT) is remarkably enhanced as measured when forskolin is administered (brought into contact) to pre-adipocytes than to mature adipocytes, said method incorporating the steps of ,

a) Seeding mammalian adipocyte precursor cells (pre-adipocytes) in wells of microplates wherein approximately $60\times10^4$ cells are seeded for 48-72 hours to get 70-80% confluence;

b) Adding forskolin at concentrations of 50µg/ml and 100µg/ml in the pre-seeded microplates of step a consisting of undifferentiated pre-adipocytes;

c) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells;

d) Adding 200 µl of freshly prepared Adipogenesis progression medium after 72 hours of incubation with the Adipogenesis induction medium in step c;

e) Incubating the cells treated with forskolin (step b), adipogenesis induction medium (step c) and adipogenesis progression medium (step d) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air;

f) Quantitatively determining by appropriate immunoassay techniques the expressions of BMP-7, BMP-4, VEGF-A and UCP-1 in the cell supernatant;

g) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells of step a;

h) Adding 200 µl of freshly prepared Adipogenesis progression medium comprising graded concentrations of forskolin(50µg/ml and 100µg/ml respectively) to the wells of step g after 72 hours of incubation with the Adipogenesis induction medium;

i) Incubating the cells treated with forskolin (step h), adipogenesis induction medium (step g) and adipogenesis progression medium (step h) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air; and

j) Quantitatively determining by appropriate immunoassay techniques the expressions of BMP-7, BMP-4, VEGF-A and UCP-1 in the cell supernatant.

[Para 0013] In yet another most preferred embodiment, the present invention relates to a method of achieving energy balance in mammalian adipose cellular systems, said method comprising step of administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to achieve effects of (a) increased inhibition of adipogenesis and (b) increased expression of secretory factors that function individually or in combination to specifically recruit brown adipocytes or brown like (beige or brite) adipocytes. In specific embodiments, the secretory factors are selected from the group

consisting of bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1).

[Para 0014] In yet another most preferred embodiment, the invention pertains to forskolin for use in therapy for obesity wherein said therapy involves achieving energy balance in mammalian adipocytes by administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to bring about the effects of (a) increased inhibition of adipogenesis and (b) increased expression of secretory factors that function individually or in combination to specifically recruit brown adipocytes or brown like (beige or brite) adipocytes. In specific embodiments, the secretory factors are selected from the group consisting of bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1)

[Para 0015] In yet another most preferred embodiment, the present invention relates to a method to induce the brown like phenotype (beige or brite adipocytes) in white adipocyte depots in mammals said method comprising step of administering effective amount of forskolin to obese mammals with depots of fully differentiated white adipocytes to achieve effect of increase in secretory factors that bring about the development of brown like phenotype (beige or brown adipocytes) within white adipocyte depots. In specific embodiment, the secretory factors are vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1). In yet another most preferred embodiment, the present invention relates to Forskolin for use in the therapy of obesity characterised in that forskolin is administered in effective amounts targeting mammalian white adipocyte depots to achieve effect of increased expression of secretory factors vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1) that cause the development of brown like phenotype (beige or brite adipocytes) in white adipocyte depots in mammals.

[Para 0016] In yet another most preferred embodiment, the present invention relates to a method of achieving energy balance in mammalian adipose cellular systems, said method comprising step of administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to bring about of the effect of enhanced expression of mitochondria uncoupling protein 1 (UCP-1) to result in

increased mitochondrial thermiogenesis in differentiated brown adipocytes and brown like (beige or brite) adipocytes.

[Para 0017] In an alternative embodiment, the present invention also relates to forskolin for use in therapy for obesity wherein said therapy involves achieving energy balance in mammalian adipocytes by administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to bring about the effects of enhanced expression of secretory factor mitochondria uncoupling protein 1 (UCP-1) to result in increased mitochondrial thermiogenesis in differentiated brown adipocytes and brown like (beige or brite) adipocytes.

## ILLUSTRATIVE EXAMPLES

[Para 0018] As illustrative examples of the most preferred embodiments outlined herein above in paragraphs [0011]-[0017], the following results are presented to show that forskolin when administered in increasing concentration is more effective in (a) preventing adipogenesis and (b) also in promoting the expression of secreted factors like BMP-7, BMP-4, VEGF-A and UCP-1 that recruit the brown adipocytes thereby creating energy balance in mammalian cell systems, when administered at the pre-adipocyte stage than once the transformation of pre-adipocytes to adipocytes has occurred.

