



(51) International Patent Classification:
C12N 5/071 (2010.01)

Published:

— with international search report (Art. 21(3))

(21) International Application Number:

PCT/EP2018/058812

(22) International Filing Date:

06 April 2018 (06.04.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

100162

06 April 2017 (06.04.2017)

LU

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

(54) Title: NOVEL HUMAN HEPATOCYTE-DERIVED MATRIX FOR STEM-CELL DIFFERENTIATION AND TISSUE REPAIR

(57) Abstract: The present invention relates to the use of immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as an *in vitro* culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type as well as a composition comprising said iHLCs and/or said ECM as a culture system. The present invention also relates to a method for preparing an iHLC derived cell-free ECM as well as a device comprising iHLCs and/or an ECM derived from said iHLC as a culture system. The present invention also comprises an *in vitro* method for obtaining target cells of a pre-selected cell type.



Novel human hepatocyte-derived matrix for stem-cell differentiation and tissue repair

FIELD OF THE INVENTION

[1] The present invention relates to the use of immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as an *in vitro* culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type as well as a composition comprising said iHLCs and/or said ECM as a culture system. The present invention also relates to a method for preparing an iHLC derived cell-free ECM as well as a device comprising iHLCs and/or an ECM derived from said iHLC as a culture system. The present invention also comprises an *in vitro* method for obtaining target cells of a pre-selected cell type.

BACKGROUND

[2] Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), represent important cell resources and hold tremendous promise for cell-based therapies, disease modeling, as well as pharmaceutical applications. In particular, hiPSCs have the potential to significantly contribute to tissue engineering and regenerative medicine. This potential is attributed to their unique capacity to self-renew and to differentiate into derivatives of all three germ layers (Thomson et al. 1998, *Science*, 282, 1145; Takahashi et al. 2007, *Cell*, 131, 861; and Yu et al. 2007, *Science*, 318, 1917).

[3] In order to grow and differentiate properly *in vitro*, hPSCs require an optimal extracellular microenvironment consisting of an extracellular matrix (ECM) as well as growth factors and cytokines. This microenvironment mimics the conditions of normal embryonic development, during which coordinated synthesis and secretion of ECM proteins direct cell proliferation, survival, differentiation as well as tissue morphogenesis (Rozario et al. 2010, *Dev Biol*, 341, 126; and Hynes 2009, *Science*, 326, 1216).

[4] hPSCs and derivatives produce their own ECM proteins, which have been used to support *in vitro* cell expansion and differentiation as well as *in vivo* tissue regeneration (Hunt et al. 2012, *Exp Cell Res*, 318, 1820; and Chen et al. 2007, *Stem Cells*, 25, 553).

[5] Compared to the ECMs derived from adult stem cells or somatic tissues, the decellularized matrices from hPSCs have a broader spectrum of signalling capacity owing to their embryonic origin. In addition, the hPSCs-derived ECMs are free from embryonic DNA and thus have reduced risk of tumor formation, significantly improving their clinical applicability.

[6] Despite these advantages, the murine soluble basement membrane extracts Matrigel (BD Biosciences and Corning) and Geltrex (Thermo Fischer Scientific) are currently used for hPSCs differentiation (Kriks et al. 2011, *Nature*, 480, 547). They even remain gold standards because they provide the best efficiency for hPSCs-differentiation *in vitro*.

[7] Additionally, the matrices provide a suitable scaffold for the survival and differentiation of grafted cells in animal models.

[8] However, these available matrices derive from murine Engelbreth-Holm-Swarm tumor (sarcoma), rendering them incompatible with clinical applications.

[9] In cell replacement therapy, one of the major current hurdles is the low survival rate of the grafted cells, probably resulting from suboptimal microenvironment, where the use of optimal scaffolds would be extremely beneficial (Uemura et al. 2010, *J Neurosci Res*, 88, 542).

[10] In conclusion, there is a tremendous need for the development of human-based matrices to replace the murine counterparts, which are compatible with clinical applications in humans and directly allow for the matching of the therapeutic-grade cells to individual patients.

SUMMARY OF THE INVENTION

[11] The present invention provides the use of immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as an *in vitro* culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type.

[12] Furthermore, the present invention envisages a composition comprising (a) immature hepatocyte-like cells (iHLCs) and/or (b) an iHLC-derived ECM as a culture system and (c) target precursor stem cells differentiating into target cells of said pre-selected cell type.

[13] Accordingly, the present invention contemplates a method for preparing an immature hepatocyte-like cell (iHLC) derived cell-free extracellular matrix (ECM), comprising (a) cultivating iHLCs *in vitro* under conditions allowing for formation of an iHLC-derived extracellular matrix (ECM), (b) decellularizing the iHLC-derived ECM and (c) obtaining the iHLC-derived cell-free extracellular matrix (ECM).

[14] An immature hepatocyte-like cell (iHLC) derived extracellular matrix, which may be obtained or obtainable by the method mentioned above may also be contemplated by the invention.

[15] The present invention further encompasses a device comprising immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as a culture system.

[16] Accordingly, the present invention provides an *in vitro* method for obtaining target cells of a pre-selected cell type, which may comprise (a) providing immature hepatocyte-like cells (iHLCs) and/or an iHLC-derived extracellular matrix (ECM), (b) adding target precursor stem cells in a

suitable cell culture medium and allowing their differentiation into the target cells of a pre-selected cell type, (c) obtaining the target cells of said pre-selected cell type.

[17] The present invention may also comprise a target cell of a pre-selected cell-type, which may be obtained or obtainable by the *in vitro* method described above for use in a method for cell grafting in a patient in need thereof.

BRIEF DESCRIPTION OF THE FIGURE

[18] Fig. 1: Optimized protocol for the differentiation of hiPSC to iHLCs.

The cells are initially differentiated on Geltrex and at day 22 passaged onto Collagen I. This is described in **Example 1**.

[19] Fig. 2: Differentiation of hiPSC to iHLCs and their expression of different markers.

A) hiPSC in phase contrast (one colony) at 10X differentiation to iHLCs (day 21) staining with anti-HNF4 and Albumin (green) as well as Nkx2.5 and AFP (red) (at 20X), markers of immature and mature hepatocytes. **B)** Semi-quantitative PCRs of markers of immature and mature hepatocytes (OATP2B1, AFP, HNF4a, CYP7A1) studied in the human cell line HepG2 as well as iHLCs at day 21. Including control beta-actin. **C)** iHLCs expressed the immature hepatocyte-specific marker AFP, HNF4 α and CYP3A7. Beside this, iHLCs also exhibit expression of marker specific for mature hepatocytes by showing expression of Albumin, α -1-antitrypsin, CYP3A4. Nevertheless, expression of the hepatocyte marker CYP7A1 was not detected for iHLCs. Results indicate that iHLCs are defined by the expression of immature and mature hepatocyte marker. This is described in **Example 2**.

[20] Fig. 3: Experimental set ups of the differentiation of hiPSCs to iHLCs and their differentiation on hiPSC-derived hepatocytes into target cells of a pre-selected cell type.

A) Experimental set up of the differentiation of hiPSCs to iHLCs. **B)** Experimental set up of the differentiation for the co-culture experiment of hiPSC onto human iHLC. BASF refers to BDNF, ascorbic acid, SHH, FGF8, whereas BAGCT refers to BDNF, GDNF, ascorbic acid, TGF β 3, dibutyl cAMP (Kriks et al. 2011, *Nature*, rosette based differentiation). **C)** Experimental set up of the control differentiation for the co-culture experiment of hiPSC onto MS5 cells. The co-culture control experiment is based on the Kriks protocol (Kriks et al. 2011, *Nature*, rosette based differentiation), where a feeder layer of a mouse stromal cell line (called MS5) is used to induce neural fate of hiPSCs. **D)** Experimental set up of the control differentiation for the co-culture experiment of hiPSC onto human iHLC matrix or geltrex. The co-culture control experiment is based on the Kriks protocol (Kriks et al. 2011, *Nature*, floor-plate based differentiation). This is described in **Example 1, 4, 5 and 6**.

[21] Fig. 4: Differentiation of hiPSC on iHLCs into neural fate.

A) Neural induction of hiPSC on human iHLCs and further maturation on P/O+ LAM/FN in comparison to neural induction of hiPSC on MS5 and further maturation on P/O+ LAM/FN at day 12 of differentiation (at 10X). Staining of N-Cadherin (N-Cadh., green) and Pax6 (red), markers of early neural fate acquisition (individual channels and merge). White arrows point at neuroectoderm rosettes. **B)** Neural induction of hiPSC on human iHLCs and further maturation on P/O+ LAM/FN in comparison to neural induction of hiPSC on MS5 and further maturation on P/O+ LAM/FN at day 50 of differentiation (at 10X). Staining of TH (tyrosine hydroxylase, marker of dopaminergic neurons, green) and TuJ1 (beta3 Tubulin, mature neuronal marker, red), markers of terminal neuronal fate acquisition (individual channels and merge). This is described in **Example 7**.

[22] Fig. 5: Hepatocyte-derived matrix in comparison to Geltrex-based differentiation.

A) Feeder-free system of hiPSCs plated on hepatocyte-derived matrix for neural induction and further maturation on P/O+ LAM/FN versus neural induction on Geltrex and further maturation on P/O+ LAM/FN at day 27 of differentiation. TH (green) and FoxA2 (red) are markers of dopaminergic neurons, with FoxA2 being an important factor for these neurons survival *in vivo*. **B)** Feeder-free system of hiPSCs plated on hepatocyte-derived matrix for neural induction and further maturation on P/O+ LAM/FN versus neural induction on Geltrex and further maturation on P/O+ LAM/FN at day 50 of differentiation. TH (green) and Map2 (red) are markers of dopaminergic neurons and mature neurons respectively. This is described in **Example 8**.

[23] Fig. 6: Matrix-based system: Geltrex versus hepatocyte matrix.

hiPSC on hepatocyte matrix (ECM extract) of human iHLC in comparison to Geltrex at day 7, 13 and 21 of differentiation. Real-time PCR results of FoxA2, Otx2, Lmx1a, TH, beta3 Tubulin (TuJ1) and Map2, markers of early and late neural/neuronal fate acquisition (individual channels and merge). This is described in **Example 9**.

[24] Fig. 7: No induction into neural fate for human hepatocyte cell line HepG2.

A) Differentiation of hiPSCs on HepG2 cells in comparison to neural induction of MS5. Images of Phase contrast at 4X at day 5, 11 and 18. **B and C)** Differentiation of hiPSCs on HepG2 cells in comparison to neural induction of MS5 cells. Real-time PCR analysis of the genes: Pax6 and Otx2, early neural fate markers, at day 18 of differentiation (**B**) as well as quantitative analysis of real-time PCR results at day 11 and 18 (**C**). This is described in **Example 10**.

[25] Fig. 8: Phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5, HepG2 cells (HepG2), hiPSCs-derived cardiomyocytes (CM) and hiPSCs-derived mesenchymal progenitor cells (MPCs).

HiPSCs were plated either on MS5 cells (positive control), HepG2, CMs and MPCs, and observed at days 1, 4, 5, 7 and 13 post-differentiation. Neuro-epithelial rosettes were observed in the MS5 conditions at day 11 but not in the other conditions, where only morphological changes were detected. In MS5, neuro-epithelial rosettes were picked at day 11 and re-plated onto P/O+LAM/FN coated-plates and showed typical rosette structures at day 13. In the other conditions, no rosettes were detected at day 13 either, demonstrating a lack of neural induction. This is described in **Example 11**.

[26] Fig. 9: Semi-quantitative PCR and quantification of midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and other cell types at day 11 and day 18.

Neural markers Otx2 and Map2 are only present in the MS5 condition. However, the neural marker Pax6 is also observed at day 18 in the HepG2 conditions, indicating that HepG2 can support early neural induction but very inefficiently. This is described in **Example 11**.

[27] Fig. 10: Immunocytochemistry on day 11 of midbrain dopaminergic (mDA) neuron differentiation of hiPSC on MS5, HepG2 and iPSC-derived CMs and MPCs.

Expression of Pax6, Nestin protein and nuclear counterstain DAPI show that hiPSC colonies at day 11 of neural differentiation are only differentiating to neural cells on the MS5 (positive control). This is described in **Example 11**.

[28] Fig. 11: Protocol of hiPSC differentiation on Geltrex +N2B27/LDN/SB (positive control), +N2B27 (negative control) and HepG2-conditioned medium. This is described in **Example 12**.

[29] Fig. 12: Phase contrast pictures of hiPSC differentiation on Geltrex +N2B27/LDN/SB (positive control), +N2B27 (negative control) and HepG2-conditioned medium.

At day 8, neural differentiation is only happening in the positive control, where dual SMAD inhibition was applied. HepG2 conditioned medium cannot replace dual SMAD inhibition for neural induction. This is described in **Example 12**.

[30] Fig. 13: Scheme of differentiation of hiPSCs to definitive endoderm.

hiPSCs were differentiated to iHLCs through definitive endoderm induction, which finished at day 8. This is described in **Example 13**. These definitive endodermal cells were further used as

supporting cells for the neural differentiation of hiPSCs in comparison to MS5 support (see **Fig. 14**).

[31] Fig. 14: Phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and definitive endodermal cells.

Neural induction of hiPSCs on MS5 and definitive endoderm was observed at day 7 and day 11 post-differentiation. Neuro-epithelial rosettes were observed in the MS5 conditions but not in the definitive endodermal cells conditions, where only morphological changes were detected. This is described in **Example 14**.

[32] Fig. 15: Immunocytochemistry on day 11 of midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and definitive endoderm.

Red staining show Nestin expression, marker of neural fate, in hiPSC colonies differentiated on MS5 versus on definitive endodermal cells. Arrow heads point at Nestin positive cells in hiPSC colonies differentiated on definitive endoderm. These data show that neural induction is happening in hiPSCs differentiated on endodermal cells but at a very low efficiency in comparison to hiPSCs differentiated on MS5. This is described in **Example 14**.

[33] Fig. 16: Phase pictures during mDA neuron differentiation of hiPSC on MS5 and definitive endodermal cells.

At day 13 of differentiation, only few neuro-epithelial structures were obtained in the definitive endoderm condition. Arrow heads point at hiPSC colonies. This is described in **Example 14**.

[34] Fig. 17: Semi-quantitative PCR of midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and definitive endoderm (DE) at day 11 and day 13.

We observe that hiPSCs differentiated on definitive endoderm do not express or at very low amount, the typical neural markers such as Pax6, Sox2 and Otx2, as well as the neuronal marker Map2 of mature neurons, in comparison to the MS5 condition. This is described in **Example 14**.

[35] Fig. 18: Quantification of semi-quantitative PCR of midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and definitive endoderm at day 11 and day 13 (see Figure 17). Again typical markers such as Otx2, Pax6 and Sox2, as well as the neuronal marker Map2 of mature neurons are not expressed or at very low amount in hiPSCs differentiated on definitive endoderm. This is described in **Example 14**.

[36] Fig. 19: Decellularization protocols of iHLCs and HepG2 to be used for the neural differentiation support of hiPSCs.

Both iHLCs and HepG2 cells were decellularized, in order to test whether the HepG2 matrix supports hiPSC neural induction using the protocol described here. This is described in **Example 15**.

[37] Fig. 20: Phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on Geltrex, HepG2 matrix and iHLC matrix.

Typical neural morphology was observed at day 14, even at day 6 in hiPSCs differentiated on Geltrex, HepG2 matrix, iHLC matrix except Collagen I, where the cells detached and died. This is described in **Example 16**.

[38] Fig. 21: Immunocytochemistry on day 11 of midbrain dopaminergic (mDA) neuron differentiation of hiPSC on Geltrex, HepG2 matrix and iHLC matrix.

Expression of Nestin protein (red) and nuclear counterstain DAPI shows that hiPSC colonies at day 11 of neural differentiation are all differentiating to neural cells on the three different matrix with optimal results obtained with the iHLC matrix based on colonies shape (elongated neural cells). This is described in **Example 16**.

[39] Fig. 22: Semi-quantitative PCR during midbrain dopaminergic (mDa) neuron differentiation of iPSCs on Geltrex, HepG2 matrix and iHLC matrix at day 11, 18 and 22.

Expression of En1 and Nurr1 (key dopaminergic markers) show that differentiation of hiPSCs on iHLC matrix is the most efficient culture system for dopaminergic differentiation, looking at day 22 of differentiation. This is described in **Example 16**.

[40] Fig. 23: Quantification of semi-quantitative PCR during midbrain dopaminergic (mDa) neuron differentiation of hiPSCs on Geltrex, HepG2 matrix and iHLC matrix at day 11, 18 and 22 (see Figure 22).

Again, the expression of En1 and Nurr1 as key dopaminergic markers increases constantly from day 11 to day 22 in the differentiation of hiPSCs on iHLC matrix, pointing out that said matrix is the most efficient culture system for dopaminergic differentiation. This is described in **Example 16**.

[41] Fig. 24: Decellularization protocols of iHLCs and HepG2 for neural differentiation support of hiPSCs without dual SMAD inhibition.

HepG2 cells and iHLCs were decellularized as described here, to test whether the iHLC and HepG2 matrix supports hiPSC neural induction without dual SMAD inhibition. This is described in **Example 17**.

[42] Fig. 25: Phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on Geltrex, HepG2 matrix and iHLC matrix without dual SMAD inhibition.

Morphology changes to neural fate were observed at day 5 in the conditions with HepG2 matrix and iHLC matrix, whereas in the condition with Geltrex the typical neural morphology was first detected at day 7. This is described in **Example 17**.

[43] Fig. 26: qPCR analysis of midbrain dopaminergic (mDA) neuron differentiation in a matrix-based system on Geltrex HepG2 matrix and iHLC matrix without dual SMAD inhibition.

Expression of neuronal markers (Nestin, Pax6, Lhx2) did not differ between the three conditions at day 3, 7 and 11 but there was an increased expression for Nestin, Pax6, Brachyury and Lhx2 for the positive control (Geltrex with dual SMAD inhibition -Geltrex LDN/SB-). Thus, dual SMAD inhibition is therefore necessary for efficient neural induction. This is described in **Example 17**.

[44] Fig. 27: Decellularization protocols of iHLCs and HepG2 for neural maturation support of hiPSCs-derived neural progenitor cells (hNPCs).

HepG2 cells and iHLCs were decellularized as described here, to test whether the matrices support mDA neuron maturation. This is described in **Example 18**.

[45] Fig. 28: Immunocytochemistry of midbrain dopaminergic (mDA) neuron maturation of hNPCs on P/O+ LAM/FN, HepG2 matrix and iHLC matrix

Co-expression of the marker of dopaminergic neurons TH (green) and the neuronal marker Map2 protein (red) shows that human NPCs matured under all conditions. The highest expression of TH was observed with the iHLC matrix, suggesting a better supporting DA neuron-maturation effect of the iHLC matrix. This is described in **Example 18**.

[46] Fig. 29: Comparison of detected peptides of core matrisome proteins and matrisome associated proteins in iHLCs, HepG2 cells, hiPSCs and MS5 by proteomic analysis.

Marked red ECM proteins show high peptide number in iHLCs as well as in MS5 in comparison to HepG2 and hiPSCs. Those were selected as candidate proteins. This is described in **Example 19**.

[47] Fig. 30: Quantification of core matrisome proteins in iHLCs, HepG2 cells and hiPSCs by proteomic analysis. Marked in red low peptide number. This is described in Example 19.

[48] Fig. 31: Graphic presentation of quantification of core matrisome proteins in iHLCs and HepG2 cells by proteomic analysis.

Beside the selected candidate proteins with neural supporting effect, iHLCs showed a high amount of Lamb1, Lamc1 and Lama1 in comparison to HepG2 cells. This is described in **Example 19**.

[49] Fig. 32: Quantification of matrisome associated proteins in iHLCs, HepG2 cells and hiPSCs by proteomic analysis. Marked in red low peptide number. This is described in **Example 19**.

[50] Fig. 33: Graphic presentation of quantification of matrisome associated proteins in iHLCs and HepG2 cells by proteomic analysis.

In order to identify matrisome associated proteins, which can support DA neural induction, matrisome associated proteins in iHLCs were quantified in comparison to HepG2 cells. iHLCs showed a high amount of FGF2, a proliferating factor, in comparison to HepG2 cells. This is described in **Example 19**.

[51] Fig. 34: Semi-quantitative PCR in hiPSCs, HepG2 cells and iHLCs for laminin chains.

Lamb1, Lamb2, Lamb3 and Lamc1 were highly expressed in HepG2 cells and iHLCs in comparison to iPSCs, suggesting that these Laminin chains could be candidates for neural support. Lama1, Lamb3 and Lamc2 are specifically expressed in iHLC suggesting a potential role in the support for DA neuron differentiation. This is described in **Example 19**.

[52] Fig. 35: Confirmation of candidate proteins from the proteomic analysis by semi-quantitative PCR.

Expression of Perlecan, Thbs1, Nid1, Ltbp1, Col5a1 and Vcan was detected in iHLCs. Specific ECM proteins responsible for the dopaminergic neural support could be Ltbp1 and Col5a1, exclusively present in iHLCs, in comparison to HepG2. This is described in **Example 19**.

[53] Fig. 36: Venn diagram of significantly up-regulated core matrisome and matrisome associated proteins in iHLCs in comparison to hiPSCs, HepG2 cells and MSCs.

Immature HLCs were analysed by LC-MS/MS in comparison to hiPSCs, HepG2 cells and MSCs. Relations of significantly regulated proteins are illustrated by a venn-diagram. 18 proteins were significantly higher expressed in iHLCs in comparison to all control groups.

[54] Fig. 37: List of significantly up-regulated core matrisome and matrisome associated proteins in iHLCs in comparison to hiPSCs, HepG2 cells and MSCs.

iHLCs showed a significantly higher expression of 18 proteins compared to the control cell types (MSC, HepG2 or hiPSC). Proteins involved in either TGF β -signalling (FBN2, IGFBP2 and 5, LTBP1), BMP-signalling (GDF6) or CNS development (AGRN, HAPLN1, LAMB1) could be detected.

Furthermore proteins, which were only up-regulated relative to two control cell types, could be also assigned to either TGF β -signalling or CNS development. SFRP1, significantly up-regulated in relative to MSC and HepG2 cells, is involved in dopamine neuron development. In contrast iHLCs showed a higher expression of FBLN1, THBS1 (TGF β -signalling) and COL3A1, COL4A1, TNC (CNS development) compared to hiPSC and HepG2. Beside this, MDK and VTN were significantly higher expressed in iHLCs in comparison to MSC and hiPSCs and are associated with CNS development as well.

[55] Fig. 38: Venn diagram of significantly down-regulated core matrisome and matrisome associated proteins in iHLCs in comparison to hiPSCs, HepG2 cells and MSCs.

Immature HLCs were analysed by LC-MS/MS in comparison to hiPSCs, HepG2 cells and MSCs. Relations of significantly regulated proteins are illustrated by a venn-diagram. 7 proteins were significantly lower expressed in iHLCs in comparison to all control groups.

[56] Fig. 39: List of significantly down-regulated core matrisome and matrisome associated proteins in iHLCs in comparison to hiPSCs, HepG2 cells and MSCs.

iHLCs showed a significantly lower expression of 7 proteins compared to the control cell types. Neither of these 7 proteins are involved in TGF β -, BMP- signalling or CNS development.

Proteins significantly lower expressed in iHLCs in comparison to only two control groups were found to be involved in biological processes. Proteins associated with TGF β -signalling (FBN1, LTBP2) and CNS development (SDC1) were down-regulated in iHLCs in comparison to MSCs and HepG2 cells. Beside this, LAMA1, which is involved in CNS development, was lower expressed in iHLCs in comparison to MSCs and hiPSCs.

DETAILED DESCRIPTION OF THE INVENTION

[57] The solution of the present invention is described in the following, exemplified in the appended examples, illustrated in the Figures and reflected in the claims.

[58] The present invention solves the limitation mentioned in the prior art by producing a human matrix to obtain therapeutic-grade cells for personalized medicine. The purpose of the invention is to create a novel human matrix, which is very beneficial and efficient for standard hPSCs differentiation to therapeutically relevant cell types and for its potential use as scaffold in cell grafting. Due to its high efficiency comparable and even surpassing the mouse counterparts, in the prior art no other human matrix with this capacity may have been reported so far.

[59] The human matrix of the present invention derived from cultured human hepatocytes may be used for 2D and 3D *in-vitro* differentiation of hiPSC into cells of clinical relevance.

[60] In particular, the matrix of the present invention may have the potential to induce a neural effect by differentiating hiPSC on an extracellular matrix derived from hiPSCs-derived immature hepatocyte like cells (iHLCs). Hepatocytes, preferably iHLCs may be considered as a good source for hiPSC neural induction.

[61] The present invention provides the use of immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as an *in vitro* culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type. The use of iHLCs as an *in vitro* culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type may be preferred in the present invention.

[62] “Immature hepatocyte-like cells (iHLCs)” as used in this context are not fully grown or developed hepatocytes on the one hand expressing markers of immature hepatocytes such as alpha-1-fetoprotein (AFP), HNF4 α , Nkx2.5, OATP2B1, CYP3A7 that distinguish the cells from mature, fully developed hepatocytes or hepatocyte cell-lines (in the present invention the human cancer cell line HepG2 cells was used mainly as a model of “mature hepatocytes”, yet not representing entirely a specific developmental stage), and on the other hand also expressing mature hepatocyte markers such as albumin, α -1-antitrypsin, CYP3A4, but not expressing CYP7A1.

Despite the expression of said combination of markers including immature and mature hepatocyte markers, iHLCs are closer to an “immature” stage, because AFP is not lost yet. AFP is a key marker lost during terminal differentiation, therefore since iHLCs still express it, the term “immature” needs to be used herein for HLCs. Eventough being called “immature” HLCs, differentiation of target precursor stem cells into target cells of a pre-selected cell type on iHLCs and/or an ECM derived from said iHLCs is even more efficient using more “mature” iHLCs including longer iHLC differentiation such as definitive endoderm induction, followed by hepatocyte differentiation, maturation and maintenance to develop more mature iHLCs. Thus,

said iHLCs of the present invention are defined by expressing markers of immature and mature hepatocyte markers.

In a preferred embodiment, the specific combination of immature and mature markers in iHLCs combined with the proteomics profile / matrix proteins such as Lama1, Lama5, Lamb1, Lamb2, Lamb3, Lamc1, Lamc2, Perlecan, Thbs1, Nid1, Vcan, Ltbp1, Col5a1, Agrn, Fbn2, Gdf6, Hapln1, Igfbp2, Igfbp5, Mdk, Vtn, Col3A1, Col4A1, Fbln2, Tnc, and Sfrp1 of the iHLCs, which are of even more importance in terms of uniqueness as indicated elsewhere herein, may perfectly define the iHLCs of the present invention.

The particular differentiation protocol of said iHLCs of the present invention is provided in [76]-[78], in **Example 1** as well as depicted in **Fig. 1** and **3A**. Preferably, iHLCs are derived from human induced pluripotent stem cells (hiPSCs).

[63] In this context, “hepatocytes” refer to a parenchymal liver cell of the main tissue of the liver (e.g. liver cells). Hepatocytes make up the majority of the liver's cytoplasmic mass and are involved in protein synthesis and storage, carbohydrate metabolism, cholesterol, bile salt and phospholipid synthesis and the detoxification, modification and excretion of exogenous and endogenous substances.

[64] iHLCs contemplated by the present invention may be derived from human or non-human induced pluripotent stem cells (iPSCs), human or non-human adult stem cells, human parthenogenetic or non-human embryonic stem cells (ESCs), or a hepatocyte cell-line or primary hepatocytes. However, non-human iPSCs, non-human adult stem cells or non-human ESCs being differentiated into iHLCs may also be preferred such as murine ESC-derived immature hepatocytes-like cells.

[65] The term “stem cell” refers to a master cell that can reproduce indefinitely to form the specialized cells of tissues and organs. A stem cell can divide to produce two daughter stem cells, or one daughter stem cell and one progenitor cell, which then proliferates into the tissue's mature, fully formed cells. In a developing embryo, stem cells can differentiate into all the specialized cells—ectoderm, endoderm and mesoderm. Stem cells may be totipotent or pluripotent or multipotent and may be capable of differentiating into one or more different cell types. In mammals, two broad types of stem cells may be listed: embryonic stem cells (ESCs) and adult stem cells.

[66] “Embryonic stem cells” refer to pluripotent stem cells and are isolated from the inner cell mass of blastocysts, which may refer to an early-stage preimplantation embryo. In general, embryonic stem cells can generate all cell types in the body, whereas adult stem cells are multipotent and can produce only a limited number of cell types. In this context, „human parthenogenetic ESCs” as used herein may refer to ESCs, which may be derived from embryos, which are not capable of developing into a human being, since the embryos are developed from an oocyte without fertilization. In other words, mammalian parthenogenetic

embryos are not viable, but can develop into blastocysts from which ESCs, thus parthenogenetic ESCs have been derived in mouse and human. Said parthenogenetic ESCs may differentiate into hepatocytes which may be able to repopulate the liver, provide normal liver function, and/or facilitate long-term survival (Espejel et al. 2014, *Stem Cells*, 32(7):1983-8).

[67] „Adult stem cells“ are found in various tissues and refer to undifferentiated cells, that multiply by cell division to replace dying cells and regenerate damaged tissues.

[68] The term “totipotent” refers to the ability of a cell to differentiate into any type of cell in a differentiated organism, as well as a cell of extra embryonic materials, such as placenta, etc.

[69] The term “pluripotent” as used herein refers to the ability of a cell being capable of differentiating into any differentiated cell type. Pluripotent stem cells do not develop into a complete organism. A pluripotent cell does not give rise to the placenta or other tissues that are vital for foetal development. Pluripotency may distinguish embryonic stem cells from adult stem cells found in adults.

[70] The term “multipotent” refers to the ability of a cell being capable of differentiating into at least two differentiated cell types.

[71] The term “hepatocyte cell-line” refers to a cell line which is capable of culturing over a long period of time (i.e., years) without losing characteristic activity of a hepatocyte. iHLCs, which may not refer to a cell line itself, may be derived from said hepatocyte-cell line.

[72] The term “primary hepatocyte” as used herein refers to a primary cell taken directly from fresh liver samples and are established for growth *in vitro*. Primary hepatocytes have undergone very few population doublings and are therefore more representative of the main functional component of the tissue from which they are derived in comparison to continuous (tumor or artificially immortalized) cell lines thus representing a more representative model to the *in vivo* state.

[73] Also indicated elsewhere herein, iHLCs are preferably derived from human induced pluripotent stem cells (hiPSCs). Thus, iHLCs may also refer to hiPSCs-derived immature hepatocyte-like cells (iHLCs).

[74] “Human pluripotent stem cells”, also called hPSC refer to undifferentiated cells having complete differentiation versatility, i.e., the capacity to grow into any of the mammalian body's cell types, except those needed to develop a fetus. In addition, those cells have growth ability, can be self-renewing, and can remain dormant or quiescent within a tissue. Human pluripotent stem cells (hPSCs) include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Induced pluripotent stem cells (iPSCs) may be generated directly from adult cells by the iPSC technology pioneered by Shinya Yamanaka's from Kyoto, Japan in 2006. In 2006, Shinya Yamanaka showed that the introduction of four specific genes which encode transcription factors may convert adult cells into pluripotent stem cells. In this context, induced pluripotent stem cells are preferably human induced pluripotent stem cells (hiPSCs) and may

differentiate on hiPSC derived iHLCs as a co-culture system into target cells of a pre-selected cell type.

[75] It is of interest to recapitulate the key stages of liver development in differentiation cultures, including the induction of definitive endoderm, the specification of the endoderm to a hepatic fate, the generation of hepatic progenitors known as hepatoblasts and the differentiation of hepatoblasts to mature hepatocytes. In most studies, differentiation is induced in a monolayer format with the sequential addition of pathway agonists and antagonists that are known to regulate the early stages of development including endoderm induction and hepatic specification (Si-Taye et al. 2010, Dev Cell 18, 175-189).

[76] The differentiation of hiPSCs into iHLCs including these key stages of liver development is described in **Example 1**. The present invention may envisage the differentiation of hiPSC into iHLCs being determined by definitive endoderm induction. Definitive endoderm induction may be more than 4 days, less than 10 days, or 4-10 days, 6-8 days, or at least 4, 5, 6, 7, or at least 8 days long. Preferably, definitive endoderm induction is 8 days. The present invention may also provide RPMI medium comprising 1% B27 w/o insulin supplemented with 100ng ml⁻¹ FGF2 and 100ng ml⁻¹ Activin A for definitive endoderm differentiation, which determines the differentiation of hiPSC into iHLCs. In this context, definitive endodermal cells refer to cells from the definitive endoderm being formed during gastrulation and initially appear as a single-cell thick, epithelial sheet that covers the bottom surface of the developing embryo. Definitive endodermal cells may be obtained by subjecting hiPSCs to a suitable protocol (**Example 13**) or definitive endoderm may be obtained by other types of pluripotent cell lines or cells showing the potential to differentiate into definitive endoderm. Definitive endodermal cells may be induced in RPMI medium and/or SMEM medium, preferably in RPMI medium supplemented with Activin A and FGF2. Definitive endoderm cells may be mammalian cells, preferably definitive endoderm cells are human cells. Definitive endoderm cells may express or fail to significantly express certain markers, preferably one or more markers selected from SOX17, CXCR4, MLXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 are expressed in definitive endoderm cells. Preferably, definitive endodermal cells are characterized by the expression of the following markers SOX17, FoxA2, CXCR4 and down regulation of the marker SOX7. The present invention may comprise the use of iHLCs being differentiated from hiPSCs, wherein said hiPSCs may be first differentiated into definitive endodermal cells using RPMI medium supplemented with Activin A and FGF2 before differentiating into iHLCs, as an in vitro culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type.

[77] Preferably, after hiPSCs are differentiated to definitive endodermal cells, they may further differentiate into hepatocytes. Said hepatocyte differentiation may be more than 4 days, less than 10 days, or 4-10 days, 6-8 days, or at least 4, 5, 6, 7, or at least 8 days long. Preferably,

said hepatocyte differentiation is 8 days long. The present invention may also provide DMEM/F12 (1:1) medium supplemented with 10 % KOSR, 100 ng ml⁻¹ human hepatocyte growth factor (HGF) and/or 1% DMSO for hepatocyte differentiation from definitive endodermal cells. Hepatocyte differentiation may be achieved by having an SOX17 expression level of more than 60%, less than 100%, or about 60%-100%, 70%-100%, 80%-100%, 90%-100% or about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100%.

[78] The differentiation into said iHLCs, which is divided into maturation and maintenance may be more than 1 day, less than 10 days, or 1-10 days, 2-8 days, 4-6 days, or at least 1, 2, 3, 4, 5, or at least 6 days long. Preferably, differentiation into said iHLCs is 6 days long. Preferably, iHLC maturation to become fully developed iHLCs is 3 days long. The present invention may also contemplate DMEM/F12 (1:1) medium supplemented with 10⁻⁷ M Dexamethasone for said hepatic maturation to become iHLCs. iHLC maintenance is preferably 3 days long. The present invention may also provide William's E medium supplemented with 10% Hyclone, 1 µg ml⁻¹ human Insulin, 1.8 % DMSO, 4.7 µg ml⁻¹ Hydrocortisone for further iHLC maintenance (**Fig. 3A** and **1**).

[79] Additionally, the present invention may provide the use of said iHLCs for the differentiation of target precursor stem cells into target cells of a pre-selected cell type.

