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Generation of *in vivo* traceable hepatocyte-like cells from human iPSCs

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Abstract

In this chapter, we describe a protocol for differentiation of human induced pluripotent stem cells (iPSCs) into hepatocyte-like-cells (HLCs) and their transduction with a lentivirus for gene transfer. Here, we engineer them to express the human sodium iodide symporter, which can be exploited as a radionuclide reporter gene, thereby enabling these cells to be tracked *in vivo* by Single-Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET) imaging. Differentiation of HLCs from iPSCs involves three steps: induction of iPSCs to definitive endoderm, differentiation to a hepatic progenitor cell population, and maturation of immature HLCs. Once proliferation of hepatic progenitors has ceased and an immature HLC population is generated, lentiviral transduction can be performed. The immature hepatic gene expression profile/morphology at the stage of transduction will be compatible with further maturation following transgene expression either *in vitro* or *in vivo*, with expression of the transgene retained. We detail how transgenic cells can be imaged *in vivo*. Whilst we provide a protocol for the NIS reporter gene, the cell engineering aspects of this protocol are transferable for use with other (reporter) genes if desired.

Key words

Reporter gene, hepatocyte-like cells, hiPSCs, SPECT, PET, cell tracking, *in vivo* imaging, hepatic differentiation, sodium iodide symporter.

1. Introduction

1.1. hiPSCs as an alternative source of hepatocytes for cell therapy

Since the first allogeneic adult hepatocytes were transplanted in 1997 *(1)* the potential advantages of hepatocyte transplants (HTx) have contributed to the maintained interest in its long-term potential as an alternative therapy to whole organ transplant. In particular, the reduced invasiveness of HTx and retention of the native liver architecture are important advantages. The latter specifically provides an insurance in the event of graft failure or immune rejection, as well as retains the potential for spontaneous regeneration. This is an advantage in instances of acute liver failure where the livers of some patients may go on to fully regenerate, providing they can survive the initial fulminant course *(2)*. Despite maintained interest in HTx, as with whole organ transplant, wider application is circumvented by donor availability. Given that primary hepatocytes are isolated from adult livers rejected for transplant available hepatocytes can be limited in both quantity and quality due to the sources available. Additionally, whilst *in vivo* within their native liver niche hepatocytes exhibit regenerative capacity, *ex vivo* their differentiated function and proliferative ability rapidly diminishes, necessitating HTx following fresh isolation or use of rapidly cryopreserved hepatocytes *(3)*. Cryopreservation, though considerably optimised in recent years and an advantage to allow repeat infusions from the same donor, detrimentally impacts cell viability, metabolic function, albumin production and attachment *(4–6)*. These factors all highlight the significant need for alternative sources of hepatocytes suitable for transplant, along with a means of improving post-transplant cell survival, in order for this promising therapeutic approach to benefit more patients in the future.

One potential substitute source is the use of stem cell-derived hepatocytes, with human induced pluripotent stem cell (hiPSC)-derived hepatocyte-like-cells (HLCs) showing particular promise. Like their predecessor, human embryonic stem cells (hESCs), hiPSCs have unlimited self-renewal ability and can differentiate into cells of all three germ layers (mesoderm, ectoderm, and endoderm) by recapitulation of embryonic developmental signalling pathways for the cell type of interest. The ability for hiPSCs to self-renew means that they can be harnessed to produce a potentially unlimited supply of hepatocyte-like cells (HLCs), making them a promising alternative source to primary hepatocytes. Whilst hiPSCs and hESCs can be differentiated into HLCs following the same protocols *(7)*, hiPSCs present fewer ethical concerns. Despite the existence of procedures for hESC line generation without the destruction of embryos *(8)* the majority of hESC lines are obtained from either the inner cell mass or epiblast of surplus blastocysts (an early-stage embryo) produced for the purposes of *in vitro* fertilisation *(9)*. This results in destruction of the blastocyst, making hiPSCs a preferable pluripotent stem cell source. HLCs differentiated from hiPSCs could also act as an autologous cell therapy offering a low immune rejection risk, as they can be reprogrammed using a patient's own somatic cells. Even where hiPSCs are considered for use as an allogeneic alternative to hESCs, they can be generated from donors whose genetic characteristics and health records are well-established.

Differentiation of pluripotent stem cells (PSCs) towards the hepatic lineage has been performed using both PSC aggregates and monolayer culture approaches for initial fate induction. PSC aggregates are used to form embryoid bodies (EBs) which are subsequently differentiated following addition of growth factor, cytokines and small molecule cocktails *(10)*. To date, EB protocols suffer from inefficient and low levels of differentiation as well as

spontaneous differentiation, yielding impure cultures encompassing cells of other lineages *(11–13)*.

Conversely, monolayer cultures which utilise a sequential, stepwise introduction of appropriate growth factors/cytokines/small molecules ensure all PSCs are evenly and consistently exposed to the differentiation inducing cocktail. These have been widely used to reproducibly yield HLCs with high levels of purity using a three-step differentiation process, first differentiating cells towards definitive endoderm, then stimulating cells to differentiate along the hepatic pathway producing an intermediate highly proliferative hepatic progenitor population before subsequently yielding a monolayer of immature HLCs which go on to develop a characteristic polyhedral morphology as they mature. Immature HLCs can be dissociated for further *in vitro* maturation either on collagen-I coated plates, within 3D scaffolds or in co-cultures or transplanted *in vivo*.

