

phenotypic variability. This is partially due to a routine use of Matrigel as a neurogenesis supporting substrate.

[005] Another drawback associated with known methods is a lack of neuronal network function of the produced organoids, which thus significantly limits any investigation into neuronal functionality and plasticity. Disease modeling and drug development is also restricted due to the remote phenotypic resemblance of known produced neuronal organoid structures compared to normal brain tissue.

[006] Thus, there is a need for methods for generating neuronal organoids based on a use of chemically defined components in order to allow consistency in the structures produced. Furthermore, there is a need to produce neuronal organoids capable of forming functional neuronal networks in order to meaningfully mimic natural neural structures.

[007] The present invention provides methods permitting both robust and reproducible neural differentiation in a well-defined 3D cell culture system, which further provides a sound basis for investigations into the formation, and plasticity characteristics, of a functional neuronal network.

[008] The organoids of the present invention further offer a valuable tool in drug development. For example, such organoids can meaningfully decrease the typically exorbitant costs associated with both preclinical and clinical drug development, at least because of a decreased need for animal-based experimentation and a reduced number of patients required in clinical trials when predicting post marketing outcome. In addition, novel biologicals (e.g., non-coding RNA therapeutics) and genome editing (e.g., using CRISPR-based platforms) can be tested efficiently in human models. The rapid developments in this field therefore benefit from the availability of highly predictive human organoid models such as those provided by the present invention.

[009] **SUMMARY**

[0010] The present disclosure relates to a method for producing a bioengineered neuronal organoid (BENO) from pluripotent stem cells (PSCs), the method comprising:

- (A) providing a source of PSCs;
- (B) culturing the PSCs of step (A), embedded in a matrix immersed in cell culture medium;
- (C) culturing the PSCs in said matrix of step (B) in cell culture medium comprising a Rho-associated kinase inhibitor (ROCKi) and FGF-2;
- (D) culturing the forming BENO originating from PSCs and matrix of step (C) in cell culture medium comprising retinoic acid and one or more inhibitors of SMAD signaling to induce neurogenesis;

- (E) culturing the forming BENO of step (D) in cell culture medium comprising TGF-beta and FGF-2 to enhance genesis of stromal cells and neurogenesis;
- (F) culturing the forming BENO of step (E) in cell culture medium comprising TGF-beta and one or more inhibitors of notch signaling to enhance genesis of stromal cells and neurodifferentiation.

[0010a] In a first aspect, there is provided a method for producing a bioengineered neuronal organoid (BENO) from pluripotent stem cells (PSCs), the method comprising:

- (A) culturing PSCs, embedded in a matrix immersed in serum-free cell culture medium;
- (B) culturing the PSCs in said matrix of step (A) in cell culture medium comprising a Rho-associated kinase inhibitor (ROCKi) and FGF-2;
- (C) culturing the forming BENO originating from PSCs and matrix of step (B) in cell culture medium comprising retinoic acid and one or more inhibitors of SMAD signaling to induce neurogenesis;
- (D) culturing the forming BENO of step (C) in cell culture medium comprising TGF-beta and FGF-2 to enhance genesis of stromal cells and neurogenesis;
- (E) culturing the forming BENO of step (D) in cell culture medium comprising TGF-beta and one or more inhibitors of notch signaling to enhance genesis of stromal cells and neurodifferentiation,

wherein the matrix comprises collagen.

[0011] In some examples, the matrix does not comprise Matrigel. In other examples, the matrix does not comprise Matrigel or other components of natural origin having a non-defined composition. In preferred examples, the matrix comprises collagen. In most preferred examples, the matrix comprises type I collagen. In some examples, the matrix is collagen. In some examples, the matrix is collagen I.

[0012] In some examples, the BENO is produced within a 3D environment, preferably wherein the 3D environment is defined by the matrix.

[0013] In some examples, the stromal cells comprise glial cells.

[0014] In some examples, the medium of step (D) comprises at least two inhibitors of SMAD signaling, preferably wherein the inhibitors of SMAD signaling comprise noggin and SB 431542. In some examples, the inhibitors of SMAD signaling are noggin and SB 431542.

[0015] In some examples, the inhibitor of notch signaling of step F is DAPT.

[0016] In one example, the inhibitors of SMAD signaling are noggin and SB 431542 and the inhibitor of notch signaling of step F is DAPT.

[0017] In some examples, the matrix is collagen employed at a concentration between 0.05 mg/ml and 50 mg/ml, preferably between 0.1 mg/ml and 10 mg/ml, more preferably between 0.5 mg/ml and 5 mg/ml, most preferably at a concentration of 1 mg/ml.