[80] As used herein, "target precursor stem cells" refer to stem cells that are capable of differentiating into specific target cells. In this context, the precursor stem cells of the present invention may be capable of differentiating into neural cells, but not restricted to these cells.

[81] The term "target cells of a pre-selected cell type" refers to cells being differentiated to from a cell type being selected in advance.

[82] The differentiation of hiPSCs, which may refer to target precursor stem cells, on hiPSCs-derived iHLCs into neural cells is illustrated in **Figure 3B** or described in **Example 4**. The differentiation of hiPSCs on hiPSCs-derived iHLCs may refer to rosette-based differentiation according to Kriks et al., *Nature*, 2011. Irradiated MS5 cells may further be used as a control for the differentiation of hiPSCs (**Fig. 3C** and **Example 5**). Murine MS5 cells are mouse bone-marrow stromal cells and are the original co-culture system for neural induction in the literature. Rosette based differentiation of hiPSCs on hiPSCs-derived iHLCs may be more efficient than rosette based differentiation of hiPSCs on MS5 cells in inducing the formation of neuroectoderm rosettes. In this context, a neuroectoderm rosette refers to neuroectodermal cells in a columnar rosette structure containing neural progenitor cells (NPCs). Neuroectodermal cells are cells of the ectoderm, the outermost of the three primary germ layers of an embryo, the others being the mesoderm and the endoderm, which gives rise to the central and peripheral nervous systems, including some glial cells. An enhanced neuroectodermal specification may be detected by the increase of the expression of neuroectodermal markers such as Pax6 and N-Cadherin once neural differentiation is initiated, thereby leading to more NPC generations. The differentiation of

target precursor stem cells into target cells of a pre-selected cell type may be preferred when target precursor stem cells are preferably plated on said iHLCs in comparison to a differentiation of target precursor stem cells on MS5 cells.

[83] The present invention may provide for neural induction done by rosette based differentiation of hiPSC on iHLCs KSR medium supplemented with 1 μM dorsomorphine and 10 μM SB431542, 100 ng ml^{-1} SHH C24II and 100 ng ml^{-1} FGF8. The neural induction of hiPSC rosette based differentiation may be more than 5 days, less than 20 days, or 5-20 days, 10-20 days, 15-20 days, or at least 5, 10, 11, 12, 13, 14, 15, 16, 17, or at least 18 days, preferably neural induction of hiPSC rosette based differentiation is 18 days. The present invention may envisage neural differentiation done by rosette based differentiation of hiPSC on iHLCs in Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2mM L-glutamine supplemented with 100 ng ml^{-1} SHH C24II, 100 ng ml^{-1} FGF8, 20 ng ml^{-1} BDNF, 0.2 mM ascorbic acid (abbreviated "BASF"), followed by further differentiation in Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2mM L-glutamine supplemented with 20 ng ml^{-1} BDNF, 20 ng ml^{-1} GDNF, 0.2 mM ascorbic acid, 1 ng ml^{-1} TGF β 3 and 0.5 mM dibutyryl cAMP (abbreviated "BAGC") (**Example 4**). Differentiation into target cells of a pre-selected cell type, preferably into neural cells done by rosette based differentiation is in total more than 30 days, less than 60 days, or 30-60 days, 40-60 days, 40-55 days, 40-50 days, 45-55 days, 45-50 days, or at least 30 days, at least 40 days, at least 45 days, at least 50 days, most preferably 50 days. (**Fig. 3B**).

[84] iHLCs functioning as feeder cells for the differentiation of target precursor stem cells into target cells of a pre-selected cell type may also induce neural fate more efficiently in comparison to HepG2 cells, hiPSCs-derived cardiomyocytes (CMs) and/or mesenchymal progenitor cells (MPCs), feeder cells not being capable of differentiating target precursor stem cells into target cells of a pre-selected cell type.

[85] Further, the present invention may envisage the use of an extracellular matrix (ECM) derived from said iHLCs as an in vitro culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type. The use of an extracellular matrix (ECM) derived from said iHLCs as an in vitro culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type may also be preferred in the present invention.

[86] "An extracellular matrix (ECM)" refers to a collection of extracellular molecules secreted by cells that provides essential physical/structural scaffolding for the cellular constituents and comprises glycoproteins (e.g. fibronectins and laminins), fibrillar proteins such as collagens and elastins giving structural support and elasticity to the tissue, and proteoglycans and the like. Since the concentration of structural proteins such as collagen in the ECM may be higher in comparison to conditioned medium, an "ECM" as used herein may also refer to a "concentrated solution of ECM proteins", thus being highly concentrated as indicated elsewhere herein Said

extracellular matrix of the present invention may be characterized by the expression of Lama1, Lama5, Lamb1, Lamb2, Lamb3, Lamc1, Lamc2, Perlecan, Thbs1, Nid1, Vcan, Ltbp1, Col5a1, Agrn, Fbn2, Gdf6, Hapln1, Igfbp2, Igfbp5, Mdk, Vtn, Col3A1, Col4A1, Fbln2, Tnc, and Sfrp1. In this context, the ECM of the present invention may preferably be produced by iHLCs being differentiated preferably from hiPSC, thus being also called "iHLC-derived ECM". "iHLC matrix" or "hiPSCs-derived iHLC matrix" can be used interchangeably with the term iHLC-derived ECM (a hepatocyte matrix). In this context "derived" or "derived from" is intended that the matrix may be obtained from iHLCs.

[87] iHLCs being preferably derived from hiPSCs may be a valuable source of human ECM for *in vitro* differentiation of target precursor stem cells into target cells of a pre-selected cell type.

[88] Therefore, the present invention may also envisage an iHLCs-derived ECM, wherein said ECM may be substantially cell-free. Preferably, said cell-free iHLC-derived ECM may be used for the plating of undifferentiated hiPSCs and their differentiation into neural cells.

[89] Preferably, after the differentiation of hiPSCs into iHLCs (as described in **Example 1**), iHLCs may secrete extracellular molecules, which may form an ECM and which may need to be decellularized, meaning isolating the ECM from its inhabiting cells, preferably iHLCs by use of a specific decellularization buffer (**Example 3**). This decellularized or cell-free iHLC matrix may then be used for hiPSC neural differentiation.

[90] The term "decellularized" or "decellularization" refers to the process used in biomedical engineering to isolate the ECM in a cell culture from its inhabiting cells. Here, the ECM derived from said iHLCs is preferably isolated from the hiPSC derived iHLCs by a specific decellularization buffer containing 20 mM ammonium hydroxide (NH₄OH), 0.5% Triton X-100 in PBS and steril filtered 0,22 µm (**Example 3**). The replication of the hepatocyte de-cellularization and the use of the remaining ECM derived from said hepatocytes, preferably derived from hiPSC derived iHLCs, may be considered as being important for culturing and differentiating target precursor stem cells into target cells of a pre-selected cell type.

[91] Since empirical evidence has been obtained that hiPSCs-derived iHLCs may be a valuable source of human ECM for *in vitro* induction of neurons, the differentiation of hiPSC on hiPSCs-derived iHLC matrix into neural cells may be performed, wherein said iHLC matrix may need to be decellularized before. The differentiation of hiPSCs on a cell-free iHLC matrix is illustrated in **Figure 3D** or described in **Example 6**. Preferably, the differentiation of hiPSCs on hiPSCs-derived iHLC matrix may refer to floor-plate based differentiation according to Kriks et al., *Nature*, 2011. A Geltrex matrix may further be used as a control for the differentiation of hiPSCs. A Geltrex matrix refers to a soluble form of reduced growth factor (RGF) basement membrane extract (BME) purified from murine Engelbreth-Holm-Swarm tumor. New manufacturing methods are now available, which allow for the removal of the lactate dehydrogenase-elevating virus (LDEV), which may be of concern in certain applications such as

xenografts or other clinically focused research. Floor-plate based differentiation of hiPSCs on hiPSCs-derived iHLC matrix into neural cells may be more efficient than floor-plate based differentiation of hiPSCs on mouse sarcoma-derived Geltrex matrix. The differentiation of target precursor stem cells into target cells of a pre-selected cell type may be preferred when target precursor stem cells are plated on an ECM derived from said iHLCs in comparison to a differentiation of target precursor stem cells on Geltrex matrix.

[92] The present invention may provide for neural induction done by floor-plate based differentiation of hiPSC on hiPSC derived iHLC matrix KSR medium supplemented with 100 nM LDN193189, 10 μ M SB431542, 100 ng ml⁻¹ SHH C24II, 2 μ M Purmorphamine 100 ng ml⁻¹ FGF8 and 3 μ M CHIR99021 (**Example 6**) and N2B27 1:1 medium (N2 medium comprises DMEM F12, 1x N2, 5 μ g/ml insulin, 2 mM L-glutamine, 1x non-essential amino acids, 10 μ M 2-mercaptoethanol, 50 U/ml Penicillin, 50 μ g/ml Streptomycin and B27 medium comprises Neurobasal, 1x B27, 2 mM L-glutamine, 50 U/ml Penicillin, 50 μ g/ml Streptomycin) supplemented with 100 nM LDN193189 and 10 μ M SB431542, with 100 ng ml⁻¹ SHH C24II (Miltenyi) and 100 ng ml⁻¹ FGF8 (Miltenyi) (**Example 15**). Neural induction (also called floor-plate induction or FP induction) of hiPSCs done by floor-plate based differentiation with KSR medium may be more than 1 day, less than 15 days, or 1-15 days, 5-15 days, 10-15 days, or at least 1, 3, 5, 6, 7, 8, 9, 10, or at least 11 days, preferably neural induction initiated by KSR medium is 11 days. Neural induction of hiPSCs done by floor-plate based differentiation with N2B27 1:1 medium may be more than 1 day, less than 20 days, or 1-20 days, 5-20 days, 10-20 days, 10-15 days, or at least 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, or at least 14, preferably neural induction initiated by N2B27 1:1 medium is 14 days. The present invention may also envisage neural differentiation done by floor-plate based differentiation of hiPSC on iHLC matrix in Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2mM L-glutamine supplemented with 3 μ M CHIR99021 and 20 ng ml⁻¹ BDNF, 20 ng ml⁻¹ GDNF, 0.2 mM ascorbic acid, 1 ng ml⁻¹ TGF β 3, 0.5 mM dibutyl cAMP, 10 μ M DAPT (abbreviated "BAGCT") (**Example 6 and 15**). Differentiation into target cells of a pre-selected cell type, preferably into neural cells done by floor-plate based differentiation is in total more than 30 days, less than 60 days, or 30-60 days, 40-60 days, 40-55 days, 40-50 days, 45-55 days, 45-50 days, or at least 30 days, at least 40 days, at least 45 days, at least 50 days, most preferably 50 days (**Fig. 3D** and **Fig. 19**).

However, the differentiation of hiPSCs on iHLC matrix in N2B27 1:1 medium was more efficient in neural induction, preferably in induction of midbrain dopaminergic neurons (mDAs) in comparison to KSR medium. Thus, the differentiation into target cells of a pre-selected cell type, preferably into neural cells, more preferably into dopaminergic neurons done by floor-plate based differentiation with N2B27 1:1 medium is the preferred differentiation.

[93] The present invention may also comprise iHLCs being characterized by the expression of α -fetoprotein (uniprot accession number P02771), albumin (uniprot accession number

P02768), HNF4 α (uniprot accession number P41235), α -1-antitrypsin (uniprot accession number P01009), CYP3A4 (uniprot accession number P08684), CYP3A7 (uniprot accession number P24462) and do not express CYP7A1 (uniprot accession number P22680).

[94] By certain signals for example bone morphogenetic protein (BMP), pre-hepatic cells become proliferative and begin to form the liver bud. These pre-hepatic cells are also designated as hepatoblasts and a number of studies have indicated that hepatoblasts give rise to hepatocytes. HNF-4 α being a liver transcription factor, which controls the expression of several hepatic genes, plays an important role in the early development of the liver and is highly expressed in the precursors of hepatocytes (hepatoblasts), thus referring to a marker of immature hepatocytes (Tanaka et al. 2009, *Mechanisms of Development*, vol. 126, Issues 8–9, pp. 665–676). Other studies have shown that various diffusible cytoplasmic markers, such as alpha-fetoprotein (AFP) may also be markers of immature hepatocytes. According to Hanayuki Okura et al. 2010, *Tissue Engineering*, vol. 16, markers of immature hepatocytes may also comprise besides AFP CYP3A7 to identify immature hepatocytes from mature hepatocytes characterized by the expression levels of CYP3A4, G6P, alpha-1-antitrypsin, glutamine synthetase, CYP1B1 and/or CK18. Mature hepatocytes may also be characterized by the expression levels of apolipoprotein-F, CYP1A1 and/or CYP1A2. In this context "immature hepatocytes" as used herein may refer to a hepatocyte lineage cell that may express AFP, CYP3A7 and/or HNF-4 α but that does not express appreciable levels of functional albumin, CYP3A4, CYP1B1, G6P, alpha-1-antitrypsin, glutamine synthetase, CK18, apolipoprotein-F, CYP1A1 and/or CYP1A2. In some embodiments, immature hepatocytes must undergo maturation to acquire the functionality of mature hepatocytes. iHLCs on the other hand may express albumin, α -1-antitrypsin, CYP3A4, being a marker of mature hepatocytes, besides HNF4 α , Nkx 2.5, α -fetoprotein (AFP), CYP3A7 and OATP2B1, which may refer to markers of immature hepatocytes. Further iHLCs may not express CYP7A1 enzyme, also being a marker of mature hepatocytes (Cai et al. 2007). Said different expression signature may distinguish iHLCs from normal immature hepatocytes. Thus, these cells may be best described as an immature hepatocyte like cell.

[95] In contrast to mature hepatocytes, iHLCs preferably express AFP, which are not expressed by mature hepatocytes any longer. In contrast to HepG2 cells, iHLCs are less mature, they are not a cell line and do not express CYP7A1, being expressed by HepG2 cells. In this context "HepG2 cells" refer to an immortalized, widely used human hepatocellular carcinoma cell line that is highly differentiated and displays many of the genotypic features of mature hepatocytes. Yet, since HepG2 cells is a cancer cell line, those cells are mainly a "model of mature hepatocytes", not representing entirely a specific developmental stage.

[96] The present invention may comprise iHLCs preferably being characterized by an expression level of AFP of more than 50%, less than 100%, or about 50%-100%, 60%-100%,

70%-100%, 80%-100%, 90%-100%, or at least 50%, 60%, 70%, 80%, or at least 90% or about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 100%. The present invention may comprise iHLCs preferably being characterized by an expression level of albumin of more than 50%, less than 100%, or about 50%-100%, 60%-100%, 70%-100%, 80%-100%, 90%-100%, or at least 50%, 60%, 70%, 80%, or at least 90%, or about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 100%. The present invention may comprise iHLCs preferably being characterized by an expression level of α -1-antitrypsin of more than 50%, less than 100%, or about 50%-100%, 60%-100%, 70%-100%, 80%-100%, 90%-100%, or at least 50%, 60%, 70%, 80%, or at least 90%, or about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 100%. The present invention may comprise iHLCs preferably being characterized by an expression level of CYP3A7 of more than 50%, less than 100%, or about 50%-100%, 60%-100%, 70%-100%, 80%-100%, 90%-100%, or at least 50%, 60%, 70%, 80%, or at least 90%, or about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 100%. In this context, the expression level is preferably with regard to the plurality of said iHLCs, which may be used for the characterization of said iHLCs. This characterization of said iHLCs is described in **Example 2**.

[97] The present invention may contemplate target precursor stem cells preferably being selected from human or non-human induced pluripotent stem cells (iPSCs), human or non-human adult stem cells, human parthenogenetic or non-human embryonic stem cells (ESCs), a hepatocyte cell-line or primary hepatocytes. Preferably, target precursor stem cells may be hiPSCs.

[98] The present invention may contemplate iHLCs and target precursor stem cells preferably being human cells. Further, the present invention may envisage iHLCs and target precursor stem cells being derived preferably from the same human donor. In this context, "human donor" refers to a human patient used for donating his/her cells for personalized medicine. Said patient preferably donates hiPSCs being differentiated into iHLCs and hiPSCs being preferably differentiated into neural cells on iHLCs and/or on iHLC matrix. The human donor preferably is a human or non-human animal, generally a mammal. Particularly, a mammal may be a rabbit, a mouse, a rat, a Guinea pig, a hamster, a dog, a cat, a pig, a cow, a goat, a sheep, a horse, a monkey, an ape or preferably a human, most preferably an adult.

[99] Further, the present invention may envisage a pre-selected cell type being preferably a non-hepatocyte cell type. A pre-selected cell type, which is not derived from hepatocytes, is

preferably a neural cell type (ectodermal origin), which may be induced by the differentiation of hiPSCs on iHLCs and/or iHLC matrix (endodermal origin). In this context, ectodermal origin refers to deriving from ectoderm and endodermal origin refers to deriving from endoderm.

[100] The term “pre-selected” or “pre-selected cell type” means selecting in advance or choosing beforehand a cell type from a variety of cells from different germline lineages (e.g. endoderm such as pancreas, lung and liver; mesoderm such as muscle, dermis, endothelium and the circulatory system including heart and spleen; ectoderm such as brain).

[101] Consequently, the present invention contemplates target cells of a pre-selected cell type preferably being neural cells. Said target cells of the present invention may be mature neural cells, dopaminergic neurons, cholinergic neurons, GABAergic neurons, glutamatergic neurons, serotonergic neurons, sensory neurons, astrocytes and/or oligodendrocytes. Preferably, said target cells of the present invention are mature neural cells, specifically dopaminergic neurons. Most preferably, said target cells of the present invention are dopaminergic neurons.

[102] Consequently, the present invention provides a pre-selected cell type preferably being a neural cell type and target cells preferably being mature neural cells expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM; specifically dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6, sensory neurons expressing POU4F1 and/or peripherin, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP. The present invention may provide a pre-selected cell type preferably being a neural cell type and target cells preferably being mature neural cells preferably expressing Map-2 and TuJ1, most preferably Map-2. Preferably, the present invention may comprise target cells being dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2. The present invention may provide a pre-selected cell type preferably being a neural cell type and target cells preferably being mature neural cells preferably expressing Map-2 and TuJ1, specifically dopaminergic neurons preferably expressing Nurr1, En1, TH and/or FoxA2, most preferably expressing Nurr1 and/or En1. The present invention may contemplate a pre-selected cell type preferably being a neural cell type and target cells preferably being mature neural cells preferably expressing Map-2 and TuJ1, specifically dopaminergic neurons preferably expressing Nurr1, En1, TH and/or FoxA2, most preferably expressing TH. The present invention may contemplate a pre-selected cell type preferably being a neural cell type and target cells preferably being mature neural cells preferably expressing Map-2 and TuJ1, specifically dopaminergic neurons preferably expressing Nurr1, En1, TH and/or FoxA2, most preferably expressing FoxA2.

[103] The present invention may envisage target cells being mature neural cells, specifically dopaminergic neurons, which may be induced more efficiently on hiPSCs-derived iHLCs in comparison to MS5 cells. The present invention may envisage target cells being mature neural cells, specifically dopaminergic neurons, which may be induced more efficiently on iHLC-derived matrix in comparison to HepG2 matrix and/or Geltrex matrix.

[104] Mature neural cells preferably express Map2 (Microtubule-associated protein 2) being a cytoskeletal protein and being expressed weakly in neuronal progenitor cells (NPCs) but increases during neuron development process. In general, its expression is confined to neurons and reactive astrocytes. Mature neural cells also preferably express the neuron-specific TuJ1 (class III β -tubulin, also called beta3 Tubulin). TuJ1 may be present in newly generated immature postmitotic neurons and differentiated neurons and in some mitotically active neuronal precursors. TuJ1 as referred to herein may be used as pan-neuronal marker of mature neurons, which may have multiple targets (somatic, nuclear, dendritic, spine and axonal proteins) and consequently may label across all parts of the neuron. In general, pan-neuronal markers may be used for study neuronal morphology.

[105] Dopaminergic neurons, in particular midbrain dopaminergic neurons preferably express TH (tyrosine hydroxylase), which may be an enzyme involved in the synthesis of dopamine and norepinephrine. Further, dopaminergic neurons, in particular midbrain dopaminergic neurons preferably express the transcription factor FoxA2 (Forkhead-Box-Protein A2). FoxA2 is preferably necessary for neuronal survival. Most preferably, FoxA2 is required for adult dopaminergic neurons maintenance. Nurr1 may also be critical for the development and maintenance of midbrain dopaminergic (DA) neurons. According to Luo 2012, Int Rev Neurobiol;102:1-22, loss of Nurr1 function early during development in mice leads to the absence of midbrain DA neurons. Additionally, the researchers have shown that reduction of Nurr1 function in adulthood leads to a slowly progressive loss of striatal DA and markers for mDA neurons, supporting its important roles in the maintenance of dopaminergic neuronal survival and function. En1 may be expressed highly by all dopaminergic neurons, in particular midbrain dopaminergic neurons from the moment they start to differentiate and continuously into adulthood. Furthermore, the deletion of one En1 allele may lead to massive cell death of dopaminergic neurons in young adult mice, highlighting En1 as a potential gene in the molecular cascade, which may lead to progressive cell death in the neuropathology of Parkinson, being the selective and progressive degeneration of dopaminergic neurons. Such data points to the core importance of En1 in the maintenance of mdDA neurons.

[106] As mentioned before, neural induction in the differentiation of hiPSCs to midbrain dopaminergic neurons in a matrix based differentiation system may be initiated more efficiently in N2B27 1:1 medium supplemented with the dual SMAD inhibition LDN-193189 (LDN) and SB431542 (SB) (**Fig. 19** or **Example 15**). Preferably, neural induction in the differentiation of

hiPSCs to mDAs in a matrix based differentiation system may be initiated more efficiently in N2B27 1:1 medium supplemented with 100 nM LDN and 10 μ M SB. Neural induction or neural identity in the differentiation of hiPSCs to midbrain dopaminergic neurons may be acquired through the use of inhibitors, preferably through dual SMAD inhibition.

[107] As used herein, the term “neural cell” may refer to any cell that *in vivo* would become part of the nervous system (CNS and/or PNS) and in culture (*in vitro*) is obtained by methods of the present inventions. Neural cells preferably being the target cells of the present invention may derive from a neural cell type. Neural cells may refer to neural stem cells (NSCs), neural progenitor cells (NPCs), all mature neural cells in general, neurons including cholinergic neurons, GABAergic neurons, glutamatergic neurons, serotonergic neurons, patternable (i.e. a cell capable of undergoing further differentiation), neuronal populations of motor neurons and dopaminergic neurons, sensory neurons and/or astrocytes and/or oligodendrocytes. Neural cells may be divided into neural cells of early differentiation, which may include neural stem cells and/or NPCs and neural cells of late differentiation, which may include mature neural cells, specifically dopaminergic neurons, cholinergic neurons, GABAergic neurons, glutamatergic neurons, serotonergic neurons, motor neurons, sensory neurons, astrocytes and/or oligodendrocytes. The term “neural” refers to a neural cell and/or a neural cell type.

[108] The term “mature neural cells” refers to fully developed and differentiated neural cells of late differentiation. Mature neural cells may be characterized among others by different markers of late neuronal fate acquisition such as Dcx, Map2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM. Mature neural cells may comprise dopaminergic neurons, cholinergic neurons, GABAergic neurons, glutamatergic neurons, serotonergic neurons, sensory neurons as well as astrocytes and/or oligodendrocytes. The defined neural cell type whether a mature neural cell may become a neuron, specifically a dopaminergic neuron, a cholinergic neuron, a GABAergic neuron, a sensory neuron, a motor neurons, a serotonergic neuron, a glutamatergic neuron or an astrocyte or an oligodendrocyte, has not yet been determined by markers characterizing dopaminergic, cholinergic, GABAergic neurons etc. and/or markers characterizing astrocytes or oligodendrocytes. In this context, the term “markers of late neuronal fate acquisition” refers to markers of neural cells of late differentiation. *Vice versa* markers of early neural fate acquisition refer to markers of neural cells of early differentiation (e.g. N-Cadherin, Pax6, Otx2, Nestin etc.). In this context, the term “neuronal” refers to a neuronal cell (a neuron).

[109] In general, neurons refer to a nerve cell, which is an electrically excitable cell that processes and transmits information through electrical and chemical signals. These signals between neurons occur via synapses, specialized connections with other cells. Neurons can connect to each other to form neural networks. Neurons are the core components of the brain and spinal cord of the central nervous system (CNS), and of the ganglia of the peripheral

nervous system. Neurons consist of a cell body (or soma) including the nucleus, a number of branching dendrites and an axon and various synapses at the axon terminal. Neurons may include but are not limited to dopaminergic neurons, sensory neurons, motor neurons, cholinergic neurons, serotonergic neurons, GABAergic neurons and/or glutamatergic neurons.

[110] In particular, “a dopaminergic (DA) neuron” refers to a dopamine-producing nerve cell, wherein dopamine is a common neurotransmitter. The main source of dopamine (DA) in the mammalian central nervous system results in dopaminergic neurons of the midbrain. They occur in a ‘harsh’ region of the brain called the substantia nigra pars compacta. This region is DA-rich and characterized by redox available neuromelanin and a high iron content. A loss of dopaminergic neurons may be associated with one of the most prominent human neurological disorders, Parkinson's disease (PD). Dopaminergic neurons may be characterized, among others by the expression of Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2.

[111] “A cholinergic neuron” refers to a neuron that synthesizes, stores and releases acetylcholine (ACh). Such neurons may be characterized, among others by the expression of NGF and/or ChAT.

[112] “A GABAergic neuron” refers to a neuron, which produces GABA (Gamma aminobutyric acid, a principal inhibitory neurotransmitter in the central nervous system), and is the predominant inhibitory neuron in the mammalian CNS. Approximately 60-75% of all synapses in the CNS are GABAergic. GABAergic neurons may be characterized, among others by the expression of GAD67 and/or vGAT.

[113] “A glutamatergic neuron” is a neuron producing glutamate, which is one of the most common excitatory neurotransmitters in the central nervous system. Glutamatergic neurons may be characterized, among others, by the expression of vGLUT1.

[114] “A serotonergic neuron” refers to a neuron synthesizing the neurotransmitter serotonin (5-HT), which is found in the gastrointestinal tract, blood platelets, and the central nervous system (CNS). Serotonergic neurons may be characterized, among others by the expression of TPH and/or SERT.

[115] “A motor neuron” refers to a neuron, whose cell body is located in the spinal cord and whose fiber (axon) projects outside the spinal cord to directly or indirectly control effector organs, mainly muscles. Motor neurons' axons are efferent nerve fibers that carry signals from the spinal cord to the effectors to produce effects. Types of motor neurons are alpha motor neurons, beta motor neurons, and gamma motor neurons. Motor neurons may be characterized, among others by the expression of HB9, SMN, ChAT and/or NKX6.

[116] “A sensory neuron” refers to a neuron that converts a specific type of stimulus into action potentials or graded potentials. Sensory neurons may be characterized, among others by the expression of POU4F1 and/or peripherin.

[117] “An astrocyte” refers to a sub-type of glial cells being non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for neurons in the central nervous system. Astrocytes perform many functions, including provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord. Astrocytes may express, among others, GFAP and/or Tapal.

[118] “An oligodendrocyte” refers to a sub-type of glial cells. Their main functions are to provide support and insulation to axons in the central nervous system of some vertebrates by creating the myelin. Oligodendrocytes may be characterized, among others by the expression of O1, O4, CNPase, and/or MBP.

[119] Further, the present invention envisages the pre-selected neural cell type being preferably induced by the addition of SHH activators, and/or FGF activators, and/or BMP inhibitors and/or TGF β inhibitors.

[120] Sonic hedgehog (SHH) is a member of the Hedgehog family of secreted signaling proteins that has a prominent role in patterning during early mammalian development. Among the other members of the Hedgehog family, SHH is expressed during early embryogenesis in a variety of tissues. In particular, SHH may directly act on cells to specify neural cell fates, may regulate proliferation and survival of oligodendrocytes precursors and neural crest cells and stimulates proliferation, differentiation and axon growth (Finco et al. 2015, *Annual Review of Physiology*; 77:105-129). Thus, the present invention may contemplate a pre-selected neural cell type being induced by SHH, which may function as an activator of dopaminergic neuron specification.

[121] FGF is a fibroblast growth factor, a family consisting of 22 FGF ligands. They function as heparin-binding proteins, structurally related to signaling molecules. Since binding of the FGF to the FGF-receptor induces receptor tyrosine auto-phosphorylation, FGFs play a tremendous role in the processes of proliferation and differentiation of wide variety of cells and tissues (Zhang et al. 2008, *Mol. Endocrinol*, 22(1): 167-175).

In particular, as used mostly herein, the growth factor FGF8 with its two variants FGF8a and FGF8b may be expressed in the mid-hindbrain region during development. Further, FGF8 signaling is important for neural crest delamination and migration (Olsen et al. 2006, *GenesDev*. 20, 185-198 and Vitelli et al. 2002, *Development* 129, 4605-4611). The present invention may contemplate a pre-selected neural cell type being induced by FGF, preferably by FGF8 and/or FGF2, which may function as an activator for dopaminergic neuron specification.

[122] Hence, specific neurons, preferably dopaminergic (DA) neurons that reside in the ventral midbrain depend both on SHH and FGF, preferably SHH and FGF8 for their development (Hynes and Rosenthal 1999, *Curr. Opin. Neurobiol.* 9, 26-36).

Thus, the present invention may contemplate a pre-selected neural cell type being induced by SHH activators and/or FGF activators, preferably by SHH activators and/or FGF8 activators and/or FGF2 activators.

[123] BMP is a bone morphogenetic protein that refers to a multi-functional growth factor and belongs to the transforming growth factor beta (TGF β) superfamily. The TGF β superfamily of ligands include besides BMPs, GDFs, Activin, Nodal and TGF β s. As soon as a TGF β superfamily ligand binds to a TGF β type II receptor, signaling begins. Each class of ligand binds to a specific type II receptor, thus for a BMP ligand a type II receptor may refer to BMPRII and for a TGF β ligand a type II receptor may refer to TGF β RII. The type II receptor is a serine/threonine receptor kinase, which catalyzes the phosphorylation of the Type I receptor. A type I receptor may refer to ALK2 and ALK3 for a BMP signaling pathway (Alberts et al. 2002, *Molecular Biology of the Cell*. New York, NY: Garland Scienc).

[124] Further, BMP signaling is important in heart, neural and cartilage development and also plays a crucial role in postnatal bone formation (Chen et al. 2004, *Growth Factors*, 22(4):233-41). Elegant studies in frog identified BMP inhibitors including chordin (Sasai, et al. 1994, *Cell* 79(5):779), follistatin (Hemmati-Brivanlou, et al. 1994, *Cell* 77(2):283), and noggin (Smith, et al. 1992, *Cell* 70(5):829) as the critical neural inducing factors. Among other, LDN-193189 may also be a selective BMP signaling inhibitor, which may inhibit the transcriptional activity of the BMP type I receptors ALK2 and ALK3 and may prevent phosphorylation of their downstream targets SMAD1, SMAD5 and SMAD8. In contrast, LDN-193189 may have little effect on ALK4, ALK5, and ALK7, which may be inhibited by a specific TGF β inhibitor. The LDN inhibitor of BMP type I receptors ALK2 and ALK3 may be used for the induction of neural fate in the present invention. Thus, the present invention may contemplate a pre-selected neural cell type being induced by BMP inhibitors, preferably by the ALK2/3 inhibitor LDN-193189.

[125] TGF β includes transforming growth factor-beta as well as functional equivalents, derivatives and analogs thereof. The TGF-beta isoforms are a family of multifunctional, disulfide-linked dimeric polypeptides that affect activity, proliferation and differentiation of various cells types. TGF-beta is a polypeptide produced in a latent propeptide form having, at this time, no identified biological activity. To be rendered active and, the propeptide form of TGF-beta must be cleaved to yield active TGF β . Among others, the specific inhibitor which may block the TGF β pathway is preferably the potent inhibitor SB431542 (Tocris). SB-431542 is a small molecule inhibitor that was identified as an inhibitor of Activin receptor-like kinase ALK5, which is the TGF β type I receptor. Besides inhibiting ALK5 it also inhibits the Activin type I receptor ALK4 and the Nodal type I receptor ALK7 (Gareth et al. 2002, *Molecular Pharmacology*, 62 (1) 65-74). SB431542 is a drug shown to enhance neural induction in an embryoid body (EB) based hESC neural induction protocol (Smith, et al. 2008, *Dev Biol* 313(1):107) and which also blocks the Activin and Nodal signaling pathway. The inhibition of

ALK4, ALK5, and ALK7 prevents the phosphorylation of their downstream targets SMAD2 and SMAD3. This specific inhibitor of TGFb may be among others used for the induction of neural fate in the present invention. Thus, the present invention may contemplate a pre-selected neural cell type being induced by TGFb inhibitors, preferably by the ALK4/5/7 inhibitor SB431542.

[126] The present invention may provide a pre-selected neural cell type being induced by BMP inhibitors and/or TGFb inhibitors. Preferably, a pre-selected neural cell type may be induced by LDN-193189 (LDN) and SB431542 (SB), both being preferably referred to dual SMAD inhibition. Thus, dual SMAD inhibition as referred to herein may be determined by LDN-193189 (LDN) and SB431542 (SB). LDN-193189 (LDN) and SB431542 (SB) may also refer to as indirect SMAD protein inhibitors. Preferably, LDN-193189 (LDN) and SB431542 (SB) may also refer to indirect inhibitors of SMAD proteins 1, 2, 3, 5, and 8. Dual SMAD inhibition may therefore be essential for inducing neural fate. The present invention may envisage a pre-selected cell type being the neural cell type being induced by dual SMAD inhibition.

[127] The term "SMAD inhibition" refers to the direct and/or indirect inhibition of the Small Mothers Against Decapentaplegic proteins. Several lines of evidence demonstrate a crucial role for SMAD signaling during neural induction. Generally, SMAD proteins are downstream of TGFb superfamily ligands, and when their specific receptors are activated, they stimulate the phosphorylation of the receptor-regulated SMADs. Receptor-regulated-SMADs may refer to SMAD1, SMAD2, SMAD3, SMAD5 and SMAD 8, with SMADs 2 and 3 specifically being activated by Activin/Nodal and TGF-beta type I receptors. SMADs 1, 5, and 8 specifically being activated by BMP type I receptors. Dual SMAD inhibition may take a confluent culture of hiPSCs and rapidly may differentiate them into early neurectoderm. This rapid differentiation may be caused by blocking the two signaling pathways that utilize SMADs for transduction: BMP and TGFb. In this context, dual SMAD inhibition may refer to the inhibitors LDN 193189 and SB431542, which may indirectly inhibit SMAD proteins.

[128] The present invention may also contemplate a composition comprising (a) immature hepatocyte-like cells (iHLCs) and/or (b) an iHLC-derived extracellular matrix (ECM) as a culture system and (c) target precursor stem cells differentiating into target cells of a pre-selected cell type.

[129] Contemplated by the invention is a composition described herein, wherein said target precursor stem cells are preferably selected from human or non-human iPSCs, human or non-human adult stem cells, and human parthenogenetic or non-human ESCs, a hepatocyte cell-line or primary hepatocytes. Preferably, the present invention may comprise a composition, wherein said target precursor stem cells may be hiPSCs.

[130] The present invention may also comprise a composition described herein, wherein said target cells of the composition are preferably mature neural cells expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or

CD56/NCAM, specifically are dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6 as well as sensory neurons expressing POU4F1 and/or peripherin. Further, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP. Preferably, said target cells of the composition contemplated by the invention are dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2.