**RESULT 1-Prevention of adipogenesis**

**Table A**

| CONCENTRATION (µg/ml) | % inhibition of adipogenesis when forskolin is added at the pre-adipocyte stage (before differentiation into the adipocyte stage) | % inhibition of adipogenesis when forskolin is added after differentiation of pre-adipocytes to the adipocyte stage |
|---|---|---|
| 6.25 | 10.2 | 1.2 |
| 12.50 | 12.8 | 6.8 |
| 25 | 19.7 | 10.6 |
| 50 | 35.5 | 12.9 |
| 100 | 41.8 | 18.5 |

[Para 0019] Table A shows that at each tested concentration of forskolin, the administration of forskolin at the mammalian pre-adipocyte stage has a profound effect on preventing adipogenesis that when administered after the differentiation of pre-adipocytes to adipocytes. Double or more than

double the % inhibition of adipogenesis was observed when forskolin was administered at the pre-adipocyte stage as compared to administration at the adipocyte stage.

**RESULT 2-Expression of secretory proteins that recruit brown adipocytes**

**A. BMP-7**

[Para 0020] The biological role of BMP-7 as a recruiter of the brown adipocyte lineage has been discussed in the following scientific literature.

1. Mathew Harms and Patrick Seale, "Brown and beige fat: development, function and therapeutic potential", Nature Medicine, Volume 19, Number 10, October 2013, pages 1252-1263;
2. BMP7 Activates Brown Adipose Tissue and Reduces Diet-Induced Obesity at Sub thermoneutrality .Mariëtte R. Boon Published: September 16, 2013; PLOS One.
3. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Tseng et al. Nature. 2008 Aug 21; 454(7207):1000-4. doi: 10.1038/nature07221.
4. Transcriptional Control of Brown Fat Development; Kajimure et al. Cell Metabolism; Volume 11, Issue 4, 7 April 2010, Pages 257–262.

[Para 0021] Immunoassays (Enzyme linked immunosorbent assay) for the quantification of BMP-7 in the cell culture supernatant when forskolin (50µg/ml and 100 µg/ml) was administered at the pre-adipocyte stage and once the differentiation to adipocytes occurred indicated that forskolin profoundly increased BMP-7 expression in at the pre-adipocyte stage than at the adipocyte stage.

[Para 0022] Thus, in correlation with the literature cited above, it may be deduced that forskolin evinces greater potential for brown fat conversion of pre-adipocytes (**Fig.1**) rather than fully differentiated white adipocytes.The example exemplified by **Fig.1** provides substantiation to the disclosed most preferred embodiment that forskolin directs the selective differentiation of mammalian pre-adipocytes to brown adipocytes by allowing the expression of secretory factor BMP-7.

**B. BMP-4**

[Para 0023] Acting along with BMP-7, BMP-4 is a new adipokine and acts on adipogenesis and white to brown transition (Qian S W et al Proc Natl Acad Sci USA 110: E798-807, 2013). Immunoassays (Enzyme linked immunosorbent assay) for the quantification of BMP-4 in the cell culture supernatant when forskolin (50µg/ml and 100µg/ml) was administered at the pre-adipocyte stage and once the differentiation to adipocytes occurred indicated that forskolin profoundly increased BMP-4 expression in at the pre-adipocyte stage than at the adipocyte stage.

[Para 0024] Thus, in correlation with the literature cited above, it may be deduced that forskolin evinces greater potential for conversion of white pre-adipocytes to the brite/beige adipocyte (brown adipocyte like) (**Fig.2**) by the combined increased expressions and biological actions of secretory factors BMP-4 and BMP-7. The example exemplified by Fig.2 provides substantiation to the most preferred embodiment that forskolin brings about the transformation of white pre-adipocytes to brite or beige adipocytes.