1.2. The three-step process for generating HLCs

HLC differentiation protocols focus on recapitulating the Nodal and Wnt/β-catenin signalling events that occur during embryonic development. Nodal, a member of the transforming growth factor beta (TGFβ) superfamily, stimulates transcription of a host of target genes involved in initiation of endodermal development (the germ layer from which the liver derives) *(14)*. Mimicking of this signalling process *in vitro* is habitually achieved through addition of Activin A (a Nodal analogue) to culture media for the first few days of PSC differentiation. Studies have shown that Wnt/β-catenin signalling is also critical for endoderm formation as well as a multitude of developmental intracellular signalling transduction cascades *(14–16)*. To recapitulate activation of Wnt/β-catenin signalling pathways

differentiation protocols usually rely on addition of Wnt3a or the small molecule CHIR99021 to culture media during the early stages of differentiation. CHIR99021 enables Wnt/β-catenin signalling by selectively inhibiting glycogen synthase kinase 3β (GSK3β). GSK3β ordinarily forms part of the β-catenin destruction complex, so inhibiting its activity prevents degradation of β-catenin thereby facilitating sustained β-catenin transport into the nucleus and ensuring downstream signalling *(17)*. Wnt signalling functions upstream of GSK3β by binding to its cell-surface receptor, Frizzled, and its coreceptor low density lipoprotein receptor protein 5/6 (LRP5/6). Receptor-binding activates the Dishevelled family proteins, which recruit axin to the plasma membrane thereby also inhibiting assembly of the β-catenin destruction complex, facilitating sustained β-catenin translocation into the nucleus *(14)*. Both use of Wnt3a and CHIR99021 are considered to effectively enable Wnt/β-catenin signalling in HLC differentiation protocols, although CHIR99021 comes at the advantage of lower cost relative to Wnt3a.

During gastrulation, after triggering development of definitive endoderm, modulation of Nodal and Wnt signalling regulates endodermal tube patterning *(18)*. The mesoderm lineage cells, cardiac mesoderm (CM) and septum transversum mesenchyme (STM), adjacent to the endodermal tube stimulate further patterning producing the foregut, midgut and hindgut domains. The portion of the foregut that develops into the liver bud borders the CM and STM, as a result of growth factor secretions by these bordering mesoderm populations [fibroblast growth factors (FGFs) by CMs and bone morphogenic proteins (BMPs) by STM *(18–20)*] the adjacent foregut cells become committed to the hepatic lineage *(18–20)*. As such, most differentiation protocols incorporate FGFs and BMPs into culture media during step 2, yielding an immature hepatocyte progenitor population representative of embryonic hepatoblast cells that colonise the liver bud, these cells are capable of high levels of cell expansion and proliferation.

In the developing liver, haematopoietic cells that migrate into the liver bud produce oncostatin M (OSM), an interleukin-6 family cytokine *(21)*. This has been shown to play an important role in inducing hepatocyte specific functions including upregulating albumin expression, synthesis of lipids and glycogen, ammonia clearance and detoxification *(22, 23)*. Hepatocyte growth factor (HGF) also stimulates expression of mature hepatocyte marker genes and glycogen accumulation, in a manner that involves activation of signalling pathways distinct from OSM *(22, 24)*. As such, PSC differentiation protocols typically incorporate either or both OSM and HGF within the media culture cocktail to induce maturation of hepatic progenitors in step 3.

HLCs yielded from differentiation protocols differ notably across research groups as a result of variations between PSC lines and culture conditions, including differences in basal medium, extracellular matrices (ECMs) used for culturing cells and selection of growth factors and their timepoints for introduction. This makes efforts to improve factors such as engraftment following transplant *in vivo* difficult to compare between studies as results are confounded by variations in HLC quality. The protocol presented here follows the 3 step differentiation process (**Figure 1**) and has been demonstrated to work for a number of PSC lines *(25, 26)* although some adaptation may be required for new PSC lines (*e.g.* initial colony seeding density may require optimisation). Tips for optimisation of new lines are included in the Notes section.

1.3 Use of radionuclide reporter genes for HLC tracking *in vivo*

To track transplanted HLCs non-invasively in real-time *in vivo* a signal specific for the administered HLCs which distinguishes them from their *in vivo* environment and can be captured by a suitable imaging modality must be generated. To achieve this, cells can either be directly or indirectly labelled *(27)*. Directly labelling cells *in vitro* involves using a contrast agent, such as nanoparticles, near infrared dyes or radiolabelled antibodies which are taken up by the cells through normal cellular processes (*e.g.* phagocytosis, *via* internalising receptors or assisted by *e.g.* transfection agents etc). Whilst easily achieved direct cell labelling is disadvantaged by events which may be of functional interest for tracking viable HLCs. For example, dilution of the contrast agent if transplanted cells proliferate will result in diminished signal contrast over time per individual cell, despite a surviving cell population remaining present, as the contrast agent will be distributed amongst progeny cells. Moreover, there is a possibility that the wrong object is visualized. For example, cells directly labelled *in vitro* with paramagnetic particles to facilitate magnetic resonance imaging (MRI) *in vivo* can die after transplantation with the nanoparticles becoming phagocytosed by macrophages, therefore the particle-based label would remain present and be detected by MRI, albeit now reporting on the macrophages rather than HLCs *(28)*. Finally, as direct labelling agents often rely on passive diffusion, even if cells are not dying or proliferating, the label may diffuse out of cells over time *in vivo*, limiting the observation time.

