Hwang, Varghese et al. 2008, Jukes, Both et al. 2008, Yamashita, Krawetz et al. 2008). Recent studies have reported the use of defined culture media with specific pathway agonists and antagonists to direct differentiation (Nakayama, Duryea et al. 2003, Darabi, Gehlbach et al. 2008, Tanaka, Jokubaitis et al. 2009). In mESCs, Tanaka et al (2009) showed that the combination of Wnt signaling with BMP inhibition resulted in the generation of paraxial mesoderm with chondrogenic potential, identified by the expression of PDGFRalpha and a lack of expression of Flk-1. This mesoderm also displayed some cardiac potential but showed no capacity to generate hematopoietic cells indicating that dependency on BMP signaling distinguishes different types of mesoderm.

[0008] Oldershaw et al. (Oldershaw, Baxter et al. 2010) used a serum free protocol. No tissues were obtained in vitro or in vivo with the method of Oldershaw.

[0009] Umeda et al (Umeda, Zhao et al. 2012) used a method using PDGF stimulation that produced nodules comprising Runx2 expressing cells.

[0010] Osteoarthritis is a degenerative disease that mainly affects the joint-lining articular cartilage of the joint. Articular cartilage has very limited capacity to regenerate itself upon injury, thus cell and tissue replacement strategies are the only means of replacing this tissue effectively. Methods of producing human cartilage from pluripotent stem cells are currently lacking, despite great need for such tissues for drug discovery and cartilage replacement strategies in patients with joint diseases such as osteoarthritis.

Summary

[0011] An aspect, the present invention refers to a method for generating articular-like nonhypertrophic chondrocyte cells, hypertrophic chondrocyte-like cells, or cartilage-like tissue, the method comprising: a. culturing a CD56+ and PDGFRα+ primitive streak-like mesoderm population, for at least 1, 2, 3 or 4 days, with a paraxial mesoderm specifying cocktail comprising: i. an FGF agonist; ii. a BMP inhibitor; and iii. a TGFβ inhibitor; to generate a CD73+CD105+, CD73+PDGFRβ+, or CD73+CD105+PDGFRβ+ paraxial mesoderm population; b. culturing the paraxial mesoderm population at a high cell density with a TGFβ agonist to produce a Sox9+and collagen 2+ chondrocyte precursor population; and c. either i. further culturing the high cell density Sox9+, collagen 2+ chondrocyte precursor population with the TGFβ agonist for at least 10 days to produce an articular like non-hypertrophic chondrocyte cells and/or cartilage like tissue; or ii. culturing the high cell density Sox9+ collagen2+ chondrocyte precursor population with a BMP4 agonist for a period of time of at least 3 weeks to produce a hypertrophic chondrocyte like cells and/or cartilage like tissue. In one example of the application describes a method for generating chondrocyte lineage cells and/or cartilage like tissue, optionally articular like non-hypertrophic chondrocyte cells and/or cartilage like tissue and/or hypertrophic chondrocyte like cells and/or cartilage like tissue, the method comprising:

(a) culturing a primitive streak-like mesoderm cell population (e.g. stage 2), optionally a CD56+, PDGFRalpha+ primitive streak-like mesoderm cell population, with a paraxial mesoderm specifying cocktail comprising:

(i) a FGF agonist;

(ii) a BMP inhibitor, optionally Noggin, LDN-193189, and/or Dorsomorphin; and

(iii) optionally one or more of a TGFbeta inhibitor, optionally SB431542; and a Wnt inhibitor, optionally IWP2 (N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d]pyrimidin-2-yl)thio]-acetamide; Sigma); Dickkopf-related protein 1 (DKK1; R & D Systems), and/or XAV939 (3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-one; Sigma);

to specify a paraxial mesoderm cell population expressing cell surface CD73, CD105 and/or PDGFR-beta;

(b) generating a chondrocyte precursor population from the paraxial mesoderm cell population expressing cell surface CD73, CD105 and/or PDGFR-beta, the generating the chondrocyte precursor population comprising:

(i) culturing the paraxial mesoderm cell population expressing cell surface CD73, CD105 and/or PDGFR-beta at a high cell density in serum free or serum containing media;