**C. VEGF-A**

[Para 0025] VEGF-A over expression leads to an increase in brown adipose tissue (BAT) thermogenesis and also promotes a "BAT-like" phenotype in white adipose tissue depots. In diet-induced obese mice, introducing VEGF-A locally in BAT rescues capillary rarefaction, ameliorates brown adipocyte dysfunction, and improves deleterious effects on glucose and lipid metabolism caused by a high-fat diet challenge. These results demonstrate a direct positive role of VEGF-A in the activation and expansion of BAT. VEGF-A over expression also exerts its action on macrophages by increasing the recruitment of M2 anti-inflammatory macrophages to fat depots. The decreased obesity and the anti-inflammatory milieu induced by VEGF-A in adipose tissue is responsible for the reduction of insulin resistance in transgenic mice (Bagchi et al, "Vascular endothelial growth factor is important for brown adipose tissue development and maintenance", *FASEB J. 27, 3257-3271 (2013).* Immunoassays (Enzyme linked immunosorbent assay) for the quantification of VEGF-A in the cell culture supernatant when forskolin (50µg/ml and 100µg/ml) was administered at the pre-adipocyte

stage and once the differentiation to adipocytes occurred indicated that forskolin profoundly increased VEGF-A expression in the pre-adipocyte stage than at the adipocyte stage. Thus, in correlation with the literature cited above, it may be deduced that forskolin evinces greater potential for conversion of white pre-adipocytes to the brown adipocyte like (brite or beige) cells **(Fig. 3)** among white adipocyte depots in the mammalian body.

**D. Uncoupling Protein-1(UCP-1)**

[Para 0026] A system of thermogenesis that evolved to protect the body from hypothermia is based upon the uncoupling of oxidative phosphorylation in brown adipocytes by the mitochondrial uncoupling protein (UCP-1). It has been shown that up-regulation of UCP1 by genetic manipulations or pharmacological agents can reduce obesity and improve insulin sensitivity (International Journal of Obesity (2008) 32, S32–S38; doi:10.1038/ijo.2008.236 UCP1: its involvement and utility in obesity. L P Kozak and R Anunciado-Koza). Immunoassays (Enzyme linked immunosorbent assay) for the quantification of UCP-1 in the cell culture supernatant when forskolin (50µg/ml and 100µg/ml) was administered at the pre-adipocyte stage and once the differentiation to adipocytes occurred indicated that forskolin profoundly increased UCP-1 expression in the pre-adipocyte stage than at the adipocyte stage. Thus, in correlation with the literature cited above, it may be deduced that forskolin evinces greater potential for conversion of pre-adipocytes to the BAT-like or brown adipocytes and enhanced UCP-1 expression in these cells can be expected to enhance bringing about energy balance through appropriate energy expenditure **(Fig. 4)**.

[Para 0027] It is already reported that administration of forskolin in humans apparently does not cause clinically significant side effects (Shonteh Henderson et al, Effect of Coleus forskolii supplementation on body composition and haematological profiles in mildly overweight women, J Int Soc Sports Nutr. 2005; 2(2): 54–62). The study elucidates that supplementation with forskolin dietary supplement Forslean® [250 mg of 10% Coleus forskolii extract, 25 mg of forskolin] two times a day for 12 weeks apparently had no clinical side effects. It may thus be inferred that the illustrative in-vitro examples

included herein above to achieve energy balance in mammalian adipocyte systems is also applicable in vivo studies in animals (mammals) including human beings.

[Para 0028] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

We claim,

1. A method of achieving mammalian energy balance using forskolin in a process of adipogenesis inhibition wherein forskolin is added separately to pre-adipocytes before differentiation and also to mature adipocytes to comparatively evaluate adipogenesis inhibition potential, said process comprising steps of:

a) Seeding mammalian adipocyte precursor cells (pre-adipocytes) in wells of microplates wherein approximately $60\times10^4$ cells are seeded for 48-72 hours to get 70-80% confluence;

b) Adding forskolin at concentrations of 50µg/ml and 100µg/ml in the pre-seeded microplates of step a consisting of undifferentiated pre-adipocytes;

c) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells;

d) Adding 200 µl of freshly prepared Adipogenesis progression medium after 72 hours of incubation with the Adipogenesis induction medium in step c;

e) Incubating the cells treated with forskolin (step b), adipogenesis induction medium (step c) and adipogenesis progression medium (step d) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air;

f) Fixing the cells of step e by adding 100 µl of 10% formalin and staining using the Oil Red O technique;

g) Reading the optical density of cells of step f at 492 nm in a microplate reader and expressing the results as inhibitory concentration (IC50) values using the graph pad prism software;

h) Calculating the percentage inhibition of adipogenesis in the cells of steps f and g using the formula, C-T/T×100, wherein C is the absorbance of Oil Red O in differentiating/undifferentiated cells and T is the absorbance of Oil Red O in sample treated differentiating/undifferentiated cells.

i) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells of step a.

j) Adding 200 µl of freshly prepared Adipogenesis progression medium comprising graded concentrations of forskolin(50µg/ml and 100µg/ml respectively) to the wells of step i after 72 hours of incubation with the Adipogenesis induction medium;

k) Incubating the cells treated with forskolin (step j), adipogenesis induction medium (step i) and adipogenesis progression medium (step j) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air;

l) Fixing the cells of step l by adding 100 µl of 10% formalin and staining using the Oil Red O technique;

m) Reading the optical density of cells of step m at 492 nm in a microplate reader and expressing the results as inhibitory concentration (IC50) values using the graph pad prism software;

n) Calculating the percentage inhibition of adipogenesis in the cells of steps m and n using the formula, C-T/T×100, wherein C is the absorbance of Oil Red O in differentiating/undifferentiated cells and T is the absorbance of Oil Red O in sample treated differentiating/undifferentiated cells; and

o) Comparing percentage inhibition of adipogenesis in the cells of steps h and o.

2. A method of mammalian energy balance using forskolin in a process of promoting the expression of secreted factors that selectively recruit brown adipose tissue (BAT) like bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1) wherein said expression of secreted factors that selectively recruit brown adipose tissue (BAT) is remarkably enhanced as measured when forskolin is administered (brought into contact) to pre-adipocytes than to mature adipocytes, said method incorporating the steps of ,

a) Seeding mammalian adipocyte precursor cells (pre-adipocytes) in wells of microplates wherein approximately 60×104 cells are seeded for 48-72 hours to get 70-80% confluence;

b) Adding forskolin at concentrations of 50µg/ml and 100µg/ml in the pre-seeded microplates of step a consisting of undifferentiated pre-adipocytes;

c) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells;

d) Adding 200 µl of freshly prepared Adipogenesis progression medium after 72 hours of incubation with the Adipogenesis induction medium in step c;

e) Incubating the cells treated with forskolin (step b), adipogenesis induction medium (step c) and adipogenesis progression medium (step d) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air;

f) Quantitatively determining by appropriate immunoassay techniques the expressions of BMP-7, BMP-4, VEGF-A and UCP-1 in the cell supernatant;

g) Adding 200 µl of freshly prepared Adipogenesis induction medium to the wells of step a;

h) Adding 200 µl of freshly prepared Adipogenesis progression medium comprising graded concentrations of forskolin (50µg/ml and 100µg/ml respectively) to the wells of step g after 72 hours of incubation with the Adipogenesis induction medium;

i) Incubating the cells treated with forskolin (step h), adipogenesis induction medium (step g) and adipogenesis progression medium (step h) for 48 hours in a humidified atmosphere (37 deg. C.) of 5% CO2 and 95% air; and

j) Quantitatively determining by appropriate immunoassay techniques the expressions of BMP-7, BMP-4, VEGF-A and UCP-1 in the cell supernatant.

3. A method of achieving energy balance in mammalian adipose cellular systems, said method comprising step of administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to achieve effects of (a) increased inhibition of adipogenesis and (b) increased expression of secretory factors that function individually or in combination to specifically recruit brown adipocytes or brown like (beige or brite) adipocytes.

4. The method according to claim 3 wherein the secretory factors are selected from the group consisting of bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1).

5. Forskolin for use in therapy for obesity wherein said therapy involves achieving energy balance in mammalian adipocytes by administering forskolin in effective amounts targeted towards

mammalian pre-adipocytes to bring about the effects of (a) increased inhibition of adipogenesis and (b) increased expression of secretory factors that function individually or in combination to specifically recruit brown adipocytes or brown like (beige or brite) adipocytes.