The limitations associated with direct cell labelling are why for medium to long term cell tracking studies indirect cell labelling through the use of reporter genes is the preferred strategy *(29)*. Indirect labelling requires cells to be genetically engineered to ectopically express a reporter gene, rendering them different from the surrounding cells *in vivo*. Reporter gene constructs normally integrate permanently into cells and pair a promoter/enhancer sequence to a reporter gene. Following translation of the reporter protein cells can be detected

either directly (*e.g.* in optical fluorescence imaging) or following the administration of an imaging probe capable of interacting with the reporter protein and subsequently accumulating signal within the transgenic cell population (such as a radiolabelled substrate). As imaging reporter genes tend to be driven by constitutive promoters, these strategies facilitate detection of only cells with intact transcriptional machinery, so the imaged signal correlates with the viable cell population. This process can be performed on multiple occasions, allowing cells to be tracked longitudinally, with progeny cells resulting from proliferation also capable of being tracked.

Radionuclide imaging relies on the concept that after administration of radiolabelled compounds to animals they enter the circulation and are transported to organs around the body. The radiolabelled elements are then absorbed specifically at anatomical sites with affinity for the administered compound and are retained at that location for a sufficient time to allow for recording of a good contrast image (for more in-depth information see *(29)*).

The protocol detailed here uses the human sodium iodide symporter (hNIS) as a radionuclide reporter gene. hNIS can take up a range of radioisotopes in the form of anionic substrates. Notably, this includes radioisotopes compatible with both PET and SPECT imaging. The radiotracers suitable for use with hNIS are widely available, making them easily accessible for serial imaging studies. Additionally, these radiotracers often do not require complex radiochemistry, with some routinely used in the clinic for medical imaging, meaning their metabolic and clearance properties have been well documented*(30)*. As an endogenous human protein expressed in only limited tissues, hNIS can enable good contrast imaging. Given that those limited tissues with endogenous mouse NIS expression (*e.g.* salivary glands, stomach, thyroid) are not regions of interest for HLC transplants any background radiotracer

uptake is unlikely to interfere with longitudinal HLC tracking. Furthermore, as a human

protein (with homology with rat and mouse NIS) it has not been reported to stimulate host

immune responses in humans (or rats or mice) *(31, 32)*. Other reporter genes, including

radionuclide reporter genes could also be utilised, the advantages and disadvantages of which

have been comprehensively reviewed elsewhere *(29, 33)*.

Fruhwirth et al have shown that as low as 1000 hNIS expressing cancer cells (within a cell

mass/pellet of 10^6 cells) are capable of detection *(34, 35)*. Such low detection limits suggest

radionuclide-afforded hNIS based imaging is a very well-suited modality for detection of

small populations of surviving cells, as may be the case with transplanted HLCs as well as for

detection of any potential 'off-target' populations that accumulate elsewhere. Whilst the

highest resolution *in vivo* images in rodents can be achieved by preclinical radionuclide

imaging approaches utilising reporter genes such as hNIS-mGFP, the protocol detailed here is

compatible with alternative transgene reporter constructs. Alternatives could for example be

optical reporters *(36)*, which at the expense of imaging depth, 3D information or resolution

(depending on the specific modality) may turn out to be easier accessible or cheaper to use.

1.4 Choice of lentiviral genetic engineering for iPSC-derived progeny

The majority of preclinical studies tracking stem cells or their differentiated progeny have

used a lentivirus for transfer of the reporter gene *(29)*. Lentiviruses have a number of

properties that make them favourable in this respect. Firstly, they are able to efficiently

deliver a large payload of genetic material enabling compatibility with even very large

reporter constructs, which may, in addition to the reporter gene, include genes for selection of

successfully transduced cells (*e.g.* fluorescent reporters for flow cytometric sorting or antibiotic resistance genes) *(37)*. In contrast to other retroviruses, lentiviruses are capable of infection and pro-viral integration of both dividing and non-dividing cells *(38, 39)*, so integration of the construct into the genome can yield stable expression of the reporter protein whether transduction is at the hiPSC, progenitor (*e.g.* hepatoblast) or mature differentiated cell (HLC) stage. Coupled with these benefits is the limitation that reporter integration is more or less distributed randomly within the genome, although typically at sites actively being transcribed *(40)*. This runs the risk of insertional mutagenesis, including oncogenic transformation, and it is unknown whether errant integration will impact hiPSC ability to differentiate successfully along the hepatic lineage. Furthermore, in some cases reporter gene expression has been reported to reduce following long-term propagation of replicating cells as a result of epigenetic silencing *(41, 42)*. This is obviously a disadvantage for any longitudinal imaging strategy or differentiating stem cell populations, as it would result in reduced imaging signal over time, thus limiting the applicability of earlier quantification. Silencing can be attenuated by treating cells with DNA methyltransferase inhibitors *(41, 42)*, however as DNA methylation plays a critical role in the pluripotency of PSCs and their ability to differentiate to different germ layers the requirement for such treatments should be avoided where possible *(43)*. One means to overcome, at least partially, issues of transgene silencing is the choice of the promoter.

Here, we detail a lentiviral transduction approach to enable expression of the hNIS fused to monomeric green fluorescent protein (hNIS-mGFP) in HLCs. Use of a fusion protein enables transduction efficiency to easily be assessed by flow cytometry and/or fluorescence imaging. HiPSCs are first differentiated into immature HLCs before transduction once they are already committed to the hepatic lineage, but are likely to have limited residual proliferative ability *in*

vitro (minimising the possibility of downstream reporter silencing). Following transduction, cells are dissociated and reseeded for further maturation on a variety of extracellular matrices, within 3D-scaffolds, co-culture formats, or transplanted immediately *in vivo*. Given that cells have already undergone significant differentiation, the reporter gene expression is considered to have a limited impact on cell phenotype or function and hNIS-mGFP expression has been shown to be retained for at least 100 days in HLCs *in vitro* **(26)**.

