Patent Claims

1. A method for producing engineered skeletal muscle tissue from pluripotent stem cells, comprising the steps of
   (i) inducing mesoderm differentiation of the pluripotent stem cells by culturing pluripotent stem cells in a basal medium comprising an effective amount of (a) FGF2, (b) a GSK3 inhibitor, (c) a SMAD inhibitor, and (d) a serum-free additive comprising transferrin, insulin, progesterone, putrescine and selenium or a bioavailable salt thereof;
   (ii) inducing the myogenic specification by culturing the cells obtained in step (i) in a basal medium comprising an effective amount of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, and (c) a serum-free additive as in (i), followed by
   continuing the cultivation in the medium with the addition of an effective amount of (d) HGF, followed by
   culturing the cells in a basal medium comprising an effective amount of (a) a gamma secretase/NOTCH inhibitor, (b) HGF, (c) a serum-free additive as in (i), and (d) knockout serum replacement (KSR);
   (iii) expanding and maturing the cells into skeletal myoblasts and satellite cells by culturing the cells obtained in step (ii) in a basal medium comprising an effective amount of (a) HGF, (b) a serum-free additive as in (i), and (c) knockout serum replacement (KSR);
   (iv) maturing the cells into skeletal myotubes and satellite cells by culturing the cells obtained in step (iii), which are dispersed in an extracellular matrix, under mechanical stimulation in a basal medium, comprising an effective amount of (a) a serum-free additive as in step (i), and (b) an additional serum-free additive comprising albumin, transferrin, ethanolamine, selenium or a bioavailable salt thereof, L-carnitine, fatty acid additive, and triiodo-L-thyronine (T3);
   thereby producing engineered skeletal muscle tissue.

2. The method of claim 1, wherein the skeletal muscle tissue generates a contraction force of at least 0.6 millinewtons (mN) upon a stimulus of 100 Hz, preferably at least 0.7 mN, more preferably at least 0.8 mN, more preferably at least 0.9 mN, more preferably at least 1 mN, more preferably at least 1.2 mN, more preferably at least 1.3 mN, more preferably at least 1.4 mN, more

preferably at least 1.5 mN, more preferably at least 1.6 mN, more preferably at least 1.7 mN, more preferably at least 1.8 mN, more preferably at least 1.9 mN, and most preferably at least 2 mN.

3. The method of claim 1 or 2, wherein the pluripotent stem cells are of primate origin, in particular human pluripotent stem cells; and/or wherein the pluripotent stem cells are selected from induced pluripotent stem cells, embryonic stem cells, parthenogenetic stem cells, pluripotent stem cells produced via nuclear transfer, and pluripotent cells produced via chemical reprogramming, in particular wherein the pluripotent stem cells are induced pluripotent stem cells.

4. The method of any one of claims 1-3, wherein step (i) is carried out for 24 to 132 hours, preferably for 48 to 120 hours, more preferably for 60 to 114 hours, even more preferably for 72 to 108 hours, more preferably for 84 to 102 hours, and most preferably for about 96 hours.

5. The method of any one of claims 1-4, wherein in step (i), the GSK3 inhibitor is selected from the group consisting of CHIR99021, CHIR98014, SB216763, TWS119, tideglusib, SB415286, 6-bromoindirubin-3-oxime and a valproate salt, preferably wherein the GSK3 inhibitor is CHIR99021; and/or
wherein in step (i), the SMAD inhibitor is selected from the group consisting of LDN193189, K02288, LDN214117, ML347, LDN212854, DMH1, preferably wherein the SMAD inhibitor is LDN193189.

6. The method of any one of claims 1-5, wherein in step (i), the effective amount of FGF2 is 1-15 ng/ml, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml; and/or
the serum-free additive provides in the medium a final concentration of 50-500 µg/ml transferrin, 1-20 µg/ml insulin, 0.001-0.1 µg/ml progesterone, 5-50 µg/ml putrescine and 6-600 nM selenium or a bioavailable salt thereof, in particular sodium selenite, and/or
the GSK3 inhibitor is CHIR99021, and the effective amount is 1-20 µM, preferably 2-19 µM, more preferably 3-18 µM, even more preferably 4-17 µM, even more preferably 5-16 µM, even more preferably 6-15 µM, even more preferably 7-14 µM, even more preferably 7.5-13 µM, even more preferably 8-

12 µM, even more preferably 9-11 µM, and most preferably about 10 µM; and/or
the SMAD inhibitor is LDN193189, and the effective amount is 0.05-5 µM, preferably 0.1-2.5 µM, more preferably 0.2-1 µM, even more preferably 0.25-0.8 µM, even more preferably 0.3-0.75 µM, even more preferably 0.35-0.7 µM, even more preferably 0.4-0.6 µM, even more preferably 0.45-0.55 µM, and most preferably about 0.5 µM.

7. The method of any one of claims 1-6, wherein the serum-free additive in step (i) is 0.1-10% (v/v) N2 additive, preferably 0.3-7.5% (v/v) N2 additive, more preferably 0,5-5% (v/v) N2 additive, more preferably 0.75%-2% (v/v) N2 additive, more preferably 0.9%-1.2% (v/v) N2 additive, and most preferably about 1% (v/v) N2 additive.

8. The method of any one of claims 1-7, wherein the basal medium in step (i), step (ii), step (iii), and/or in step (iv) is selected from DMEM, DMEM/F12, RPMI, IMDM, alphaMEM, Medium 199, Hams F-10, Hams F-12, wherein the basal medium is preferably DMEM, in particular wherein the basal medium is supplemented with pyruvate and/or non-essential amino acids, and/or comprises 1 g/l glucose.