6. The method according to claim 3 wherein the secretory factors are selected from the group consisting of bone morphogenetic protein-7 (BMP-7), bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1).

7. A method to induce the brown like phenotype (beige or brite adipocytes) in white adipocyte depots in mammals said method comprising step of administering effective amount of forskolin to obese mammals with depots of fully differentiated white adipocytes to achieve effect of increase in secretory factors that bring about the development of brown like phenotype (beige or brown adipocytes) within white adipocyte depots.

8. The use according to claim 7 wherein the secretory factors are vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1).

9. Forskolin for use in the therapy of obesity characterised in that forskolin is administered in effective amounts targeting mammalian white adipocyte depots to achieve effect of increased expression of secretory factors vascular endothelial growth factor-A (VEGF-A) and mitochondrial uncoupling protein (UCP1) that cause the development of brown like phenotype (beige or brite adipocytes) in white adipocyte depots in mammals.

10. A method of achieving energy balance in mammalian adipose cellular systems, said method comprising step of administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to bring about of the effect of enhanced expression of mitochondria uncoupling protein 1 (UCP-1) to result in increased mitochondrial thermiogenesis in differentiated brown adipocytes and brown like (beige or brite) adipocytes.

11. Forskolin for use in therapy for obesity wherein said therapy involves achieving energy balance in mammalian adipocytes by administering forskolin in effective amounts targeted towards mammalian pre-adipocytes to bring about the effects of enhanced expression of secretory factor mitochondria uncoupling protein 1 (UCP-1) to result in increased mitochondrial thermiogenesis in differentiated brown adipocytes and brown like (beige or brite) adipocytes.

Fig.1

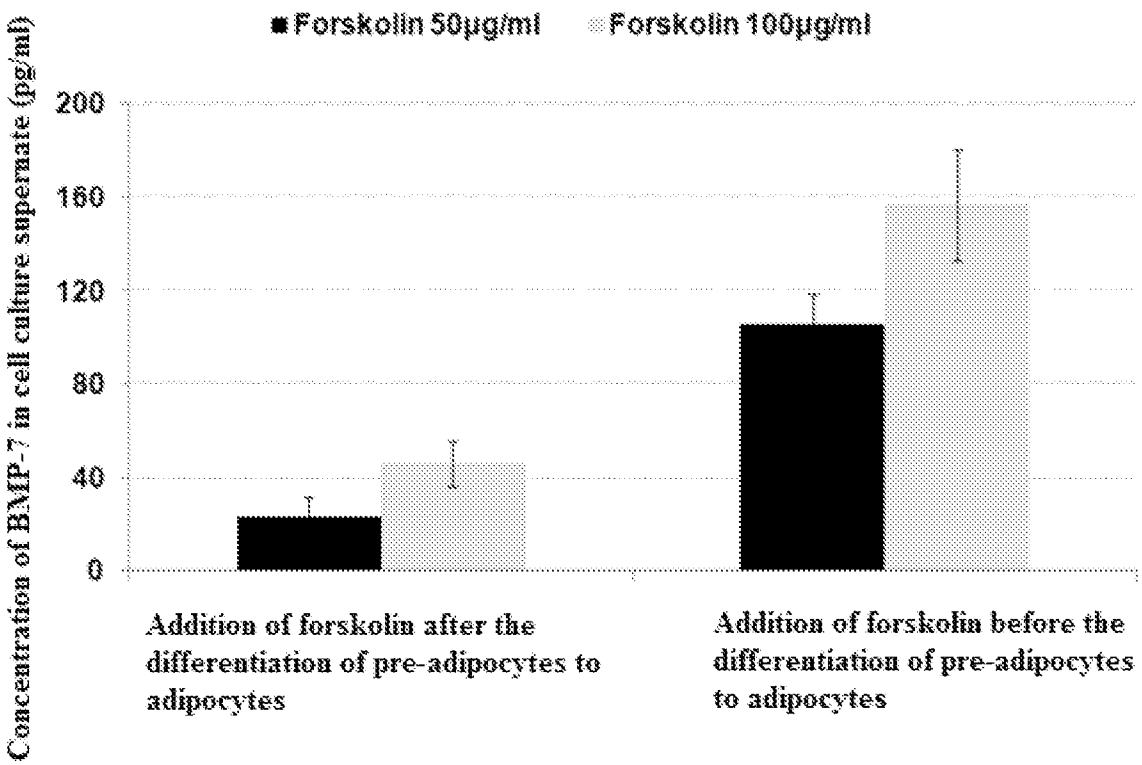

Forskolin 50µg/ml
Forskolin 100µg/ml
Concentration of BMP-7 in cell culture supernate (pg/ml)
200
160
120
80
40
0
Addition of forskolin after the differentiation of pre-adipocytes to adipocytes
Addition of forskolin before the differentiation of pre-adipocytes to adipocytes