[131] The present invention may also envisage a composition described herein, comprising said target precursor stem cells and/or target cells of a pre-selected cell type in a 2D- or 3D-cell culture. Preferably, the present invention may envisage a composition comprising said target precursor stem cells and/or target cells of a pre-selected cell type in 3D-cell culture.

[132] Normally, the cultivation of cells on a flat plastic surface leads to an artificial, two-dimensional cell layer. Normal cells in the human body develop in a three-dimensional environment, surrounded by other cells, membranes and adhesion proteins etc. Preferably, the present invention provides a 3D- cell culture system, offering an *in vivo* morphology and a particular environment for more realistic cell biology.

[133] Further, the present invention may also provide a method for preparing an iHLC-derived cell-free ECM, comprising (a) cultivating iHLCs *in vitro* under conditions allowing for formation of an iHLC-derived ECM, (b) decellularizing the iHLC-derived ECM and (c) obtaining the iHLC-derived cell-free ECM. In this context, the term “cultivating” may refer to letting cells grow and differentiate under certain conditions that may also enable or allow the process of forming an ECM, preferably an iHLC-derived ECM. The term “decellularizing” in this context, may include washing iHLCs once with PBS and then adding the decellularization buffer to the cells, which may contain 20mM ammonium hydroxide, 0.5% Triton X-100 in PBS and which is preferably steril-filtered. The decellularization buffer may isolate the ECM derived from said iHLCs from its inhabiting cells, said iHLCs (**Example 3**).

[134] The present invention may also comprise an immature hepatocyte-like cell (iHLC) derived extracellular matrix preferably obtained or obtainable by the method as mentioned above. Preferably, the differentiation of hiPSC on iHLC matrix is the most efficient culture system for dopaminergic differentiation compared to HepG2 matrix. According to Shin et al., 2006, *Stem Cells*; 24:125-138, neural induction was induced for human embryonic stem cells on a feeder layer from mouse embryonic fibroblasts (MEF) with a conditioned medium from HepG2 cells. The present invention disproved Shin et al. 2006 that neither HepG2 cells and/or HepG2 conditioned medium nor HepG2 matrix may be used as an *in vitro* culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type. HepG2 cells or HepG2 conditioned medium or HepG2 matrix may not induce neural fate in comparison

to iHLCs and/or an ECM derived from said iHLCs of the present invention. Preferably, the present invention may envisage an iHLC-derived ECM obtained or obtainable by the method as mentioned above for dopaminergic neuron differentiation in comparison to a HepG2 matrix. A HepG2 matrix may refer to the extracellular matrix derived from (produced from) HepG2 cells being a human hepatocellular carcinoma cell line.

[135] Additionally, the present invention may comprise an iHLC-derived extracellular matrix preferably characterized by the expression of Lama1, Lama5, Lamb1, Lamb2, Lamb3, Lamc1, Lamc2, Perlecan, Thbs1, Nid1, Vcan, Ltbp1, Col5a1, Agrn, Fbn2, Gdf6, Hapln1, Igfbp2, Igfbp5, Mdk, Vtn, Col3A1, Col4A1, Fbln2, Tnc, and Sfrp1.

[136] An iHLC matrix may provide a specific combination of factors or ECM components for neural induction, specifically for dopaminergic neuron induction. This specific combination of factors or ECM components may refer to the specific signature of the iHLC matrix. In this context, the term “signature of the iHLC matrix” may refer to specific ECM proteins (candidate proteins) being expressed in the iHLC matrix, which may give the matrix the ability to induce neural fate, preferably dopaminergic neurons. Lama1, Lama5, Lamb1, Lamb2, Lamb3, Lamc1, Lamc2, Perlecan (Hspg2), Thbs1, Nid1, Vcan, Ltbp1, Col5a1, Agrn, Fbn2, Gdf6, Hapln1, Igfbp2, Igfbp5, Mdk, Vtn, Col3A1, Col4A1, Fbln2, Tnc, and Sfrp1, but not limited to may be considered as those candidate proteins (ECM proteins), which may represent the specific signature of the iHLC matrix.

[137] In particular, Lama1, Lama5, Lamb1, Lamc1, Ltbp1, Thbs1, Nid1, Agrn, Tnc, Fbln1, Vtn, Fbn2, Hapln1, Igfbp2 and Igfbp5 may refer to ECM glycoproteins, whereas Perlecan and/or Vcan are preferably proteoglycans and Col4a1 and Col3a1 may refer to collagens. ECM glycoproteins, collagens and proteoglycans may refer to core matrisome proteins (proteins of the core matrisome). In this context, the term “matrisome” may refer to the ensemble of more than 1000 genes encoding ECM proteins (core matrisome proteins) and ECM-associated proteins (matrisome associated proteins). Additionally, Mdk, Sfrp1, Gdf6 may refer to a matrisome-associated protein, which may comprise ECM-affiliated proteins, ECM regulators and secreted factors, in particular Mdk, Sfrp1 and Gdf6 may refer to a secreted factor.

[138] The present invention may further comprise an iHLC-derived ECM being preferably characterized by Lamb1, Lamc1 and/or Lama1 in comparison to HepG2 cells. The present invention may further comprise an iHLC-derived ECM being preferably characterized by Agrn, Fbn2, Gdf6, Hapln1, Igfbp2, Igfbp5, Lamb1 and Ltbp1 in comparison to HepG2 cells, hiPSCs and mesenchymal stem cell (MSC). Here, Fbn2, Igfbp2, Igfbp5 and Ltbp1 are involved in TGFβ-signalling, Gdf6 in BMP-signalling and Agrn, Hapln1 and Lamb1 in CNS development. The present invention may further comprise an iHLC-derived ECM being preferably characterized by Mdk and Vtn in comparison to MSC and hiPSCs. Mdk and Vtn are associated with CNS development. The present invention may further comprise an iHLC-derived ECM being

preferably characterized by Col3a1, Col4a1, Fbln1, Thbs1, and Tnc in comparison to hiPSCs and HepG2 cells. Col3a1, Col4a1, and Tnc are involved in CNS development, whereas Fbln1 and Thbs1 are involved in TGF β -signalling. The present invention may further comprise an iHLC-derived ECM being preferably characterized by Sfrp1 in comparison to MSC and HepG2 cells. Sfrp1 is involved in dopamine neuron development. The present invention may also comprise an iHLC-derived ECM being preferably characterized by FGF2 in comparison to HepG2 cells. FGF2 may also refer to a matrisome-associated protein of an extracellular matrix, in particular to a secreted factor as mentioned above.

[139] Further, the present invention may provide an iHLC-derived ECM supporting neural induction characterized by Lama5, Lamb1, Lamb2, Lamc1, Perlecan, Thbs1, Nid1, Vcan, Agrn, Hapln1, Fbn2, Igfbp2, Igfbp5, Mdk, Vtn, Col3a1, Col4a1, Tnc, Fbln1. Additionally, the present invention may comprise an iHLC-derived ECM supporting dopaminergic neuron induction characterized by Lama1, Lamb3, Lamc2, Ltbp1, Col5a1, Sfrp1, Tnc.

[140] The iHLC-derived ECM contemplated by the invention is preferably further (a) highly concentrated, (b) growth factor reduced, (c) phenol-red free and/or (d) suitable for human embryonic stem cell (hESC) cultivation.

[141] In this context, the term “highly concentrated” may refer to higher protein concentration (HC), which provides greater matrix stiffness and scaffold integrity. A preferred protein concentration may comprise more than 10 mg/mL, less than 30 mg/mL, or 10-30 mg/mL, 15-25 mg/mL, 18-21 mg/mL, or at least 10, 15, 18, 19, 20, or at least 21 mg/mL. Preferably, 18 mg/mL to 21 mg/mL. Further, the term “growth factor reduced (GFR)” may refer to applications, where more highly defined basement membrane preparation may be desired. Here, a preferred protein concentration may comprise more than 5 mg/mL, less than 20 mg/mL, or 5-20 mg/mL, 7-15 mg/mL, 8-12 mg/mL, or at least 5 mg/mL, 8, 9, 10, 11, or at least 12 mg/mL. Preferably, 8 mg/mL to 12 mg/mL. The term “phenol-red free” may refer to assays that require color detection (e.g., colorimetric, fluorescence). A phenol red free medium may not include Phenol Red pH-indicator. A preferred protein concentration of a phenol-red free medium may comprise more than 5 mg/mL, less than 20 mg/mL, or 5-20 mg/mL, 7-15 mg/mL, 8-12 mg/mL, or at least 5 mg/mL, 8, 9, 10, 11, or at least 12 mg/mL. Preferably, 8 mg/mL to 12 mg/mL. Although, the iHLC-derived ECM of the present invention may comprise certain candidate proteins as mentioned above, it may also comprise some characteristics of the standard Corning Matrigel matrix of the literature, which may also be highly concentrated, growth factor reduced and/or phenol-red free. A Corning Matrigel matrix may refer to a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins, including Laminin (a major component of said Matrigel), Collagen IV, heparin sulfate proteoglycans, entactin/nidogen, and a number of growth factors.

Additionally, said ECM derived from said iHLCs may provide the reproducibility and consistency essential for human embryonic and/or induced pluripotent feeder-free stem cell culture. The present invention may envisage an ECM derived from said iHLCs comprising a perfect combination of specific ECM components for the induction of neural fate and standard formulation of ECMs known from the literature.

[142] The present invention may encompass a device comprising immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLC as a culture system. Preferably, the device is a vial or a cell-culture dish. The vials of the present invention may comprise centrifuge tubes comprising different sizes. The present invention may include centrifuge tubes (e.g. polypropylene centrifuge tubes) comprising 10, 15, 20 25, 30, 35, 40 or 50 ml working volume. The present invention may envisage cell-culture dishes comprising petri dishes of different sizes. The present invention may include petri-dishes comprising 3, 4, 5, 6, 7, 8, 9, or 10 ml working volume. Additionally, the present invention may envisage cell-culture dishes comprising flasks of different sizes. The present invention may include flasks comprising 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300 or greater than 300 ml working volume. The vials and the cell-culture dishes may include the ECM preferably derived from hiPSC derived iHLCs, which may provide a 3D-cell culture system, offering an *in vivo* morphology and a particular environment for more realistic cell biology

[143] The present invention may envisage a device comprising target precursor stem cells of the present invention or target cells of a pre-selected cell type of the present invention. Said device may comprise target precursor stem cells, wherein said target precursor stem cells are preferably selected from human or non-human iPSCs, human or non-human adult stem cells, and human parthenogenetic or non-human ESCs, a hepatocyte cell-line or primary hepatocytes. Preferably, the device may comprise hiPSCs as target precursor stem cells. Further, the device may comprise target cells of a pre-selected cell type, wherein said pre-selected cell type is a non-hepatocyte cell type, preferably a neural cell type. Further, the device may comprise target cells of a pre-selected cell type, wherein said target cells are mature neural cells expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM; specifically dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6, sensory neurons expressing POU4F1 and/or peripherin, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP. Preferably, the device comprises target cells of a pre-selected cell type, wherein said target cells preferably are

dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2.

[144] The invention may also contemplate an *in vitro* method for obtaining target cells of a pre-selected cell type, comprising (a) providing immature hepatocyte-like cells (iHLCs) and/or an iHLC-derived ECM from the present invention, (b) adding target precursor stem cells from the present invention in a suitable cell culture medium and allowing their differentiation into the target cells of a pre-selected cell type defined by the invention; (c) obtaining the target cells of the pre-selected cell type.

[145] In this context “providing iHLC and/or iHLC-derived ECM” as used herein may refer to the differentiation of target precursor stem cells into iHLCs (**Example 1**) and the obtainment of an extracellular matrix derived from said iHLCs, which may be decellularized with a specific decellularization buffer containing 20mM ammonium hydroxide, 0.5% Triton X-100 (**Example 3**). “A suitable cell culture medium” as used herein, may refer to the medium, which may be used for the differentiation of target precursor stem cells on iHLCs (**Example 4**) and/or on hiPSCs-derived iHLC matrix (**Example 6** and **Example 15**) into the target cells of a pre-selected cell type. Here, neural induction in a rosette based differentiation (e.g. differentiation of hiPSCs on iHLCs) may be achieved with KSR medium containing Knock out DMEM, 15% Knock out serum replacement (KOSR), 2 mM L-glutamine, non-essential amino acids and 10 μ M β -mercaptoethanol supplemented with 1 μ M dorsomorphine (Tocris) and 10 μ M SB431542 (Miltenyi) and 100 ng ml⁻¹ SHH C24II (Miltenyi) and 100 ng ml⁻¹ FGF8 (Miltenyi), as it is described in **Example 4**. Neural induction of midbrain dopaminergic neurons (mDA) in a floor-plate based differentiation (e.g. differentiation of hiPSCs on iHLC matrix) may be achieved with KSR medium supplemented with 100 nM LDN193189 (Stemgent), 10 μ M SB431542 (Miltenyi), 100 ng ml⁻¹ SHH C24II (Miltenyi), 2 μ M Purmorphamine (Stemgent), 100 ng ml⁻¹ FGF8 (Miltenyi) and 3 μ M CHIR99021 (Miltenyi), as it is described in **Example 6**. Further, neural induction of midbrain dopaminergic neurons (mDA) of hiPSCs on iHLC matrix may also be achieved with N2B27 1:1 medium supplemented with 100 nM LDN193189 and 10 μ M SB431542, with 100 ng ml⁻¹ SHH C24II (Miltenyi) and 100 ng ml⁻¹ FGF8 (Miltenyi), as it is described in **Example 15**.

[146] The present invention may further provide the *in vitro* method, wherein said target cells are mature neural cells optionally expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM, specifically dopaminergic neurons expressing TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6, sensory neurons expressing POU4F1 and/or peripherin, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP. Preferably, the present invention may provide an *in vitro* method,

wherein said target cells obtained or obtainable by the *in vitro* method are dopaminergic neurons.

[147] Further, the present invention may contemplate a target cell of a pre-selected cell type obtained or obtainable by the *in vitro* method mentioned above for use in a method of cell grafting in a patient in need thereof. Cell grafting or neural transplantation involves a process for transplanting genetically modified donor cells, preferably neural cells from the present invention, into the central nervous system (CNS) to treat specific diseases. Conditions for successful transplantation may include: 1) viability of the implant; 2) retention of the graft at the site of transplantation; and 3) minimum amount of pathological reaction at the site of transplantation.

[148] The patient may be a non-human animal, a mammal, including rabbits, rats, rabbits, pigs, mice, hamsters, dogs, cats, cows, goats, sheeps, horses, monkeys, apes and/or humans. The patient may be any subject as defined herein, preferably a human subject. In one embodiment, the subject is an adult.

[149] Additionally, the present invention may contemplate a target cell preferably characterized by the increased expression of FoxA2, TH, Map2, Nestin, Pax6, Otx2, En1, Lmx1A and/or Nurr1 in comparison to a target cell obtained by cultivation in MS5 or Geltrex.

[150] Further, said target cell of the present invention may be used in a method for treating a neurodegenerative disease, including without limitation Alzheimer's Disease, Parkinson, Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease, Muscular Dystrophy, preferably Parkinson. Additionally, said target cell of the present invention may be used in a method for treating a neurodegenerative disease, including without limitation Alzheimer's Disease, Parkinson, Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease, Muscular Dystrophy in a subject. The subject may be a non-human animal, a mammal, including rabbits, rats, rabbits, pigs, mice, hamsters, dogs, cats, cows, goats, sheeps, horses, monkeys, apes and/or humans, preferably a human. In one embodiment, the subject is an adult.

[151] The present invention may comprise target cells, specifically dopaminergic neurons expressing Nurr1 and/or En1 for use in a method for treating a neurodegenerative disease, including without limitation Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease, Muscular Dystrophy, preferably Parkinson's Disease. Neurodegenerative disease may refer to a wide range of diseases and/or disorders of the central and peripheral nervous system, such as Parkinson's disease, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), denervation atrophy, otosclerosis, stroke, dementia, multiple sclerosis, Huntington's disease, encephalopathy associated with acquired immunodeficiency disease (AIDS), and other diseases associated with neuronal cell toxicity and cell death.

[152] Definitions:

[153] The term “*in vitro*” refers to studies that are conducted using components of an organism that have been isolated from their usual biological surroundings, such as cells, or biological molecules. For example, cells can be studied in artificial culture medium, proteins can be examined in solutions. Colloquially called “test-tube experiments”, these studies in biology, medicine and its sub-disciplines are traditionally done in test tubes, flasks, petri dishes, etc.

[154] As used herein the term, “*in vivo*” refers to the natural environment (e.g., an animal or a cell) and to processes or reactions that occur within a natural environment, such as embryonic development, cell differentiation, neural tube formation, etc.

[155] The term “culture system” as used herein refers to the use of different media containing different ingredients and supplements for differentiating cells in particular.

[156] The term “cell culture” refers to any *in vitro* culture of cells. Included within this term may be continuous cell lines (e.g., with an immortal phenotype), primary cell cultures, finite cell lines (e.g., non-transformed cells), and any other cell population maintained *in vitro*, including oocytes and embryos.

[157] The term “cultivation” refers to the process of cultivating cells, cell strains or lines in the laboratory preferably on matrices or feeder cells that the cells are able to grow, differentiate, induce certain markers and/or mature to fully developed cells.

[158] The term “derived from” may be used interchangeably with the term “originated from” or “obtained from”. For example, an extracellular matrix may be obtained preferably from iHLCs or iHLCs originating preferably from hiPSCs.

[159] The term “substantially” may be used interchangeably with the term “significantly” or “extremely”. For example, the ECM of the present invention being substantially cell-free means that the ECM is extremely isolated of its inhabiting cells, preferably of said iHLCs of the present invention.

[160] The term “expressing” may be used interchangeably with the term “characterized by the expression” or “characterized by the expression level”.

[161] The term “expression” may be used interchangeably with the term “expression level”.

[162] The term “fate” in general refers to a cell with a genetically determined lineage whose progeny cells are capable of becoming a variety of cell types or a few specific cell types depending upon *in vivo* or *in vitro* culture conditions. In other words, a cell's predetermined fate is determined by its environment to be destined for a particular differentiation pathway such that a cell becomes one cell type instead of another cell type. In this context, for example, “neural fate” may refer to hiPSCs which may become a neural cell instead of a muscle cell or a skin cell after differentiation of hiPSCs on hiPSCs-derived iHLCs and/or iHLC matrix.

[163] An “activator” may refer to a protein (transcription factor) that increases gene transcription of a certain gene/set of genes or which activates a signaling process or a

differentiation process of cells. In this context, “SHH and/or FGF activators” as used herein may refer to the proteins SHH and/or FGF, preferably to SHH and/or FGF8 that activate neural differentiation of hiPSCs into midbrain dopaminergic neurons.

[164] On the contrary, an “inhibitor” may refer to a protein that decreases gene transcription of a certain gene/set of genes or which inhibits a signaling cascade. In this context, “BMP and/or TGFb inhibitors” may be inhibitors that may inhibit BMP and/or TGFb signaling, which may be acquired for neural identity. Preferably, a BMP inhibitor may be LDN, a TGFb inhibitor may be SB.

[165] The term “increased” preferably means that an expression level of a protein is higher for specific cells which may be cultivated under certain conditions of the present invention than an expression level of a protein for cells being cultivated under conditions known in the prior art.

[166] The term “plurality of iHLCs” may refer to a specific amount of iHLCs in a well of a 48 well plate (about 100.000 cells in a well of a 48 well plate) or in a well of a 96 well plate (about 30.000 cells in a well of a 96 well plate), which is used for the characterization of said iHLCs in immunocytochemistry, preferably in immunocytochemistry pictures of about 200-500 cells are taken and the expression of iHLCs markers is examined. The characterization of said iHLCs is described in **Example 2**.

[167] “Feeder cells” or “feeders” may refer to cells of one tissue type that are co-cultured with cells of another (second) tissue type, to provide an environment in which the cells of the second tissue type can grow. The feeder cells are optionally from a different species as the cells they are supporting. Herein, the feeder cells may be MS5 cells, hiPSCs-derived iHLCs, HepG2 cells, hiPSC derived cardiomyocytes or hiPSC derived mesenchymal progenitor cells (MPCs), preferably hiPSCs-derived iHLCs. The cells, which are co-cultured with those feeders may be human or non-human iPSCs, human or non-human adult stem cells, human parthenogenetic or non-human ESCs, preferably hiPSCs.

[168] The term “feeder free” or “feeder-free matrix-based system” refers to the differentiation of cells of a tissue type not being co-cultured with other cells of another tissue type that provide an environment in which the cells being differentiated are able to grow. Feeder-free matrices may be Geltrex or Matrigel matrices. The iHLC matrix of the present invention may also be considered as feeder free. Feeder free may be used interchangeably with the term cell-free or decellularized. Feeder-free cells, preferably hiPSCs do not grow as round colonies with defined edges likes cells on feeder layers. Commonly, they grow as a monolayer. Thus, specialized culture media and specific enzymes have made it possible to expand those cells, preferably hiPSCs in the absence of feeder cells (e.g. iHLCs). Since feeder cells often interfere in certain differentiation protocols by producing pluripotency-enhancing factors, feeder-free matrices may be preferred in the present invention.

[169] It is noted that as used herein, the singular forms “a”, “an”, and “the”, include plural references unless the context clearly indicates otherwise. Thus, for example, reference to “a reagent” includes one or more of such different reagents and reference to “the method” includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[170] Unless otherwise indicated, the term “at least” preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[171] The term “and/or” wherever used herein includes the meaning of “and”, “or” and “all or any other combination of the elements connected by said term”.

[172] The term “about” or “approximately” as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range. It includes, however, also the concrete number, e.g., “about 20” includes 20.

[173] The term “less than” or in turn “greater than” includes the concrete number. For example, less than 20 means less than or equal to. Similarly, more than or greater than means more than or equal to, or greater than or equal to, respectively.

[174] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or “including” or sometimes when used herein with the term “having”. When used herein “consisting of” excludes any element, step, or ingredient not specified.

[175] The term “including” means “including but not limited to”. “Including” and “including but not limited to” are used interchangeably.

[176] It should be understood that this invention is not limited to the particular methodology, protocols, material, reagents, and substances, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[177] All publications cited throughout the text of this specification (including all patents, patent application, scientific publications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. To the

extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

[178] The content of all documents and patent documents cited herein is incorporated by reference in their entirety.

[179] A better understanding of the present invention and of its advantages will be had from the following examples, offered for illustrative purposes only. The examples are not intended to limit the scope of the present invention in any way.

38
EXAMPLES

[180] Hereinafter, the present invention is described in more detail and specifically with reference to the Examples, which however are not intended to limit the present invention.

[181] Example 1: Differentiation of hiPSCs to immature hepatocyte like cells (iHLCs).

[182] First, hiPSC need to be differentiated into iHLCs for further differentiation of hiPSCs on iHLCs (**Example 4**) or on an iHLC-matrix (**Example 6** or **Example 15**) into neural fate.

[183] hiPSCs were dissociated by accutase and seeded on geltrex coated dishes in mTeSR1 supplemented with 10 μ M Rock inhibitor Y-27632. Definitive endoderm differentiation was initiated by changing the medium to RPMI containing 1% B27 w/o insulin supplemented with 100 ng ml⁻¹ FGF2 (Miltenyi) and 100 ng ml⁻¹ Activin A (Miltenyi). For hepatocyte differentiation, medium was changed at day 8 to DMEM/F12 1:1 containing 10% Knock out serum replacement (KOSR), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, non-essential amino acids supplemented with additional D-glucose to a final concentration 4.5 mg L⁻¹, 100 ng ml⁻¹ human HGF (Miltenyi) and 1%DMSO (Sigma). At day 16 day, human HGF and DMSO were removed and medium was supplemented with 1 μ M dexamethasone (Sigma) for 3 days. For hepatic maturation, cells were cultured at day 19 in William's E medium supplemented with 10% FBS (fetalclone II, Hyclone, Thermo Scientific), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 0.023 IE ml⁻¹ human insulin (Sigma), 4.7 μ g ml⁻¹ hydrocortisone (Sigma), 80 μ g ml⁻¹ gentamicin (Sigma) and 1.8 % DMSO (Sigma) until the desired maturation state (**Fig. 3A**). For co-culture based midbrain dopaminergic (mDA) neuron differentiation and matrix based mDA neuron differentiation experiments, iHLCs were seeded at day 22 on Collagen I (10 μ g/cm²) coated dishes (50.000 cells/cm²) in William's E medium supplemented with 10% FBS (fetalclone II, Hyclone, Thermo Scientific), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 0.023 IE ml⁻¹ human insulin (Sigma), 4.7 μ g ml⁻¹ hydrocortisone (Sigma), 80 μ g ml⁻¹ gentamicin (Sigma) and 1.8 % DMSO (Sigma). Cells were used on the following day for mDA co-culture based differentiation experiments and after 3 days for matrix based mDA differentiation experiments (**Fig. 1**).

[184] Example 2: Markers of immature hepatocytes expressed in iHLCs differentiated from hiPSCs.

[185] After differentiation of hiPSC into iHLCs, iHLCs were characterized by the expression of marker of immature hepatocytes.

[186] Briefly, hiPSCs were obtained and differentiated into iHLC as described in **Example 1**.

On day 21 of differentiation iHLCs were subjected to immunocytochemistry using antibodies against markers of immature and mature hepatocytes HNF4a, Albumin (green) and Nkx2.5 and α -fetoprotein (AFP) (red) (**Fig. 2A**).

[187] Further, iHLCs were characterized and compared to HepG2 cells. HepG2 cells were obtained in parallel and mRNA was isolated from HepG2 as well as from iHLCs. Real-time PCR was then performed for other markers of immature hepatocytes such as OATP2B1, AFP, HNF4a, CYP3A7 as well as for mature hepatocyte markers such as albumin, α -1-antitrypsin, CYP3A4 and CYP7A1 (**Fig. 2B** and **C**). Beta-actin was used as a control in **Fig. 2B**.

[188] iHLCs expressed all markers of immature hepatocytes except CYP7A1 which was expressed in mature HepG2 cells, a human hepatocyte cell line.

[189] Example 3: Decellularization of hiPSCs-derived iHLCs and/or HepG2 matrix.

[190] For the differentiation of hiPSC on an iHLCs matrix (**Example 6** or **15**) or on a HepG2 matrix (**Example 15**), the matrix needs to be decellularized first.

[191] For decellularization of the extracellular matrix, iHLCs and/or HepG2 cells were seeded 3 days before decellularization in the appropriate medium at a density of 5×10^4 cells cm^2 . For decellularization, decellularization buffer (20 mM ammonium hydroxide, 0.5% Triton X-100 in PBS, steril filtered 0,22 μm) was preheated to 37 °C. hiPSCs-derived iHLCs were washed once with PBS and decellularization buffer was added to the cells, following incubation for 5 min at RT. Buffer was carefully taken off and cells were washed 3 times with PBS. Matrix was stored in PBS until hiPSCs were seeded.

[192] Example 4: Differentiation of hiPSCs on iPSC-derived iHLCs, according to Kriks et al. (2011), rosette based differentiation.

[193] hiPSCs-derived iHLCs were treated with 10 $\mu\text{g ml}^{-1}$ mitomycin C in final hepatocyte maturation medium (see above) for 1.5 hours at 37 °C. Cells were washed twice with PBS and stored in hepatocyte medium until seeding of hiPSCs. On day 0 of differentiation hiPSCs were seeded in clumps at a density of 1500 cells per cm^2 on mitomycin C treated hepatocytes in KSR containing Knock out DMEM, 15% Knock out serum replacement, 2 mM L-glutamine, non-essential amino acids and 10 μM β -mercaptoethanol. Cells were co-cultured with mitomycin treated hepatocytes for 18 days in KSR supplemented with 1 μM dorsomorphine (Tocris) and 10 μM SB431542 (Miltenyi) from day 2 to day 6 and 100 ng ml^{-1} SHH C24II (Miltenyi) and 100 ng ml^{-1} FGF8 (Miltenyi) from day 6 to day 18 of differentiation. On day 18 of differentiation, neural rosettes were mechanically isolated and plated on dishes pre-coated with polyornithine (15 $\mu\text{g ml}^{-1}$) and laminin (4 $\mu\text{g ml}^{-1}$) in Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2 mM L-glutamine supplemented with 100 ng ml^{-1} SHH C24II, 100 ng ml^{-1} FGF8, 20 ng ml^{-1} BDNF (Miltenyi), 0.2 mM ascorbic acid (Sigma). After 7 days, cells were dissociated by accutase and

seeded in droplets at a density of 20.000 cells per 20 μ l on dishes pre-coated with polyornithine (15 μ g ml^{-1}) laminin (4 μ g ml^{-1}) and fibronectin (1 μ g ml^{-1}) (P/O+LAM/FN) in N2/B27 supplemented with 100 ng ml^{-1} SHH C24II, 100 ng ml^{-1} FGF8, 20 ng ml^{-1} BDNF, 0.2 mM ascorbic acid. Final differentiation was performed by culturing cells in N2/B27 containing 20 ng ml^{-1} BDNF, 20 ng ml^{-1} GDNF, 0.2 mM ascorbic acid, 1 ng ml^{-1} TGF β 3 (Miltenyi) and 0.5 mM dibutyryl cAMP (Sigma) from day 32 of differentiation until the desired maturation stage (**Fig. 3B**).

[194] Example 5: Differentiation of hiPSCs on irradiated MS5 cells (as a control), according to Kriks et al. (2011); rosette based differentiation.

[195] Differentiation of hiPSCs on the mouse stromal cell line MS5 was performed as described above (differentiation of iPSCs on iPSC-derived iHLCs), except from co-culture of iPSCs on irradiated MS5 for 11 days instead of 18 days. MS5 cells function as a control for the differentiation of hiPSC into neural fate.

[196] In brief, irradiated MS5 cells were plated at a density of 50.000 cells per cm^2 in alpha-MEM containing 10% FBS and 2 mM L-glutamine. hiPSCs were seeded in clumps at a density of 1500 cells per cm^2 on MS5 cells in KSR containing Knock out DMEM, 15% Knock out serum replacement, 2 mM L-glutamine, non-essential amino acids and 10 μ M β -mercaptoethanol, supplemented with 1 μ M dorsomorphine (Tocris) and 10 μ M SB431542 (Miltenyi) from day 2 to day 6 and 100 ng ml^{-1} SHH C24II (Miltenyi) and 100 ng ml^{-1} FGF8 (Miltenyi) from day 6 to day 11 of differentiation. On day 11, neural rosettes were mechanically isolated and plated on dishes pre-coated with polyornithine (15 μ g ml^{-1}) and laminin (4 μ g ml^{-1}) in Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2 mM L-glutamine supplemented with 100 ng ml^{-1} SHH C24II, 100 ng ml^{-1} FGF8, 20 ng ml^{-1} BDNF (Miltenyi), 0.2 mM ascorbic acid (Sigma). On day 25, dissociation of cells by accutase was performed and cells were seeded in droplets at a density of 20.000 cells per 20 μ l on dishes pre-coated with P/O+LAM/FN in N2/B27 supplemented with 100 ng ml^{-1} SHH C24II, 100 ng ml^{-1} FGF8, 20 ng ml^{-1} BDNF, 0.2 mM ascorbic acid.

After 7 days final differentiation was achieved by culturing cells in N2/B27 containing 20 ng ml^{-1} BDNF, 20 ng ml^{-1} GDNF, 0.2 mM ascorbic acid, 1 ng ml^{-1} TGF β 3 (Miltenyi) and 0.5 mM dibutyryl cAMP (Sigma) until the desired maturation stage (**Fig. 3C**).

[197] Example 6: Differentiation of hiPSCs on human iPSC-derived hepatocyte matrix and Geltrex, according to Kriks et al. (2011); floor-plate based differentiation.

[198] Human iPS cells were dissociated by accutase and seeded at a cell density of 5×10^4 cells per cm^2 on human iPS-derived hepatocyte matrix or Geltrex (Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix, Thermo Fisher Scientific) coated dishes in mTeSR1 supplemented with 10 μ M Rock inhibitor. After cells reached a confluence of 80-

90%, medium was changed to KSR containing Knock out DMEM, 15% Knock out serum replacement, 2 mM L-glutamine, non-essential amino acids and 10 μ M β -mercaptoethanol supplemented with 100 nM LDN193189 (Stemgent) and 10 μ M SB431542 (Milttenyi) at day 0 of differentiation. On day 1, medium was changed to KSR containing 100 nM LDN193189 (LDN), 10 μ M SB431542 (SB), 100 ng ml⁻¹ SHH C24II (Milttenyi) (SHH), 2 μ M Purmorphamine (Stemgent) (Pur) and 100 ng ml⁻¹ FGF8 (Milttenyi). Medium was changed on day 3 to KSR supplemented with 100 nM LDN193189, 10 μ M SB431542, 100 ng ml⁻¹ SHH C24II (Milttenyi), 2 μ M Purmorphamine (Stemgent), 100 ng ml⁻¹ FGF8 (Milttenyi) and 3 μ M CHIR99021 (Milttenyi) (CHIR). KSR medium was gradually shifted to N2/B27 medium containing Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2 mM L-glutamine, starting from day 5 on (Day 5: 75% KSR, 25% N2/B27; Day 7: 50% KSR, 50% N2/B27; Day 9 25% KSR, 75% N2/B27) with exposure to the following factors from day 5 to day 6 to 100 nM LDN193189, 10 μ M SB431542, 100 ng ml⁻¹ SHH C24II, 2 μ M Purmorphamine, 100 ng ml⁻¹ FGF8, 3 μ M CHIR99021 and day 7 until day 10 to 100 nM LDN193189, 3 μ M CHIR99021. On day 11, medium was changed completely to N2/B27 supplemented with 3 μ M CHIR99021, 20 ng ml⁻¹ BDNF (Milttenyi), 20 ng ml⁻¹ GDNF (Milttenyi), 0.2 mM ascorbic acid (Sigma), 1 ng ml⁻¹ TGF β 3 (Milttenyi), 0.5 mM dibutyryl cAMP (Sigma) and 10 μ M DAPT (Milttenyi). On day 13, cells were mechanically dissociated and seeded on dishes pre-coated with polyornithine (15 μ g ml⁻¹) and laminin (4 μ g ml⁻¹) with a splitting ratio of 1:2 in N2/B27 supplemented with 20 ng ml⁻¹ BDNF, 20 ng ml⁻¹ GDNF, 0.2 mM ascorbic acid, 1 ng ml⁻¹ TGF β 3, 0.5 mM dibutyryl cAMP and 10 μ M DAPT. After 7 days, cells were dissociated by accutase and plated on dishes pre-coated with P/O+LAM/FN under high cell density conditions (3 x 10⁵ cells per cm²) in final differentiation medium (Neurobasal, 1% N2, 1% B27 w/o Vitamin A and 2 mM L-glutamine containing BDNF, 20 ng ml⁻¹ GDNF, 0.2 mM ascorbic acid, 1 ng ml⁻¹ TGF β 3, 0.5 mM dibutyryl cAMP, 10 μ M DAPT) until the desired maturation stage (**Fig. 3D**).

[199] Example 7: Immunofluorescence images of neural induction on MS5 cells and iHLCs co-cultured with hiPSCs.

[200] The differentiation of hiPSC on iHLCs was compared to the differentiation of hiPSCs on MS5 cells. Briefly, hiPSCs were obtained and co-cultured on human iHLC in comparison to MS5 cells (as a control) for neural induction. After neural induction, hiPSCs were reseeded on P/O+LAM/FN for further maturation.