[0018] In some examples, retinoic acid is employed at an effective concentration between 0.01 μ M and 100 μ M, preferably between 0.1 μ M and 10 μ M, more preferably between 0.5 μ M and 5 μ M, most preferably at a concentration of 1 μ M. In some examples, noggin is employed at an effective concentration of 0.1 ng/ml – 1 μ g/ml, preferably 1 ng/ml – 500 ng/ml, more preferably 10 ng/ml – 200 ng/ml, most preferably 50 ng/ml. In some examples, SB 431542 is employed at an effective concentration of 0.1 μ M – 1 mM, preferably 1 μ M – 100 μ M, more preferably between 5 μ M and 50 μ M, most preferably 10 μ M. In some examples, TGF-beta is employed at an effective concentration of between 0.1 ng/ml and 100 ng/ml, preferably between 0.3 ng/ml and 30 ng/ml, more preferably between 1 ng/ml and 10 ng/ml, most preferably at a concentration of 5 ng/ml. In some examples, FGF-2 is employed at an effective concentration of 0.1 ng/ml and 1 μ g/ml, preferably between 1 ng/ml and 100 ng/ml, more preferably between 5 ng/ml and 50 ng/ml, most preferably at a concentration of 10 ng/ml. In some examples, DAPT is employed at an effective concentration of between 0.01 μ M and

100 μ M, preferably between 0.1 μ M and 10 μ M, more preferably between 0.5 μ M and 5 μ M, most preferably at a concentration of 2.5 μ M.

[0019] In some examples, retinoic acid is employed at a concentration of between 0.5 μ M and 5 μ M, noggin is employed at a concentration of 10 ng/ml – 200 ng/ml, SB 431542 is employed at a concentration of between 5 μ M and 50 μ M, TGF-beta is employed at a concentration of between 1 ng/ml and 10 ng/ml, FGF-2 is employed at a concentration of between 5 ng/ml and 50 ng/ml, and DAPT is employed at a concentration of between 0.5 μ M and 5 μ M.

[0020] In some examples, retinoic acid is employed at a concentration of between 0.5 μ M and 5 μ M, noggin is employed at a concentration of 10 ng/ml – 200 ng/ml, SB 431542 is employed at a concentration of between 5 μ M and 50 μ M, TGF-beta is employed at a concentration of between 1 ng/ml and 10 ng/ml, FGF-2 is employed at a concentration of between 5 ng/ml and 50 ng/ml, DAPT is employed at a concentration of between 0.5 μ M and 5 μ M, and the matrix is collagen employed at a concentration between 0.1 mg/ml and 10 mg/ml.

[0021] In some examples, retinoic acid is employed at a concentration of 1 μ M, noggin is employed at a concentration of 50 ng/ml, SB 431542 is employed at a concentration of 10 μ M, TGF-beta is employed at a concentration of 5 ng/ml, FGF-2 is employed at a concentration of 10 ng/ml, and DAPT is employed at a concentration of 2.5 μ M.

[0022] In some examples, retinoic acid is employed at a concentration of 1 μ M, noggin is employed at a concentration of 50 ng/ml, SB 431542 is employed at a concentration of 10 μ M, TGF-beta is employed at a concentration of 5 ng/ml, FGF-2 is employed at a concentration of 10 ng/ml, DAPT is employed at a concentration of 2.5 μ M, and the matrix is collagen employed at a concentration between 0.5 mg/ml and 5 mg/ml

[0023] In some example, the PSCs are animal cells. In some example, the PSCs are mammal cells. In some example, the PSCs are rodent (e.g., mouse or rat) or human cells. In preferred examples, the PSCs are human PSCs. The PSCs of the present disclosure are not produced using a process involving a modification to the germ line genetic identity of a human being or involving a use of a human embryo for industrial or commercial purposes.