Fig.2

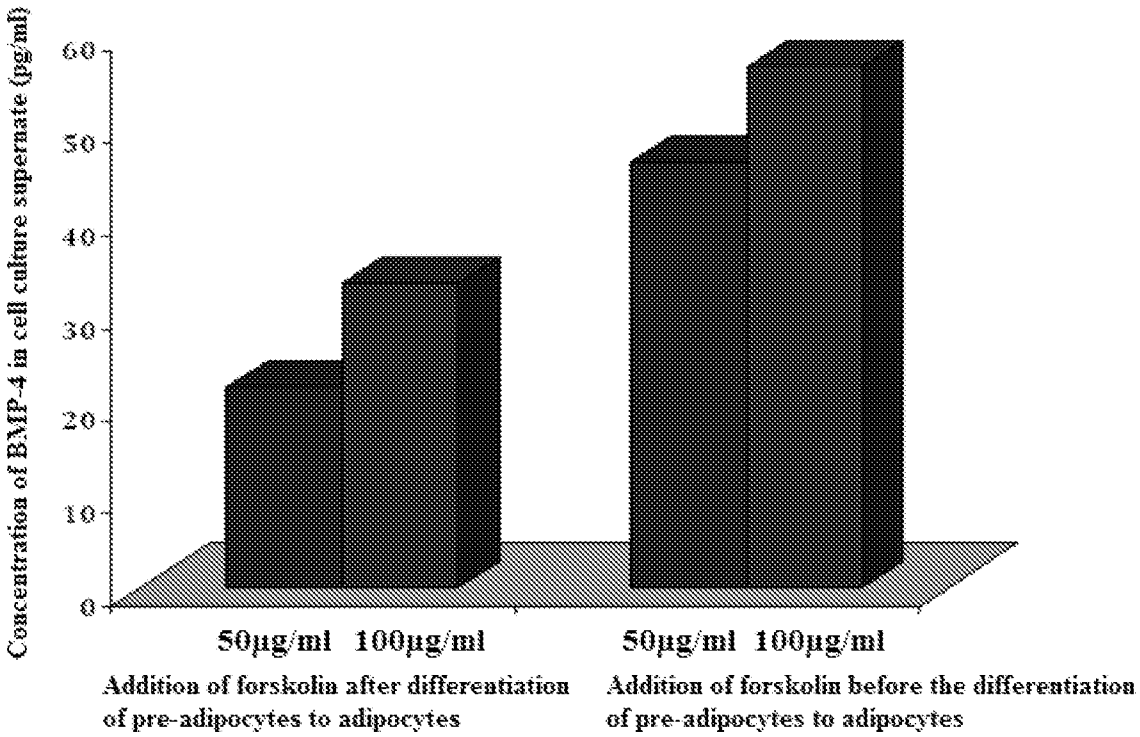

Concentration of BMP-4 in cell culture supernate (pg/ml)
60
50
40
30
20
10
0
50μg/ml 100μg/ml
50μg/ml 100μg/ml
Addition of forskolin after differentiation of pre-adipocytes to adipocytes
Addition of forskolin before the differentiation of pre-adipocytes to adipocytes