9. The method of any one of claims 1-8, wherein in step (ii), the cultivation is carried out in the presence of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, and (c) the serum-free additive for 36 to 60 hours, preferably for 42 to 54 hours, and most preferably for about 48 hours; and/or
the cultivation is carried out in the presence of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, (c) the serum-free additive, and (d) HGF for 36 to 60 hours, preferably for 42 to 54 hours, and most preferably for about 48 hours; and/or the cultivation is carried out in the presence of (a) a gamma-secretase/NOTCH inhibitor, (b) HGF, (c) the serum-free additive, and (d) knockout serum replacement (KSR) for 72 to 120 hours, preferably for 76 to 114 hours, more preferably for 84 to 108 hours, even more preferably for 90 to 102 hours, and most preferably for about 96 hours.

10. The method of any one of claims 1-9, wherein in step (ii), the gamma-secretase/NOTCH inhibitor is selected from the group DAPT, RO4929097, semagacestat (LY450139), avagacestat (BMS-708163), dibenzazepine (YO-

01027), LY411575, IMR-1, L685458, preferably wherein the gamma-secretase/NOTCH inhibitor is DAPT.

11. The method of any one of claims 1-10, wherein in step (ii), the effective amount of FGF2 is 15-30 ng/ml, preferably 17.5-25 ng/ml, more preferably 18-22 ng/ml, even more preferably 19-21 ng/ml, and most preferably about 20 ng/ml; and/or the effective amount of HGF is 1-15 ng/ml, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml; and/or
the gamma-secretase/NOTCH inhibitor is DAPT, and the effective amount is 1-20 µM, preferably 2-19 µM, more preferably 3-18 µM, even more preferably 4-17 µM, even more preferably 5-16 µM, even more preferably 6-15 µM, even more preferably 7-14 µM, even more preferably 7.5-13 µM, even more preferably 8-12 µM, even more preferably 9-11 µM, and most preferably about 10 µM;
the KSR is used in an amount of 5-20% (v/v), preferably 6-17.5% (v/v), more preferably 7-15% (v/v), more preferably 8%-12% (v/v), more preferably 9%-11% (v/v), and most preferably about 10% (v/v) KSR; in particular wherein the KSR is used in the presence of a reducing agent such as beta-mercaptoethanol and/or alpha-thioglycerol.

12. The method of any one of claims 1-11, wherein in step (iii),
the effective amount of HGF is 1-15 ng/ml, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml; and/or
the KSR is used in an amount of 5-20% (v/v), preferably 6-17.5% (v/v), more preferably 7-15% (v/v), more preferably 8%-12% (v/v), more preferably 9%-11% (v/v), and most preferably about 10% (v/v) KSR; in particular wherein the KSR is used in the presence of a reducing agent such as beta-mercaptoethanol and/or alpha-thioglycerol.

13. The method of any one of claims 1-12, wherein in step (iv), the additional serum-free additive provides in the medium a final concentration of 0.5-50 mg/ml albumin, 1-100 µg/ml transferrin, 0.1-10 µg/ml ethanolamine, 17.4-1744 nM selenium or a bioavailable salt thereof, in particular sodium selenite, 0.4-40

µg/ml L-carnitine, 0.05-5 µl/ml fatty acid additive, 0.0001-0.1 µg/ml triiodo-L-thyronine (T3).

14. The method of any one of claims 1-13, wherein the additional serum-free additive in step (iv) is 0.1-10% (v/v) B27, preferably 0.5-8% (v/v), more preferably 1-6% (v/v), even more preferably 1.5-4% (v/v), and most preferably about 2% (v/v) B27.

15. The method of any one of claims 1-14, wherein in step (iv), the mechanical stimulation is a static tension, a dynamic stimulation, or an auxotonic stimulation, preferably wherein the mechanical stimulation is a static tension.

16. The method of any one of claims 1-15, comprising, prior to step (i), a seeding step, wherein the pluripotent stem cells are seeded in a stem cell medium in the presence of a ROCK inhibitor, preferably wherein the seeding step is carried out 18-30 hours prior to step (i).

17. The method of claim 15, wherein the ROCK inhibitor is selected from the group consisting of Y27632, H-1152P, thiazovivin, fasudil, hydroxyfasudil, GSK429286A and RKI1447, preferably the ROCK inhibitor is selected from the group consisting of Y27632, H-1152P, thiazovivin, fasudil and hydroxyfasudil, more preferably the ROCK inhibitor is selected from the group consisting of Y27632 and H-1152P, particularly preferably wherein the ROCK inhibitor is Y27632.

18. The method of claim 16 or 17, wherein the ROCK inhibitor is Y27632 and is used at a concentration of 0.5-10 µM, preferably 1-9 µM, more preferably 2-8 µM, more preferably 3-7 µM, more preferably 4-6 µM, and most preferably at a concentration of about 5 µM; and/or
wherein the stem cell medium is iPS-Brew XF.

19. The method of any one of claims 15-18, wherein the pluripotent stem cells in the seeding step are first seeded into an engineered form in the presence of one or more components of an extracellular matrix in a master mix before the stem cell medium is added.

20. The method of claim 19, wherein the component of an extracellular matrix in the master mix is collagen, preferably type I collagen, more preferably of bovine origin, human origin or marine origin, in particular collagen of bovine origin, optionally wherein the extracellular matrix additionally comprises laminin and/or fibronectin.

21. The method of claim 20, wherein the pluripotent stem cells are seeded in medium at a ratio of 1-6 x 10^6 cells/ml and 0.7-1.4 mg/ml collagen.

22. The method of any one of claims 19-21, wherein the master mix comprises 5-15% (v/v) of an exudate of Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells as extracellular matrix component, preferably 7.5%-12.5% (v/v), more preferably 9-11% (v/v), and most preferably about 10% (v/v), in particular wherein the exudate is Matrigel; and/or wherein the pH of the master mix is pH 7.2 to pH 7.8.

23. The method of any one of claims 19-21, wherein the master mix comprises stromal cells, wherein the stromal cells generate the extracellular matrix components collagens, laminin, fibronectin, and/or proteoglycans; and/or wherein the pH of the master mix is pH 7.2 to pH 7.8.