[201] After 12 days of differentiation immunocytochemistry using antibodies against N-Cadherin (N-Cadh., green) and Pax6 (red) was applied. In another embodiment, antibodies against TH (green) and TuJ1 (red) were applied after 50 days of differentiation. Nuclei were stained with DAPI in both embodiments. A detailed description of the hiPSC differentiation on iHLCs and MS5 cells is provided in **Example 4** and **5**.

[202] Neuro-ectoderm rosettes obtained from hiPSCs can clearly be depicted by immunofluorescence stained with markers of early neural fate acquisitions such as N-Cadherin and Pax6 in iHLC co-culture system compared to MS5 cells (**Fig. 4A**). This data shows that rosette based differentiation of hiPSCs on hiPSCs-derived iHLCs was more efficient than rosette based differentiation of hiPSCs on MS5 cells in inducing the formation of neuroectoderm rosettes.

[203] In addition, stronger expression of markers of dopaminergic neurons (TH, green) and mature neurons (TuJ1, red) can be illustrated in hiPSC on human iHLC in comparison to MS5 at day 50 of differentiation (**Fig. 4B**). This data reveals that neurons derived from hiPSCs co-cultured with hiPSCs-derived iHLCs are more mature and denser compared to the mouse stromal cells MS5 functioning as a control.

[204] Example 8: Immunofluorescence images of neural induction on hepatocyte-derived matrix in comparison to Geltrex-based differentiation.

[205] After decellularization the remaining ECM derived from iHLCs was used to culture hiPSCs, showing that the iHLC-derived matrix provided an improved inducing neural effect in comparison to Geltrex-based differentiation.

[206] Briefly, hiPSCs were obtained and plated on hepatocyte-derived matrix (iHLC matrix) and on Geltrex matrix (as a control) for neural induction. After neural induction, hiPSCs were reseeded on P/O+ LAM/FN for further maturation. After 27 days as well as after 50 days of differentiation immunocytochemistry using antibodies against TH and FoxA2 and/or Map2 was applied. A detailed description of hiPSC differentiation on iHLC matrix and on Geltrex matrix is provided in **Example 6**.

[207] Markers of dopaminergic neurons such as TH (green) and FoxA2 (red), with FoxA2 being an important factor for neuronal survival were more powerful in expression on hepatocyte-derived matrix at day 27 of differentiation. Markers of dopaminergic and mature neurons such as TH (green) and Map2 (red) were also more powerful in expression on hepatocyte-derived matrix at day 50 of differentiation (**Fig. 5A and B**).

[208] Example 9: Expression of markers of early and late neuronal fate acquisition on hepatocyte versus Geltrex matrix.

[209] To confirm the data of **Example 8**, differentiation of hiPSC on hiPSCs-derived iHLC matrix into a neural cells, was performed again in comparison to the Geltrex matrix, which is currently used for feeder-free hiPSC neuronal differentiation. Additionally, real time PCR was conducted for different neural markers at different days of hiPSC differentiation.

[210] hiPSC were obtained and plated on hepatocyte-derived matrix of human iHLC and on Geltrex matrix (as a control) for neural induction. After neural induction, hiPSCs were reseeded

on P/O+ LAM/FN for further maturation. mRNA was then isolated and semi-quantitative PCR of FoxA2, Otx2, Lmx1a, TH, beta3 Tubulin and Map2 as markers of early and late neural fate acquisition was performed at day 7, 13 and 21 of differentiation. A detailed description of the hiPSC differentiation on iHLC matrix and Geltrex matrix is provided in **Example 6**.

[211] Real time PCR shows slightly changes in relative mRNA expression of early neural fate markers such as FoxA2 and Otx2 at day 7 and day 13 for hepatocyte-derived matrix compared to Geltrex matrix. Relative mRNA expression of markers of dopaminergic neurons such as TH and late neuronal fate acquisition such as Map2 shows moderate to strong changes after 21 days of differentiation for hepatocyte-derived matrix compared to Geltrex matrix (**Fig. 6**).

[212] The data mentioned above (**Fig. 5A, B and 6**) represent groundwork for the development of a human-based matrix for application in hiPSCs neuronal cultures, as well as its use in cell replacement therapies.

[213] Example 10: No induction into neural fate for human hepatocyte cell line HepG2.

[214] As a next step, the induction of neural fate was performed by testing HepG2 cells as feeder cells in comparison to the differentiation of hiPSCs on MS5 cells. HepG2 were taken as another co-culture system to prove, if other cells besides said iHLCs of the present invention have the potential to lead to the same neural inducing effect. Here, HepG2 cells do not induce neural fate to hiPSCs.

[215] Briefly, hiPSCs were obtained and co-cultured on human HepG2 cells in comparison to MS5 cells for differentiation into neural fate. Images of phase contrast at day 5, 11 and 18 were taken. A detailed description of the methods used is provided in **Example 4** (instead of hiPSC differentiation on iHLCs, a differentiation of hiPSC on HepG2 cells was performed) and **5**. Neuroectoderm rosettes were depicted at day 11 and day 18 in MS5 and no neural induction was identified in HepG2 based co-culture system (**Fig. 7A**).

[216] Furthermore, mRNA was isolated from HepG2 as well as from MS5 cells and real-time PCR analysis of the genes Pax6 and Otx2 was performed at day 18 of differentiation. Gapdh was used as a housekeeping gene for normalization. Real-time PCR analysis reveals that early neural fate markers such as Pax6 and Otx2 were expressed for the differentiation of hiPSCs on MS5 cells compared to HepG2 (**Fig. 7B**). Quantitative analysis of real-time PCR was also performed at day 11 and 18 for Pax6 and Otx2 genes (**Fig. 7C**). It can be concluded that differentiation of hiPSCs on HepG2 cells did not induce neural fate at all.

[217] Example 11: Differentiation of hiPSCs on hiPSCs-derived cardiomyocytes, (mesoderm), mesenchymal progenitor cell (mesoderm) or HepG2 cells (endoderm).

[218] To confirm the data of Example 10, other cell types from different germline lineages, mainly derived from hiPSCs, were tested, whether those cell types may be able to induce neural fate as well as it was seen for said hiPSCs-derived iHLCs of the present invention.

[219] To test whether the neural support is also happening with other cell types, using a co-culture system, hiPSCs were plated either on MS5 cells (positive control) or on other cell types such as hiPSCs-derived cardiomyocytes (CMs), mesenchymal progenitor cells (MPCs) or HepG2 cells (HepG2) and observed at days 1, 4, 5, 7 and 13 post-differentiation. Neuro-epithelial rosettes were observed in the MS5 conditions at day 11 but not in the other conditions, where only morphological changes were detected. In MS5, neuro-epithelial rosettes were picked at day 11 and re-plated onto Poly-L-ornithine (15 µg/ml) and laminin (5 µg/ml) (P/O+LAM) coated-plates and showed typical rosette structures at day 13. In the other conditions, no rosettes were detected at day 13 either, demonstrating a lack of neural induction for HepG2 cells, CMs and/or MPCs (**Fig. 8**).

[220] Next, semi-quantitative PCR for Otx2, Nestin, Pax6 and Map2 (Gapdh referred as a control) and quantification of midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and other cell types such as HepG2 cells, CMs or MPCs at day 11 and day 18 were performed. It was observed that the marker of dopaminergic neurons Otx2 and the marker of mature neurons Map2 are only present in the MS5 condition. However, the early neural fate marker Pax6 is also observed at day 18 in the HepG2 conditions, indicating that HepG2 can support early neural induction but very inefficiently (**Fig. 9**).

[221] Further, immunocytochemistry on day 11 of mDA neuron differentiation of hiPSC on MS5 cells, HepG2 cells and iPSC-derived CMs and MPCs was carried out. Expression of Pax6 and Nestin protein, both markers of early neural fate, shows that hiPSC colonies at day 11 of neural differentiation are only differentiating into neural cells on the MS5 (positive control) (**Fig. 10**).

[222] It can be concluded that neural induction is not supported by other cell types such as HepG2 cells, CMs and/or MPCs. However, neural induction is supported in the present invention by hiPSCs-derived iHLCs and iHLC matrix.

[223] Example 12: Differentiation of hiPSCs on Geltrex using conditioned medium from HepG2 cells.

[224] According to Shin et al., 2006, *Stem Cells*; 24:125-138, neural induction was induced for human embryonic stem cells on a feeder layer from mouse embryonic fibroblasts (MEF) with *inter alia* MEDII medium, which is a conditioned medium from HepG2 cells. To disprove the data according to Shin et al. (2006) that neural induction is not induced using said hiPSCs-derived iHLCs of the present invention co-culturing with HepG2-conditioned medium, differentiation of hiPSC on Geltrex using conditioned medium from HepG2 cells was performed

to test whether the HepG2 conditioned medium is able to support hiPSC-neural induction and can replace SMAD inhibition (LDN and SB) for neural induction.

[225] Geltrex using N2B27 1:1 medium supplemented with 100nM ALK2/3 inhibitor LDN-193189 (LDN) and 10 μ M ALK4/5/7 inhibitor SB431542 (Miltényi) was used as the positive control, whereas Geltrex using only N2B27 1:1 medium was used as a negative control.

[226] First, HepG2 conditioned medium was produced as followed: Coating of dishes for HepG2 conditioned medium with 10 μ g/cm² Collagen I. Then, HepG2 cells were seeded (50.000 cells per cm²) in 6 ml N2B27 1:1 medium on a 6 cm dish (1,75 x 10⁵ cells/ml). N2B27 1:1 (N2 medium: DMEM/F12/, 1x N2, 5 μ g/ml insulin, 2 mM L-Glutamine, 1x non-essential amino acids, 10 μ M 2-mercaptoethanol, 50 U/ml Pen, 50 μ g/ml Strep; B27 medium: Neurobasal, 1x B27, 2 mM L-Glutamine, 50 U/ml Pen, 50 μ g/ml Strep) conditioned medium was collected every 3 days from HepG2 cells and iHLCs, mixed 1:1 with fresh N2B27 1:1 medium and used for differentiation experiments.

[227] Next, hiPSCs were dissociated by accutase and seeded on Geltrex coated dishes in mTeSR1 supplemented with 10 μ M Rock inhibitor Y-27632. After three days, cells were 60-70% confluent and medium was changed to N2B27 1:1 medium only, to N2B27 1:1 medium supplemented with 100 nM LDN and 10 μ M SB and to N2B27 1:1 HepG2 conditioned medium

[228] At day 8 of differentiation N2B27 1:1 conditioned medium was supplemented with 100 ng ml⁻¹ SHH C24II (Miltényi) and 100 ng ml⁻¹ FGF8 (Miltényi). At day 14 of differentiation cells were dissociated mechanically in clumps and were seeded on Poly-L-ornithine (15 μ g/ml) and laminin (5 μ g/ml) coated dishes in N2B27 1:1 conditioned medium supplemented with 100 ng ml⁻¹ SHH C24II, 100 ng ml⁻¹ FGF8 and 100 ng ml⁻¹ FGF2 (**Fig. 11**).

[229] At day 8 of differentiation massive cell proliferation and cell death in the conditions N2B27 only (negative control) and HepG2 conditioned medium occurred, whereas in condition with LDN and SB (positive control) cells differentiated and neural differentiation could be detected. Here, phase contrast pictures of hiPSC differentiation on the positive control, the negative control and HepG2-conditioned medium were performed. Geltrex using conditioned medium from HepG2 cells did not induce neural fate, thus conditioned medium from HepG2 cells cannot replace dual SMAD inhibition for neural induction (**Fig. 12**).

[230] This data shows that neural induction is not happening using conditioned medium from HepG2 cells.

[231] Example 13: Differentiation of hiPSCs to definitive endoderm.

[232] To test whether the neural support also occurs on less mature iHLC, in order to be able to shorten iHLC differentiation, first differentiation of hiPSC to definitive endodermal cells was performed.

[233] hiPSCs were dissociated by accutase and seeded on geltrex coated dishes in mTeSR1 supplemented with 10 μ M Rock inhibitor Y-27632. Definitive endoderm differentiation was initiated by changing the medium to RPMI containing 1% B27 w/o insulin supplemented with 100 ng ml⁻¹ FGF2 (Miltenyi) and 100 ng ml⁻¹ Activin A (Miltenyi). After 8 days cells were seeded on geltrex coated dishes and used for co-culture based differentiation of hiPSCs. The differentiation of hiPSCs to iHLCs through definitive endoderm induction was finished at day 8 (**Fig. 13**). These definitive endodermal cells were further used as supporting cells for the neural differentiation of hiPSCs in comparison to MS5 support (**Example 14**).

[234] Example 14: Differentiation of hiPSC on hiPSCs-derived definitive endodermal cells.

[235] To test whether the neural support also occurs on less mature iHLC, in order to be able to shorten iHLC differentiation, differentiation of hiPSC on hiPSCs-derived definitive endodermal cells was performed.

[236] First, phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on MS5 and definitive endodermal cells were taken. For that, hiPSCs were plated either on MS5 cells or on hiPSCs-derived definitive endodermal cells and observed at day 7 and day 11 post-differentiation. Neuro-epithelial rosettes were observed in the MS5 conditions but not in the definitive endodermal cells conditions, where only morphological changes were detected. In both conditions, cells were picked and re-plated onto P/O+LAM/FN coated-plates (**Fig. 14**).

[237] Further, immunocytochemistry was performed on day 11 of mDA neuron differentiation of iPSC on MS5 and definitive endoderm. The cells were stained for Nestin expression (red), a marker of neural fate. Neural induction occurs in hiPSCs differentiated on endodermal cells but at a very low efficiency in comparison to hiPSCs differentiated on MS5 (**Fig. 15**).

[238] After re-plating of hiPSCs-derived cells differentiated on MS5 or definitive endoderm cells as mentioned above, phase contrast pictures were taken again at day 13 of differentiation, pointing out that only few neuro-epithelial structures were obtained in the definitive endoderm condition (**Fig. 16**).

[239] To confirm the data so far, semi-quantitative PCR of mDA neuron differentiation of iPSC on MS5 and definitive endoderm (DE) at day 11 and day 13 was conducted. PCR was performed for typical neural markers such as Otx2, Nestin, Pax6, Oct4, Sox2 and Nanog, as well as for the neuronal marker Map2 of mature neurons, whereas Gapdh referred as a control. Said neural markers and the neuronal marker Map2 of mature neurons were not expressed in hiPSCs differentiated on definitive endoderm in comparison to MS5 on day 11 and day 13 (**Fig. 17**).

[240] Additionally, quantification of semi-quantitative PCR of mDA neuron differentiation was performed at day 11 and day 13 for said neural markers listed above (**Fig. 18**).

[241] It can be concluded that neural induction occurs on less mature iHLCs but very inefficiently, thus mature iHLCs including longer iHLC differentiation are required for an efficient neural support.

[242] Example 15: Differentiation of hiPSCs to mDA neurons in a matrix based differentiation system.

[243] Since it is already known that HepG2 cells and conditioned medium from HepG2 cells did not induce neural fate efficiently, hiPSC were differentiated on HepG2 matrix to test whether the HepG2 matrix is able to support hiPSC-neural induction.

[244] First, decellularization of iHLCs and HepG2 matrix for neural differentiation support of hiPSCs was performed as already mentioned in **Example 3**. HiPSCs were dissociated by accutase and seeded on human iHLCs matrix, HepG2 matrix or geltrex coated dishes in mTeSR1 supplemented with 10 μM Rock inhibitor Y-27632. Neural induction was initiated by changing the medium to N2B27 1:1 medium supplemented with 100 nM LDN193189 (Stemgent) and 10 μM SB431542 (Miltenyi) (day 0 of differentiation). At day 8 of differentiation medium was supplemented, beside 100 nM LDN193189 and 10 μM SB431542, with 100 ng ml⁻¹ SHH C24II (Miltenyi) and 100 ng ml⁻¹ FGF8 (Miltenyi). At day 14 of differentiation cells were dissociated mechanically in clumps and were seeded on Poly-L-ornithine (15 $\mu\text{g/ml}$) and laminin (5 $\mu\text{g/ml}$) coated dishes in N2B27 1:1 medium supplemented with 100 nM LDN193189, 10 μM SB431542, 100 ng ml⁻¹ SHH C24II, 100 ng ml⁻¹ FGF8 and 100 ng ml⁻¹ FGF2. At day 23 cells were dissociated by accutase and seeded on Poly-L-ornithine (15 $\mu\text{g/ml}$), laminin (5 $\mu\text{g/ml}$) and fibronectin (1 $\mu\text{g/ml}$) coated dishes in N2B27 1:1 medium supplemented with 20 ng ml⁻¹ BDNF (Miltenyi), 20 ng ml⁻¹ GDNF (Miltenyi), 0.2 mM ascorbic acid (Sigma), 1 ng ml⁻¹ TGF β 3 (Miltenyi), 0.5 mM dibutyryl cAMP (Sigma) and 10 μM DAPT (Miltenyi) until desired maturation (**Fig. 19**).

[245] Example 16: Differentiation of hiPSC on iHLC matrix vs. HepG2 matrix with dual SMAD inhibition.

[246] To test whether the HepG2 matrix is able to support hiPSC-neural induction even if the cells themselves and/or conditioned medium of HepG2 cells are unable to do so (**Example 10, 11 and 12**), phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on Geltrex, HepG2 matrix and iHLC matrix, all matrices using dual SMAD inhibition, were taken. hiPSCs were differentiated on Geltrex, HepG2 matrix, iHLC matrix or Collagen I and observed at day 0, day 6 and day 14 of differentiation. In view of the application of dual SMAD inhibition, typical neural morphology was observed in all three conditions (Geltrex, HepG2 and iHLC matrix) (**Fig. 20**).

[247] Further, immunocytochemistry on day 11 of mDA neuron differentiation of hiPSC on Geltrex, HepG2 matrix and iHLC matrix was conducted. The cells were stained for Nestin expression (red), a marker of neural fate, having a differentiation into neural cells on all three matrices with optimal results obtained with the iHLC matrix (**Fig. 21**).

[248] Semi-quantitative PCR during mDa neuron differentiation of iPSCs on Geltrex, HepG2 matrix and iHLC matrix at day 11, 18 and 22 was also performed for typical neural markers such as Otx2, Nestin, Pax6 and Oct4, for key dopaminergic markers such as Nurr1, En1 and Lmx1a, as well as for the neuronal marker Map2 of mature neurons, whereas Gapdh referred as a control. The support of neural induction of HepG2 matrix as used herein is efficient at early stages but there is a clear lack of support for long-term dopaminergic neuron differentiation in comparison to the iHLC-derived matrix, since expression of key dopaminergic neural markers such as En1 and Nurr1 was clearly stronger in the differentiation of hiPSCs on iHLC matrix at day 22. (**Fig. 22**).

[249] Additionally, quantification of semi-quantitative PCR during mDA neuron differentiation was also conducted at day 11, 18 and 22 for said neural markers and dopaminergic markers listed above (**Fig. 23**).

[250] This reveals that the support of neural induction on HepG2 matrix is efficient at early stages but there is a clear lack of support for long-term dopaminergic neuron differentiation in comparison to the iHLC-derived matrix, which clearly supports dopaminergic neuron differentiation.

[251] Example 17: Differentiation of hiPSC on iHLC matrix vs. HepG2 matrix without dual SMAD inhibition.

[252] Further, it was of interest if the matrices (iHLC, HepG2 and Geltrex matrix) have already been tested to support neural induction without the neural conditions (dual SMAD inhibition) in medium.

[253] First, decellularization of iHLCs and HepG2 matrix for neural differentiation support of hiPSCs was performed as already mentioned in **Example 3**. For the differentiation of hiPSC, hiPSCs were dissociated by accutase and seeded on human iHLCs matrix, HepG2 matrix or geltrex coated dishes in mTeSR1 supplemented with 10 μ M Rock inhibitor Y-27632 as described in **Example 15**. At day 8 of differentiation until day 11 of differentiation N2B27 1:1 medium (N2 medium: DMEM/F12/, 1x N2, 5 μ g/ml insulin, 2 mM L-Glutamine, non-essential amino acids, 10 μ M 2-mercaptoethanol, 50 U/ml Pen, 50 μ g/ml Strep; B27 medium: Neurobasal, 1x B27, 2 mM L-Glutamine, 50 U/ml Pen, 50 μ g/ml Strep) was supplemented with just 100 ng ml⁻¹ SHH C24II (Miltenyi) and 100 ng ml⁻¹ FGF8 (Miltenyi). The differentiation was stopped at day 11 (**Fig. 24**).

[254] To question whether these matrices support neural induction without dual SMAD inhibition, phase contrast pictures during midbrain dopaminergic (mDA) neuron differentiation of iPSC on Geltrex, HepG2 matrix and iHLC matrix were taken. hiPSCs were differentiated on Geltrex, HepG2 matrix, iHLC matrix without dual SMAD inhibition. Morphology changes to neural fate were observed at day 5 in the conditions with HepG2 matrix and iHLC matrix, whereas in the condition with Geltrex the typical neural morphology was first detected at day 7 (**Fig. 25**).

[255] Further, qPCR analysis of mDA neuron differentiation in a matrix-based system on Geltrex, HepG2 matrix and iHLC matrix without dual SMAD inhibition was conducted at day 3, 7 and 11, having Geltrex with dual SMAD inhibition (Geltrex LDN/SB) as a positive control. Expression of neuronal markers (Nestin, Pax6, Lhx2) was detected for all three conditions as well as for the positive control. The Expression of neuronal markers (Nestin, Pax6 and Lhx2) did not differ between the conditions at day 3, 7, and 11 in comparison to the positive control with Geltrex having dual SMAD inhibition included (**Fig. 26**).

[256] It can be concluded that dual SMAD inhibition is necessary for neural induction. The matrices only play the role of support when supplemented with dual SMAD inhibition.

[257] Example 18: Differentiation of hiPSCs-derived neural progenitor cells (NPCs) on P/O+ LAM/FN (gold standard), iHLC or HepG2 matrix.

[258] Next, it was observed if the iHLC matrix is able to support neuronal maturation at the later stages, preferably dopaminergic maturation. Hereto, human neural progenitor cells (NPCs) were seeded on iHLC matrix, HepG2 matrix and/or on P/O LAM/FN coated dishes.

[259] First, decellularization of iHLC and HepG2 matrix for neural differentiation support of hNPCs was performed as already mentioned in **Example 3**. Differentiation of human hiPSCs-derived neural progenitor cells (hNPCs) on iHLC and HepG2 matrix was done as described as followed (**Fig. 27**): Human NPCs were dissociated by accutase and seeded on human iHLCs matrix, HepG2 matrix or on P7O LAM/FN (gold standard) coated dishes in N2B27 1:1 medium supplemented with 20 ng ml⁻¹ BDNF (Miltenyi), 20 ng ml⁻¹ GDNF (Miltenyi), 0.2 mM ascorbic acid (Sigma), 1 ng ml⁻¹ TGFβ3 (Miltenyi), 0.5 mM dibutyryl cAMP (Sigma) and 10 μM DAPT (Miltenyi).

[260] Further, to check whether the matrices are able to support neuronal differentiation at the later stages (neuronal maturation), immunocytochemistry of mDA neuron maturation of hNPCs on P/O+LAM/FN, HepG2 matrix and iHLC matrix was performed for 7 days. Co-expression of the marker of dopaminergic neurons TH (green) and the neuronal marker Map2 of mature neurons (red) was detected, pointing out that hNPCs matured under all three conditions. The highest expression of TH was observed with the iHLC matrix, suggesting a better supporting DA neuron-maturation effect of the iHLC matrix (**Fig. 28**).

[261] iHLC matrix is able to support dopaminergic neuron maturation more efficiently in comparison to HepG2 matrix and the gold standard P/O LAM/FN.

[262] Example 19: Proteomics data and their confirmation by PCR.

[263] To identify “neural-inducing” candidate proteins in iHLCs in comparison to HepG2 and MS5, proteomics analysis was performed.

[264] First, detected peptides of core matrisome proteins and matrisome associated proteins were compared in iHLCs, HepG2 cells, hiPSCs and MS5 by proteomic analysis. For that, in order to identify ECM proteins of the iHLCs with DA neuron supporting effect, ECM proteins, which showed a high peptide number in iHLCs as well as in MS5 in comparison to HepG2 and hiPSCs, were selected as candidate proteins (marked in red, Hspg2, Thbs1, Nid1, Col5a1, Ltp1, Col5a2, Vcan, Emilin-2, Emilin-1 and Fbln2) (**Fig. 29**).

[265] Next, quantification and graphical presentation of quantification of core matrisome proteins in iHLCs, HepG2 cells and hiPSCs by proteomic analysis were done, with high amount of Lamb1, Lamc1 and Lama1 in comparison to HepG2 cells. Further, the same was performed for the matrisome-associated proteins in iHLCs, HepG2 and hiPSCs by proteomic analysis, with having high amount of FGF2 in comparison to HepG2 cells (**Fig. 30, 31, 32, 33**).

[266] Additionally, due to high amounts of different laminin chains in the iHLCs in comparison to HepG2 cells by the proteomics analysis, semi-quantitative PCR in hiPSCs, HepG2 cells and iHLCs for the different forms of the laminin α , β and γ chains was conducted with Gapdh being the control. Lama5, Lamb1, Lamb2, Lamc1 were highly expressed in HepG2 cells and iHLCs in comparison to iPSCs, suggesting that these laminin chains could be further candidate proteins for neural support. Lama1, Lamb3 and Lamc2 are specifically expressed in iHLC suggesting a potential role in the support for dopaminergic neuron differentiation (**Fig. 34**).

[267] In order to confirm the expression of the candidate proteins from the proteomics analysis in iHLCs, semi-quantitative PCR was performed in MS5, HepG2 and iHLCs. It was found that the expression of Perlecan, Thbs1, Nid1, Ltp1, Col5a1 and Vcan as specific candidate proteins is responsible for neural induction, being detected in iHLCs. The expression of Ltp1 and Col5a1 as specific candidate proteins is responsible for dopaminergic neural support, being exclusively present in iHLCs in comparison to HepG2 (**Fig. 35**).

[268] In summary, neural support is certainly happening through the ECM protein combination, which includes at least: Perlecan (Hspg2), Thbs1, Nid1, Ltp1, Col5a1, Vcan, Lamb1, Lamb2, Lamb3, Lamc1, Lamc2, Lama1 and/or Lama5. A matrix supporting neural induction contains Lama5, Lamb1, Lamb2, Lamc1, Perlecan, Thbs1, Nid1 and Vcan, whereas a matrix supporting dopaminergic neuron induction contains Lama1, Lamb3, Lamc2, Ltp1 and Col5a1.

[269] Example 20: Up- and downregulation of core matrisome and matrisome associated proteins in iHLCs in comparison to hiPSCs, HepG2 cells and MSCs.

[270] Immature HLCs were analyzed by liquid chromatography (LC) with mass spectrometry (MS)/mass spectrometry (MS) (LC-MS/MS) (MS: Quexactive HF mass spectrometer, Thermo Fisher Scientific, Waltham, USA; HPLC: Ultimate 3000 RSLC nano-HPLC, Dionex, Sunnyvale, USA) in comparison to hiPSCs, HepG2 cells and MSCs. Relations of significantly up-regulated proteins are illustrated by a venn-diagram. 18 proteins were significantly higher expressed in iHLCs in comparison to all control groups (**Fig. 36**). 18 proteins being significantly higher expressed by iHLCs in comparison to the controls also include proteins involved in either TGF β -signalling (FBN2, IGFBP2 and 5, LTBP1), BMP-signalling (GDF6) or CNS development (AGRN, HAPLN1, LAMB1). Furthermore proteins, which were only up-regulated relative to two control cell types, could be also assigned to either TGF β -signalling or CNS development. SFRP1, significantly up-regulated in relative to MSC and HepG2 cells, is involved in dopamine neuron development. In contrast iHLCs showed a higher expression of FBLN1, THBS1 (TGF β -signalling) and COL3A1, COL4A1, TNC (CNS development) compared to hiPSC and HepG2. Beside this, MDK and VTN were significantly higher expressed in iHLCs in comparison to MSC and hiPSCs and are associated with CNS development (**Fig. 37**).

Additionally, relations of significantly down-regulated proteins are illustrated again by a venn-diagram. 7 proteins were significantly lower expressed in iHLCs in comparison to all control groups (**Fig. 38**). Neither of these 7 proteins being lower expressed in iHLC in comparison to control groups are involved in TGF β -, BMP- signalling or CNS development. Proteins significantly lower expressed in iHLCs in comparison to only two control groups were found to be involved in biological processes. Proteins associated with TGF β -signalling (FBN1, LTBP2) and CNS development (SDC1) were down-regulated in iHLCs in comparison to MSCs and HepG2 cells. Beside this, LAMA1, which is involved in CNS development, was lower expressed in iHLCs in comparison to MSCs and hiPSCs (**Fig. 39**).

52
CLAIMS

1. Use of immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as an in vitro culture system for the differentiation of target precursor stem cells into target cells of a pre-selected cell type.
2. Use according to claim 1, wherein said iHLC-derived ECM is substantially cell-free.
3. Use according to any one of the preceding claims, wherein said iHLCs are derived from human or non-human induced pluripotent stem cells (iPSCs), human or non-human adult stem cells, human parthenogenetic or non-human embryonic stem cells (ESCs), or a hepatocyte cell-line or primary hepatocytes.
4. Use according to any one of the preceding claims, wherein said iHLCs are characterized by the expression of α -fetoprotein, albumin, α -1-antitrypsin, and do not express CYP7A1.
5. Use according to any one of the preceding claims, wherein said target precursor stem cells are selected from human or non-human induced pluripotent stem cells (iPSCs), human or non-human adult stem cells, human parthenogenetic or non-human embryonic stem cells (ESCs).
6. Use according to any one of the preceding claims, wherein said iHLCs and target precursor stem cells are human cells.
7. Use according to any one of the preceding claims, wherein said iHLCs and target precursor stem cells are derived from the same human donor.
8. Use according to any one of the preceding claims, wherein said pre-selected cell type is a non-hepatocyte cell type.
9. Use according to any one of the preceding claims, wherein said target cells of a pre-selected cell type are neural cells.
10. Use according to any one of the preceding claims, wherein said pre-selected cell type is the neural cell type and said target cells are mature neural cells expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM; specifically dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A, and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic

neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6, sensory neurons expressing POU4F1 and/or peripherin, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP.

11. Use according to claim 10, wherein the pre-selected neural cell type is induced by addition of SHH activators, and/or FGF activators, and/or BMP inhibitors and/or TGFb inhibitors.

12. A composition comprising (a) immature hepatocyte-like cells (iHLCs) and/or (b) an iHLC-derived extracellular matrix (ECM) as a culture system and (c) target precursor stem cells differentiating into target cells of a pre-selected cell type.

13. Composition according to claim 12, wherein said target precursor stem cells are selected from human or non-human induced pluripotent stem cells (iPSCs), human or non-human adult stem cells, and human parthenogenetic or non-human embryonic stem cells (ESCs).

14. Composition according to claim 12 or 13, wherein said target cells are mature neural cells expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM; specifically dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6, sensory neurons expressing POU4F1 and/or peripherin, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP.

15. Composition according to any one of claims 12 to 14, comprising said target precursor stem cells and/or target cells of a pre-selected cell type in a 2D- or 3D-cell culture.

16. A method for preparing an immature hepatocyte-like cell (iHLC) derived cell-free extracellular matrix (ECM), comprising

- (a) cultivating iHLCs *in vitro* under conditions allowing for formation of an iHLC-derived extracellular matrix (ECM),
- (b) decellularizing the iHLC-derived ECM, and
- (c) obtaining the iHLC-derived cell-free extracellular matrix (ECM).

17. An immature hepatocyte-like cell (iHLC) derived extracellular matrix obtained or obtainable by the method according to claim 16.

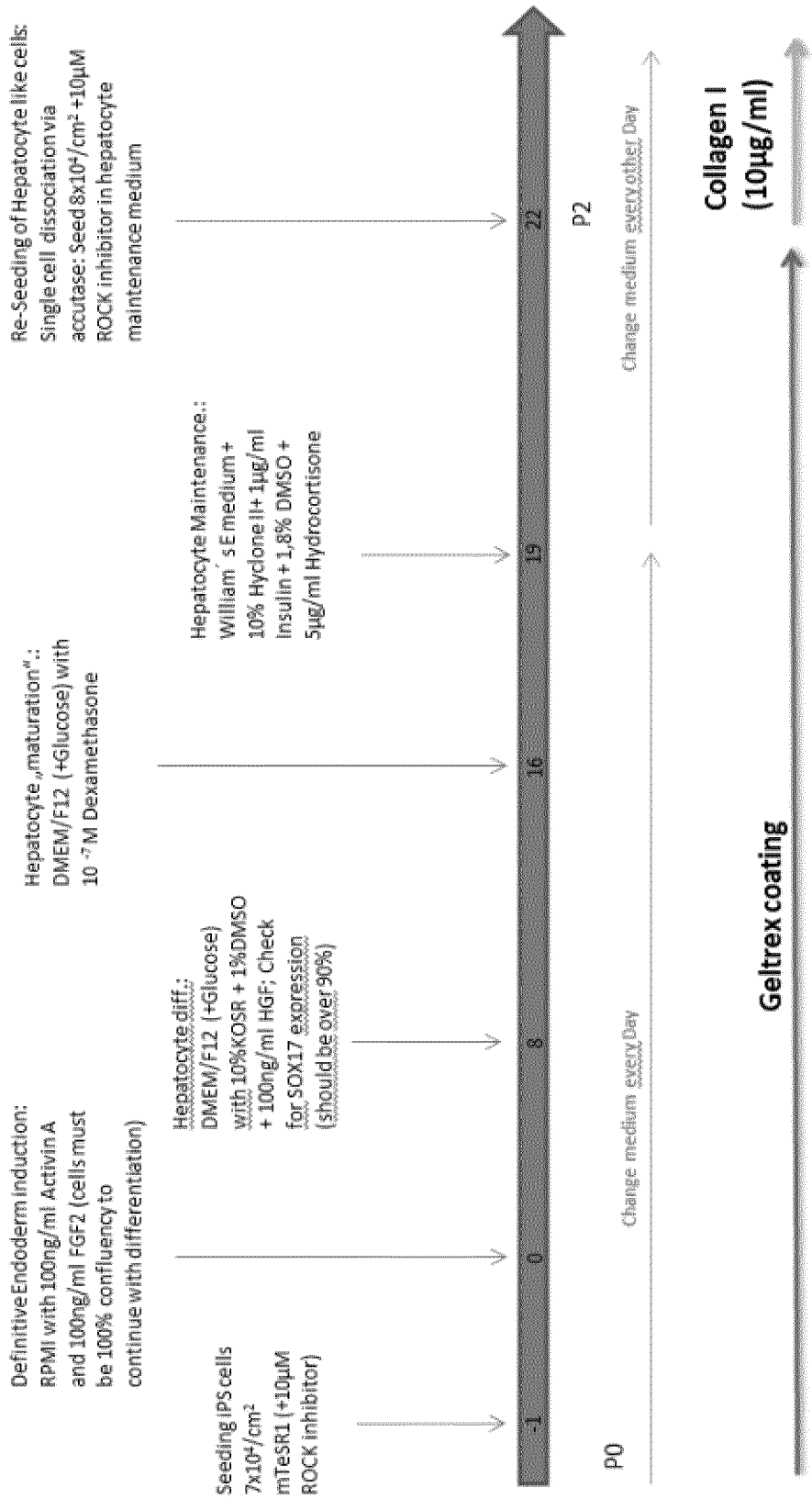
18. The iHLC-derived extracellular matrix according to claim 17, wherein said extracellular matrix is characterized by the expression of Lama1, Lama5, Lamb1, Lamb2, Lamb3, Lamc1, Lamc2, Perlecan, Thbs1, Nid1, Vcan, Ltbp1 and Col5a1.
19. The iHLC-derived extracellular matrix according to claim 18, wherein said extracellular matrix is (a) highly concentrated, (b) growth factor reduced, (c) phenol-red free and/or (d) suitable for human embryonic stem cell (hESC) cultivation.
20. A device comprising immature hepatocyte-like cells (iHLCs) and/or an extracellular matrix (ECM) derived from said iHLCs as a culture system.
21. The device according to claim 20, wherein said device is a vial or a cell-culture dish.
22. The device according to claim 20 or 21, further comprising target precursor stem cells as defined in any one of claims 5 to 7 or target cells of a pre-selected cell type as defined in any one of claims 8 to 11.
23. An *in vitro* method for obtaining target cells of a pre-selected cell type, comprising
(a) providing immature hepatocyte-like cells (iHLCs) and/or an iHLC-derived extracellular matrix (ECM) optionally according to any one of claims 2 to 4 and/or 6 to 7;
(b) adding target precursor stem cells optionally according to any one of claims 5 to 7 in a suitable cell culture medium and allowing their differentiation into target cells of a pre-selected cell type optionally according to any one of claims 8 to 11;
(c) obtaining the target cells of the pre-selected cell type.
24. The *in vitro* method according to claim 23, wherein said target cells are mature neural cells optionally expressing Dcx, Map-2, Synapsin 1, TuJ1, NSE, Map2a, Gap43, NF, CD24, CDH2/CD325, synaptophysin, and/or CD56/NCAM; specifically dopaminergic neurons expressing Nurr1, En1, TH, AADC, Dat, Otx-2, FoxA2, LMX1A and/or VMAT2, cholinergic neurons expressing NGF and/or ChAT, GABAergic neurons expressing GAD67 and/or vGAT, glutamatergic neurons expressing vGLUT1, serotonergic neurons, motor neurons expressing HB9, SMN, ChAT and/or NKX6, sensory neurons expressing POU4F1 and/or peripherin, astrocytes expressing GFAP and/or Tapal, or oligodendrocytes expressing O1, O4, CNPase, and/or MBP.
25. A target cell of a pre-selected cell type obtained or obtainable by the *in vitro* method according to claim 23 for use in a method for cell grafting in a patient in need thereof.