2. Materials

2.1. Plasmid production

1. Luria Broth (LB) agar antibiotic selection plates: Add 1.5 g of agar per 100 mL of LB and autoclave. Allow liquid to cool to room temperature and then add antibiotics to the solution to a final concentration of 100μg/mL ampicillin or 50μg/mL kanamycin depending on the resistance gene of the plasmid to be spread on the plate. Pour or pipette into non-tissue culture treated petri dishes so that the depth of liquid is at least 1cm (between 12-15mL per dish). Leave to set at room temperature. Plates can either be used immediately or sealed with parafilm and stored upside down at 4°C for up to 2 months.

2. Super Optimal broth with Catabolite repression (SOC) media: add 0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in deionised H_2O and autoclave. Allow to cool before adding 20 mM Glucose, sterilise through a 0.22μm filter. Store at 4°C for up to two months or freeze as aliquots at -20°C for long term storage.

3. Glycerol stock solution: Add pure glycerol (>99% v/v) to an equal volume of endotoxin free distilled H_2O in an autoclave-able bottle and autoclave. Store at room temperature.

4. Bacterial plate incubator set to 37°C, 5% CO_2

5. Shaking bacteria incubator with temperature control, set to 37°C, 5% CO_2

6. Spectrophotometer (*e.g.* SPECTROstar Nano, BMG LABTECH)

7. Chemically competent *E.Coli* suitable for transforming plasmids with direct repeats (such as lentiviral vectors) such as Stbl3 (New England Biolabs) or similar

8. Plasmid DNA maxiprep kit (Qiagen)

2.2. Virus production

1. 25kD Linear polyethylenimine (PEI) stock solution (final concentration 1µg/µL): dissolve 100 mg of powder in 100 mL of endotoxin free dH_2O that has been heated to ~80°C. Allow solution to cool to room temperature and then neutralise to pH 7.0 with hydrochloric acid (HCl) by stirring and slowly adding dropwise. Typically, the solution will be basic and will require adjusting with HCl first, sodium hydroxide can also be used to adjust the pH to 7.0 if it becomes too acidic. Re-check the pH after allowing the solution 10 minutes to mix to ensure no drift has occurred. Sterile filter the solution in a tissue culture hood (0.22µm) and store at -20°C or -80°C in aliquots of 0.5-1mL. Working stocks can be kept at 4°C for up to 2 months.

2. Basal media for HEK 293T cell culture: Dulbecco's Modified Eagle's Medium with 1g/L of glucose supplemented with 10% (v/v) fetal bovine serum (FBS, Apollo Scientific), penicillin (100 IU/mL) and streptomycin (0.1mg/mL) (Sigma) and 2mM *L*-glutamine (Sigma).

3. For large batch virus production: Millicell HY 5-layer T1000 cell culture flask (MerckMillipore) and Millicell HY 3-layer T600 cell culture flask (MerckMillipore)

2.3. Maintenance and differentiation of iPSCs to HLCs

2.3.1.   Tissue culture incubator conditions

1. For maintenance of hiPSC colonies: Normoxic tissue culture incubator- 5% CO_2, 37°C, humidified

2. For hepatic differentiation under hypoxic conditions: Hypoxic tissue culture incubator- 5% CO_2 with variable Oxygen control set to 5%, 37°C, humidified

2.3.2.   Preparation of coated well plates and dishes

1. Vitronectin coated wells for routine iPSC passage and maintenance: Add 500-700μL of Vitronectin XF solution (STEMCELL Technologies), prepared as a 10 μg/mL solution in PBS to the number of wells intended for cell seeding in a 6-well plate. Agitate the plate and ensure the entire surface of the wells are covered and leave for 1 hour at room temperature or seal with parafilm and store at 4°C for up to 2 weeks. Aspirate Vitronectin layer immediately prior to adding passaged hiPSC colonies. Do not wash the well with PBS.

2. Mouse embryonic fibroblast (MEF) media: Advanced DMEM F12 supplemented with 10% FBS, 2mM L-glutamine, 0.1mM β-mercaptoethanol and 100IU penicillin/streptomycin.

3. 0.1% gelatin solution: Dissolve gelatin powder in endotoxin-free H_2O (0.5g in 500mL autoclaved milliQ water is sufficient purity). Either use a stir bar and heat plate to

enable the gelatin to dissolve or autoclave the solution. Allow to cool to room temperature and then filter the solution through a 0.22μm pore.

4. Gelatin coated well plates or 10cm dishes for iPSC differentiation: To coat wells/dishes add a sufficient volume of 0.1% gelatin solution to cover the surface area and incubate at room temperature for 1 hour (6mL for a 10cm dish and 1.5-2mL for a well of a 6 well plate). Aspirate gelatin solution and replace with mouse embryonic fibroblast (MEF) media by pipetting the media gently at the edges of the well/dish. Incubate in the normoxic cell culture incubator overnight to allow the incorporation of proteins into the gelatin. Plates can be kept in the normoxic incubator for up to 2 weeks until required providing there is sufficient media for the plate/wells not to dry out (top up as needed with MEF media). When ready to seed cells aspirate the MEF media immediately prior to addition of the cell suspension. Do not wash the well/plate.

5. Collagen-I coated wells for immature HLC maturation: Prepare a 0.01M acetic acid solution by dilution of acetic acid glacial in PBS. Dilute rat tail collagen Type I solution to a concentration of 0.1mg/mL. Coat wells with a sufficient volume to cover the surface area and agitate as necessary to ensure an even distribution of the collagen layer. Seal the plate with parafilm and leave at 4°C overnight. Wash wells once with PBS prior to cell seeding.