24. The method of any one of claims 19-23, wherein the stem cell medium is added to the master mix in the engineered form after about 1 hour, wherein the stem cell medium comprises KSR and FGF2.

25. The method of claim 24, wherein the stem cell medium comprises 5-20% (v/v), preferably 6-17.5% (v/v), more preferably 7-15% (v/v), more preferably 8%-12% (v/v), more preferably 9%-11% (v/v), and most preferably about 10% (v/v) KSR; and/or
wherein the stem cell medium comprises 1-15 ng/ml FGF2, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml FGF2.

26. The method of any one of claims 19-25, wherein step (iii) is carried out for 7-11 days, preferably for 8-10 days, and most preferably for about 9 days.

27. The method of any one of claims 1-18, wherein after step (iii), the skeletal myoblasts and satellite cells are seeded into an engineered form in an additional step prior to step (iv) in the presence of one or more components of an extracellular matrix in a master mix.

28. The method of claim 27, wherein the component of an extracellular matrix in the master mix is collagen, preferably type I collagen, more preferably of bovine origin, human origin or marine origin, in particular collagen of bovine origin, optionally wherein the extracellular matrix additionally comprises laminin and/or fibronectin.

29. The method of claim 28, wherein the skeletal myoblasts and satellite cells are seeded in medium at a ratio of 1-6 x 10^6 cells/ml and 0.7-1.4 mg/ml collagen.

30. The method of any one of claims 27-29, wherein the master mix comprises 5-15% (v/v) of an exudate of Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells as an extracellular matrix component, preferably 7.5%-12.5% (v/v), more preferably 9-11% (v/v), and most preferably about 10% (v/v), in particular wherein the exudate is Matrigel; and/or
wherein the pH of the master mix is pH 7.2 to pH 7.8.

31. The method of any one of claims 27-29, wherein the master mix comprises stromal cells, wherein the stromal cells produce the extracellular matrix components collagens, laminin, fibronectin, and/or proteoglycans; and/or
wherein the pH of the master mix is pH 7.2 to pH 7.8.

32. The method of any one of claims 27-31, wherein after about one hour, a medium as used in step (iii) is added to a master mix in an engineered form, wherein the medium additionally comprises an effective amount of a ROCK inhibitor;
in particular wherein the ROCK inhibitor is selected from the group consisting of Y27632, H-1152P, thiazovivin, fasudil, hydroxyfasudil, GSK429286A and RKI1447, preferably the ROCK inhibitor is selected from the group consisting of Y27632, H-1152P, thiazovivin, fasudil and hydroxyfasudil, more preferably the ROCK inhibitor is selected from the group consisting of Y27632 and H-1152P, particularly preferably wherein the ROCK inhibitor is Y27632.

33. The method of claim 32, wherein the ROCK inhibitor is Y27632 and is used at a concentration of 0.5-10 µM, preferably 1-9 µM, more preferably 2-8 µM, more preferably 3-7 µM, more preferably 4-6 µM, and most preferably at a concentration of about 5 µM.

34. The method of any one of claims 27-33, wherein after about one day the medium is replaced with a medium as used in step (iii), and the cells are subsequently further cultured in this medium for an additional 5-9 days, preferably 6-8 days, most preferably about 7 days.

35. The method of any one of claims 19-34, wherein the engineered form has the form of a ring, ribbon, strand, patch, pouch, or cylinder, wherein optionally individual skeletal muscle tissues are fused.

36. The method of any one of claims 1-35, wherein step (iv) is carried out for at least 19 days, preferably at least 28 days, more preferably for at least 56 days, even more preferably is carried out for at least 120 days, and in particular is carried out for at least 240 days.

37. The method of any one of claims 1-36, wherein the method does not comprise a differentiation- or maturation-related transgene, preferably wherein the method does not comprise a myogenic transgene, more preferably wherein the method does not comprise the transgene Pax7 or MyoD.

38. The method of any one of claims 1-37, wherein the method does not comprise a skeletal myoblast enrichment step, preferably not an enrichment step by cell selection, more preferably not an enrichment step by antibody-based cell selection.

39. A method for producing skeletal myoblasts, skeletal myotubes, and satellite cells from pluripotent stem cells, comprising the steps of

(i) inducing mesoderm differentiation of the pluripotent stem cells by culturing pluripotent stem cells in a basal medium comprising an effective amount of (a) FGF2, (b) a GSK3 inhibitor, (c) a SMAD inhibitor, and (d) a serum-free additive comprising transferrin, insulin, progesterone, putrescine, and selenium or a bioavailable salt thereof;

(ii) inducing myogenic specification by culturing the cells obtained in step (i) in a basal medium comprising an effective amount of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, and (c) a serum-free additive as in (i), followed by
continuing the cultivation in the medium with the addition of an effective amount of (d) HGF, followed by
culturing the cells in a basal medium comprising an effective amount of (a) a gamma-secretase/NOTCH inhibitor, (b) HGF, (c) a serum-free additive as in (i), and (d) knockout serum replacement (KSR);

(iii) maturing the cells into skeletal myoblasts and satellite cells by culturing the cells obtained in step (ii) in a basal medium comprising an effective amount of (a) HGF, (b) a serum-free additive as in (i), and (c) knockout serum replacement (KSR), followed by

(iv) maturing the cells into skeletal myotubes and satellite cells by culturing the cells obtained in step (iii) in a basal medium comprising an effective amount of (a) a serum-free additive as in step (i), and (b) an additional serum-free additive comprising albumin, transferrin, ethanolamine, selenium or a bioavailable salt thereof, L-carnitine, fatty acid additive, and triiodo-L-thyronine (T3);
thereby generating skeletal myoblasts, skeletal myotubes, and satellite cells.