26. The target cell according to claim 25, wherein said target cell is characterized by the increased expression of FoxA2, TH, Map2, Nestin, Pax6, Otx2, En1, Lmx1A and/or Nurr1 in comparison to a target cell obtained by cultivation in MS5 or Geltrex.

27. The target cell according to claim 26 for use in a method for treating a neurodegenerative disease, including without limitation Alzheimer's Disease, Parkinson, Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease, Muscular Dystrophy.

Figure 1

Differentiation of human iPS cells to immature hepatocyte-like cells (iHLCs)

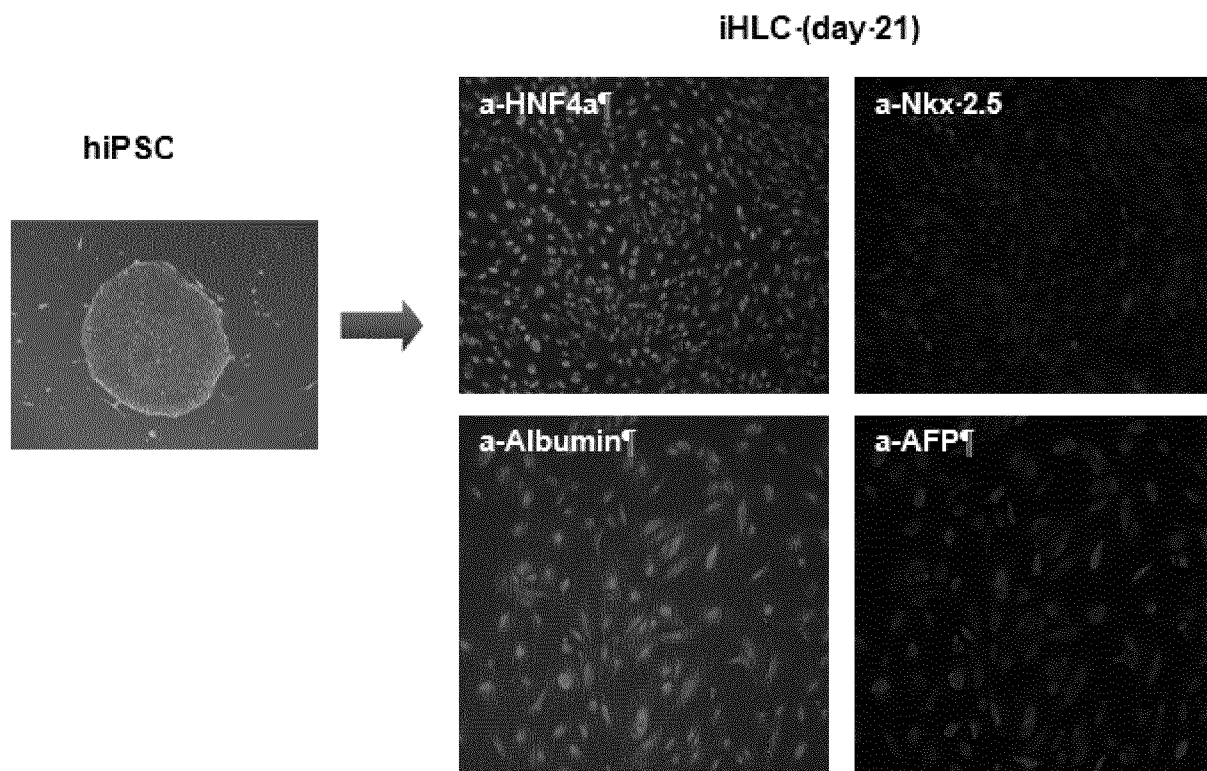


Note: Use single cell adapted iPS cells (at least 4x splitted as single cells)

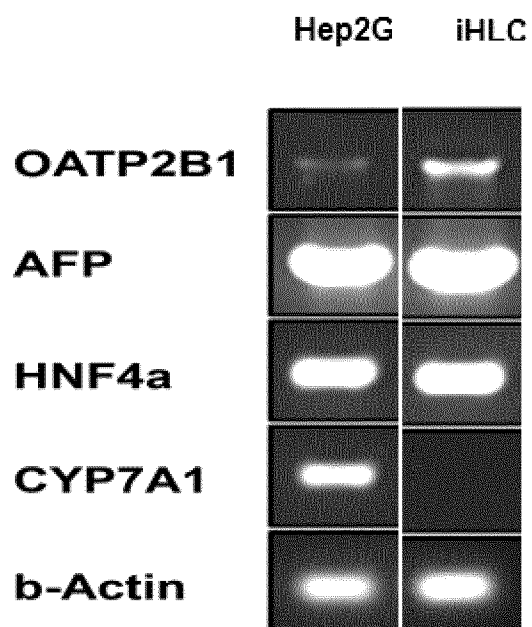
2/43

Figure 2

A



B



3/43

Figure 2 (cont.)

C

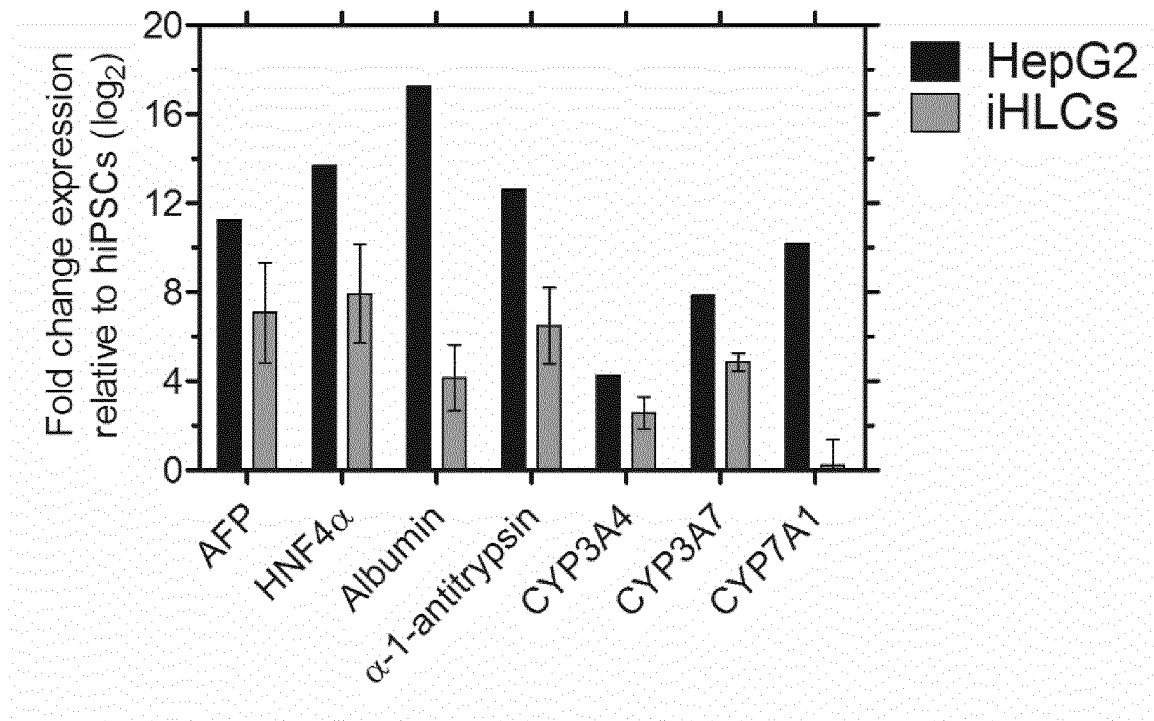
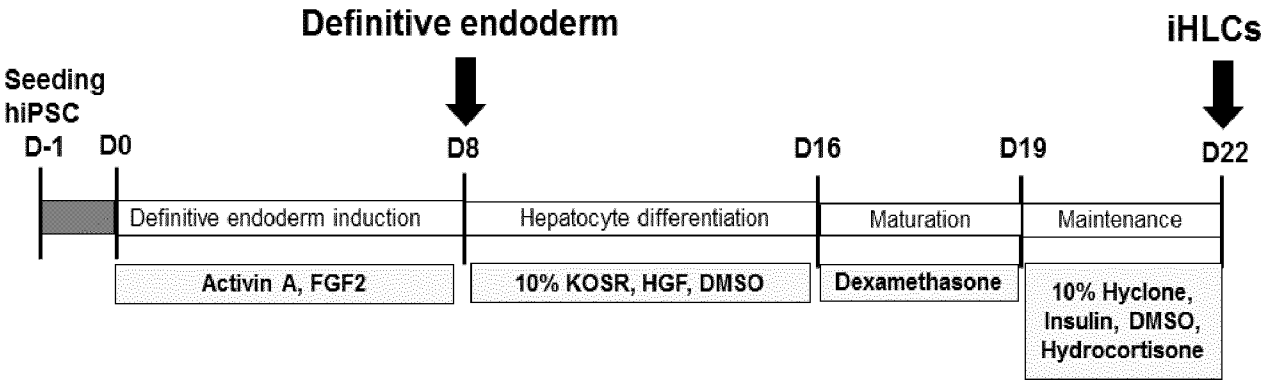
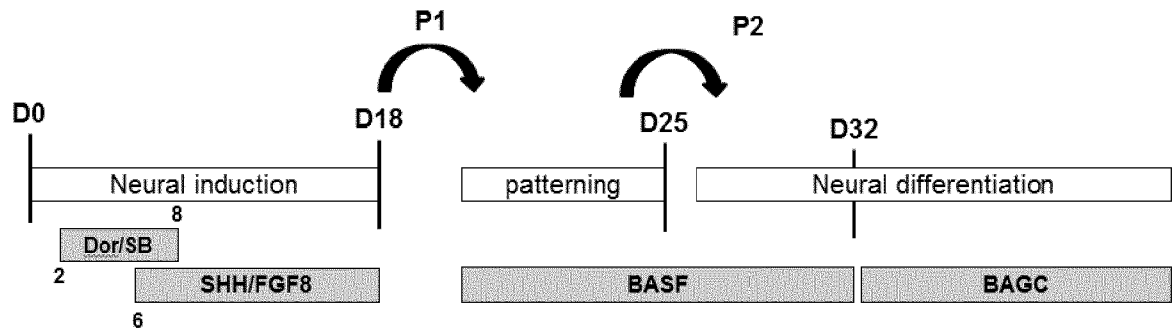


Figure 3

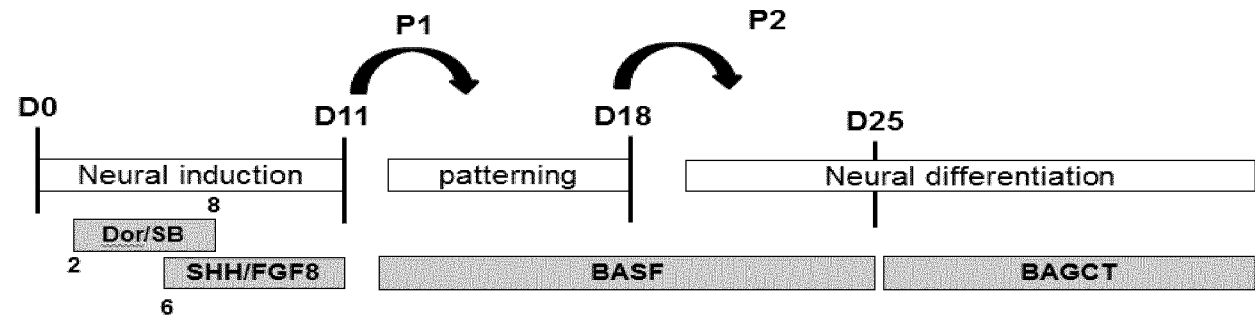
A



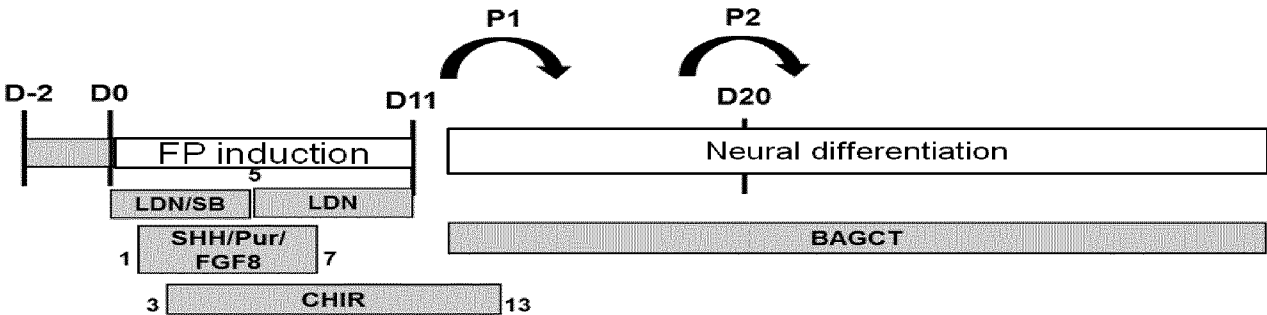
B



C



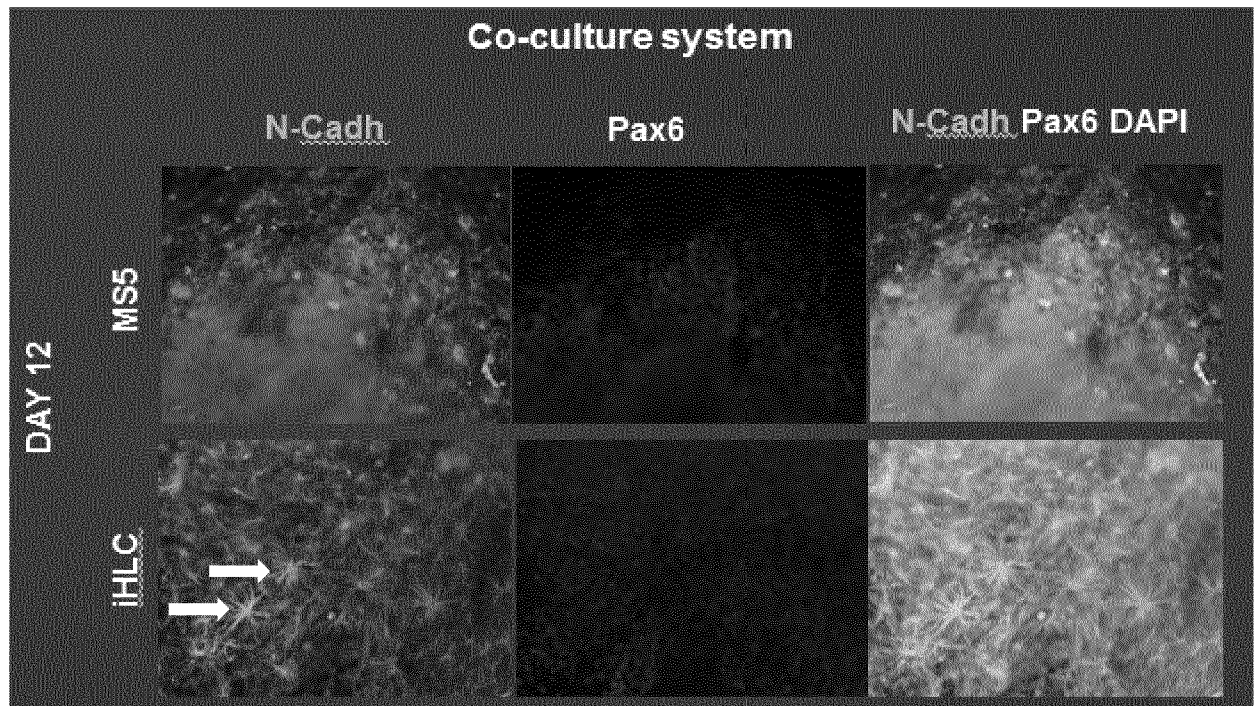
D



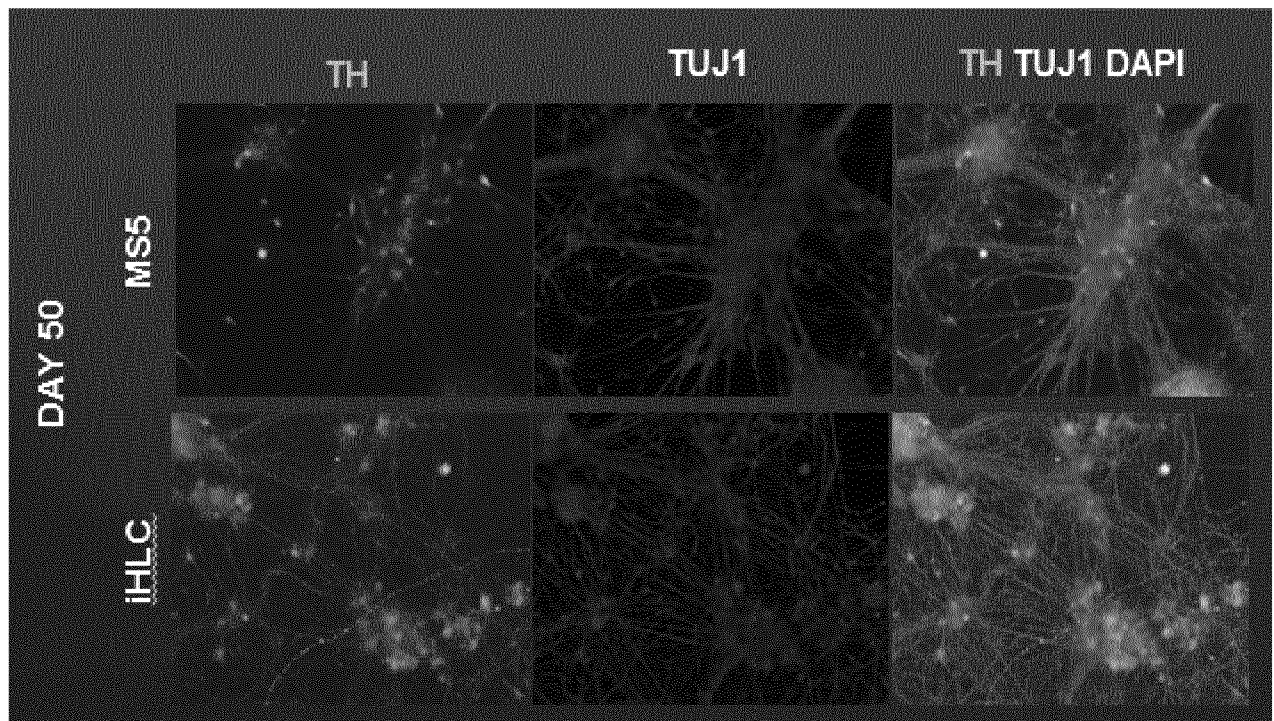
5/43

Figure 4

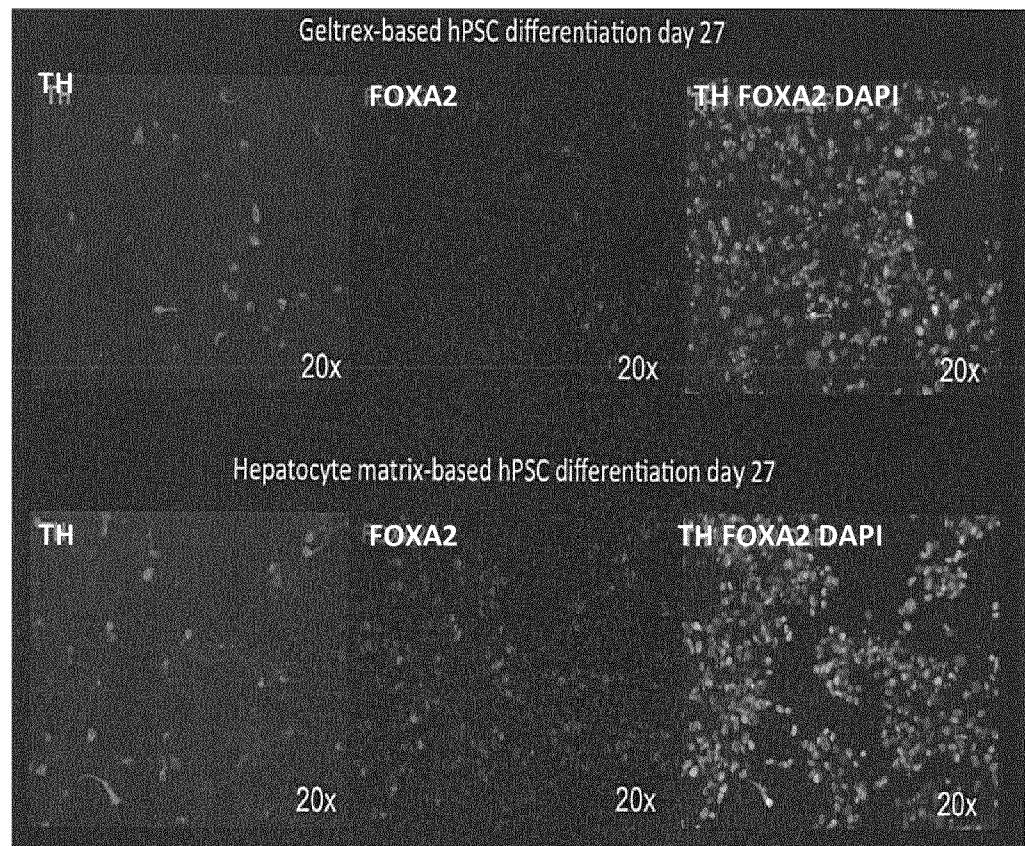
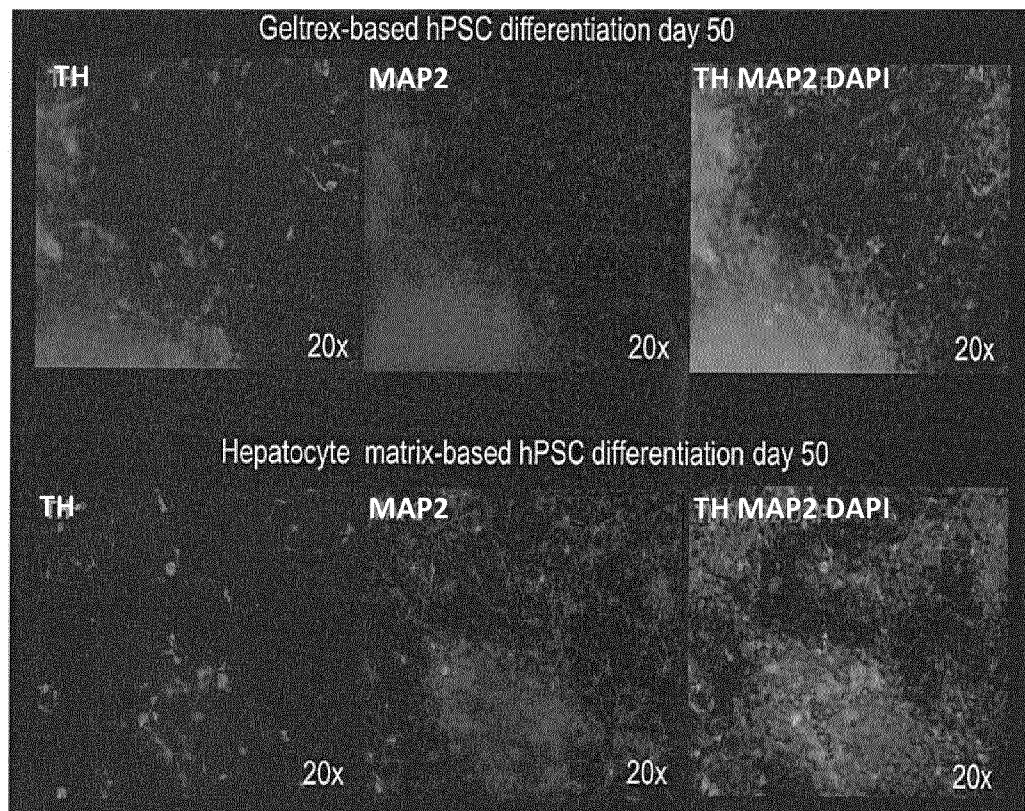
A



B

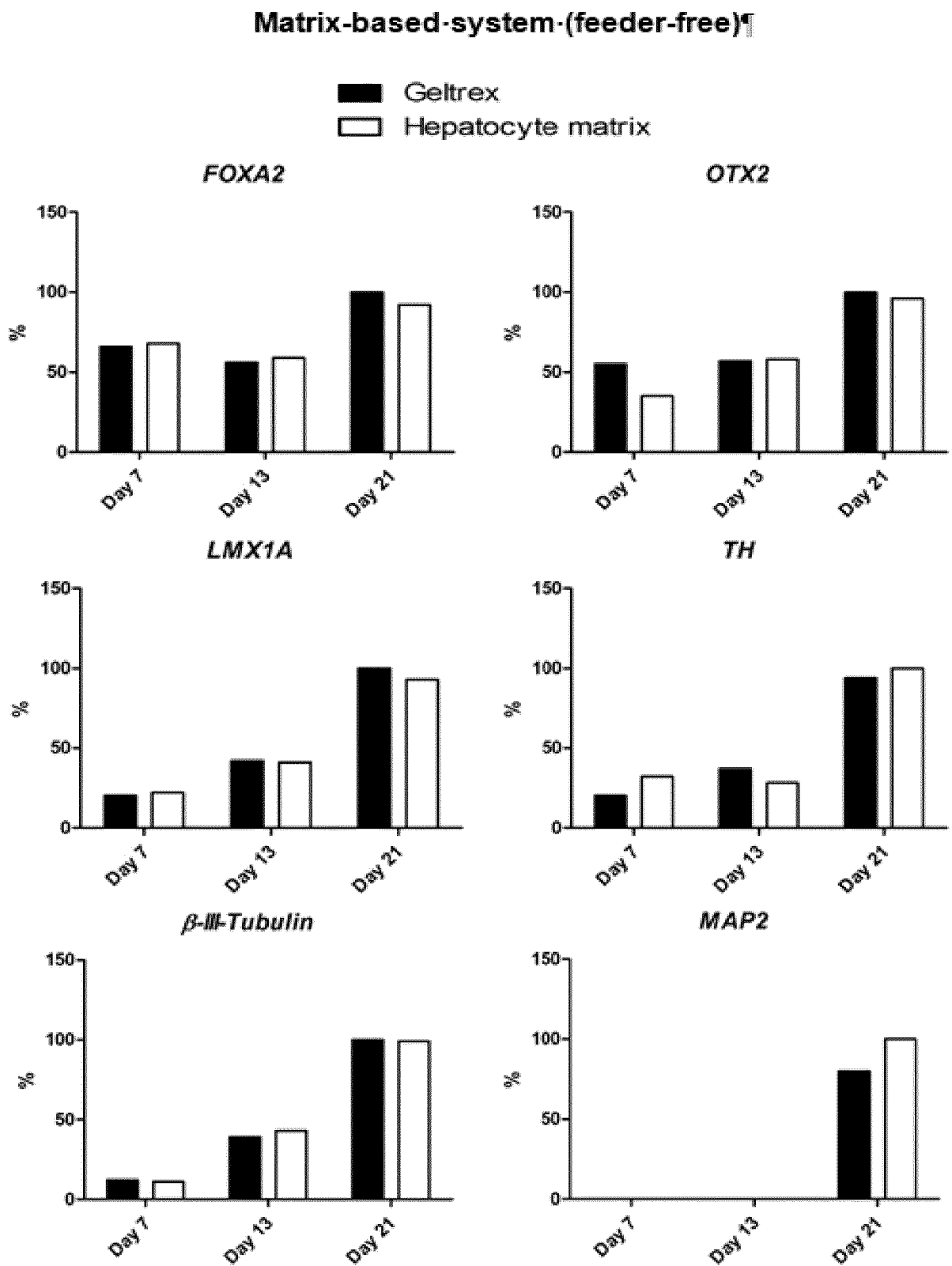


6/43

Figure 5**A****B**

7/43

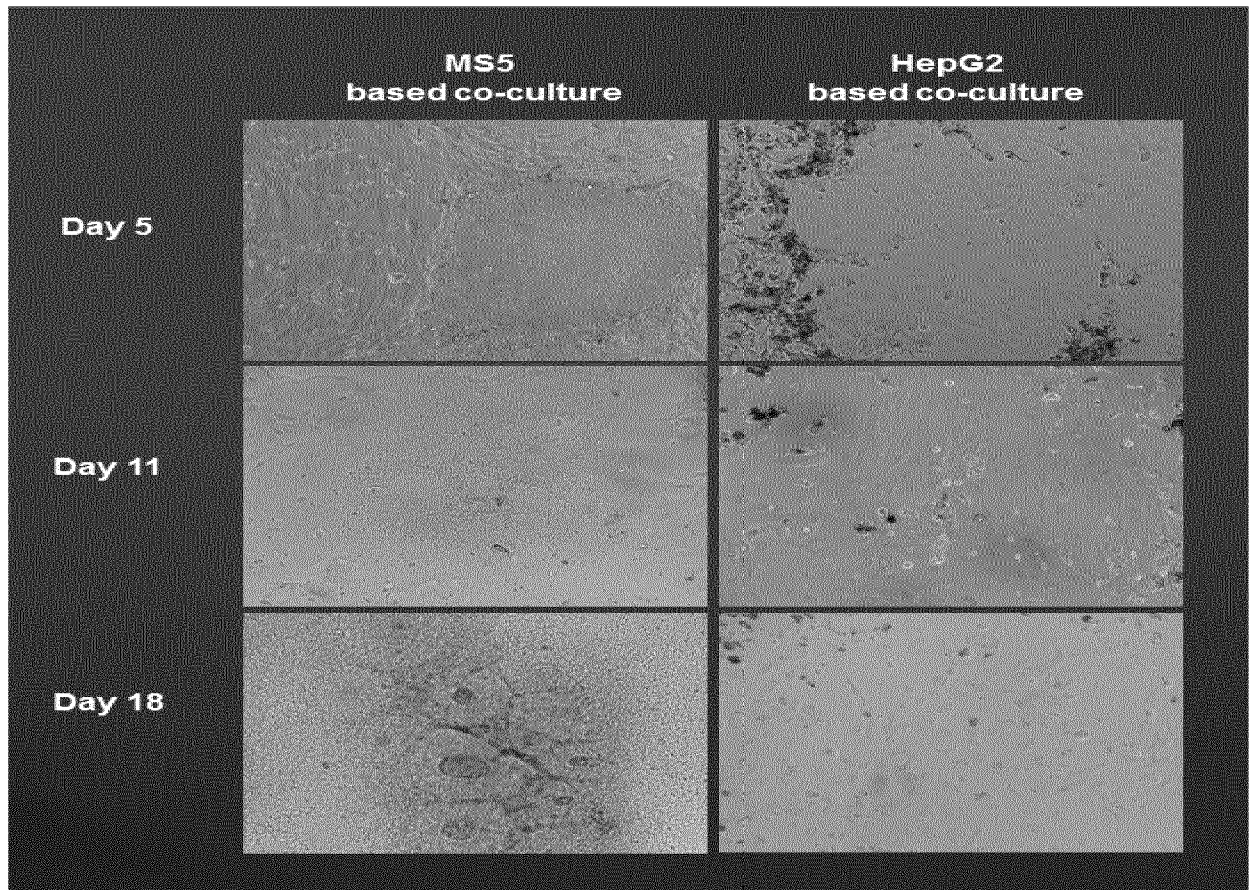
Figure 6



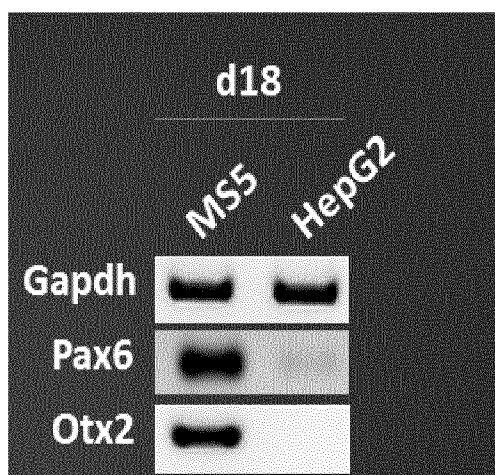
8/43

Figure 7

A



B



C

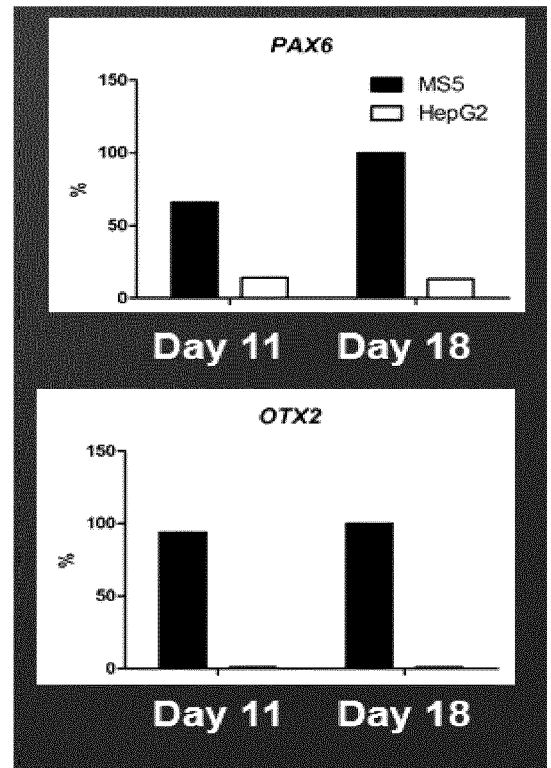


Figure 8

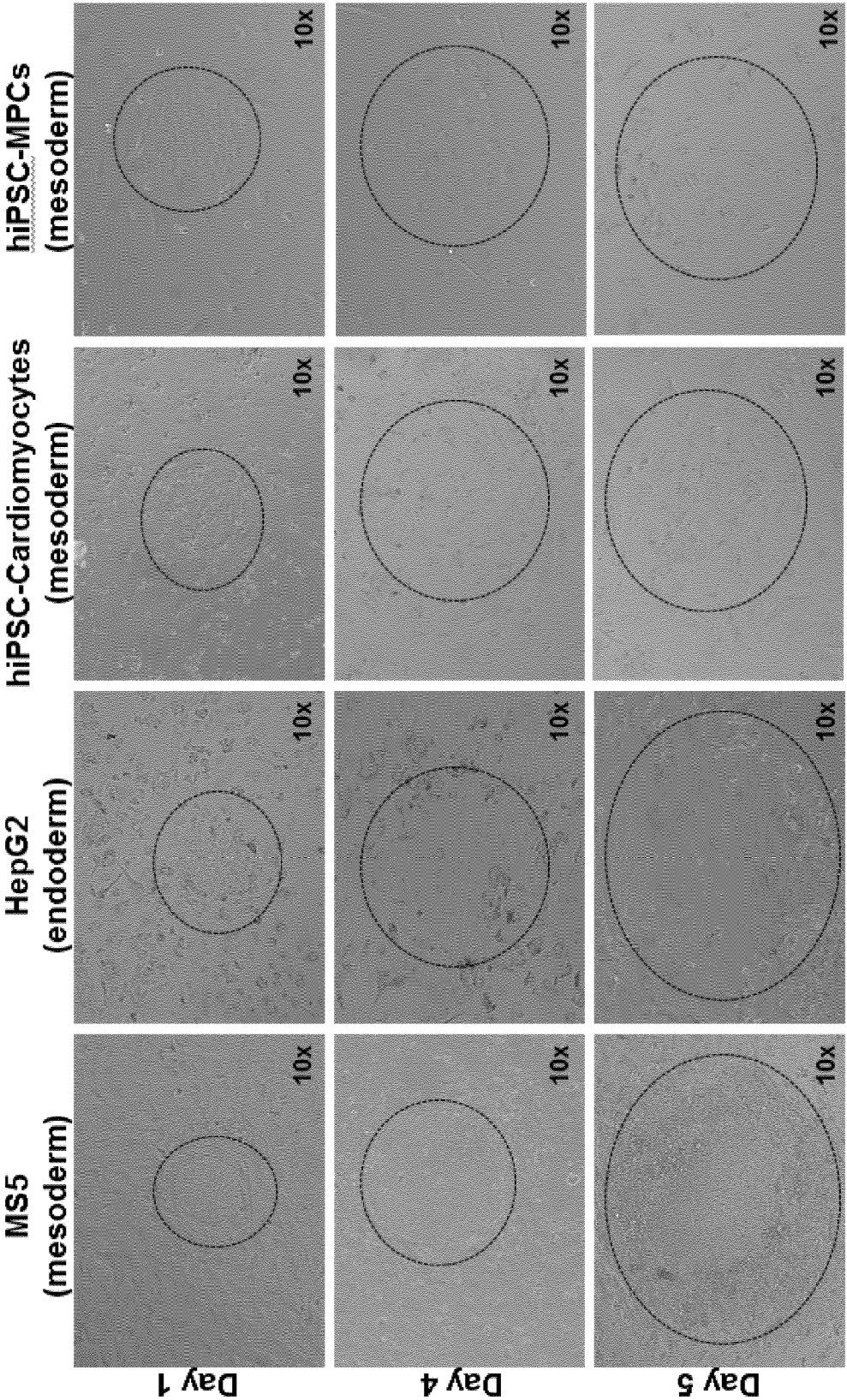


Figure 8 (cont.)