6. For passaging of hiPSC colonies: Gentle Cell Dissociation Reagent (STEMCELL Technologies)

7. For lifting immature HLCs from gelatin coated wells/plates: TrypLE Express (1× solution, ThermoFisher)

2.3.3. Growth factors for differentiation

1. 1% BSA/PBS stock solution: Dissolve 0.5g of BSA in 50mL PBS, this can be aided by leaving on a tube roller for up to an hour. Sterilize using a 0.22μm filter. For a 0.1% BSA/PBS solution dilute 1/10 with sterile PBS. For a 0.01% BSA/PBS solution dilute 1% stock 1/100 with sterile 1X PBS. Aliquots of 1%, 0.1% and 0.01% BSA/PBS can be stored at -20°C.

2. 10μg/mL Oncostatin-M stock solution: Dissolve 50μg in 5mL (10μg in 1mL) 0.01% BSA solution. Prepare 100μL aliquots and store at -20°C.

3. 50mM Ly294002 stock solution: Dissolve 5mg in 325μL DMSO. Prepare 50μL aliquots and store at -20°C.

4. 50μg/mL HGF stock solution: Dissolve 50μg in 1mL 0.01% BSA solution. Prepare 100μL aliquots and store at -20°C.

5. 3mM CHIR99021 stock solution: Dissolve 5mg in 3.32μL DMSO. Prepare 50μL aliquots and store at -20°C.

6. ROCK inhibitor (10mM Y-27632) stock solution: Dissolve 2mg in 600μL BioXtra Water. Prepare 100μL aliquots and store at -20°C.

7. 10μg/mL BMP4 stock solution: Reconstitute 10μg in 1mL 0.1% BSA solution. Prepare 200μL aliquots, and store at -20°C.

8. 100μg/mL Activin A stock solution: Reconstitute 100μg in 1mL 0.1%BSA solution. Prepare 50μL aliquots, and store at -20°C.

9. 100μg/mL FGF2 stock solution: Reconstitute 100μg in 1mL 0.1% BSA solution. Prepare 50μL aliquots, and store at -20°C.

2.3.4. hiPSC and HLC differentiation media

1. For maintenance of hiPSCs prior to differentiation: Essential 8 (E8) media (Gibco)

2. For Day 1(D1) and D2 of differentiation: Essential 6 (E6) media (Gibco)

3. Progenitor cell basal media (D3-D8): RPMI-1640 (Gibco) supplemented with 1% Penstrep, 2% MEM Non-Essential Amino Acids (MEM NEAA, 100×, Gibco) and 1x B27 supplement (50×, Gibco)

4. Hepatocyte maturation media (D9 onwards): Hepato-ZYME serum free media (Gibco) supplemented with 1% Penstrep, 2mM L-Glutamine, 2% MEM NEAA, 2% Chemically Defined Lipid concentrate (Gibco), 1% Insulin-Transferrin-Selenium (ITS) (Gibco), 10ng/mL Oncostatin-M (R&D Systems), 50ng/mL HGF (Peprotech)

2.4. HLC transplantation and *in vivo* imaging

1. *In vivo* imaging substrate (see **Note 1**)

2. For radionuclide imaging: Gamma counter (*e.g.* 1282 Compugamma, LKB-Wallac, Australia), Preclinical NanoSPECT/CT or NanoPET/CT (*e.g.* Mediso Medical Imaging

System, Budapest, Hungary), radionuclide dose calibrator (*e.g.* Capintec), animal heating chamber and heat pad/mat.

3. Surgical kit including: blunt-ended and tissue scissors, serrated and tissue forceps, haemostatic gauze, cotton buds, sutures, sterile PBS, analgesic (*e.g.* carprofen), isofluorane, 29-31 gauge insulin syringes, surgical gloves and gown.

3. Methods

3.1. Preparation of plasmid stocks

3.1.1. Transformation of plasmid DNA into *E.Coli*

1. Equilibrate the water bath to 42°C.

2. Ensure the SOC media is brought to room temperature.

3. Preheat the shaking incubator to 37°C.

4. Thaw one vial of competent stbl3 cells on ice for each plasmid to be transformed

5. Pipette up to 100ng of each diluted plasmid directly into the vial of competent cells and mix by stirring gently with the pipette tip (see **Note 2**). Do not mix cells by pipetting.

6. Incubate the vials of DNA/bacterial mix on ice for 30 minutes.

7. Bring the ice box with the incubating vials to the water bath and heat-shock the bacteria by incubating the vials for 30 seconds at 42°C (without shaking).

8. Immediately place the vials back on ice for 2 minutes.

9. Add 250µL of room temperature SOC medium to each vial.

10. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a shaking/rotating incubator (see **Note 3**).

11. Whilst the vials are shaking, incubate the antibiotic selection plates at 37°C for 1 hour slightly open in the bacterial incubator.

12. Spread 10-100μL from each transformation vial onto LB plates containing their appropriate selection antibiotic using a sterile bacterial spreader (see **Note 4**).

13. Incubate selection plates for 12-20 hours at 37°C in a bacterial incubator.

14. Inspect the plates the next day and identify 1-3 well isolated colonies suitable for inoculating liquid cultures.

15. Add the appropriate selection antibiotic to room temperature LB.

16. For each plasmid maxiprep to be undertaken transfer 300mL of the antibiotic inoculated LB to an autoclaved flat-bottomed 500mL conical flask (see **Note 5**)

17. Pick colonies and transfer to prepared flasks of LB selection media.

18. Label flasks with the strain of bacteria, date and plasmid identifier and seal loosely with a foam plug/foil.

19. Transfer conical flasks to the pre-heated shaking/rotating incubator and shake at 225rpm, 37°C for 16-20 hours.

3.1.2. Isolation of plasmid DNA from liquid cultures

1. Prepare a glycerol stock of successfully grown bacterial cultures by mixing 250μL glycerol stock solution 1:1 with the bacterial culture in a cryovial.