40. The method of claim 39, wherein a proportion of skeletal myoblasts of the amount of all available cells of at least 40% is achieved by the method, preferably at least 50%, more preferably at least 60%, most preferably at least 70%, as determined by the expression of actinin by flow cytometry.

41. The method of claim 39 or 40, wherein a proportion of satellite cells of the amount of all available cells of at least 10% is achieved by the method, preferably at least 15%, more preferably at least 20%, most preferably at least 30% is achieved, determined by the expression of Pax7 by flow cytometry.

42. The method of any one of claims 39-41, wherein the method does not include a skeletal myoblast enrichment step, preferably not an enrichment step by cell selection, more preferably not an enrichment step by antibody-based cell selection.

43. The method of any one of claims 39-42, wherein the pluripotent stem cells are of primate origin, in particular human pluripotent stem cells; and/or wherein the pluripotent stem cells are selected from induced pluripotent stem cells, embryonic stem cells, parthenogenetic stem cells, pluripotent stem cells produced via nuclear transfer, and pluripotent cells produced via chemical reprogramming, in particular wherein the pluripotent stem cells are induced pluripotent stem cells.

44. The method of any one of claims 39-43, wherein step (i) is carried out for 48 to 132 hours, preferably for 48 to 120 hours, more preferably for 60 to 114 hours, even more preferably for 72 to 108 hours, more preferably for 84 to 102 hours, and most preferably for about 96 hours.

45. The method of any one of claims 39-44, wherein in step (i), the GSK3 inhibitor is selected from the group consisting of CHIR99021, CHIR98014, SB216763, TWS119, tideglusib, SB415286, 6-bromoindirubin-3-oxime, and a valproate salt, preferably wherein the GSK3 inhibitor is CHIR99021; and/or
wherein in step (i), the SMAD inhibitor is selected from the group consisting of LDN193189, K02288, LDN214117, ML347, LDN212854, DMH1, preferably wherein the SMAD inhibitor is LDN193189.

46. The method of any one of claims 39-45, wherein in step (i), the effective amount of FGF2 is 1-15 ng/ml, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml; and/or
the serum-free additive provides a final concentration in the medium of 50-500 mg/l transferrin, 1-20 mg/l insulin, 1-30 µg/l progesterone, 5-50 µg/ml putrescine and 6-600 nM selenium or a bioavailable salt thereof, in particular sodium selenite, and/or
the GSK3 inhibitor is CHIR99021, and the effective amount is 4-18 µM, preferably 5-16 µM, more preferably 6-15 µM, even more preferably 7-14 µM, even more preferably 8-13 µM, even more preferably 9-12 µM, even more preferably 9.5-11 µM, and most preferably about 10 µM; and/or
the SMAD inhibitor is LDN193189, and the effective amount is 0.05-5 µM, preferably 0.1-2.5 µM, more preferably 0.2-1 µM, even more preferably 0.25-0.8 µM, even more preferably 0.3-0.75 µM, even more preferably 0.35-0.7 µM,

even more preferably 0.4-0.6 µM, even more preferably 0.45-0.55 µM, and most preferably about 0.5 µM.

47. The method of any one of claims 39-46, wherein the serum-free additive in step (i) is 0.1-10% (v/v) N2 additive, preferably 0.3-7.5% (v/v) N2 additive, more preferably 0,5-5% (v/v) N2 additive, more preferably 0.75%-2% (v/v) N2 additive, more preferably 0.9%-1.2% (v/v) N2 additive, and most preferably about 1% (v/v) N2 additive.

48. The method of any one of claims 39-47, wherein the basal medium in step (i), step (ii), step (iii) and/or step (iv) is selected from DMEM, DMEM/F12, RPMI, IMDM, alphaMEM, Medium 199, Hams F-10, Hams F-12, wherein the basal medium is preferably DMEM, in particular wherein the basal medium is supplemented with pyruvate and/or non-essential amino acids, and/or comprises 1 g/l glucose.

49. The method of any one of claims 39-48, wherein in step (ii), the cultivation is carried out in the presence of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, and (c) the serum-free additive for 36 to 60 hours, preferably for 42 to 54 hours, and most preferably for about 48 hours; and/or
the cultivation is carried out in the presence of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, (c) the serum-free additive, and (d) HGF for 36 to 60 hours, preferably for 42 to 54 hours, and most preferably for about 48 hours; and/or
the cultivation is carried out in the presence of (a) a gamma-secretase/NOTCH inhibitor, (b) HGF, (c) the serum-free additive, and (d) knockout serum replacement (KSR) for 72 to 120 hours, preferably for 76 to 114 hours, more preferably for 84 to 108 hours, even more preferably for 90 to 102 hours, and most preferably for about 96 hours.

50. The method of any one of claims 39-49, wherein in step (ii), the gamma-secretase/NOTCH inhibitor is selected from the group of DAPT, RO4929097, semagacestat (LY450139), avagacestat (BMS-708163), dibenzazepine (YO-01027), LY411575, IMR-1, L685458, wherein the gamma-secretase/NOTCH inhibitor is preferably DAPT.

51. The method of any one of claims 39-50, wherein in step (ii), the effective amount of FGF2 is 15-30 ng/ml, preferably 17.5-25 ng/ml, more preferably 18-

22 ng/ml, even more preferably 19-21 ng/ml, and most preferably about 20 ng/ml; and/or
the effective amount of HGF is 1-15 ng/ml, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml; and/or
the gamma-secretase/NOTCH inhibitor is DAPT, and the effective amount is 1-20 µM, preferably 2-19 µM, more preferably 3-18 µM, even more preferably 4-17 µM, even more preferably 5-16 µM, even more preferably 6-15 µM, even more preferably 7-14 µM, even more preferably 7.5-13 µM, even more preferably 8-12 µM, even more preferably 9-11 µM, and most preferably about 10 µM;
the KSR is used in an amount of 6-14% (v/v), preferably 7-13% (v/v), more preferably 8%-12% (v/v), more preferably 9%-11% (v/v), and most preferably about 10% (v/v) KSR; in particular wherein the KSR is used in the presence of a reducing agent such as beta-mercaptoethanol and/or alpha-thioglycerol.