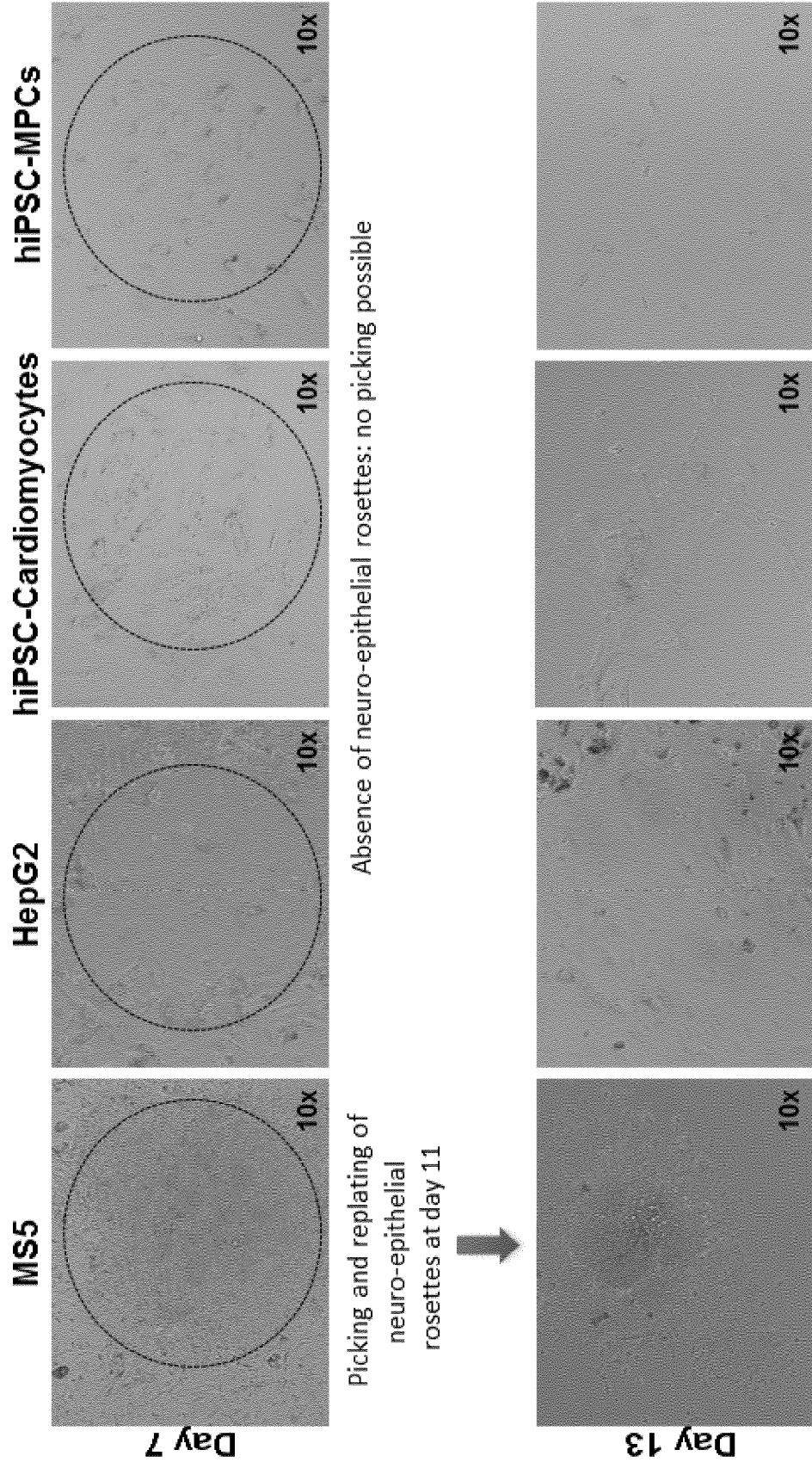


Figure 9

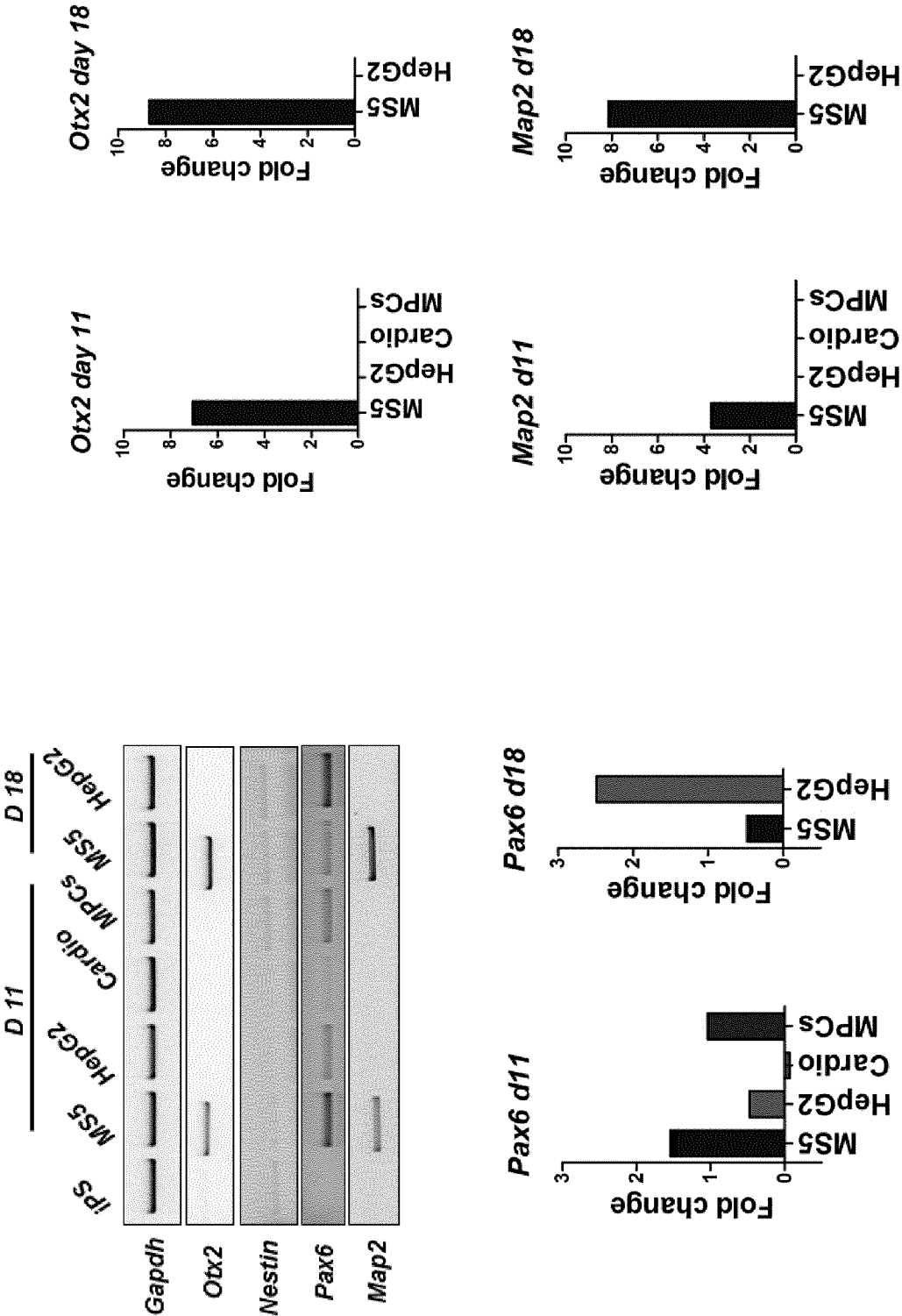


Figure 10

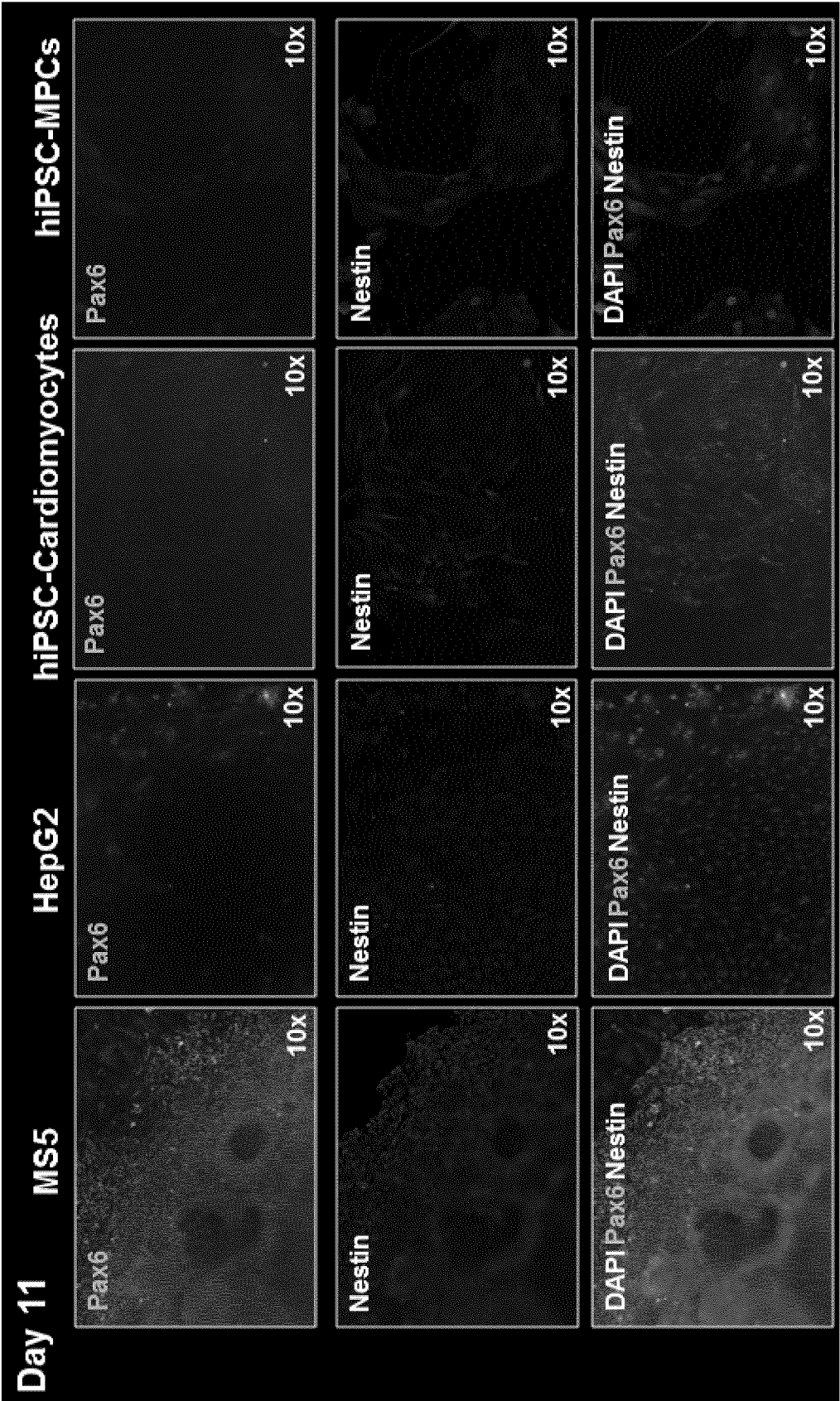


Figure 11

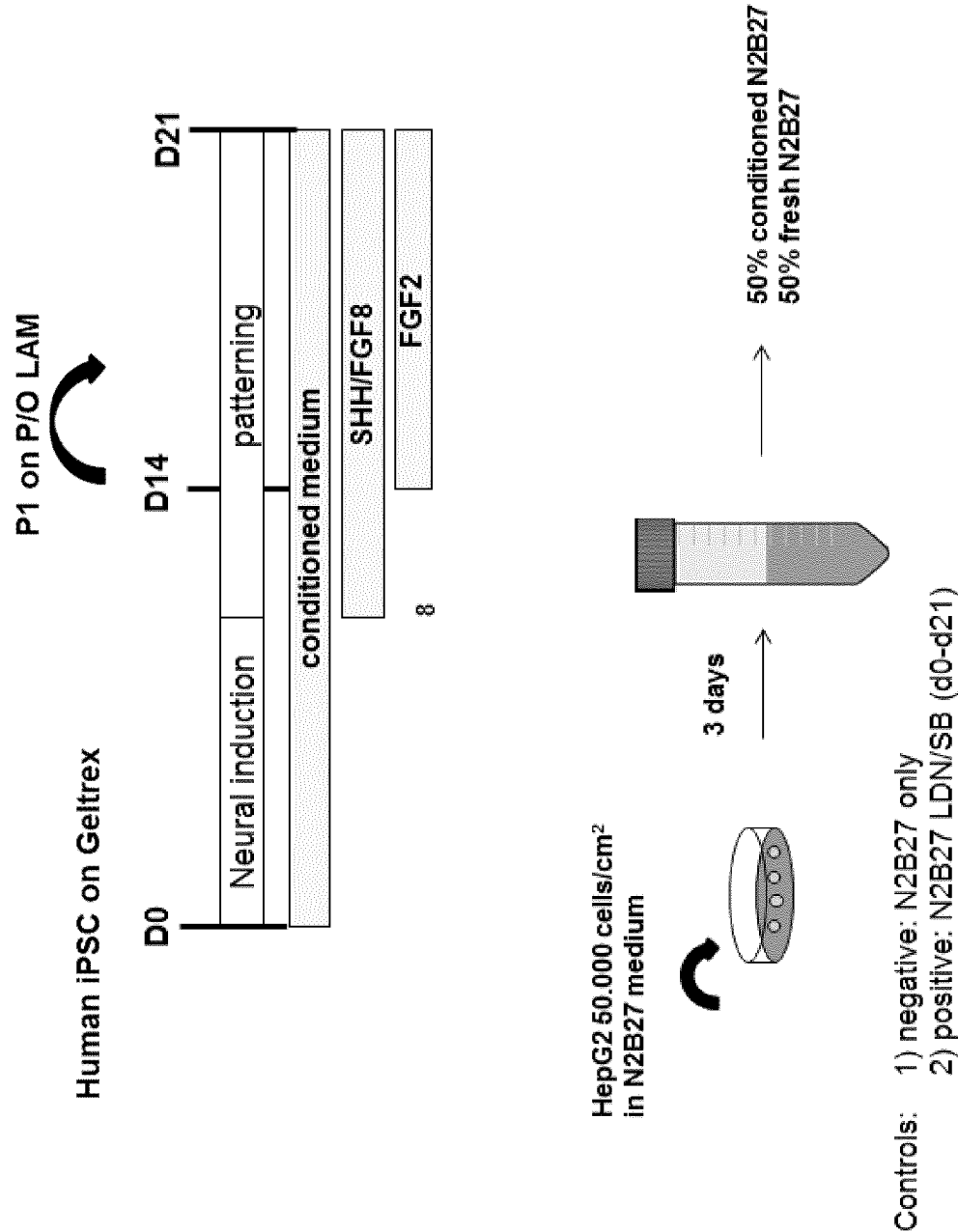


Figure 12

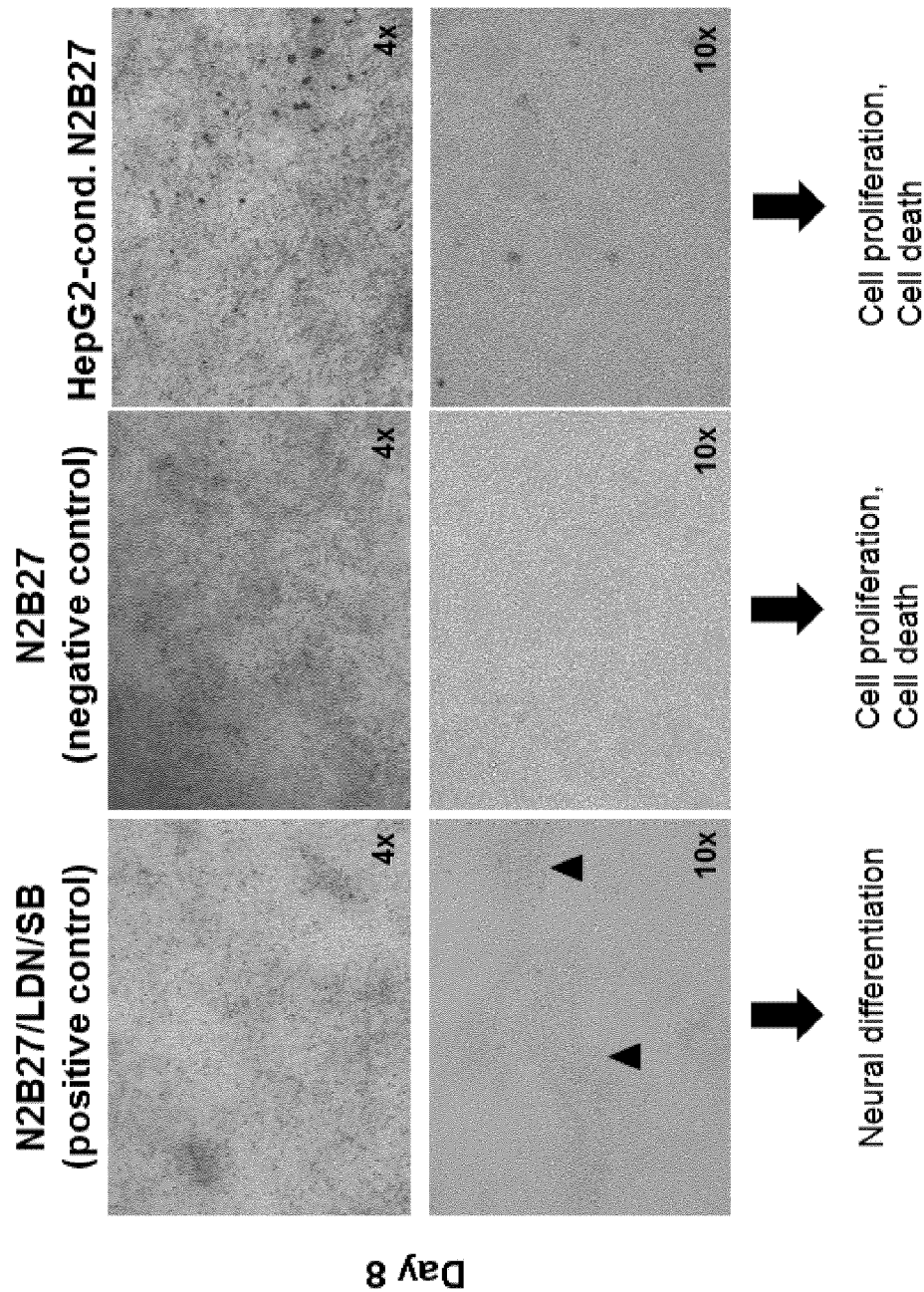
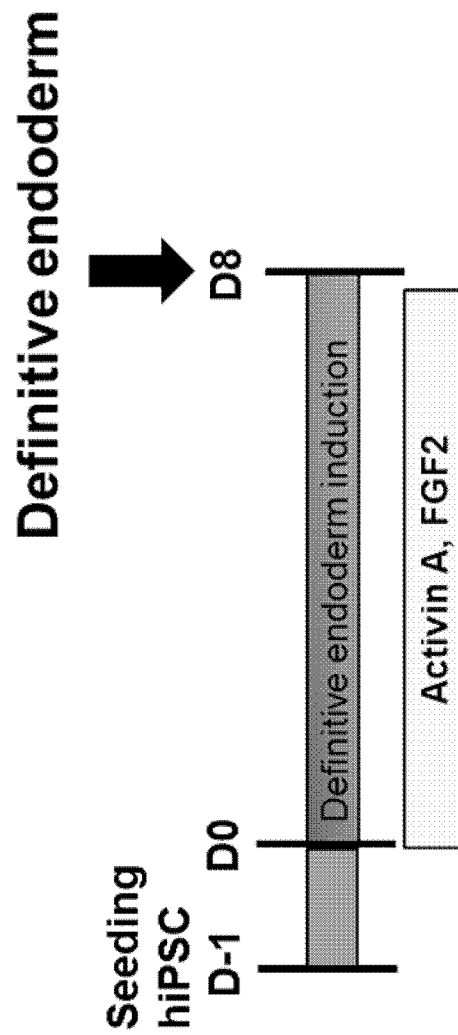
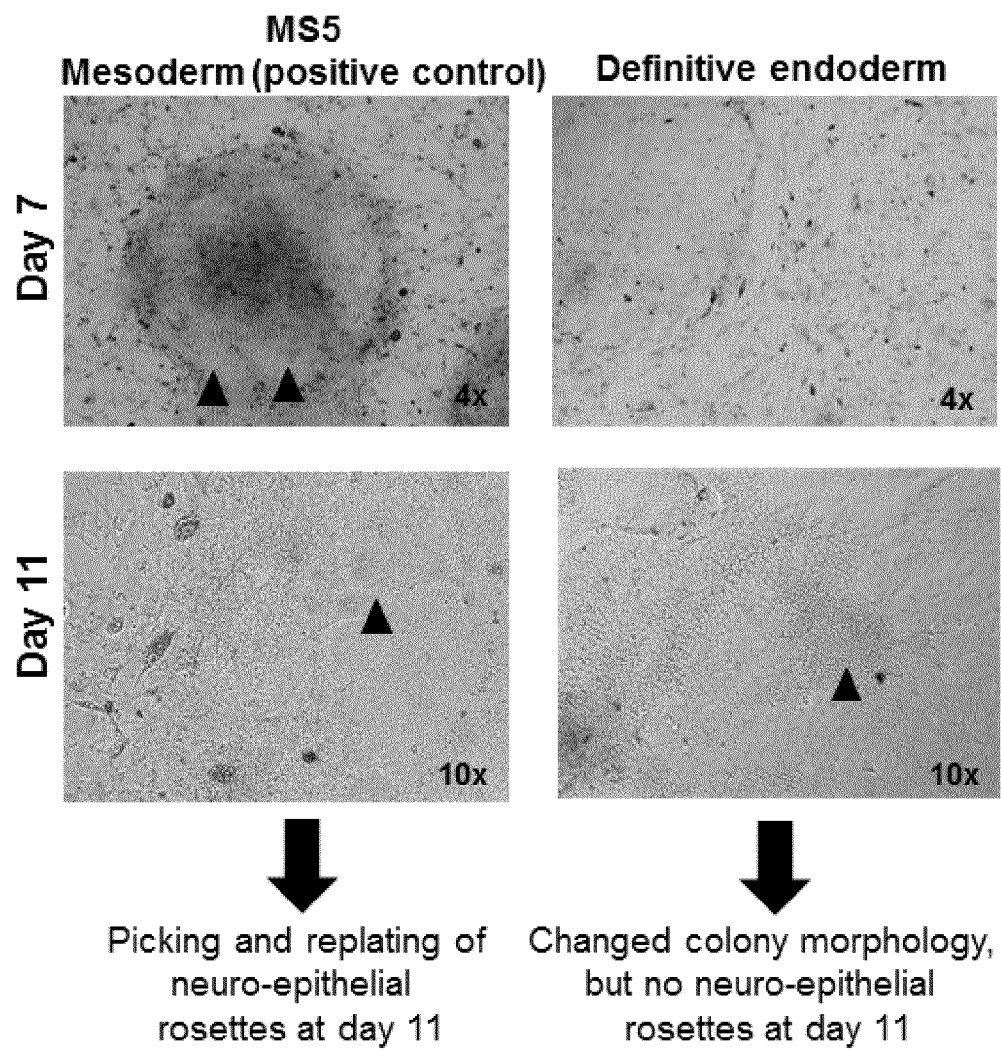