[0024] The different steps of the disclosure are performed for various periods. In one example, step (A) and step (B) are performed on day -1. In one example step (C) is performed from day -1 to day 0. In one example step (D) is performed from day 0 to day 8. In one example step (E) is performed from day 8 to day 15. In one example step (F) is performed from day 15 to at least day 28. In one example, step (A) and step (B) are performed on day -1 and step (C) is performed from day -1 to day 0. In one example, step (A) and step (B) are performed on day -1, step (C) is performed from day -1 to day 0 and step

(D) is performed from day 0 to day 8. In one example step (A) and step (B) are performed on day -1, step (C) is performed from day -1 to day 0, step (D) is performed from day 0 to day 8, and step (E) is performed from day 8 to day 15. In one example, step (A) and step (B) are performed on day -1, step (C) is performed from day -1 to day 0, step (D) is performed from day 0 to day 8, step (E) is performed from day 8 to day 15 and step (F) is performed from day 15 to at least day 28. In one example, step (A) and step (B) are performed on day -1, step (C) is performed from day -1 to day 0, step (D) is performed from day 0 to day 10, step (E) is performed from day 10 to day 15 and step (F) is performed from day 15 to at least day 28.

[0025] In one example, the disclosure provides neuronal organoids, e.g., bioengineered neuronal organoids (BENOs), characterized in that the neuronal cells of the neuronal organoid are organized in a functional neuronal network. In one example, the disclosure provides bioengineered neuronal organoids (BENOs) produced by the method of the disclosure.

[0025a] In a second aspect, there is provided a neuronal organoid, characterized in that that the neuronal cells of the neuronal organoid are organized in a functional neuronal network, and wherein the neuronal network is characterized by the synchronization of neurons.

[0025b] In a third aspect, there is provided a bioengineered neuronal organoid (BENO) produced by the method of the first aspect, characterized in that the neuronal cells of the BENO are organized in a functional neuronal network, and wherein the neuronal network is characterized by the synchronization of neurons.

[0026] In some examples, the disclosure is directed at the use of BENOs produced by methods of the disclosure as model for a disease. In some examples, the disclosure is directed at the use of BENOs produced by methods of the disclosure as models for a disease related to neural tissue. In some examples, the disclosure is directed at the use of BENOs produced by methods of the disclosure as models for a disease selected from the group consisting of stroke, brain inflammation disorders, neurodegenerative diseases, neuroinflammatory diseases, traumatic injury, channelopathy, and psychiatric diseases. In some examples, the disclosure is directed at the use of BENOs produced by methods of the disclosure as models for a disease selected from the group consisting of neurodegenerative diseases (such as Parkinson's disease, Alzheimer's disease), neuroinflammatory diseases (e.g., multiple sclerosis), traumatic injury (e.g., brain-surgery-induced injury), channelopathy (e.g., epilepsy) and psychiatric diseases (e.g., autism, schizophrenia).

[0027] In other examples, the disclosure is directed at the use of BENOs produced by methods of the disclosure as model for a disease, wherein the BENOs are in co-culture with another tissue engineering platform. In some examples, the other tissue engineering platform is selected from the group consisting of EHM (Engineered Heart Muscle), BSMs (bioengineered skeletal muscle), ESM (Engineered Skeletal Muscle), ELT (Engineered Liver Tissue), and ECT (Engineered Connective Tissue). In other examples, the other tissue engineering platform is selected from the group consisting of tumor models (e.g., tumor brain invasion, metastases spread) and leukocyte infiltration models (e.g., autoimmune disease).

[0028] In some examples, the disclosure is directed at the use of BENOs produced by methods of the disclosure in drug screening, such as drug discovery and drug refinement by phenotypic drug screening. This use of BENOs includes, but is not limited to the discovery and refinement

of drugs that may induce or enhance repair, regeneration, protection, and disease prevention in brain and neural tissue.

[0028a] In a fourth aspect, there is provided use of a BENO of the second or third aspect as model for a disease and/or drug screening.

[0029] In other examples, the disclosure is directed at a kit for practicing a method of the disclosure. In some examples, the kit contains PSCs, a matrix, suitable media and the required supplements (ROCKi, FGF-2, retinoic acid, one or more inhibitors of SMAD signaling, TGF-beta, and one or more inhibitors of notch signaling). In other examples, a kit contains a matrix, suitable media and the required supplements (ROCKi, FGF-2, retinoic acid, one or more inhibitors of SMAD signaling, TGF-beta, and one or more inhibitors of notch signaling). In other examples, a kit contains a matrix and the required supplements (ROCKi, FGF-2, retinoic acid, one or more inhibitors of SMAD signaling, TGF-beta, and one or more inhibitors of notch signaling). In other examples, a kit contains a matrix and at least 4 of the required supplements (ROCKi, FGF-2, retinoic acid, one or more inhibitors of SMAD signaling, TGF-beta, and one or more inhibitors of notch signaling).

[0029a] In a fifth aspect, there is provided a kit for performing a method of the first aspect.

[0030] BRIEF DESCRIPTION OF THE DRAWINGS

[0031] FIG. 1. Scheme of typical neural differentiation from stem cells.