52. The method of any one of claims 39-49, wherein in step (iii), the effective amount of HGF is 1-15 ng/ml, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml;
the KSR is used in an amount of 5-20% (v/v), preferably 6-17.5% (v/v), more preferably 7-15% (v/v), more preferably 8%-12% (v/v), more preferably 9%-11% (v/v), and most preferably about 10% (v/v) KSR; in particular wherein the KSR is used in the presence of a reducing agent such as beta-mercaptoethanol and/or alpha-thioglycerol.

53. The method of any one of claims 39-52, wherein step (iii) is carried out for 7-11 days, preferably 8-10 days, and most preferably for about 9 days.

54. The method of any one of claims 39-53, wherein in step (iv), the additional serum-free additive provides in the medium a final concentration of 0.5-50 mg/ml albumin, 1-100 µg/ml transferrin, 0.1-10 µg/ml ethanolamine, 17.4-1744 nM selenium or a bioavailable salt thereof, in particular sodium selenite, 0.4-40 µg/ml L-carnitine, 0.05-5 µl/ml fatty acid additive, 0.0001-0.1 µg/ml triiodo-L-thyronine (T3).

55. The method of any one of claims 39-54, wherein the additional serum-free additive in step (iv) is 0.1-10% (v/v) B27, preferably 0.5-8% (v/v), more preferably 1-6% (v/v), even more preferably 1.5-4% (v/v), and most preferably about 2% (v/v) B27.

56. The method of any one of claims 39-55, wherein step (iv) is carried out for at least 30 days, preferably at least 35 days, more preferably for at least 40 days, and even more preferably for at least to 50 days.

57. The method of any one of claims 39-56, comprising, prior to step (i), a seeding step in which the pluripotent stem cells are seeded in a stem cell medium in the presence of a ROCK inhibitor, preferably wherein the seeding step is carried out 18-30 hours prior to step (i).

58. The method of claim 57, wherein the ROCK inhibitor is selected from the group consisting of Y27632, H-1152P, thiazovivin, fasudil, hydroxyfasudil, GSK429286A, and RKI1447, preferably the ROCK inhibitor is selected from the group consisting of Y27632, H-1152P, thiazovivin, fasudil and hydroxyfasudil, more preferably the ROCK inhibitor is selected from the group consisting of Y27632 and H-1152P, wherein particularly preferably the ROCK inhibitor is Y27632.

59. The method of claim 57 or 58, wherein the ROCK inhibitor is Y27632 and is used at a concentration of 0.5-10 µM, preferably 1-9 µM, more preferably 2-8 µM, more preferably 3-7 µM, more preferably 4-6 µM, and most preferably at a concentration of about 5 µM; and/or
wherein the stem cell medium is iPS-Brew XF.

60. The method of claim 59, wherein the stem cell medium comprises 5-20% (v/v), preferably 6-17.5% (v/v), more preferably 7-15% (v/v), more preferably 8%-12% (v/v), more preferably 9%-11% (v/v), and most preferably about 10% (v/v) KSR; and/or
wherein the stem cell medium comprises 1-15 ng/ml FGF2, preferably 2.5-14 ng/ml, more preferably 5-13 ng/ml, even more preferably 7.5-12.5 ng/ml, even more preferably 8-12 ng/ml, even more preferably 9-11 ng/ml, and most preferably about 10 ng/ml FGF2.

61. An engineered skeletal muscle tissue, having multinuclear mature skeletal muscle fibers with satellite cells, and having no blood supply and/or no central nervous system control; in particular wherein the presence of skeletal muscle fibers is detected by staining of actinin and with DAPI.

62. The engineered skeletal muscle tissue of claim 61, wherein the skeletal muscle tissue is serum-free and/or does not comprise a differentiation- or maturation-related transgene, preferably wherein the skeletal muscle tissue does not comprise a myogenic transgene, more preferably wherein the skeletal muscle tissue does not comprise the Pax7 or MyoD transgene.

63. The engineered skeletal muscle tissue of claim 61 or claim 62, wherein said skeletal muscle tissue generates at least a contraction force of 0.3 millinewtons (mN) upon a stimulus of 100 Hz at 200 mA, preferably generates at least 0.4 mN, more preferably at least 0.5 mN, more preferably at least 0.6 mN, more preferably at least 0.7 mN, more preferably at least 0.8 mN, more preferably at least 0.9 mN, more preferably at least 1 mN, more preferably at least 1.2 mN, more preferably at least 1.3 mN, more preferably at least 1.4 mN, more preferably at least 1.5 mN, more preferably at least 1.6 mN, more preferably at least 1.7 mN; more preferably at least 1.8 mN; more preferably at least 1.9 mN; and most preferably at least 2 mN.

64. The engineered skeletal muscle tissue of any one of claims 61-63, wherein the skeletal muscle tissue is formed by engineering, preferably wherein it has the engineered form of a ring, a ribbon, a strand, a patch, a pouch, or a cylinder, optionally wherein individual skeletal muscle tissues are fused, particularly wherein the skeletal muscle tissue has the form of a ring.

65. Mesodermally differentiated skeletal myoblast progenitor cells, obtained according to step (i) of claim 1 or claim 39, prepared by a method according to claim 1(i) or according to claim 39(i), characterized by the expression of the genes MSGN1 and/or TBX6, wherein the expression of MSGN1 and/or TBX6 can be determined by flow cytometry and/or immunostainings; and/or the mRNA *SP5* is expressed, wherein the expression of *SP5* can be determined by RNA sequencing.