2. Label the vial and store at -80°C for use in future preps.

3. Transfer the remaining liquid broth to sterile 50mL falcon tubes and centrifuge at 6000g, 4°C for 15 mins (see **Note 6**).

4. Discard supernatant and store bacterial pellets on ice (or freeze at -20°C until ready for isolation).

5. Isolate DNA from the bacterial pellets using the maxi prep column purification kit and dissolve the DNA pellet in DNAse, RNAse, endotoxin free H$_2$O.

3.2. Lentiviral particle production

A schematic demonstrating the steps in seeding multi-layered tissue culture flasks, alongside a flowchart summary of the steps for lentiviral particle production are depicted in **Figure 2**.

3.2.1. HEK293T multi-layer flask preparation for lentiviral particle production

Subculture enough cells for the intended size of lentivirus in advance. If producing a large batch of lentivirus (*e.g.* in multiple T1000 multilayer flasks) then 293T cells will need to be expanded notably in advance. If incubator space is at a premium this can be achieved by expanding cells using T600 (3-layer) flasks (see **Note 7**).

1. Prepare a 293T cell suspension according to the cell number and culture media volumes in Table 1, and pipette into the multi-layered flask. Avoid foaming/bubbles by directing the liquid stream along the inner surface of the flask.

2. After adding the cell suspension ensure medium is evenly distributed between layers by positioning the multi-layered flask vertically upon a flat surface (**Figure 2A.1**). To redistribute the suspension more evenly first position the flask with the top layer facing towards the user (**Figure 2A.2**), then tilt the flask clockwise 45º to divide the liquid evenly between each of the layers (this position can also be maintained for transporting the flask to the incubator, **Figure 2A.3**).

3. While maintaining the tilted position, carefully lay the flask flat (on a shelf of the incubator, **Figure 2A.4**). Then gently agitate the flask back and forth and left-to-right to evenly spread cells across the surfaces of each layer, whilst avoiding spilling media between layers. If seeding multiple multilayer flasks, the agitation step can be performed once flasks are stacked. Incubate at 37°C, 5% CO_2 for ~20 hours.

3.2.2. Lentiviral particle production from pre-seeded multi-layer flasks by PEI transfection

1. Prior to transfection bring all reagents to room temperature. In a sterile tube dilute total plasmid DNA (µg) in serum-free (SF) DMEM (volume of SF-media is 10% of final volume in culture vessel) using constructs at a 4:3:1 ratio of transgene (hNIS-mGFP or similar): viral packaging (pΔ8.91): viral envelope (pVSV-G), as shown in Table 2 (see **Note 8**).

2. Add PEI to the diluted DNA and mix immediately by vortexing or pipetting. The volume of PEI used is based on a 3:1 ratio of PEI (μg):total DNA (μg), see Table 2 for calculations for each culture vessel size.

3. Incubate DNA/PEI solution for 15 minutes at room temperature, then add DNA: PEI complex to complete media (see **Note 9** and **10**).

4. Aspirate media from pre-seeded HEK293T flasks and subsequently add the media/DNA/PEI solution being careful not to dislodge the cells.

5. Return the flask to the incubator and incubate at 37°C, 5% CO_2 for 24 hours.

6. After 24-hours aspirate transfection media and replace with the volume of complete culture media indicated in Table 1 (see **Note 11**).

7. 24-hours after medium change collect the crude viral supernatant. Filter viral media through a 0.45μm filter to remove dead cells and debris.

8. To concentrate the viral particles centrifuge filtered supernatant at 10,000g, 6°C for 16 hours (see **Note 12**).

9. Discard the supernatant and carefully resuspend the pellet in PBS by gentle pipetting, OptiMEM or complete media of the cell type intended for transduction downstream (see **Note 13**). Resuspension volume can be 1/10 of crude viral media volume for a 10× concentrated solution or 1/100 for a 100× concentrated solution.

10. Aliquot the resuspended viral particles in cryovials and freeze at -80°C. Multiple freeze thaw cycles should be avoided as the titre reduces 10-15% percent each time, aliquot volumes should be made up accounting for this.

3.2.3. Quantification of viral titre

1. Seed $2×10^5$ HepG2 or 293T cells per well in a 24-well plate and leave to adhere overnight (see **Note 14**).

2. 16-18 hours after cell seeding serially dilute an aliquot of the concentrated virus in culture media using an appropriate dilution range to a final volume of 200μL per dilution (*e.g.* 1/10, 1/50, 1/100, 1/500, 1/1000, 1/5000, 1/10000 would capture the requisite information of a 100× concentrated stock).

3. Aspirate media from the seeded cells, wash each well once with PBS and add diluted viral media to each well.

4. 24h post-transduction using complete media top up the volume in each well to 500μL.

5. 48-72h post infection discard all viral supernatant according to local rules, wash wells three times with PBS and lift and collect cells from each dilution. Resuspend cells in FACS buffer and add an appropriate live dead stain (*e.g.* DAPI or Hoechst).

6. Acquire live cells on a flow cytometer to assess the proportion of cells successfully transduced (here, hNIS-mGFP).

7. Calculate viral titre by the following equation:

$$Titre\ (Transducing\ Units/mL) = \{(F \times Cn)/V\} \times DF$$

wherein F = GFP$^+$ cell frequency as determined by flow cytometry (or immunofluorescence), Cn = seeded cell number, V = viral inoculum volume and DF = dilution factor. Results of viral dilutions yielding GFP expression in the linear range (1-20%) should be averaged to calculate the titre.