Fig.3

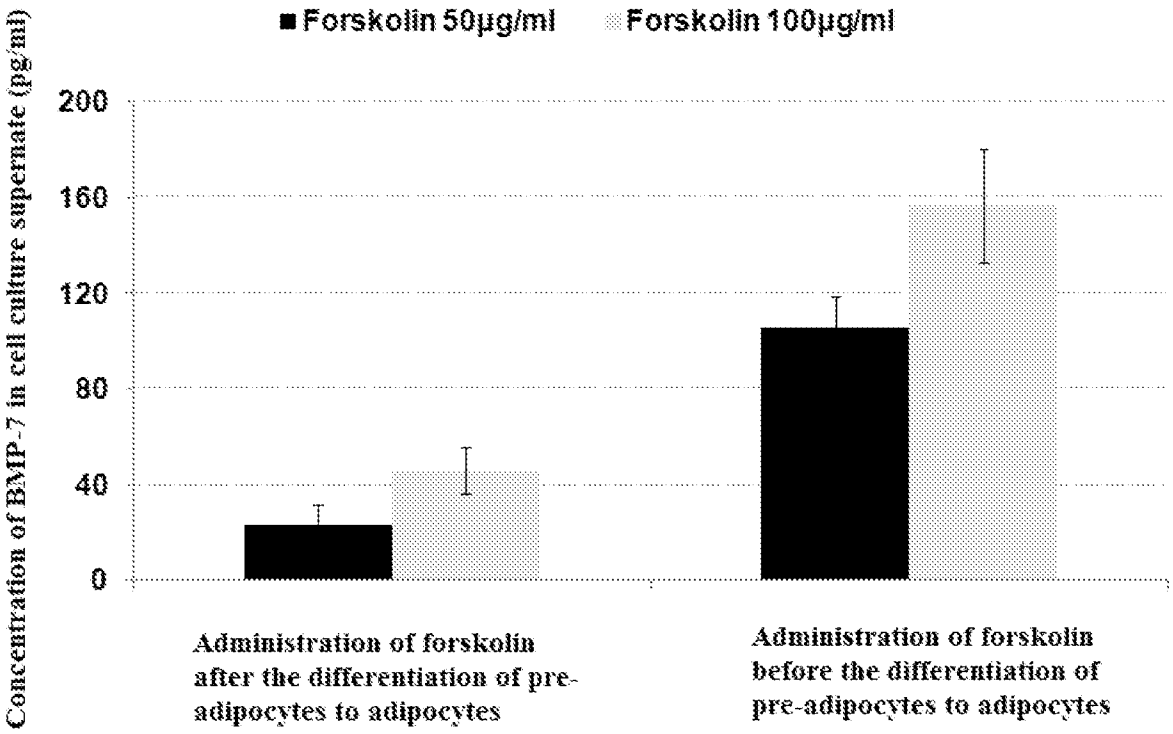
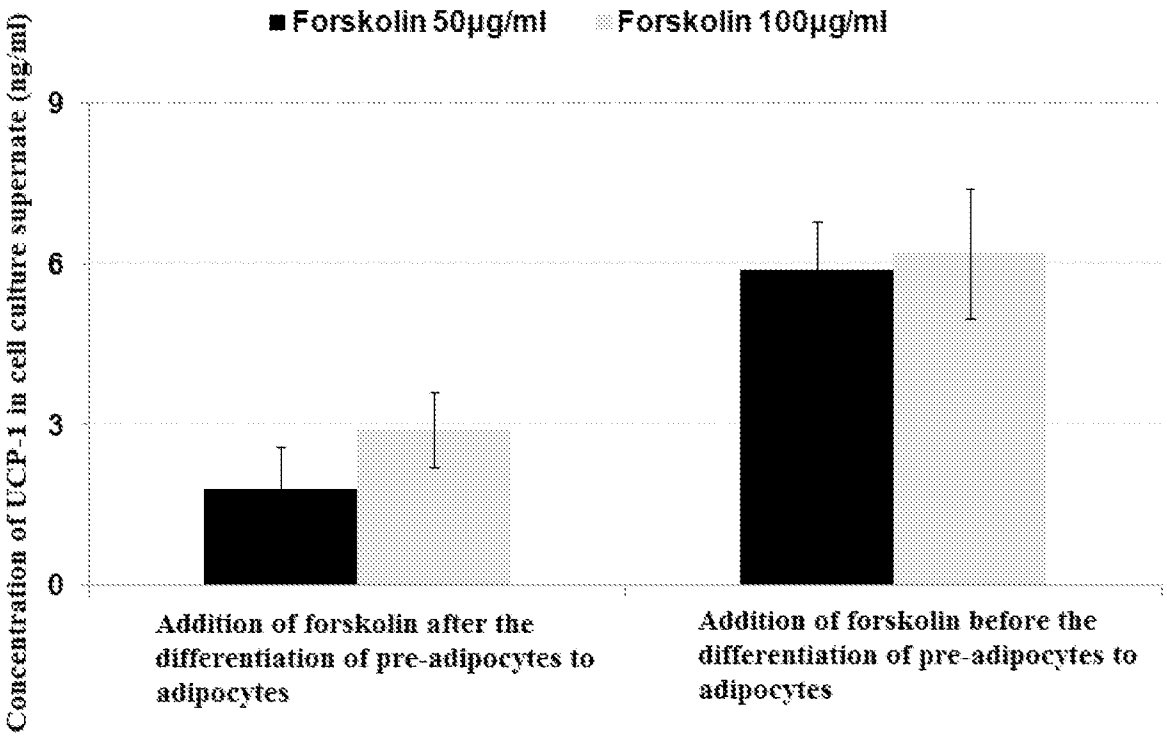