66. Myogenically specified skeletal myoblast progenitor cells, obtained according to step (ii) of claim 1 or claim 39, produced by a method according to claim 1(i) to (ii) or according to claim 39(i) to (ii), characterized by the expression of the gene PAX3, wherein the expression of PAX3 can be determined by flow cytometry and/or immunostainings; and/or the mRNA *SIM1* is expressed, wherein the expression of *SIM1* can be determined by RNA sequencing.

67. Skeletal myoblast cells, obtained according to step (iii) of claim 1 or claim 39, produced by a method according to claim 1(i) to (iii) or according to claim 39(i) to (iii), characterized by the expression of actinin, preferably wherein the expression of actinin can be determined by flow cytometry and/or immunostainings.

68. A satellite cell, obtained according to step (iii) of claim 1 or claim 39, produced by a method according to claim 1(i) to (iii) or according to claim 39(i) to (iii), characterized by the expression of the gene Pax7, wherein the expression of Pax7 can be determined by flow cytometry and/or immunostainings, more preferably wherein satellite cells further express Ki67.

69. A mixture of skeletal myoblast cells according to claim 67 and satellite cells according to claim 68, wherein a proportion of satellite cells out of the amount of all available cells of at least 10% is obtained, preferably at least 15%, more preferably at least 20%, even more preferably at least 30%, determined by the expression of Pax7 by flow cytometry; and/or wherein a proportion of skeletal myoblasts out of the amount of all available cells of at least 40% is obtained, preferably at least 50%, more preferably at least 60%, most preferably at least 70%, determined by the expression of actinin by flow cytometry.

70. Skeletal myotubes, obtained according to step (iv) of claim 1 or claim 39, prepared by a method according to claim 1(i) to (iv) or according to claim 39(i) to (iv), characterized by an anisotropic orientation of the actinin protein-containing sarcomere structure.

71. Use of a skeletal muscle tissue according to claims 61-64, and/or cells according to any one of claims 64-68, and/or skeletal myotubes according to claim 70 in an *in-vitro* drug assay; in particular wherein the drug assay is a toxicity assay or

an assay for skeletal muscle tissue function under the influence of pharmacological and gene therapeutic drug candidates.

72. A skeletal muscle tissue according to claims 61-64 and/or cells according to any one of claims 65-69, and/or of skeletal myotubes according to claim 70 for use in medicine.

73. Satellite cells according to claim 68 for use in the therapy of damaged skeletal muscle and/or in the treatment of skeletal muscle diseases, preferably genetic skeletal muscle defects, in particular Duchenne muscular dystrophy and/or Becker-Kiener muscular dystrophy, and/or lysosomal storage diseases, in particular Pompe disease, preferably wherein the skeletal muscle disease is Duchenne muscular dystrophy.

74. An *in vitro* method for testing the efficacy of a drug candidate on a skeletal muscle tissue, comprising the steps of
    (a) providing a skeletal muscle tissue according to any one of claims 60-63,
    (b) optionally inflicting damage on the skeletal muscle tissue, and
    (c) contacting the skeletal muscle tissue of step (a) or (b) with a drug candidate;

    preferably wherein the method further comprises determining the contraction force and/or the structure of the skeletal muscle tissue and/or the metabolic function and/or molecular parameters and/or protein biochemical parameters before and/or after step (c).

75. An *in vitro* method for testing the toxicity of a substance on a skeletal muscle tissue, comprising the steps of
    (a) providing a skeletal muscle tissue according to any one of claims 61-64,
    (b) contacting the skeletal muscle tissue from step (a) with a substance to be tested,

    preferably wherein the method further comprises determining the contraction force and/or skeletal muscle tissue structure and/or metabolic function and/or molecular parameters and/or protein biochemical parameters before and/or after step (b).

76. An *in vitro* method for testing the effect of nutrients and dietary supplements on skeletal muscle tissue performance, comprising the steps of

(a) providing a skeletal muscle tissue according to any one of claims 61-64,
(b) contacting the skeletal muscle tissue from step (a) with a nutrient or dietary supplement to be tested

preferably wherein the method further comprises determining the contraction force and/or the structure of the skeletal muscle tissue and/or the metabolic function and/or molecular parameters and/or protein biochemical parameters before and/or after step (b).

77. An *in vitro* method for testing the efficacy of a drug candidate on mesodermally differentiated skeletal myoblast progenitor cells, myogenically specified skeletal myoblast progenitor cells, satellite cells, skeletal myoblast cells, skeletal myotubes, or a mixture of skeletal myoblast cells and satellite cells, comprising the steps of:
(a) providing mesodermally differentiated skeletal myoblast progenitor cells, myogenically specified skeletal myoblast progenitor cells, satellite cells, skeletal myoblast cells, skeletal myotubes, or a mixture of skeletal myoblast cells and satellite cells according to any one of claims 65-70,
(b) optionally inflicting damage on the cells from step (a), and
(c) contacting the cells of step (a) or (b) with a drug candidate,

preferably wherein the method further comprises determining the expression of actinin and/or Pax7 before and/or after step (c), wherein the expression can be determined by flow cytometry and/or immunostainings.

78. An *in vitro* method for testing the toxicity of a substance on mesodermally differentiated skeletal myoblast progenitor cells, myogenically specified skeletal myoblast progenitor cells, satellite cells, skeletal myoblast cells, skeletal myotubes, or a mixture of skeletal myoblast cells and satellite cells, comprising the steps of:
(a) providing mesodermally differentiated skeletal myoblast progenitor cells, myogenically specified skeletal myoblast progenitor cells, satellite cells, skeletal myoblast cells, skeletal myotubes, or a mixture of skeletal myoblast cells and satellite cells according to any one of claims 65-70,
(b) contacting the cells of step (a) with a substance to be tested,

preferably wherein the method further comprises determining the expression of actinin and/or Pax7 before and/or after step (b), wherein the expression can be determined by flow cytometry and/or immunostainings.