(ii) culturing the high cell density CD73+, CD105+ and/or PDGFRbeta+ paraxial mesoderm cell population with a TGFbeta agonist, optionally TGFB1, TGFB2 and/or TGFB3 in serum free media to produce a high cell density Sox9+, collagen 2+ chondrocyte precursor population (e.g. Stage 3); and

(c) either:

(i) culturing the high cell density Sox9+, collagen 2+ chondrocyte precursor population with a TGFbeta agonist (optionally TGFBeta1, TGFbeta2 and/or TGFBeta3) for an extended period of time to produce articular like non-hypertrophic chondrocyte like cells and/or cartilage like tissue; or

(ii) culturing the high cell density Sox9+ collagen2+ chondrocyte precursor population with a BMP4 agonist for an extended period of time to produce hypertrophic chondrocyte like cells and/or cartilage like tissue (e.g. stage 4).

[0012] Another example includes a method for generating chondrocyte like cells and/or cartilage like tissue, optionally articular like non-hypertrophic chondrocyte like cells and/or cartilage like tissue and/or hypertrophic chondrocyte like cells and/or cartilage like tissue, the method comprising:

(a) culturing a starting population of pluripotent stem cells with a primitive streak inducing cocktail to induce a primitive streak-like mesoderm cell population expressing CD56 and/or PDGFR-alpha (e.g. stage 1);

(b) culturing the primitive streak-like mesoderm cell population expressing CD56 and PDGFR-alpha with a paraxial mesoderm specifying cocktail comprising:

(i) a FGF agonist;

(ii) a BMP inhibitor, optionally Noggin, LDN-193189, and/or Dorsomorphin; and

(iii) one or more of a TGFbeta inhibitor, optionally SB431524; and a Wnt inhibitor, optionally DKK1, IWP2 and/or XAV939;

to specify a paraxial mesoderm cell population expressing cell surface CD73, CD105 and PDGFR-beta;

(c) generating a chondrocyte precursor population from the paraxial mesoderm cell population expressing cell surface CD73, CD105 and/or PDGFR-beta, the generating the chondrocyte precursor population comprising:

(i) culturing the paraxial mesoderm cell population expressing CD73, CD105 and/or PDGFR-beta at a high cell density in serum free or serum containing media;

(ii) culturing the high cell density CD73+, CD105+ and PDGFRbeta+ paraxial mesoderm cell population with a TGFbeta agonist in serum free media to produce a high cell density Sox9+, collagen 2+ chondrocyte precursor population; and

(d) either

(i) culturing the high cell density Sox9+, collagen 2+ chondrocyte precursor population with a TGFbeta agonist for an extended period of time to produce articular like non-hypertrophic chondrocyte like cells and/or cartilage like tissue; or

(ii) culturing the high cell density Sox9+ collagen2+ chondrocyte precursor population with a BMP4 agonist for an extended period of time to produce hypertrophic chondrocyte like cells and/or cartilage like tissue.

[0013] In an example, the method is for generating articular like non-hypertrophic chondrocyte like cells and/or cartilage like tissue. In another embodiment, the method is for generating hypertrophic chondrocyte like cells and/or cartilage like tissue.

[0014] In an example, the extended period of time the high cell density Sox9+ collagen2+ chondrocyte precursor population is cultured with the TGF beta agonist is at least 3 weeks, at least 4

weeks, at least 5 weeks, at least 6 weeks, at least 7 weeks, at least 8 weeks, at least 9 weeks, at least 10 weeks, at least 11 weeks, at least 12 weeks or more to generate a non-hypertrophic chondrocyte like and/or cartilage like tissue that expresses for example lubricin and CILP2.

[0015] In an example, the extended period of time the high cell density Sox9+ collagen2+ chondrocyte precursor population is cultured with the BMP4 agonist is at least 3 weeks, at least 4 weeks, at least 5 weeks, at least 6 weeks, at least 7 weeks, at least 8 weeks, at least 9 weeks, at least 10 weeks, at least 11 weeks, at least 12 weeks or more to generate a cartilage like tissue that expresses collagen 2 or hypertrophic chondrocyte cells that express collagen 10.

[0016] In one example, there is disclosed a method of generating chondrocyte like cells comprising: (a) culturing chondrocyte precursor cells at a high cell density in serum free or serum containing media; (b) culturing the high cell density chondrocyte precursor cells with a TGFbeta agonist in serum free media; and (c) either (i) culturing the chondrocyte precursor cells with a TGFbeta agonist for an extended period of time to produce articular cartilage like chondrocyte cells; or (ii) culturing the chondrocyte precursor cells with a BMP4 agonist for an extended period of time to produce hypertrophic chondrocyte lineage cells and/or cartilage like tissue.