3.3. Maintenance and passaging of hiPSCs

1. Aspirate spent media.

2. Wash cells once with 2mL sterile PBS.

3. Add 500μL Gentle Cell Dissociation Reagent per well and transfer to cell culture incubator (5% CO_2, 37ºC) for 3 minutes.

4. Aspirate Gentle Cell solution (see **Note 15**).

5. Pipette 1mL E8 media directly onto colonies using a P1000 pipette, the force of the media ejection will aid colonies to lift off the well in small clumps (see **Note 16**).

6. Collect the media from step 5 in a sterile 15mL falcon tube and repeat, directing the stream at remaining colonies, particularly large colonies.

7. Repeat step 6, being sure to rinse the whole surface of the well (see **Note 16**)

8. If reseeding for hiPSC line maintenance, then plating smaller colonies is desired. Smaller colonies can be produced during dissociation by pipetting mediated mechanical disruption. Larger colonies will sink to the bottom of the tube, collect this in the pipette tip and pipette back into the tube between 2-6 times depending on the size of the clumps.

9. Reseed colony suspension at a dilution between 1/10 and 1/40 in a fresh vitronectin coated well of a 6-well well plate, top up with E8 to a final volume of 1.5mL. Return plated cells to the cell culture incubator and agitate the plate to ensure the colonies are evenly distributed across the well.

10. Refresh E8 medium daily (see **Note 17** and **18**).

11. If reseeding onto gelatin plates in preparation for differentiation, then larger colonies than those used for maintenance are desired (see **Figure 3**). If very large colonies are observed, we recommend disaggregating them mechanically as these will not differentiate efficiently otherwise. Depending on confluence of the well at passaging, one well of a 6 well plate is sufficient for approximately two 10cm dishes (or 6 wells of a 6-well plate).

12. hiPSCs seeded onto gelatin plates for differentiation should be placed in a hypoxic cell culture incubator (5% (v/v) O_2, 5% (v/v) CO_2, 37ºC, humidified) and allowed to acclimatise to the new matrix for 2-3 days with E8 media refreshed daily prior to induction of differentiation.

3.4. Differentiation of hiPSCs to HLCs and lentiviral transduction for *in vivo* tracking

Micrographs demonstrating the expected changes in the cell morphology and confluence of the culture dish for each step of differentiation are shown in **Figure 3** as a guide.

3.4.1. Induction of endodermal differentiation

1. Prepare day 1 differentiation media by addition of Activin A, BMP4, CHIR99021, FGF2 and LY294002 to E6 media to the final concentrations indicated in Table 3 (see **Note 19**).

2. Aspirate E8 media, replenish with day 1 media, being careful to add the media slowly to the edge of the well/dish to ensure no holes are made in the gelatin layer and the colonies are not disturbed. Return the dish/plate to the hypoxic incubator.

3. 24 hours later, prepare day 2 differentiation media by addition of Activin A, BMP4, FGF2 and LY294002 to E6 media to the final concentrations indicated in Table 3.

4. Observe the culture dish down the microscope, cells should be visibly expanding from the colonies.

5. Aspirate day 1 media, replenish with day 2 media, adding media slowly to the edge of the well/dish. Return the dish/plate to the hypoxic incubator.

3.4.2. Hepatic progenitor cell differentiation

1. On day 3 of differentiation prepare a stock of day 3-8 progenitor cell basal media.

2. Prepare day 3 differentiation media by adding Activin A and FGF2 to an aliquot of the progenitor cell basal media to the final concentrations indicated in Table 3.

3. Store remaining progenitor cell basal media at 4°C.

4. Aspirate day 2 media, replenish with day 3 media, adding media slowly to the edge of the well/dish. Return the dish/plate to the hypoxic incubator (see **Note 20**).

5. 24 hours later prepare differentiation media by bringing an aliquot of progenitor cell basal media to room temperature and adding Activin A to a final concentration of 50ng/mL.

6. Aspirate media and replenish with differentiation media prepared in step 5, adding media slowly to the edge of the well/dish. Return the dish/plate to the hypoxic incubator.

7. Repeat steps 5 and 6 for days 5-8 (see **Note 21**).

3.4.3. Maturation of hepatic progenitors

1. On day 9 of differentiation prepare a stock of hepatic maturation media by supplementing Hepato-ZYME SFM as indicated in Table 3. Store at 4°C.

2. Bring fully supplemented hepatic maturation media to room temperature, do not warm prepared media in a water bath at 37°C as this will degrade the supplements before the HLCs can benefit from them.

3. Aspirate day 8 media and replenish with fully supplemented hepatic maturation media, adding media slowly to the edge of the well/dish. Return the dish/plate to the hypoxic incubator.

4. Replenish hepatic maturation media every other day until day 18 of differentiation when a characteristic polyhedral morphology should be visible in the culture monolayer under the microscope (**Figure 3**, see **Note 22**).

3.4.5. Lentiviral transduction of HLCs

1. Ensure to keep aside at least one dish of cells as a non-transduced control.

2. Calculate the total volume of concentrated viral stock solution needed for an MOI of 5-10 for the number of dishes/wells intended for transduction using the equation:

   Cell number x MOI (5) = pfu (or TU/mL) needed (see **Note 23**).

3. Thaw the viral stock solution on wet ice.

4. Dilute thawed viral particles into a sufficient volume of hepatocyte maturation media for the number of dishes intended for transduction (3 mL viral media per 10 cm dish). Keep viral-media solution room temperature to avoid cold shock to the cells.