79. An *in vitro* method for testing the effect of nutrients and dietary supplements on mesodermally differentiated skeletal myoblast progenitor cells, myogenically specified skeletal myoblast progenitor cells, satellite cells, skeletal myoblast cells, skeletal myotubes, or a mixture of skeletal myoblast cells and satellite cells, comprising the steps of:
    (a) providing mesodermally differentiated skeletal myoblast progenitor cells, myogenically specified skeletal myoblast progenitor cells, satellite cells, skeletal myoblast cells, skeletal myotubes, or a mixture of skeletal myoblast cells and satellite cells according to any one of claims 65-70,
    (b) contacting the cells of step (a) with a nutrient or dietary supplement to be tested,

    preferably wherein the method further comprises determining the expression of actinin and/or Pax7 before and/or after step (b), wherein the expression can be determined by flow cytometry and/or immunostainings.

80. The method of any one of claims 1-60, wherein the skeletal muscle tissue generates at least a contraction force of 2 millinewtons (mN) upon a stimulus of 100 Hz, preferably at least 2.3 mN, more preferably at least 2.6 mN, even more preferably at least 3 mM, even more preferably at least 3.3 mN, even more preferably at least 3.6 mN, most preferably at least 4 mN.

81. The method of any one of claims 1-60 or 80, wherein the skeletal muscle tissue has a contraction speed of at least 3 mN/sec upon a stimulation of 100 Hz, preferably at least 4 mN/sec, more preferably at least 5 mN/sec, more preferably at least 6 mN/sec, even more preferably at least 6.5 mN/sec, even more preferably at least 7 mN/sec.

82. The method of any one of claims 1-60, 80, or 81, wherein the skeletal muscle tissue has a relaxation speed of at least 0.5 mN/sec upon termination of a stimulation of 100Hz, preferably at least 0.7 mN/sec, more preferably at least 0.9 mN/sec more preferably at least 1 mN/sec, even more preferably at least 1.2 mN/sec, even more preferably at least 1.5 mN/sec.

83. The method of any one of claims 1-60 or 80-82, wherein the basal medium in step (iv) comprises an effective amount of creatine and/or triiodo-L-thyronine (T3).

84. The method of claim 83, wherein the effective amount of creatine in the basal medium increases the contraction force of the engineered skeletal muscle compared to a maturing in step (iv) without an effective amount of creatine.

85. The method of claim 83 or 84, wherein the effective amount of T3 in the basal medium shortens the contraction speed and/or relaxation speed of the engineered skeletal muscle compared to a maturing in step (iv) without an effective amount of T3.

86. The method of any one of claims 1-60 or 80-85, wherein the basal medium in step (iv) provides a final concentration of 0.1-10 mM creatine, preferably 0.2-6 mM creatine, more preferably 0.4-4 mM creatine, even more preferably 0.6-3 mM creatine, even more preferred 0.7-2.5 mM creatine, even more preferred 0.8-2 mM creatine, even more preferred 0.85-1.5 mM creatine, even more preferred 0.9-1.2 mM creatine, and most preferred about 1 mM creatine.

87. The method of any one of claims 1-60 or 80-86, wherein the basal medium in step (iv) provides a final concentration of 0.001-1 µM triiodo-L-thyronine (T3), preferably 0.005-0.7 µM T3, more preferably 0.01-0.35 µM T3, even more preferred 0.04-0.02 µM T3, even more preferred 0.05-0.18 µM T3, even more preferred 0.06-0.15 µM T3, even more preferred 0.08-0.12 µM T3, even more preferred about 0.1 µM T3.

88. The method of any one of claims 1-60 or 80-87, wherein the skeletal muscle tissue has the property of being self-regenerating.

89. The method of claim 88, wherein the regenerative property is characterized by a recovered contractility and/or muscle recovery, preferably wherein said recovered contractility and/or muscle recovery is measured after irreversible muscle damage with cardiotoxin, more preferably wherein said recovered contractility and/or muscle recovery is measured 10-30 days after incubation with cardiotoxin.

90. The method of any one of claims 1-60 or 80-89, wherein step (iv) is carried out for at least 50 days, more preferably at least 60 days, even more preferably at least 70 days, even more preferably at least 80 days.

91. An engineered skeletal muscle tissue produced by a method according to any one of claims 1-60 or 80-90.

92. The engineered skeletal muscle tissue of any one of claims 61-64 or 91, wherein the skeletal muscle tissue generates at least a contraction force of 0.6 millinewtons (mN) upon a stimulus of 100 Hz, preferably at least 0.7 mN, more preferably at least 0.8 mN, more preferably at least 0.9 mN, more preferably at least 1 mN, more preferably at least 1.2 mN, more preferably at least 1.3 mN, more preferably at least 1.4 mN, more preferably at least 1.5 mN, more preferably at least 1.6 mN, more preferably at least 1.7 mN, more preferably at least 1.8 mN, more preferably at least 1.9 mN, more preferably at least 2 mN, more preferably at least 2.3 mN, more preferably at least 2.6 mN, even more preferably at least 3 mM, even more preferably at least 3.3 mN, even more preferably at least 3.6 mN, and most preferably at least 4 mN.

93. The engineered skeletal muscle tissue of any one of claims 61-64 or 91-92, wherein the skeletal muscle tissue has a contraction speed of at least 3 mN/sec upon a stimulation of 100 Hz, preferably at least 4 mN/sec, more preferably at least 5 mN/sec, more preferably at least 6 mN/sec, even more preferably at least 6.5 mN/sec, even more preferably at least 7 mN/sec.

94. The engineered skeletal muscle tissue of any one of claims 61-64 or 91-93, wherein the skeletal muscle tissue has a relaxation speed of at least 0.5 mN/sec upon termination of a stimulation of 100Hz, preferably at least 0.7 mN/sec, more preferably at least 0.9 mN/sec, more preferably at least 1 mN/sec, even more preferably at least 1.2 mN/sec, even more preferably at least 1.5 mN/sec.