[0017] In an example, the chondrocyte precursor cells are primary fetal chondrocytes or passaged fetal chondrocytes.

[0018] In an example, the generated cells and/or tissues are administered to a subject.

[0019] In another aspect, the present disclosure refers to an isolated population generated according to the method disclosed herein. Also described in another example is an isolated population of articular like non-hypertrophic chondrocyte like cells and/or cartilage like tissue and/or hypertrophic chondrocyte like cells and/or cartilage like tissue generated according to a method described herein.

[0020] A further example includes composition comprising the population of articular like non-hypertrophic chondrocyte like cells and/or cartilage like tissue and/or hypertrophic chondrocyte like cells and/or cartilage like tissue, and a carrier optionally PEG, hydrogel, bone scaffolding, bone substitute scaffolding and/or matrigel. In an example, the carrier is pharmaceutical grade.

[0021] In an example, the isolated population is comprised in a composition comprising a diluent or carrier, optionally a pharmaceutical diluent. In an embodiment, the diluent is culture media, optionally comprising a cryopreservation agent such as glycerol and/or DMSO, serum and albumin, such as human serum albumin.

[0022] In yet another aspect, the present disclosure refers to a cartilage or bone tissue product comprising the isolated populations of cells or composition disclosed herein, and a scaffold, preferably wherein the scaffold is a bone substitute. A further example includes a cartilage and/or bone tissue product comprising the cells or composition described herein, and a scaffold.

[0023] Another example includes method for ameliorating symptoms and/or treating a subject in need thereof comprising administering cells and/or tissue generated using a method described herein and/or transplanting a cartilage and/or bone tissue product described herein.

[0024] Also described in another example is use of cells and/or tissue generated using a method described herein and/or a cartilage and/or bone tissue product comprising said cells and/or tissue for ameliorating symptoms and/or treating a subject in need thereof. In another aspect, the present disclosure refers to the isolated population of cells as disclosed herein and/or the product disclosed herein for use in a method of ameliorating symptoms and/or treating osteoarthritis. In another aspect, the present disclosure refers to use of the isolated population of cells as disclosed herein and/or the product disclosed herein in the manufacture of a medicament for treating osteoarthritis.

[0025] A further example includes a method of generating a paraxial mesoderm cell population comprising: (a) culturing a starting population of pluripotent stem cells with a primitive streak inducing cocktail to induce a primitive streak-like mesoderm cell population expressing CD56 and PDGFR-alpha (e.g. stage 0); (b) culturing a primitive streak-like mesoderm cell population expressing CD56 and PDGFR-alpha with a paraxial mesoderm specifying cocktail comprising: (i) a FGF agonist; and (ii) a BMP inhibitor, optionally Noggin, LDN-193189, Dorsomorphin; and (iii) one or more of a TGFbeta inhibitor, optionally SB431524; and a Wnt inhibitor, optionally DKK1, IWP2, and/or XAV939; to specify a paraxial mesoderm cell population expressing cell surface CD73, CD105 and PDGFR-beta.

[0026] Methods of isolating cells and screening assays are also described. In another aspect, the present disclosure refers to an isolated paraxial chondrogenic mesoderm population of cells prepared according to the method disclosed herein.

[0027] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the disclosure are given by way of

illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

Brief description of the drawings

[0028] An embodiment of the present disclosure will now be described in relation to the drawings in which:

[0029] **Figure 1**. Serum-free differentiation of paraxial mesoderm, chondrocyte progenitors, and cartilage tissues from human pluripotent stem cells (hPSCs). (A) hPSCs are differentiated in 4 stages including the induction of a primitive streak-like mesoderm population (stage 1) using Activin A, BMP4 and basic (b) FGF from days 1 to 4 of differentiation as embryoid bodies. On day 4 (T4), mesoderm populations are monitored by the expression of CD56 and PDGFRa on the cell surface by flow cytometry (B). Day 4 mesoderm cells are specified to a paraxial mesoderm fate in monolayer culture by treatment of Dorsomorphin, a BMP inhibitor, a TGFbeta inhibitor SB431542, and bFGF, from days 4 to 6 and bFGF from days 4 to 15 (stage 2). Day 15 paraxial mesoderm cells can generate chondrocyte progenitors by plating in a high density 'spot' termed micromass, or by plating onto collagen coated membrane filters (not shown) in the presence of TGFB3 for approximately 10 days (stage 3). Chondrocyte progenitors can be specified to articular chondrocytes or growth plate-like chondrocytes in a cartilage tissue format during stage 4 of differentiation by extended stimulation with TGFB3 (articular) or BMP3 (growth plate-like) for example for 12 weeks. Tissues have been kept in culture for at least 7 months. Efficient induction of a primitive streak-like population from hESCs (C) and hIPSCs (D,E) was confirmed by the expression of CD56 and PDGFRα by flow cytometry on day 3 of differentiation. hESCs were induced to generate a primitive streak population with the following

Claims:

1. A method for generating articular-like nonhypertrophic chondrocyte cells, hypertrophic chondrocyte-like cells, or cartilage-like tissue, the method comprising:
    a. culturing a $CD56^+$ and $PDGFR\alpha^+$ primitive streak-like mesoderm population, for at least 1, 2, 3 or 4 days, with a paraxial mesoderm specifying cocktail comprising:
        i. an FGF agonist;
        ii. a BMP inhibitor; and
        iii. a TGFβ inhibitor;

    to generate a $CD73^+CD105^+$, $CD73^+PDGFR\beta^+$, or $CD73^+CD105^+PDGFR\beta^+$ paraxial mesoderm population;

    b. culturing the paraxial mesoderm population at a high cell density with a TGFβ agonist to produce a $Sox9^+$and collagen 2^+ chondrocyte precursor population; and
    c. either
        i. further culturing the high cell density $Sox9^+$, collagen 2^+ chondrocyte precursor population with the TGFβ agonist for at least 10 days to produce an articular like non-hypertrophic chondrocyte cells and/or cartilage like tissue; or
        ii. culturing the high cell density Sox9+ collagen2+ chondrocyte precursor population with a BMP4 agonist for a period of time of at least 3 weeks to produce a hypertrophic chondrocyte like cells and/or cartilage like tissue.

2. The method of claim 1, wherein the primitive streak inducing cocktail further comprises a Wnt agonist

3. The method of claim 2, wherein the Wnt agonist is selected from Wnt3a, CHIR-99021, and 6 BromoIndirubin-3'-Oxime.
4. The method of claim 1, wherein the paraxial mesoderm is specified in a monolayer culture.

5. The method of any one of claims 1 to 4, wherein the TGF-β agonist, is selected from the group consisting of TGF-β1, TGF β2, and TGF-β3, non-hypertrophic chondrocyte cells and/or cartilage like tissue are produced.

6. The method of any one of claims 1 to 4, wherein the with BMP4 agonist is selected from GDF5, GDF6, GDF7, BMP4, BMP2, BMP6, BMP7, and BMP10 to produce a hypertrophic chondrocyte like cells and/or cartilage like tissue are produced.

7. The method of any one of claims 1 to 7, wherein the FGF agonist is selected from the group consisting of FGF-2, , FGF-19, FGF 21, FGF-3, FGF-5, FGF-6, FGF-8a, FGF-16, FGF-17, FGF-18, FGF 20 and FGF-23.

8. The method of any one of claims 1 to 7, wherein the primitive streak-like mesoderm population is contacted with the FGF agonist for at least 5 days, 6 days, 7 days, 8 days, 9 days, 10, days, or 11 days or more to increase the proportion of cells expressing CD73 and/or CD105 by at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% or 65% compared to an FGF agonist untreated primitive streak-like mesoderm population.

9. The method of claim 1, wherein the paraxial mesoderm population further expresses transcription factors Meox1 and Nkx3.2 and is negative for Nkx2.5.

10. The method of claim 1, wherein the paraxial mesoderm population is plated in a high cell density format selected from a micromass culture, pellet culture or a filter culture wherein the paraxial mesoderm population is plated at a cell density of at least 10 million cells per 1 ml, 20 million cells/ml, 30 million cells/ml, 40 million cells/ml or 50 million cells/ml; and the membrane filter culture comprises about 500,000, about 750,000, about 1 million, about 1.25 million, about 1.5 million, about 1.75 million, or about 2 million cells.