Forskolin 50µg/ml
Forskolin 100µg/ml
Concentration of BMP-7 in cell culture supernate (pg/ml)
200
160
120
80
40
0
Administration of forskolin after the differentiation of pre-adipocytes to adipocytes
Administration of forskolin before the differentiation of pre-adipocytes to adipocytes

Fig.4


Forskolin 50µg/ml
Forskolin 100µg/ml
Concentration of UCP-1 in cell culture supernate (ng/ml)
9
6
3
0
Addition of forskolin after the differentiation of pre-adipocytes to adipocytes
Addition of forskolin before the differentiation of pre-adipocytes to adipocytes

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/60176

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/0775, A61P 3/04, A61P 3/06 (2016.01)
CPC - C12N 5/0653, C12N 2501/01
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) - C12N 5/0775, A61P 3/04, A61P 3/06 (2016.01)
CPC - C12N 5/0653, C12N 2501/01

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/29, 435/366, 435/377
(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar
Search terms: forskolin, adipose, adipocyte, adipogenesis, differentiate, brown, bone morphogenetic protein, BMP-7, BMP7, BMP-4, BMP4, vascular endothelial growth factor-A, VEGF-A, VEGFA, mitochondrial uncoupling protein, UCP 1, UCP-1, UCP1, energy balance

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y<br>---<br>A | US 2015/0030662 A1 (NATIONAL UNIVERSITY OF SINGAPORE, et al.) 29 January 2015 (29.01.2015) para [0015], [0024], [0025], [0040], [0055], [0057],[0058], [0059], [0073], [0077], [0078], [0079] | 7, 10-11<br>----------<br>2, 8, 9<br>----------<br>1, 3-6 |
| Y | SUN et al., Brown adipose tissue derived VEGF-A, [ modulates cold tolerance and energy expenditure. Mol Metab, July 2014, Vol 3, No 4, pp 474.483. Especially abstract | 2, 8, 9 |
| Y | QIAN et al., BMP4-mediated brown fat-like changes in white adipose tissue alter glucose and energy homeostasis. Proc Natl Acad Sci U S A, 26 February 2013, Vol 110, No 9, pp E798-807. Especially abstract | 2 |
| A | US 2005/0158706 A1 (HALVORSEN et al.) 21 July 2005 (21.07.2005) para [0010], [0046]-[0056] | 1, 3-6 |
| A | YANG et al., cAMP/PKA Regulates Osteogenesis, Adipogenesis and Ratio of RANKL/OPG mRNA Abstract - Expression in Mesenchymal Stem Cells by Suppressing Leptin. PLOS ONE, 2008, Vol 3, No 2, page e1540. Especially abstract | 1, 3-6 |

☒ Further documents are listed in the continuation of Box C. ☐

* 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 | Date of mailing of the international search report |
|---|---|
| 29 March 2016 | 22 APR 2016 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-8300 | Authorized officer:<br>Lee W. Young<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/US 15/60176

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 2013/188138 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 19 December 2013 (19.12.2013) para [0337] | 1, 3-6 |
| A | US 2015/0216935 A1 (BROWN UNIV) 06 August 2015 (06.08.2015) para [0110] | 1, 3-6 |