95. Use of a skeletal muscle tissue according to claims 61-64 or 91-94, and/or cells according to any one of claims 65-69, and/or skeletal myotubes according to claim 70 in an *in vitro* drug assay; in particular wherein the drug assay is a toxicity assay, or an assay for skeletal muscle tissue function under the influence of pharmacological and gene therapeutic drug candidates.

96. Skeletal muscle tissue according to claims 61-64 and 91-94, and/or cells according to any one of claims 65-69, and/or of skeletal myotubes according to claim 70 for use in medicine.

Claims

1. A method for producing engineered skeletal muscle tissue from pluripotent stem cells, comprising the steps of
   (i) inducing mesoderm differentiation of the pluripotent stem cells by culturing pluripotent stem cells in a basal medium comprising an effective amount of (a) FGF2, (b) a GSK3 inhibitor, (c) a SMAD inhibitor, and (d) a serum-free additive comprising transferrin, insulin, progesterone, putrescine and selenium or a bioavailable salt thereof;
   (ii) inducing myogenic specification by culturing the cells obtained in step (i) in a basal medium comprising an effective amount of (a) a gamma-secretase/NOTCH inhibitor, (b) FGF2, and (c) a serum-free additive as in (i), followed by
   continuing the cultivation in the medium with the addition of an effective amount of (d) HGF, followed by
   culturing the cells in a basal medium comprising an effective amount of (a) a gamma secretase/NOTCH inhibitor, (b) HGF, (c) a serum-free additive as in (i), and (d) knockout serum replacement (KSR);
   (iii) expanding and maturing the cells into skeletal myoblasts and satellite cells by culturing the cells obtained in step (ii) in a basal medium comprising an effective amount of (a) HGF, (b) a serum-free additive as in (i), and (c) knockout serum replacement (KSR);
   (iv) maturing the cells into skeletal myotubes and satellite cells by culturing the cells obtained in step (iii), which are dispersed in an extracellular matrix, under mechanical stimulation in a basal medium, comprising an effective amount of (a) a serum-free additive as in step (i), and (b) an additional serum-free additive comprising albumin, transferrin, ethanolamine, selenium or a bioavailable salt thereof, L-carnitine, fatty acid additive, and triiodo-L-thyronine (T3);
   thereby producing engineered skeletal muscle tissue.

2. The method of claim 1, wherein the skeletal muscle tissue generates a contraction force of at least 0.6 millinewtons (mN) upon a stimulus of 100 Hz.

3. The method of claim 1 or 2, wherein in step (iv), the mechanical stimulation is a static tension, a dynamic stimulation, or an auxotonic stimulation.

4. The method of any one of claims 1-3, comprising, prior to step (i), a seeding step, wherein the pluripotent stem cells are seeded in a stem cell medium in the presence of a ROCK inhibitor.

5. The method of any one of claims 1-4, wherein after step (iii), the skeletal myoblasts and satellite cells are seeded into an engineered form in an additional step prior to step (iv) in the presence of one or more components of an extracellular matrix in a master mix.

6. The method of claim 5, wherein the engineered form has the form of a ring, ribbon, strand, patch, pouch, or cylinder, wherein optionally individual skeletal muscle tissues are fused.

7. The method of any one of claims 1-6, wherein the method does not comprise a differentiation- or maturation-related transgene.

8. The method of any one of claims 1-7, wherein the basal medium in step (iv) comprises an effective amount of creatine and/or triiodo-L-thyronine (T3).

9. The method of any one of claims 1-8, wherein the skeletal muscle tissue has a contraction speed of at least 3 mN/sec upon a stimulation of 100 Hz.

10. The method of any one of claims 1-9, wherein the skeletal muscle tissue has a relaxation speed of at least 0.5 mN/sec upon termination of a stimulation of 100Hz.

11. An engineered skeletal muscle tissue, having multinuclear mature skeletal muscle fibers with satellite cells, and having no blood supply and/or no central nervous system control, wherein the skeletal muscle tissue is serum-free and/or does not comprise a differentiation- or maturation-related transgene.

12. The engineered skeletal muscle tissue of claim 11, wherein the skeletal muscle tissue does not comprise a myogenic transgene.

13. The engineered skeletal muscle tissue of claim 12, wherein the skeletal muscle tissue does not comprise the Pax7 or MyoD transgene.

14. The engineered skeletal muscle tissue of any of claims 11-13, wherein the skeletal muscle tissue generates at least a contraction force of 0.6 millinewtons (mN) upon a stimulus of 100 Hz.

15. The engineered skeletal muscle tissue of any of claims 10-14, wherein the skeletal muscle tissue has a contraction speed of at least 3 mN/sec upon a stimulation of 100 Hz.

16. The engineered skeletal muscle tissue of any of claims 10-15, wherein the skeletal muscle tissue has a relaxation speed of at least 0.5 mN/sec upon termination of a stimulation of 100Hz.

17. A skeletal muscle tissue according to any of claims 10-16 for use in medicine.

18. An *in vitro* method for testing the efficacy of a drug candidate on a skeletal muscle tissue, comprising the steps of
    (a) providing a skeletal muscle tissue according to any of claims 10-17,
    (b) optionally inflicting damage on the skeletal muscle tissue, and
    (c) contacting the skeletal muscle tissue of step (a) or (b) with a drug candidate.

19. An *in vitro* method for testing the toxicity of a substance on a skeletal muscle tissue, comprising the steps of
    (a) providing a skeletal muscle tissue according to any of claims 10-17,
    (b) contacting the skeletal muscle tissue from step (a) with a substance to be tested.

20. An *in vitro* method for testing the effect of nutrients and dietary supplements on skeletal muscle tissue performance, comprising the steps of
    (a) providing a skeletal muscle tissue according to any of claims 10-17,
    (b) contacting the skeletal muscle tissue from step (a) with a nutrient or dietary supplement to be tested.