11. The method of claim 1, wherein the paraxial mesoderm population is cultured with the TGFβ agonist in serum free media for about 14 days, to produce the chondrocyte precursor population that expresses the Sox9+ and collagen 2+, wherein the TGFβ agonist is TGFβb3.

12. The method of claim 1, wherein the chondrocyte precursor population is cultured with the TGFβ agonist until the population expresses lubricin or cartilage intermediate layer protein 2 (CILP2).

13. The method of any one of claims 1 to 12, wherein:
the steps are performed in vitro; and/or
the generated cells and/or tissues are combined with a carrier that is suitable for use in transplantation.

14. An isolated population generated according to the method of any one of claims 1 to 12.

15. The isolated cell population of claim 14, further comprising endothelial cells or fibroblasts.

16. A cartilage or bone tissue product comprising the isolated populations of cells or composition of claim 14 or 15, and a scaffold, preferably wherein the scaffold is a bone substitute.

17. The isolated population of cells any one of claims 14 or 15 and/or the product of claim 16 for use in a method of ameliorating symptoms and/or treating osteoarthritis.

18. The population of cells and/or tissues for the use of claim 17, wherein:
    (a) the population of cells is induced from autologous cells; and/or
    (b) the population of cells is enriched for articular like non-hypertrophic chondrocyte cells and/or cartilage like tissue,

    wherein the subject has a joint condition such as osteoarthritis, osteochondritis dissecans, polychondritis, and other chondropathies, or joint injuries affecting the cartilage.

19. The population of cells and/or tissues for the use of claim 17, wherein the population of cells is induced from autologous cells; and/or the subject has a bone condition such as a bone fracture, bone break or is in need of a bone replacement for example due to a malignancy, trauma or has achondroplasia, osteogenesis imperfect, osteoporosis or other osteopathies, optionally wherein the population of cells is enriched for hypertrophic chondrocyte like cells and/or cartilage like tissue, preferably wherein the enriched hypertrophic chondrocyte like cells and/or cartilage like tissue, is adhered to a scaffold.

20. The method of any one of claims 1 to 13, wherein the method additionally includes a step of isolating paraxial chondrogenic mesoderm population of cells comprising:
    a. contacting a population of cells comprising paraxial chondrogenic mesoderm cells with a cocktail comprising a CD73 specific binding agent, CD105 specific binding agent, and a PDGFRβ specific binding agent; and
    b. enriching for $CD73^+$, $CD105^+$ and $PDGFR\beta^+$ cells.

21. The method of claim 20 wherein the cells are enriched using flow cytometry.

22. An isolated paraxial chondrogenic mesoderm population of cells prepared according to the method of claim 20 or 21.

23. The method of claim 1, wherein the TGFβ agonist is TGFβ3 and wherein the CD56+ and PDGFRα+ primitive streak-like mesoderm cell population, is generated by iPSCs for about 1 to about 5 days with a primitive streak inducing cocktail, comprising:
    i. Activin A
    ii. an BMP4 agonist, selected from BMP4, BMP2, BMP6, BMP7, and BMP10; and
    iii. an FGF agonist, selected from bFGF, FGF-4, FGF-9, or a combination thereof.

24. The method of claim 23, wherein the PSCs wherein the iPSCs are aggregated into embryoid bodies.

25. The method of claim 1, wherein the BMP inhibitor is selected from Noggin, LDN-193189, Dorsomorphin (DM), Chordin, a type 1 BMP receptor inhibitor, a BMP ligand, soluble BMPR1a, and soluble BMPR1b.

26. The method of claim 1, wherein the TGFβ inhibitor is SB431542.

27. The method of claim 1, wherein the paraxial mesoderm specifying cocktail further comprise Wnt inhibitor, selected from DKK1, IWP2, or XAV939.

28. The method of claim 1, wherein the paraxial mesoderm population at a high cell density in serum free or serum containing media.

29. The method of claim 28, wherein the paraxial mesoderm population is cultured for less than 1 day, 1 day, 2 days, 3 days or 4 days.

30. The method of claim 1, wherein the TGF-β agonist is selected from TGF-β1, TGF-β2, and TGF-β3.

31. Use of the isolated population of cells any one of claims 14 or 15 and/or the product of claim 16 in the manufacture of a medicament for treating osteoarthritis.