Figure 13



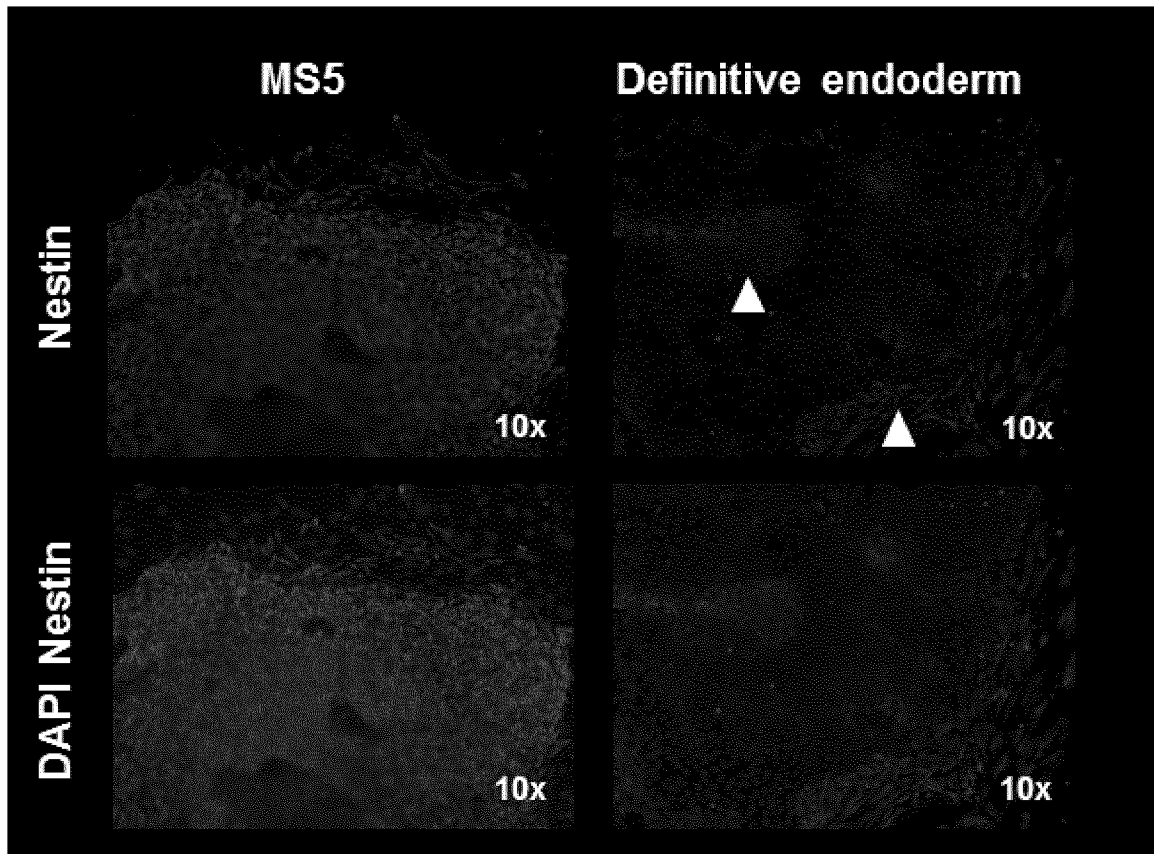
16/43

Figure 14



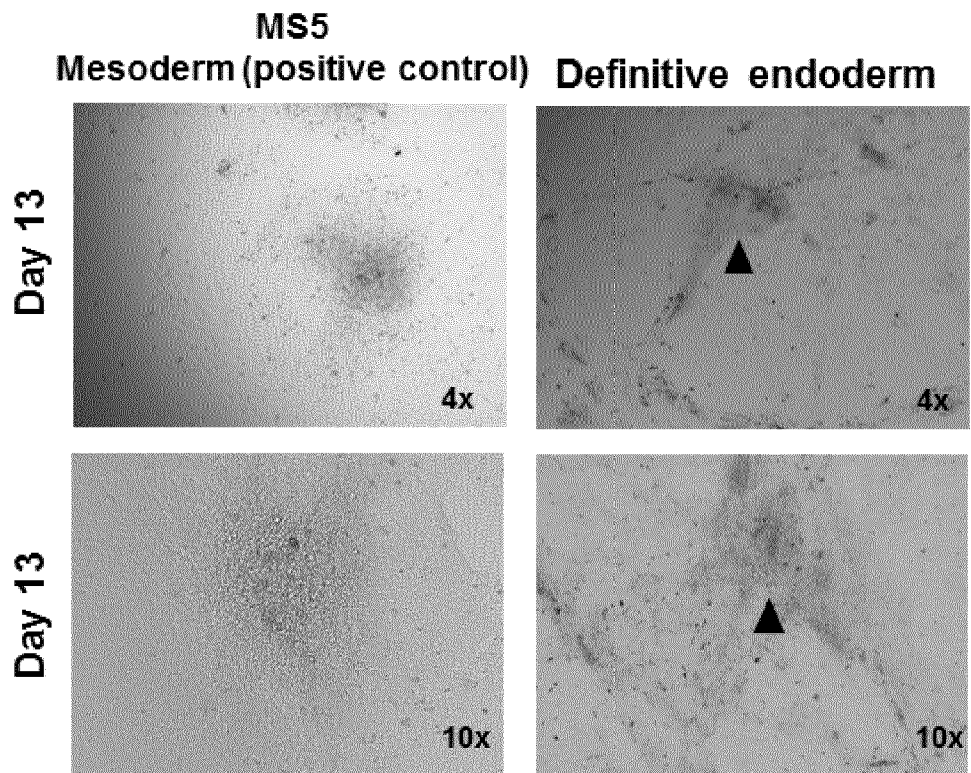
17/43

Figure 15



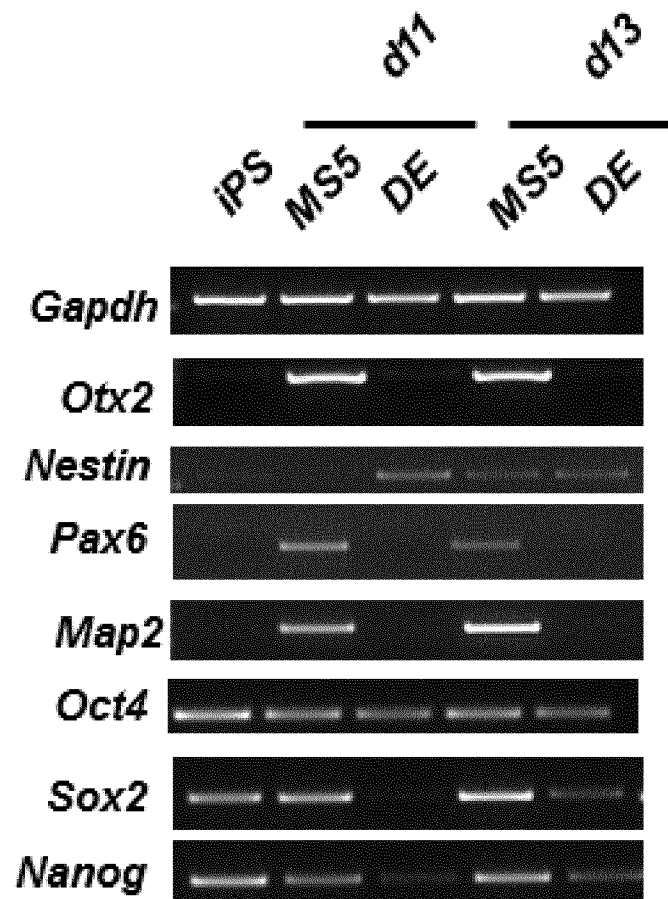
18/43

Figure 16



19/43

Figure 17



20/43

Figure 18

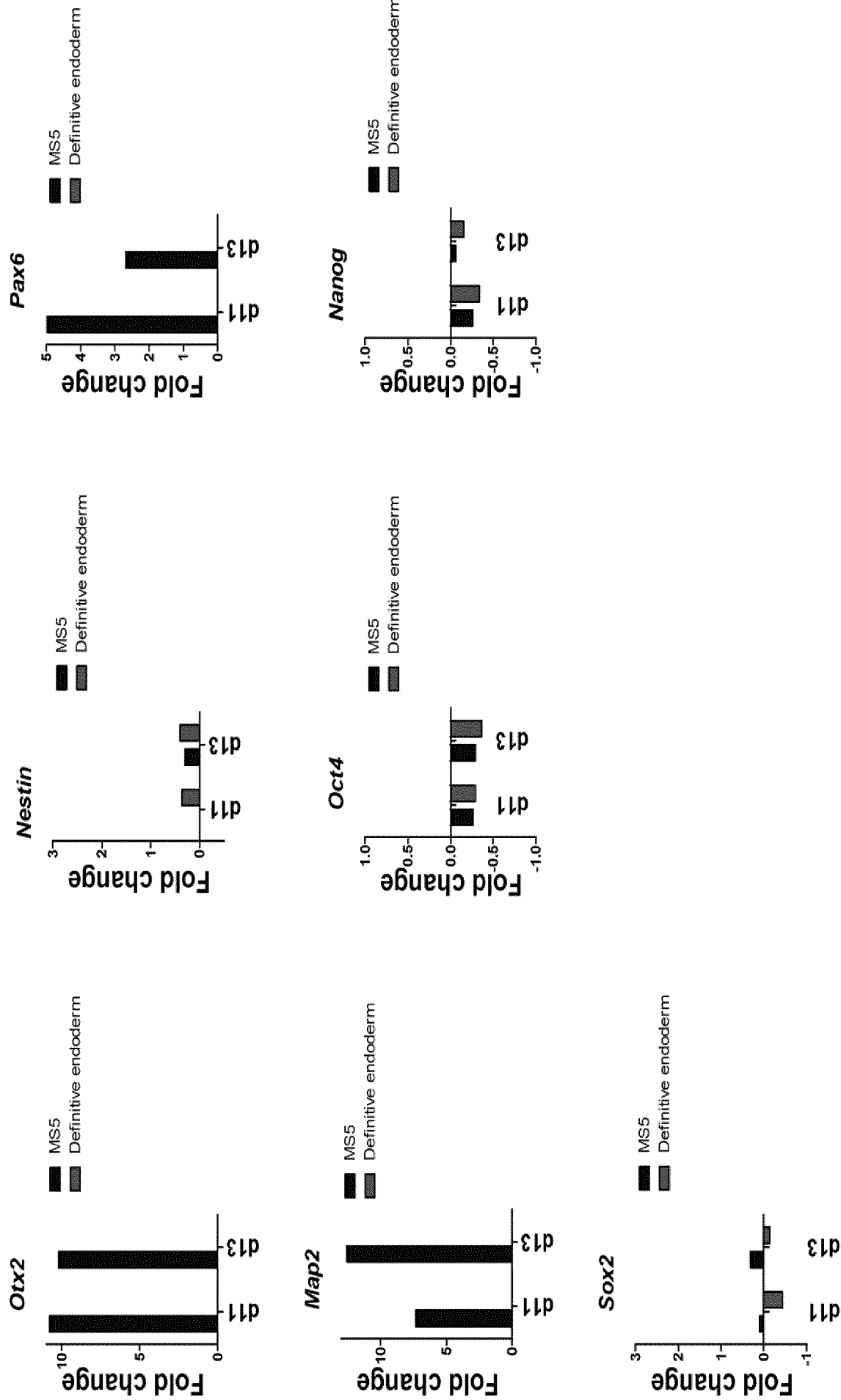


Figure 19

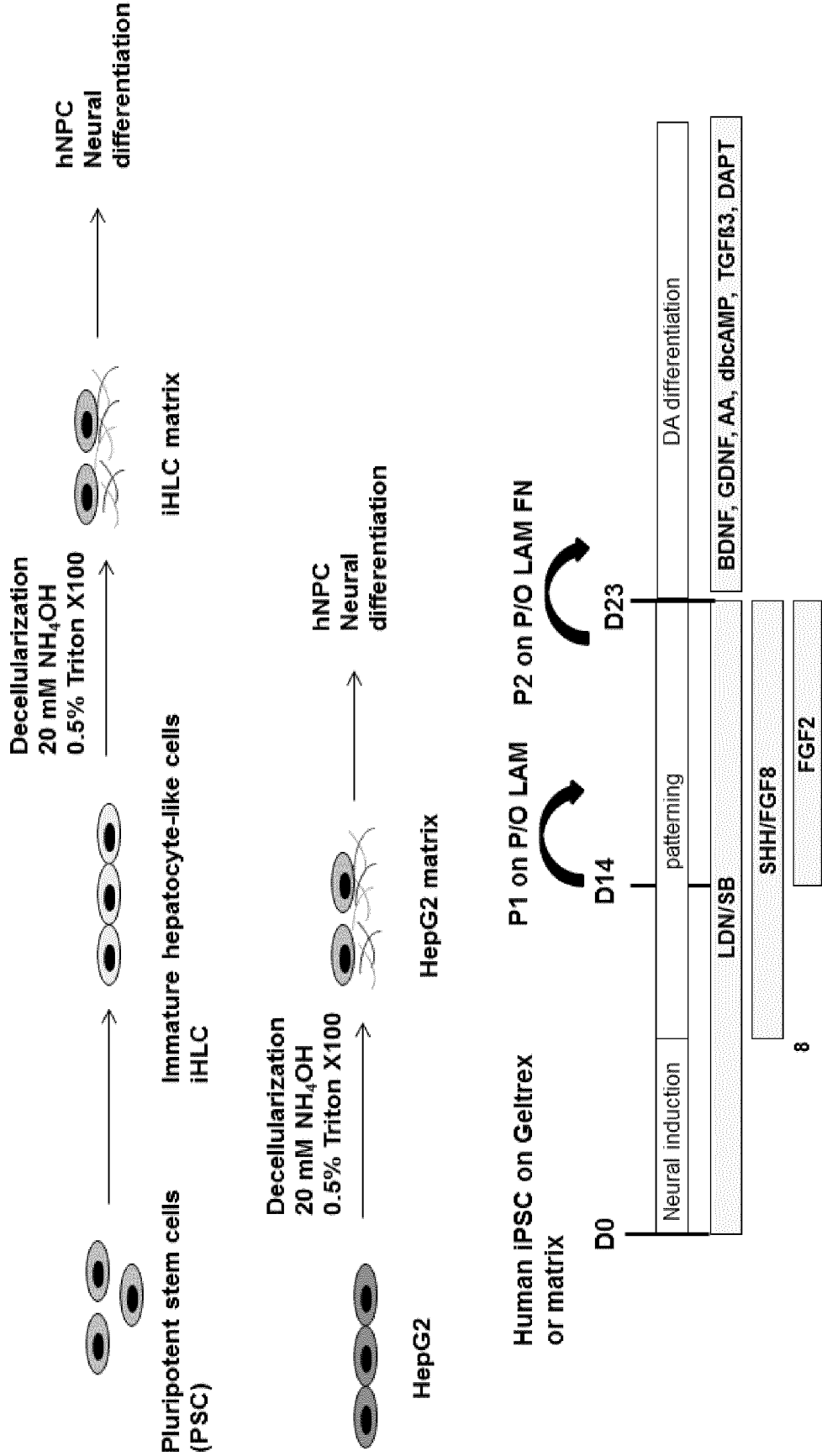


Figure 20

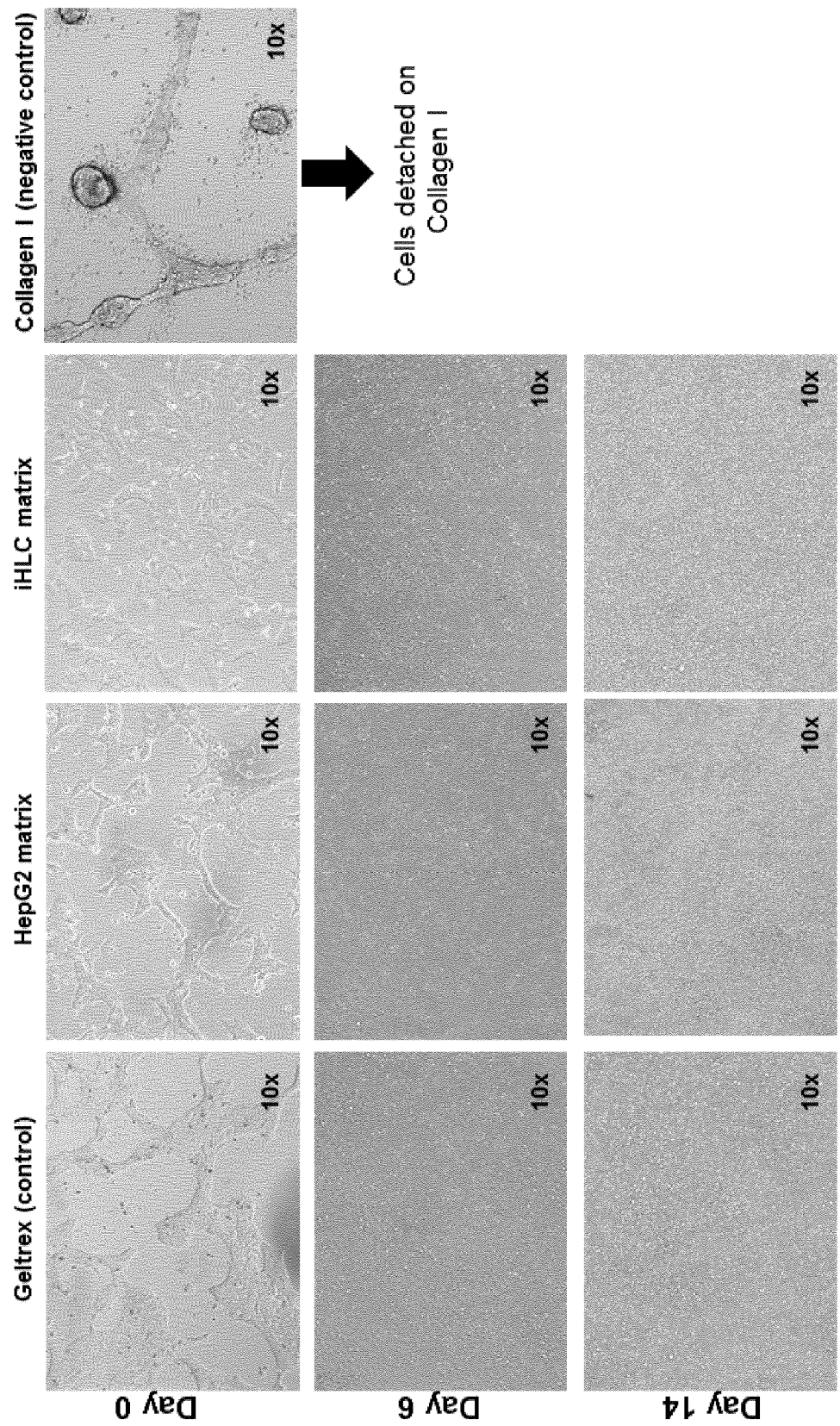
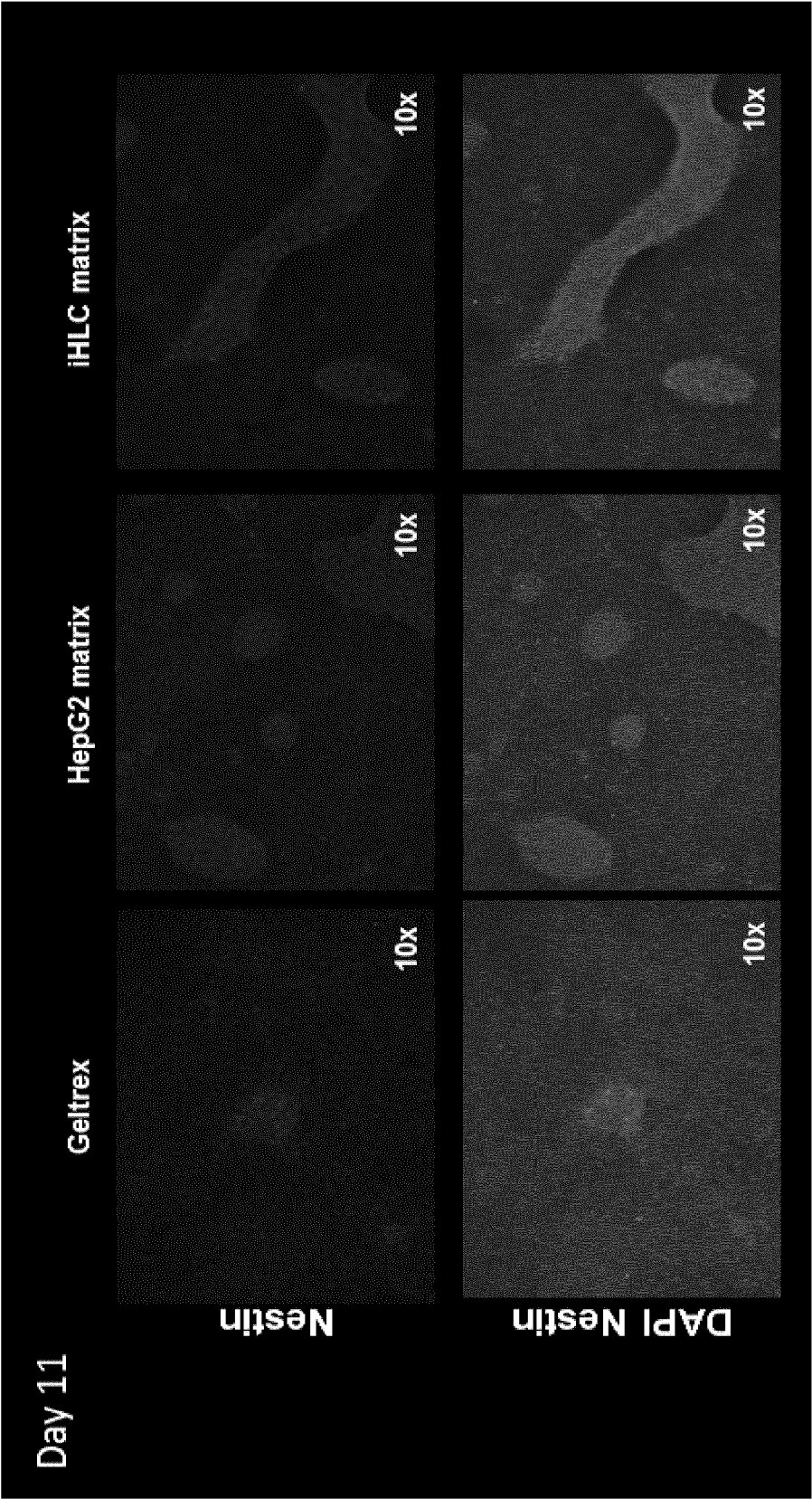


Figure 21



24/43

Figure 22

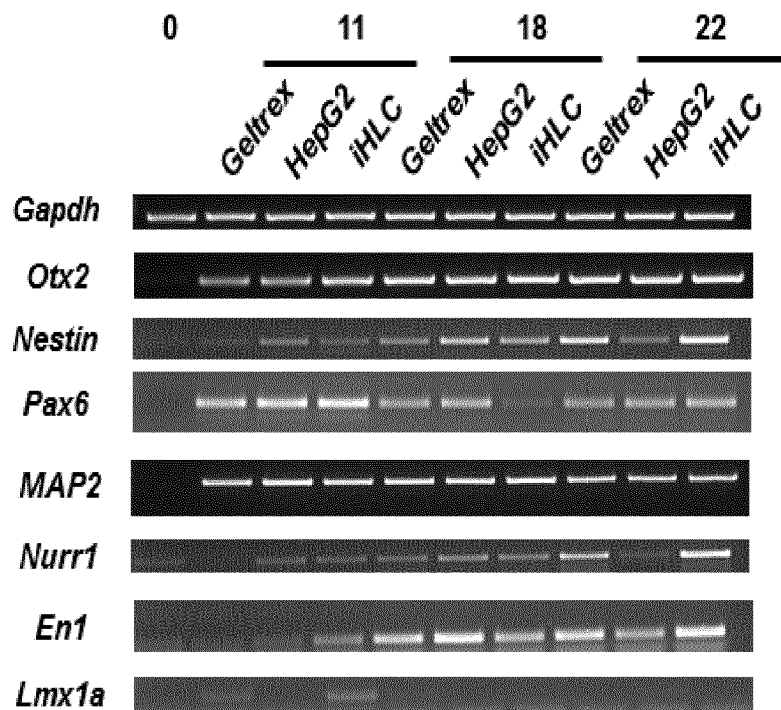


Figure 23

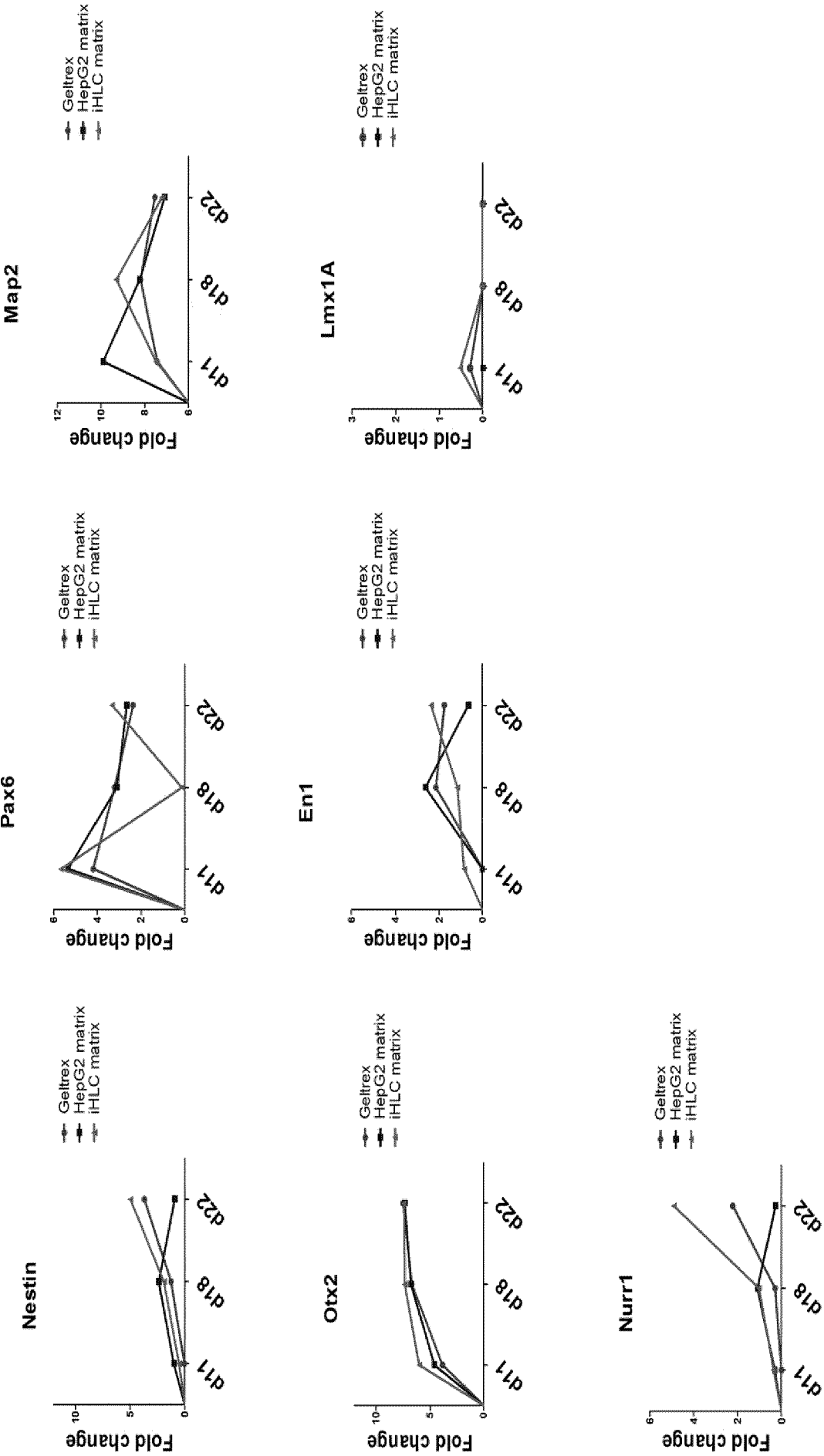


Figure 24

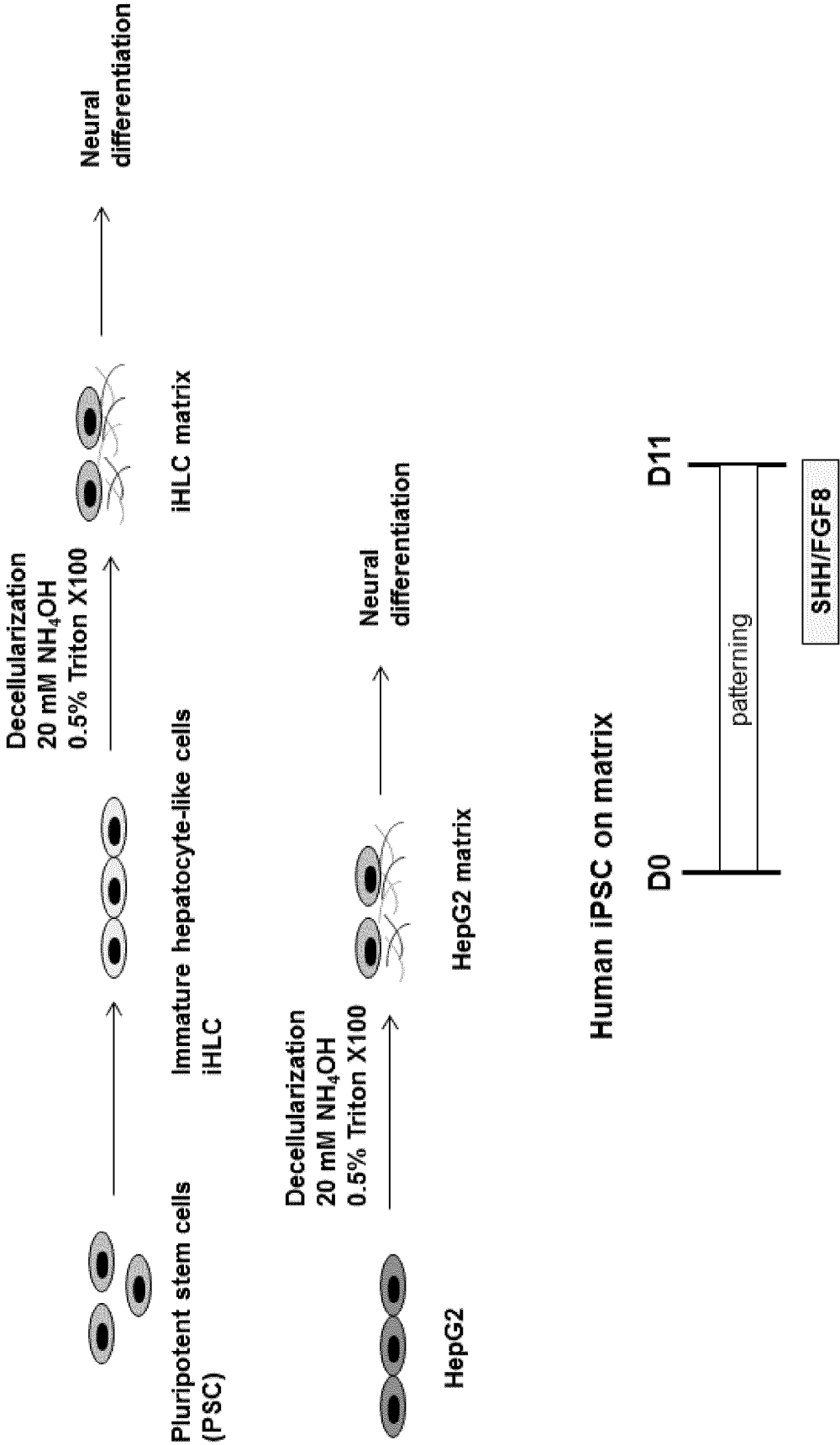


Figure 25

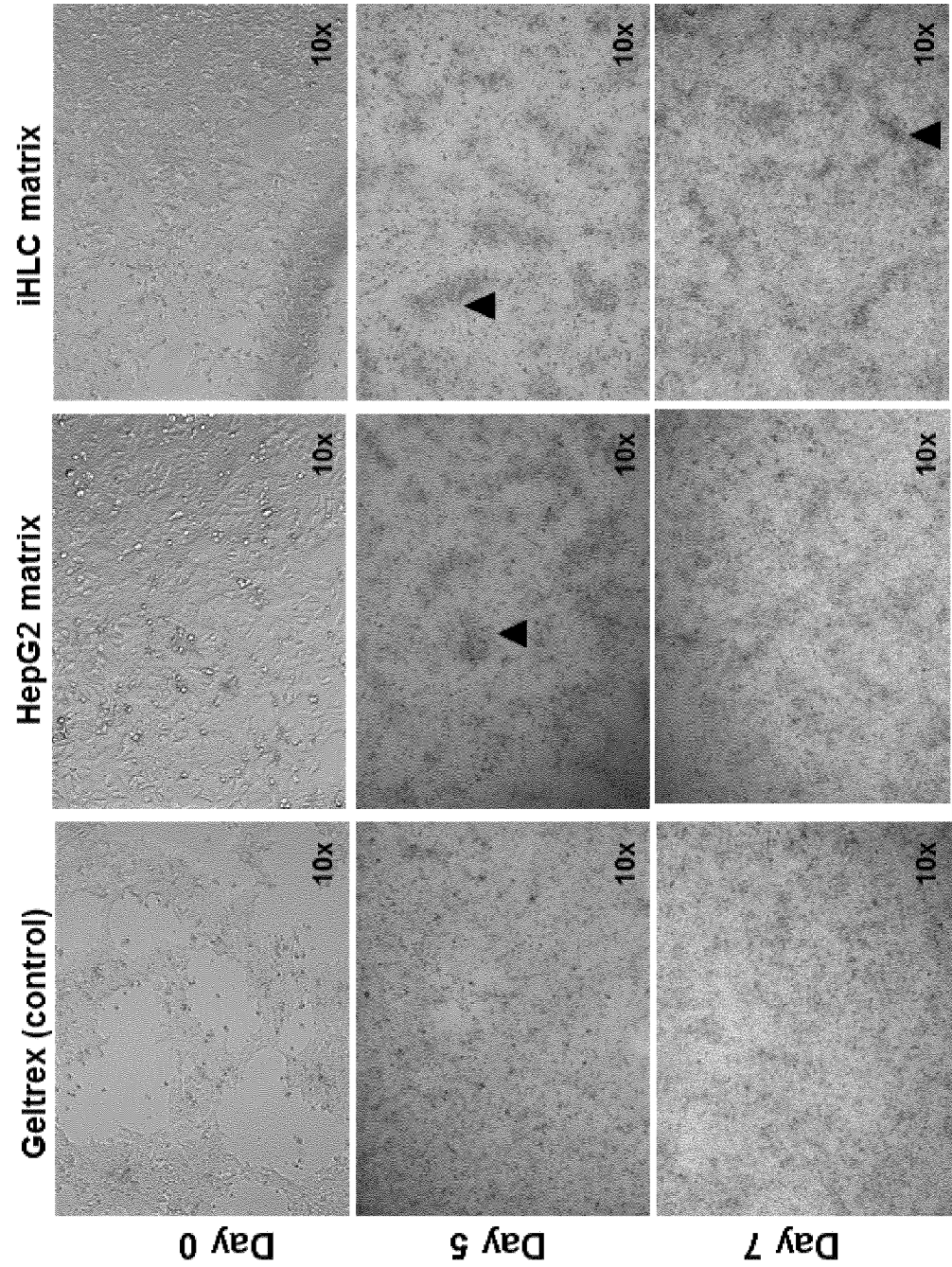


Figure 26

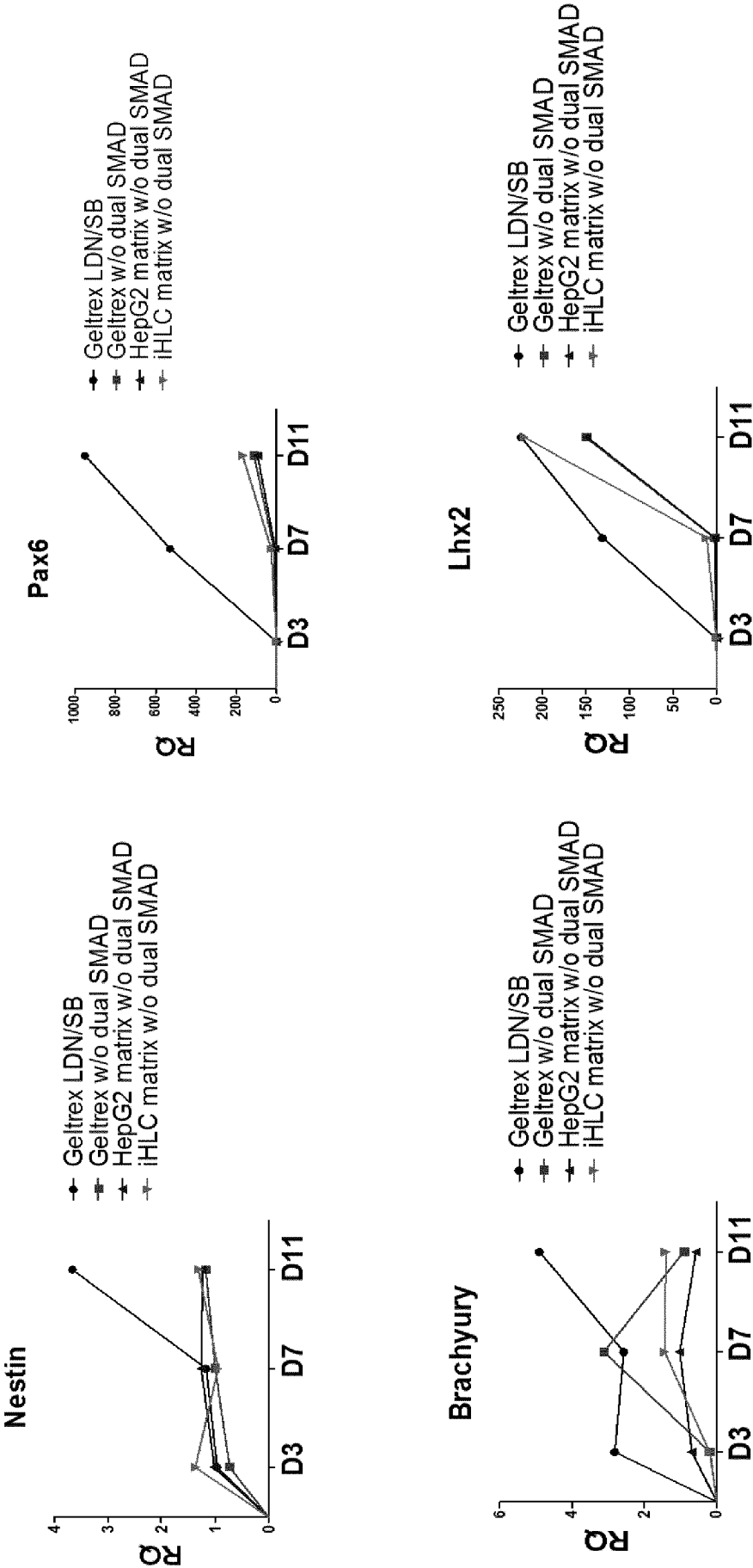


Figure 27

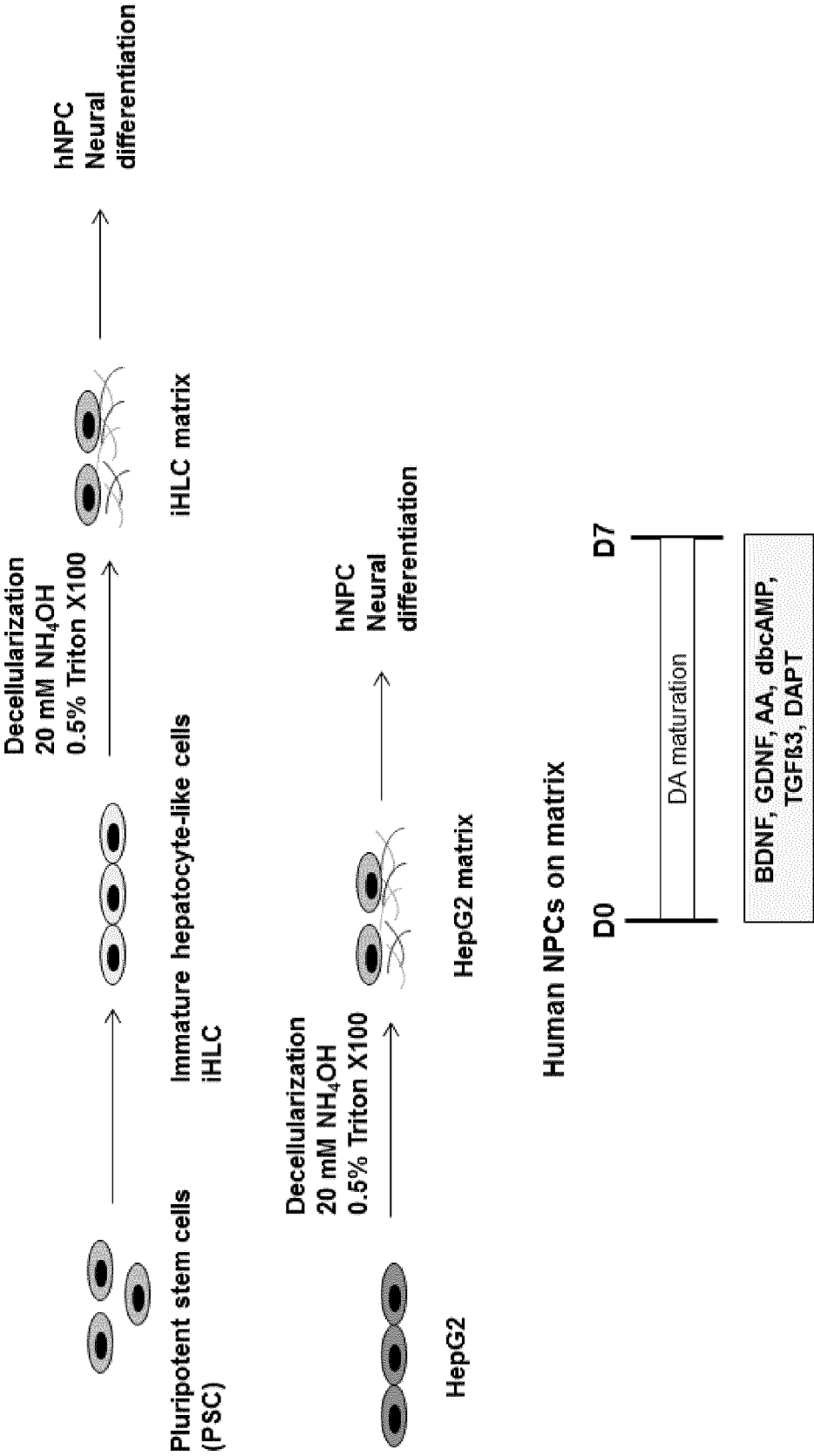
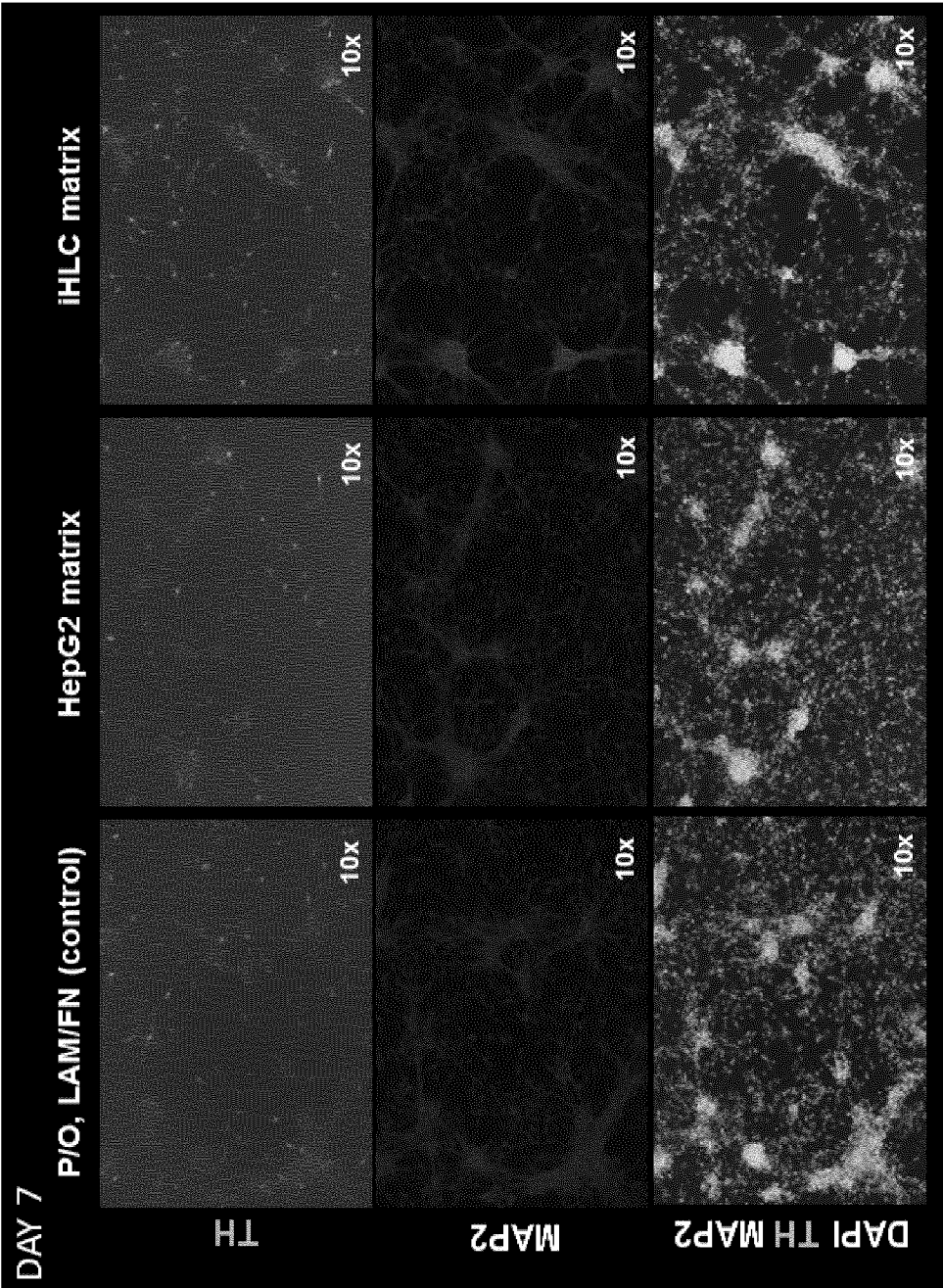


