GENERATION OF ADIPOCYTES FROM BOVINE EMBRYONIC STEM CELL LINES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 63/513,412, filed July 13, 2023, which is incorporated by reference in its entirety.

FIELD OF THE DISCLOSURE

[0002] The present disclosure generally relates to methods, cells, and compositions for preparing bovine embryonic stem cells (bESCs) and adipocytes from bESCs. In particular, provided herein are methods for generating feeder-free and serum-free bovine embryonic stem cells (bESCs) and adipocytes from bESCs along with the related compositions.

BACKGROUND OF THE DISCLOSURE

[0003] By virtue of their capacity for pluripotent differentiation and indefinite self-renewal, pluripotent stem cells (PSCs) including bovine embryonic stem cells (bESCs) represent an ideal starting material for a wide range of cellular production processes, including cellular agriculture to generate meat products *in vitro* without harming or killing live animals while simultaneously reducing land resources for farming. However, advancing stem cell technologies for cellular agriculture requires development of protocols for culture and differentiation of PSCs from domesticated animals in chemically-defined conditions without any animal serum, xenogeneic feeders, or other components.

[0004] Meat is composed of skeletal muscle cells (~90%), adipose and connective tissues (~10%), and blood (~0.3%) (Ben-Arye *et al*., Tissue Engineering for Clean Meat Production. Frontiers in Sustainable Food Systems. 2019, 3). Theoretically, these major components of meat could each be generated directly from bESCs. However, reliable sources of bESCs and methodological approaches to leverage such bESCs for meat component manufacturing (e.g., adipocyte, fibroblast, and skeletal muscle cell production) are required to reach commercially viable levels of production.

SUMMARY

[0005] The present disclosure describes methods, cells, and compositions for preparing bovine embryonic stem cells (bESCs) and adipocytes from bESCs.

[0006] In a first aspect, the present disclosure provides a method of culturing bovine embryonic stem cells (bESCs), the method comprising: culturing bESCs in a serum-free culture medium comprising an effective amount of a γ-secretase inhibitor on a matrix-coated culture vessel in feeder-free conditions; and obtaining an expanded population of bESCs expressing Oct4, Sox2, and alkaline phosphatase.

[0006a] In one example, the present disclosure provides a method of culturing bovine

embryonic stem cells (bESCs), the method includes the steps of a) culturing bESCs in a serum-free culture medium comprising an effective amount of a γ-secretase inhibitor on a matrix-coated culture vessel in feeder-free conditions; and b) obtaining an expanded population of bESCs expressing Oct4, Sox2, and alkaline phosphatase.

[0007] In one exampleof the first aspect, the γ-secretase inhibitor comprises (2S)-N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine 1,1-dimethylethyl ester (DAPT).

[0008] In one exampleof the first aspect, the matrix comprises vitronectin or collagen.

[0009] In a second aspect, the present disclosure provides a method of producing adipocytes from bovine embryonic stem cells (bESCs), the method comprising: (a) culturing bESCs in a serum-free culture medium on a matrix-coated cell culture vessel in feeder-free conditions to obtain bESCs expressing Oct4, Sox2, and alkaline phosphatase, wherein the cell culture medium comprises an effective amount of γ-secretase inhibitor; and (b) dissociating the bESCs and culturing the dissociated cells on a matrix-coated cell culture vessel under serum-free medium to obtain adipocytes.

[0009a] In a second example, the present disclosure provides a method of producing adipocytes from bovine embryonic stem cells (bESCs), the method includes the steps of (a) culturing bESCs in a serum-free culture medium on a matrix-coated cell culture vessel in feeder-free conditions to obtain bESCs expressing Oct4, Sox2, and alkaline phosphatase, wherein the cell culture medium comprises an effective amount of γ-secretase inhibitor; and (b) dissociating the bESCs and culturing the dissociated cells on a matrix-coated cell culture vessel under serum-free medium to obtain adipocytes.

[0010] In one exampleof the second aspect, the γ-secretase inhibitor comprises DAPT.

[0011] In one exampleof the second aspect, the serum-free culture medium in step (a) is mTeSR medium.

[0012] In one exampleof the second aspect, the matrix comprises vitronectin or collagen.

[0013] In one example of the second aspect, the dissociated cells are cultured in serum-free medium for 3-5 passages.

[0014] In one example of the second aspect, the serum-free medium in step (b) is Gibco StemPro™ MSC SFM.

[0015] In one example of the second aspect, the adipocytes exhibit adipocyte morphology and accumulate lipids.

[0016] In one example of the second aspect, the adipocytes express adiponectin and leptin.

[0017] In a third aspect, the present disclosure provides a method of producing adipocytes from bovine embryonic stem cells (bESCs), the method comprising: (a) culturing blastocysts on mouse embryonic fibroblasts (MEFs) in a serum-free culture medium comprising an effective amount of one or more inhibitors of the WNT pathway to obtain bESCs expressing Oct4, Sox2, and alkaline phosphatase; (b) culturing the bESCs from step (a) on a matrix-coated cell culture vessel in feeder-free conditions to form bESC spheres; and (c) dissociating cells from the bESC spheres and culturing the dissociated cells on a matrix-coated cell culture vessel under serum-free medium to obtain adipocytes.

[0017a] In a third example, the present disclosure provides a method of producing adipocytes from bovine embryonic stem cells (bESCs), the method includes the steps of (a) culturing blastocysts on mouse embryonic fibroblasts (MEFs) in a serum-free culture medium comprising an effective amount of one or more inhibitors of the WNT pathway to obtain bESCs expressing Oct4, Sox2, and alkaline phosphatase; (b) culturing the bESCs from step

(a) on a matrix-coated cell culture vessel in feeder-free conditions to form bESC spheres; and (c) dissociating cells from the bESC spheres and culturing the dissociated cells on a matrix-coated cell culture vessel under serum-free medium to obtain adipocytes.

[0018] In one example of the third aspect, the blastocysts are cultured on MEFs for 12-14 days.

[0019] In one example of the third aspect, the one or more inhibitors of the WNT pathway comprises IWR-1, CCT251545, and/or iCRT3.

[0020] In one example of the third aspect, the culture medium further comprises an Lck/Src inhibitor, Activin A, and LIF. In one example, the Lck/Src inhibitor comprises WH-4-023.

[0021] In one example of the third aspect, the matrix comprises vitronectin or collagen.

[0022] In one example of the third aspect, the bESCs in step (b) are cultured for about 5 days.

[0023] In one example of the third aspect, the dissociated cells are cultured in serum-free medium for 3-5 passages.

[0024] In one example of the third aspect, the serum-free medium in step (c) is Gibco StemPro™ MSC SFM.

[0025] In one example of the third aspect, the adipocytes exhibit adipocyte morphology and accumulate lipids.

[0026] In one example of the third aspect, the adipocytes express adiponectin and leptin.

[0027] These and other features and advantages of the present disclosure will be more fully understood from the following detailed description taken together with the accompanying claims. It is noted that the scope of the claims is defined by the recitations therein and not by the specific discussion of features and advantages set forth in the present description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figure 1** shows derivation of bESCs and their maintenance according to Methods 1-3. Images demonstrate sequential steps in the derivation of bESCs and growing them according to Methods 1, 2, and 3 on plates precoated with vitronectin, as indicated.

[0029] **Figure 2** shows bESC growth on different matrices. bESCs were generated using Method 1 and cultured in medium composed mTeSR^{TM}Plus, 4 mM Glutamax, 1/100 dilution