[0032] FIG. 2. Enhanced neurogenesis under dual SMAD signaling pathway inhibition. FIG. 2A. IF analysis of tissues treated exclusively with noggin or noggin and SB431542. Neurons were visualized using an antibody against neurofilament (FITC-green) and nuclei with DAPI (Blue). Bar graph represents 10 μ m. Quantification of mean fluorescence ratio from whole tissues is presented in the right-hand graph. FIG. 2B. qPCR analysis of neuronal markers PAX6 and MAP2 show a 21-fold increase in both markers following SB431542 treatment (n=4 tissues/group).

[0033] FIG. 3. FGF-2 enhances neuronal differentiation. FIG. 3A. Increasing input of pluripotent stem cells resulted in enhanced neurofilament staining at BENO culture day 28. FIG. 3B. Proliferative effect of FGF-2 on stem cells and NPCs. The amount of neurons as well as network complexity inside the tissues as visualized with neurofilament staining is higher in tissues treated with FGF-2 at days 8-15.

[0034] FIG. 4. Neuronal transcript analysis upon BDNF and GDNF treatment. Neither PAX6 nor MAP2 expression was enhanced upon the addition of BDNF and GDNF in culture from day 10 to day 28. BENOs were analyzed at day 28.

[0035] FIG. 5. Enhanced neurogenesis by notch inhibition. DAPT treatment from day 15 to day 28 increased the abundance of PAX6 transcripts, thus suggesting a higher amount of neuronal commitment.

[0036] FIG. 6. Evaluation of neurogenic and gliogenic potential of different protocols. FIG. 6A. Summarizes a scheme of treatment performed in the individual protocols of Example 1.

CLAIMS

1. A method for producing a bioengineered neuronal organoid (BENO) from pluripotent stem cells (PSCs), the method comprising:
 - (A) culturing PSCs, embedded in a matrix immersed in serum-free cell culture medium;
 - (B) culturing the PSCs in said matrix of step (A) in cell culture medium comprising a Rho-associated kinase inhibitor (ROCKi) and FGF-2;
 - (C) culturing the forming BENO originating from PSCs and matrix of step (B) in cell culture medium comprising retinoic acid and one or more inhibitors of SMAD signaling to induce neurogenesis;
 - (D) culturing the forming BENO of step (C) in cell culture medium comprising TGF-beta and FGF-2 to enhance genesis of stromal cells and neurogenesis;
 - (E) culturing the forming BENO of step (D) in cell culture medium comprising TGF-beta and one or more inhibitors of notch signaling to enhance genesis of stromal cells and neurodifferentiation,wherein the matrix comprises collagen.
2. The method of claim 1, wherein the matrix does not comprise Matrigel.
3. The method of claim 1, wherein the matrix does not comprise Matrigel or other components of natural origin having a non-defined composition.
4. The method of any one of claims 1-3, wherein the matrix comprises type I collagen.
5. The method of any one of claims 1-4, wherein the BENO is produced within a 3D environment, preferably wherein the 3D environment is defined by the matrix.
6. The method of any one of claims 1-5, wherein the stromal cells comprise glial cells.
7. The method of any one of claims 1-6, wherein the medium of step (D) comprises at least two inhibitors of SMAD signaling, preferably wherein the inhibitors of SMAD signaling comprise noggin and SB 431542.

8. The method of any one of claims 1-7, wherein the inhibitor of notch signaling of step F is DAPT.
9. The method of any one of claims 1-8, wherein the PSCs are human PSCs.
10. The method of any one of claims 1-9,
 - wherein step (A) is performed on day -1,
 - wherein step (B) is performed from day -1 to day 0,
 - wherein step (C) is performed from day 0 to day 10,
 - wherein step (D) is performed from day 10 to day 15,
 - wherein step (E) is performed from day 15 to at least day 28.
11. A neuronal organoid, characterized in that the neuronal cells of the neuronal organoid are organized in a functional neuronal network, and wherein the neuronal network is characterized by the synchronization of neurons.
12. A bioengineered neuronal organoid (BENO) produced by the method of any one of claims 1-10, characterized in that the neuronal cells of the BENO are organized in a functional neuronal network, and wherein the neuronal network is characterized by the synchronization of neurons.
13. Use of a BENO of claims 11 or 12 as model for a disease and/or drug screening.
14. The use of claim 13, wherein the BENO is in co-culture with another tissue engineering platform.
15. A kit for performing a method of any one of claims 1-9.