Figure 28



31/43

Figure 29

	iHLC	HepG2	hiPSC	MS5
Hspg2	95	24	25	78
Fn1	67	66	10	89
Lamb1	35	23	19	14
Lamc1	34	28	23	2
Lama5	28	38	5	0
Col1a1	34	28	0	51
Col1a2	29	14	1	40
Co2a1	22	15	0	1
Thbs1	23	0	0	10
Col18a1	22	14	15	0
Lama1	20	0	12	0
Nid1	19	8	6	17
Col5a1	9	0	0	14
Tnc	17	0	0	0
Ltbp1	17	0	0	10
Serpin H1	14	14	14	15
Col4a1	12	0	5	0
Col4a2	21	1	8	3
Col5a2	13	1	0	21
Col12a1	14	1	0	0
Fras1	13	0	1	0
Frem2	13	0	1	0
Fbn2	11	0	0	0
Agrin	12	21	7	0
Vcan	10	3	3	10
Edil3	10	0	5	0
Emilin-2	10	0	0	9
Nestin	9	44	0	12
Emilin-1	8	1	0	31
GDF15	8	0	0	0
FGF2	7	6	0	0
Fbln2	7	0	0	16
Fbln1	7	0	0	0
Col26a1	6	1	1	0

Figure 30

Division	Category	Accession	Unique peptides	Confidence score	ratio hiPSC/iHLC	ratio HepG2/iHLC
Core matrisome	ECM Glycoproteins	FN1	85	4037	0,0	0,1
Core matrisome	ECM Glycoproteins	LAMB1	33	1221	0,1	0,0
Core matrisome	ECM Glycoproteins	LAMC1	35	1505	0,1	0,0
Core matrisome	Proteoglycans	HSPG2	80	2934	0,0	0,0
Core matrisome	Collagens	COL18A1	26	853	0,0	0,0
Core matrisome	Collagens	COL1A1	52	1291	0,0	2,5
Core matrisome	ECM Glycoproteins	LAMA1	16	603	0,0	0,0
Core matrisome	ECM Glycoproteins	VTN	3	118	0,0	0,0
Core matrisome	ECM Glycoproteins	NID1	18	621	0,0	0,0
Core matrisome	Collagens	COL1A2	23	631	0,0	1,5
Core matrisome	Collagens	COL4A1	11	413	0,0	0,0
Core matrisome	ECM Glycoproteins	CYR61	13	435	0,0	0,0
Core matrisome	Collagens	COL4A2	11	311	0,0	0,0
Core matrisome	ECM Glycoproteins	PXDN	21	655	0,1	0,1
Core matrisome	Collagens	COL2A1	8	249	0,1	0,6
Core matrisome	ECM Glycoproteins	CTGF	9	279	0,0	0,0
Core matrisome	ECM Glycoproteins	MFGE8	10	288	0,1	0,0
Core matrisome	ECM Glycoproteins	LTBP1	13	432	0,0	0,0
Core matrisome	ECM Glycoproteins	THBS1	14	414	0,0	0,0
Core matrisome	Collagens	COL26A1	3	83	0,0	0,0
Core matrisome	ECM Glycoproteins	AGRN	21	668	0,1	0,2
Core matrisome	ECM Glycoproteins	TNC	9	252	0,0	0,0
Core matrisome	ECM Glycoproteins	LAMA5	34	1143	0,1	0,7
Core matrisome	ECM Glycoproteins	FRS1	8	238	0,3	0,3
Core matrisome	ECM Glycoproteins	EDIL3	5	166	0,0	0,0

Figure 30 (cont.)

Core matrisome	Collagens	COL12A1	Q99715	5	110	0,3	0,2
Core matrisome	ECM Glycoproteins	FGG	P02679	18	873	0,3	5,2
Core matrisome	ECM Glycoproteins	EMILIN2	Q9BXX0	4	133	0,0	0,0
Core matrisome	ECM Glycoproteins	MFAP2	P55001	2	59	0,0	0,0
Core matrisome	Collagens	COL5A2	P05997	2	53	0,0	0,0
Core matrisome	Proteoglycans	VCAN	P13611	6	177	0,1	0,3
Core matrisome	ECM Glycoproteins	VWA1	Q6PCB0	2	75	0,0	0,0
Core matrisome	ECM Glycoproteins	SPARC	P09486	2	64	0,1	0,0
Core matrisome	ECM Glycoproteins	TINAGL1	Q9GZM7	3	91	0,0	0,0
Core matrisome	ECM Glycoproteins	FBLN2	P98095	3	106	0,0	0,0
Core matrisome	ECM Glycoproteins	FBLN1	P23142	2	40	0,0	0,0
Core matrisome	Collagens	COL6A2	P12110	2	59	0,0	0,2
Core matrisome	ECM Glycoproteins	FGA	P02671	8	243	0,5	6,7
Core matrisome	ECM Glycoproteins	MFAP1	P55081	2	53	0,7	1,2
Core matrisome	Collagens	COL6A1	P12109	2	56	0,0	0,4
Core matrisome	ECM Glycoproteins	RELN	P78509	4	123	0,2	1,7

Figure 31

Core matrixome proteins

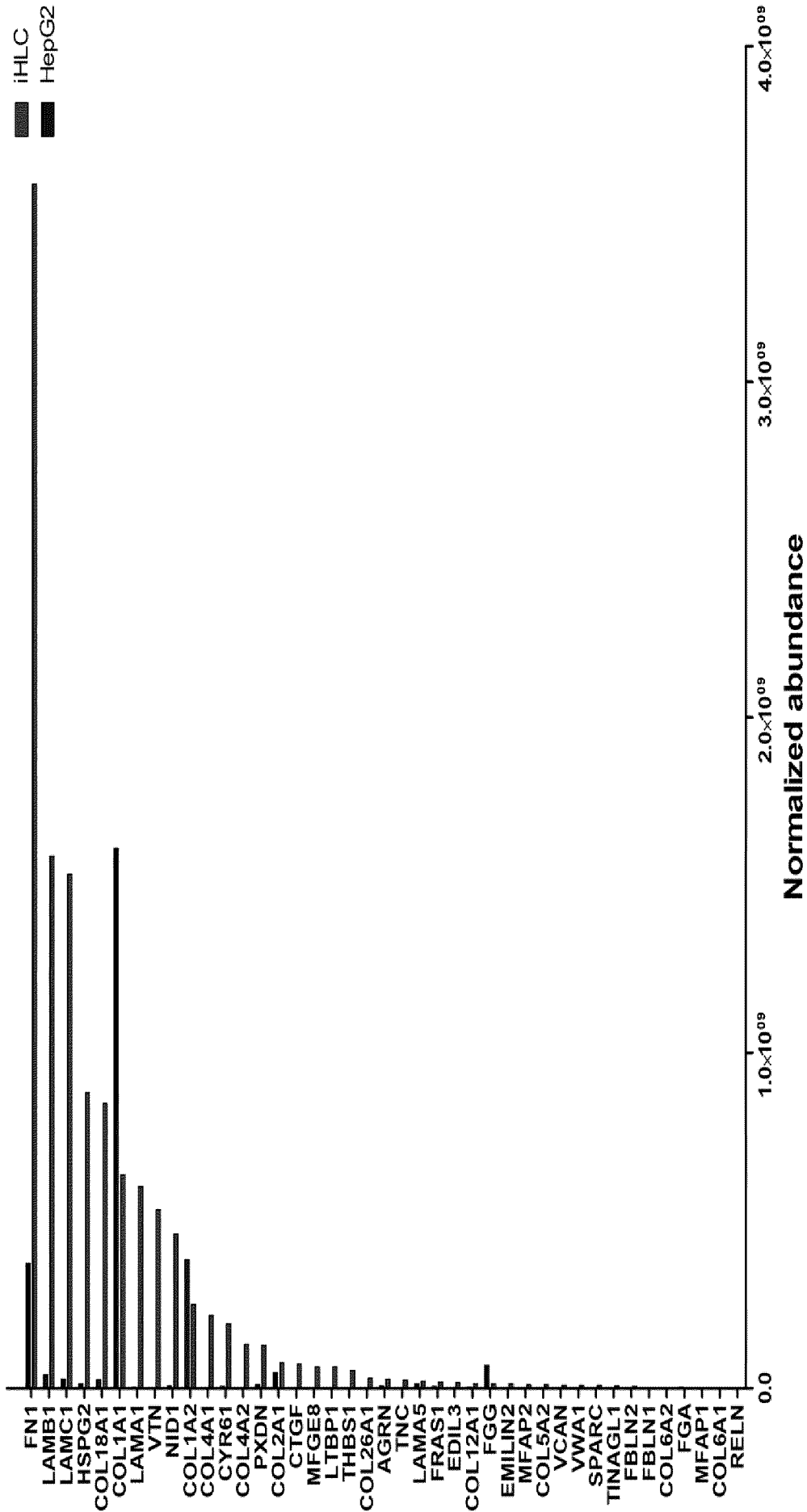


Figure 32

Division	Category	Accession	Unique peptides	Confidence score	ratio hiPSC/iHLC	ratio HepG2/iHLC
Matrisome-associated	Secreted Factors	FGF2	8	228	0,0	0,0
Matrisome-associated	ECM Regulators	SERPINC1	6	132	0,0	0,0
Matrisome-associated	ECM Regulators	SERPINH1	18	878	0,6	0,4
Matrisome-associated	Secreted Factors	HGF	21	598	0,0	0,0
Matrisome-associated	ECM-affiliated Proteins	ANXA2	14	488	0,1	0,0
Matrisome-associated	Secreted Factors	LEFTY2	4	574	0,0	0,0
Matrisome-associated	Secreted Factors	LEFTY1	3	539	0,0	0,0
Matrisome-associated	ECM Regulators	SERPINE2	6	147	0,0	0,0
Matrisome-associated	Secreted Factors	HRNR	6	259	0,2	0,1
Matrisome-associated	ECM-affiliated Proteins	ANXA5	7	245	0,1	0,1
Matrisome-associated	ECM Regulators	SERPINE1	4	115	0,0	0,0
Matrisome-associated	Secreted Factors	GDF15	4	100	0,1	0,1
Matrisome-associated	ECM-affiliated Proteins	LMAN1	15	552	1,6	6,1
Matrisome-associated	ECM-affiliated Proteins	ANXA6	17	508	0,4	1,1
Matrisome-associated	ECM-affiliated Proteins	ANXA1	4	136	0,1	0,0
Matrisome-associated	Secreted Factors	MDK	3	89	0,0	0,1
Matrisome-associated	Secreted Factors	S100A10	2	41	0,1	0,0
Matrisome-associated	ECM-affiliated Proteins	GPC4	11	542	5,2	0,0
Matrisome-associated	ECM Regulators	TGM2	9	295	0,2	2,2
Matrisome-associated	ECM-affiliated Proteins	GPC3	8	334	0,5	12,0
Matrisome-associated	Secreted Factors	S100A11	2	71	0,1	0,1
Matrisome-associated	Secreted Factors	S100A8	5	144	0,3	0,6
Matrisome-associated	Secreted Factors	S100A9	2	99	0,3	0,4
Matrisome-associated	ECM-affiliated Proteins	ANXA7	3	161	0,0	0,1
Matrisome-associated	ECM Regulators	P4HA1	7	288	0,7	2,3

Figure 32 (cont.)

Matrisome-associated	Secreted Factors	FLG2	Q5D862	3	142	0,3	0,3
Matrisome-associated	ECM Regulators	ITIH2	P19823	2	73	0,0	0,2
Matrisome-associated	ECM Regulators	CTSD	P07339	2	62	0,1	0,1
Matrisome-associated	ECM-affiliated Proteins	LGALS1	P09382	4	116	0,2	0,8
Matrisome-associated	ECM Regulators	PCSK5	Q92824	3	84	0,0	0,0
Matrisome-associated	ECM Regulators	CTSA	P10619	2	51	0,6	0,7
Matrisome-associated	ECM Regulators	CSTA	P01040	2	71	0,2	0,4
Matrisome-associated	ECM Regulators	PLOD1	Q02809	5	166	0,3	0,9
Matrisome-associated	ECM Regulators	CTSB	P07858	2	93	0,0	0,7
Matrisome-associated	Secreted Factors	HCFC1	P51610	7	238	3,1	2,0
Matrisome-associated	ECM Regulators	PLOD3	O60568	3	78	0,3	1,1
Matrisome-associated	ECM-affiliated Proteins	GPC6	Q9Y625	5	213	1,4	3,1
Matrisome-associated	ECM-affiliated Proteins	FREM2	Q5SZK8	2	61	0,2	0,0
Matrisome-associated	ECM-affiliated Proteins	ANXA11	P50995	2	152	0,2	1,0
Matrisome-associated	ECM-affiliated Proteins	ANXA4	P09525	3	118	0,4	16,4
Matrisome-associated	ECM Regulators	OGFOD1	Q8N543	2	57	6,2	6,1
Matrisome-associated	ECM-affiliated Proteins	SDC2	P34741	2	56	10,8	35,1
Matrisome-associated	ECM-affiliated Proteins	SDC1	P18827	2	83	11,0	308,3

Figure 33

Matrisome associated proteins

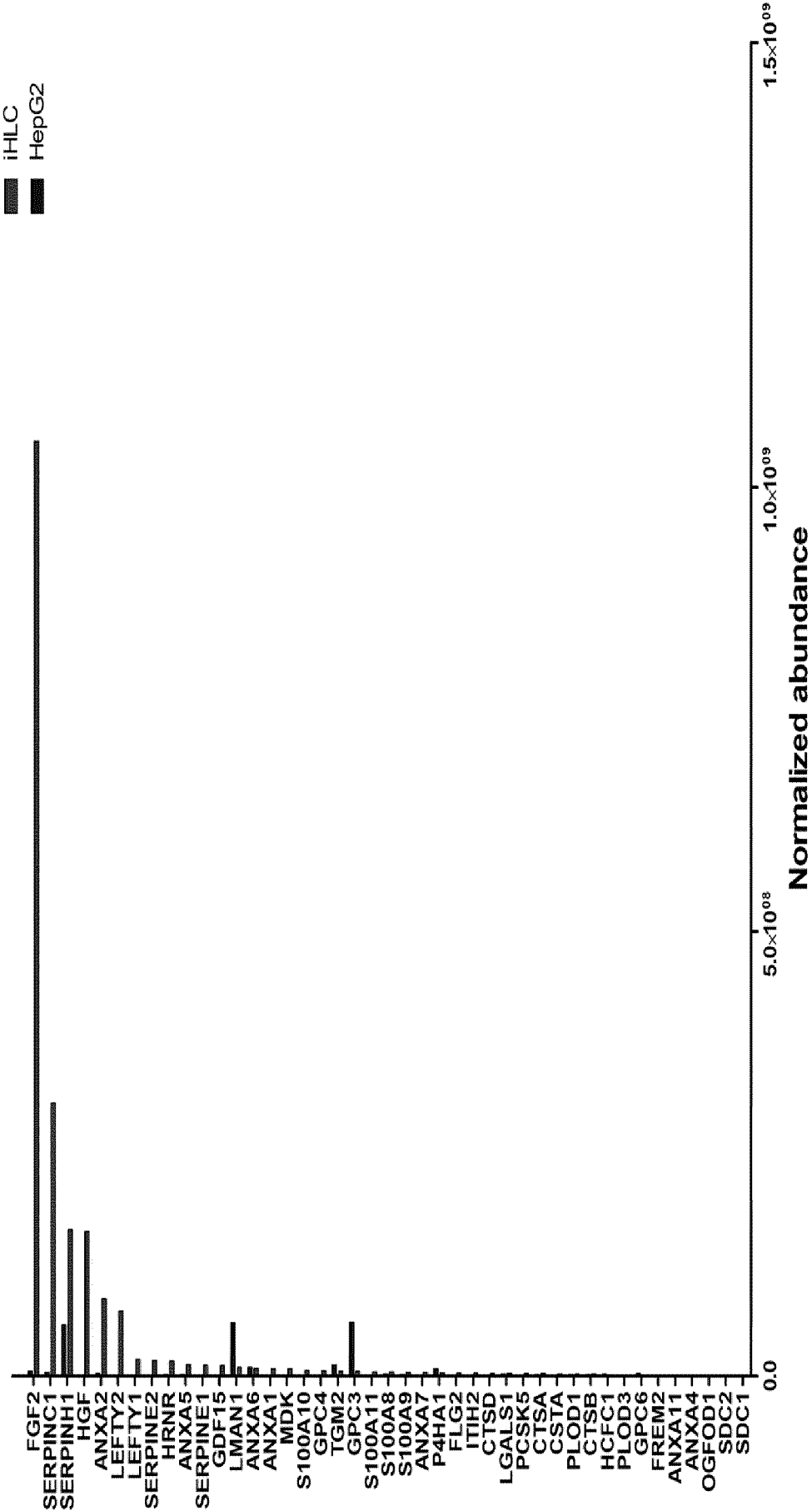
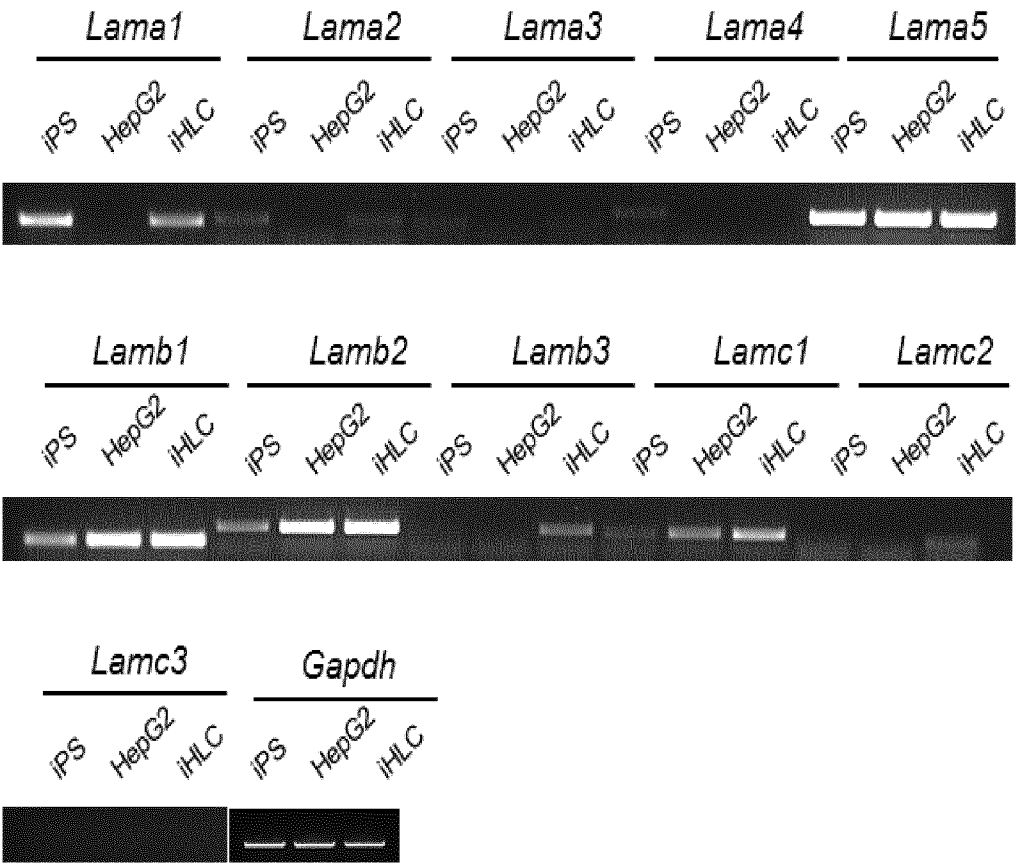
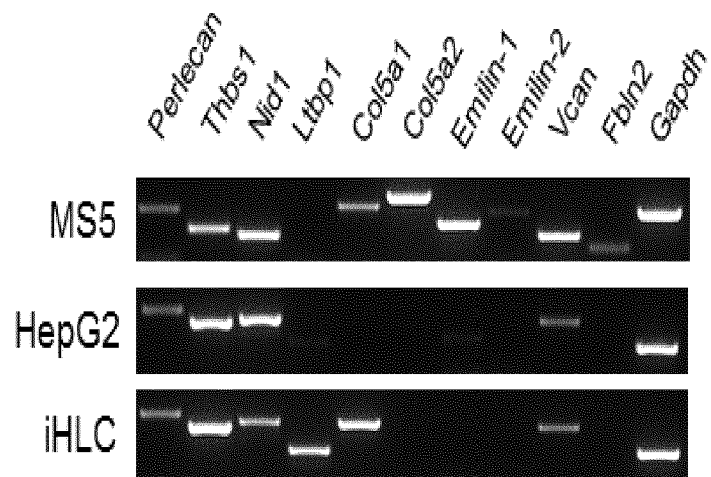


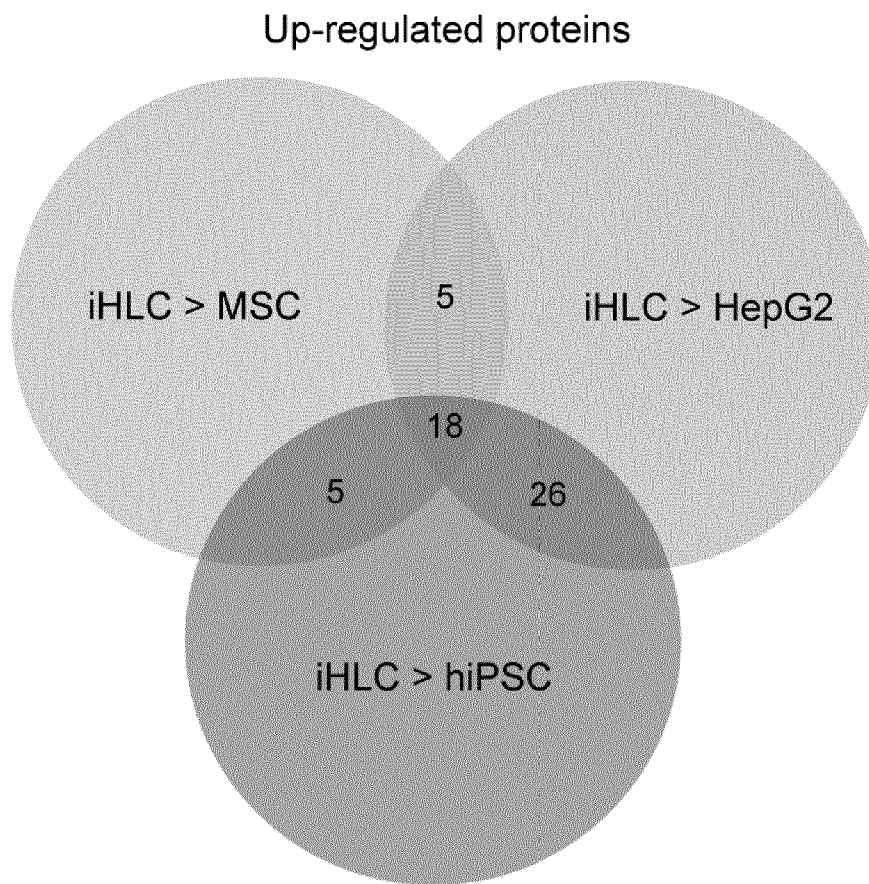
Figure 34



39/43

Figure 35

40/43

Figure 36

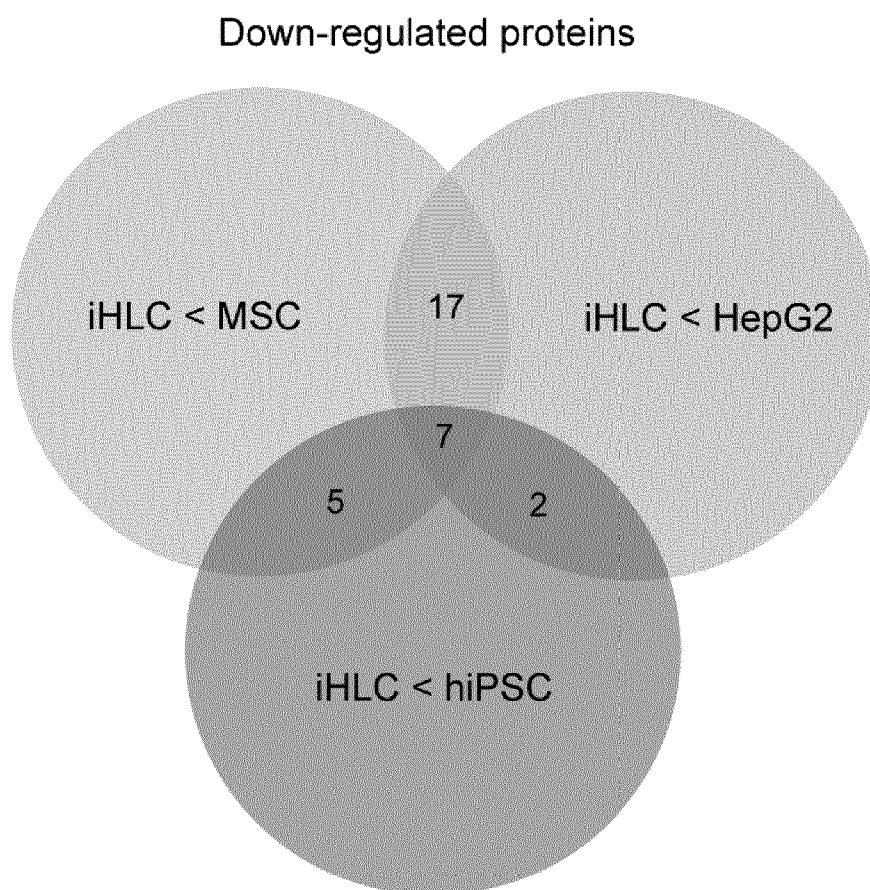
41/43

Figure 37

<u>iHLC > MSC</u> ∩ <u>iHLC > HepG2</u>	<u>iHLC > hiPSC</u> ∩ <u>iHLC > HepG2</u>	<u>iHLC > MSC</u> ∩ <u>iHLC > hiPSC</u>	<u>iHLC > MSC</u> ∩ <u>iHLC > HepG2</u> ∩ <u>iHLC > hiPSC</u>
EFEMP1	ADAM9	COL2A1	AGRN
EDIL3	AGT	CTSC	ANXA3
GPC4	ANXA1	MDK	COL18A1
MFAP2	ANXA2	VTN	CTSV
SFRP1	COL12A1	VWA1	CYR61
	COL26A1		F2
	COL3A1		FBN2
	COL4A1		FREM2
	COL5A1		GDF6
	FBLN1		HAPLN1
	FN1		IGFBP2
	FSTL1		IGFBP5
	IGFBP7		LAMA5
	LAMC1		LAMB1
	LOXL2		LTBP1
	LUM		SERPINB9
	MATN2		SERPINC1
	MMP14		SULF2
	MMP2		
	PXDN		
	S100A10		
	S100A11		
	SPARC		
	THBS1		
	THSD4		
	TIMP3		
	TNC		

42/43

Figure 38



43/43

Figure 39

<u>iHLC < MSC</u> $\cap$ <u>iHLC < HepG2</u>	<u>iHLC < hiPSC</u> $\cap$ <u>iHLC < HepG2</u>	<u>iHLC < MSC</u> $\cap$ <u>iHLC < hiPSC</u>	<u>iHLC < MSC</u> $\cap$ <u>iHLC < HepG2</u> $\cap$ <u>iHLC < hiPSC</u>
A2M	GPC3	FGF2	CD109
ANXA4	NID1	FRAS1	CTSD
COL16A1		LAMA1	FLG
COL2A1		VCAN	ITIH2
CTGF			LGALS1
CTSB			SERPINE1
CTSH			SERPINE2
FBN1			
LGALS3			
LMAN1			
LTBP2			
P4HA1			
S100A13			
SDC1			
SDC4			
SERPINA1			
SERPINB1			

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/058812A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/071
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KARIM SI-TAYEB ET AL: "Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells", HEPATOLOGY, WILEY, vol. 51, no. 1, 1 January 2010 (2010-01-01), pages 297-305, XP002667016, ISSN: 0270-9139, DOI: 10.1002/HEP.23354 [retrieved on 2009-10-01] the whole document figure 3f ----- -/--	1-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

4 June 2018

Date of mailing of the international search report

15/06/2018

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/058812

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SOOJUNG SHIN ET AL: "Long-Term Proliferation of Human Embryonic Stem Cell-Derived Neuroepithelial Cells Using Defined Adherent Culture Conditions", STEM CELLS., vol. 24, no. 1, 1 January 2006 (2006-01-01), pages 125-138, XP055387183, US ISSN: 1066-5099, DOI: 10.1634/stemcells.2004-0150	1-6, 8-17, 19-24
Y	the whole document	1-24
X	SCHULZ THOMAS C ET AL: "Directed neuronal differentiation of human embryonic stem cells", BMC NEUROSCIENCE, BIOMED CENTRAL, LONDON, GB, vol. 4, no. 1, 22 October 2003 (2003-10-22), page 27, XP021002826, ISSN: 1471-2202, DOI: 10.1186/1471-2202-4-27	1-6, 8-17, 19-24
Y	the whole document	1-24
X	WO 2009/080794 A1 (MC2 CELL APS [DK]; EL-SABBAN MARWAN [DK]; SINDET-PEDERSEN STEEN [GB]) 2 July 2009 (2009-07-02)	1-6,12, 13, 15-17, 19-23
Y	page 8, line 14 - line 26; example 4	1-24
X	KYUNG-MEE PARK ET AL: "Decellularized Liver Extracellular Matrix as Promising Tools for Transplantable Bioengineered Liver Promotes Hepatic Lineage Commitments of Induced Pluripotent Stem Cells", TISSUE ENGINEERING PART A, vol. 22, no. 5-6, 1 March 2016 (2016-03-01), pages 449-460, XP055387188, US ISSN: 1937-3341, DOI: 10.1089/ten.tea.2015.0313	1-6,12, 13,15, 20-23
Y	the whole document	1-24
X	WO 2012/094255 A2 (UNIV CALIFORNIA [US]; ZHOU PING [US]; WU JIAN [US]; NOLTA JAN [US]) 12 July 2012 (2012-07-12)	1-6,12, 13,15, 20-23
Y	paragraph [0007]; claims 1-19	1-24
X	WO 99/53021 A1 (BRESAGEN LTD [AU]; BETTESS MICHAEL DAVID [AU]; RATHJEN PETER DAVID [AU]) 21 October 1999 (1999-10-21)	1-6,8, 12,13, 15,20-23
Y	example 5	1-24
	-/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/058812

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IL-SHIN LEE ET AL: "Human neural stem cells alleviate Alzheimer-like pathology in a mouse model", MOLECULAR NEURODEGENERATION, BIOMED CENTRAL LTD, LO, vol. 10, no. 1, 21 August 2015 (2015-08-21), page 38, XP021228557, ISSN: 1750-1326, DOI: 10.1186/S13024-015-0035-6 the whole document -----	25-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/058812

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009080794	A1	02-07-2009	NONE
WO 2012094255	A2	12-07-2012	US 2014023624 A1 23-01-2014 WO 2012094255 A2 12-07-2012
WO 9953021	A1	21-10-1999	CA 2324591 A1 21-10-1999 EP 1068295 A1 17-01-2001 JP 2002517982 A 25-06-2002 NZ 507103 A 25-10-2002 WO 9953021 A1 21-10-1999