5. Aspirate spent media from HLCs.

6. Wash cells twice with PBS and aspirate washes.

7. Add viral media dropwise to the dish(es) ensuring the whole surface is covered.

8. Gently swirl the dish to ensure an even viral media layer covers the cells.

9. Leave the dish(es) to incubate at room temperature in the tissue culture hood for 15 min.

10. Add 1.5mL complete hepatocyte maturation media to each dish and transfer cells to the hypoxic incubator to incubate overnight.

11. 16-24 hours later top up dishes with 1.5mL complete hepatocyte maturation media and return cells to the hypoxic cell culture incubator.

12. 48 hours after viral transduction, check cells under a fluorescence microscope by comparing them to untransduced control cells to confirm success of transgene expression.

13. Cells can now either be a) pre-labelled with radiotracer prior to lifting from the culture dish and transplanting *in vivo* for immediate *in vivo* imaging (see 0 and 3.5),

b) passaged for further *in vitro* maturation either by seeding onto collagen-I coated dishes or within scaffolds or as part of a co-culture for later transplant or c) transplanted *in vivo*, for subsequent *in vivo* imaging facilitated by radiotracer administration i/v to the animal, post cell transplant (see 3.6.1).

3.4.6. Passaging of immature transgenic HLCs

1. Wash dishes twice with PBS and aspirate.

2. Add 6mL/dish of 1x TrypLE Express and incubate the plate at 37ºC for up to 20 minutes.

3. Check the dish under a bright field/phase contrast microscope, cells should be visibly lifting off the plate. Tap the plate gently if needed to encourage lifting.

4. Collect the cell suspension in a sterile 50mL tube and using a 10mL pipette, pipette up and down 3× to dissociate any clumps of cells.

5. Add an equal volume of Hepato-ZYME to the tube to quench the reaction.

6. Pass the re-suspended cells through a 70µm cell strainer into a fresh sterile 50mL tube.

7. Centrifuge the cells for 3 minutes at 300×g**,** 4ºC.

8. Aspirate the supernatant being careful not to disturb the pellet.

9. Resuspend the cells in complete hepatocyte maturation media and count.

10. Cells can now be analysed for transduction efficiency by flow cytometry, transplanted *in vivo* or reseeded 3.5×10^5 cells per well on collagen-I coated 12-well plates for further maturation (typically up to day 35) (see **Note 24**).

3.5. Radiolabelling traceable HLCs *in vitro* for *in vivo* transplantation

3.5.1. Testing reporter gene function of HLCs *in vitro*

1. Prepare a radiotracer stock solution of 100kBq/mL $[^{99m}Tc]TcO_4^-$ and note the time.

2. Aspirate media from HLCs.

3. Wash cells with PBS^{++} (6mL/10cm dish) and aspirate.

4. Add 3mL radiotracer stock solution per dish of hNIS-mGFP$^+$ HLCs or untransduced control HLCs and incubate plates in the hypoxic incubator for 30mins.

5. Collect supernatant containing residual radioactivity into a 50mL falcon tube and label for γ-counting later.

6. Wash dishes twice with 5mL PBS, collecting each washout in a labelled tube for γ-counting later.

7. Passage cells from the dish as described in 0, collecting all washouts and count the cells with a haemocytometer.

8. Centrifuge radiolabelled cell suspension at 300×g, 3 minutes, 4°C.

9. Collect supernatant in a labelled tube and resuspend HLCs for transplant in sterile PBS, store on ice until required. Once transplanted into mice proceed to 0 (skip radiotracer administration steps 5 and 6).

3.5.2. Calculating radiotracer uptake in pre-labelled/transplanted HLCs

1. Make up a spare stock of radiolabelled HLCs in a 1.5mL from cells not intended for transplantation. Note cell number and use as a non-transplanted imaging phantom and γ-counter cell control.

2. Use radiotracer stock solution to prepare calibration standards (*e.g.* of 0, 1, 3, 10, 30 and 70kBq if using $[^{99m}Tc]TcO_4^-$) in labelled 1.5mL tubes.

3. γ-count cell stock and collected washouts and supernatants from *in vitro* labelling steps.

4. Quantify uptake of radiotracer in cells by γ-counting collected supernatant, washouts and cells using the following equation (see **Note 25**):

Cell uptake (% total radioactivity measured) =

$$\left[\frac{activity\ in\ collected\ cells\ (CPM)}{\begin{pmatrix} activity\ in\ collected\ cells\ (CPM) + activity\ in\ PBS\ wash\ 1\ (CPM) + \\ activity\ in\ PBS\ wash\ 2\ (PM) + activity\ in\ washouts\ from\ cell\ passaging\ (CPM) \\ + activity\ in\ collected\ supernatants\ (CPM) \end{pmatrix}} \right] x\ 100$$

5. Normalise results to dissociated cell number per dish to account for variations between dishes/batches of differentiation in future experiments as shown in **Figure 4**.

3.6. Tracking transplanted HLCs *in vivo* by nanoSPECT/CT imaging

In vivo imaging and *ex vivo* organ biodistribution analyses of transgene expressing HLCs should always be compared to either sham mice or mice transplanted with untransduced HLCs to ensure detected signals can clearly be attributed to the transplanted cell population. For details relating to achievable contrast by radionuclide reporter gene imaging, we refer to *(27, 29)*. We further recommend validation of the radionuclide reporter gene function prior to using the cells *in vivo* (see *in vitro* radiotracer uptake assay in 3.5.1 and quantification in 3.5.2). To monitor HLC biodistribution immediately after administration, they can also be pre-labelled with radiotracers similar to the functional reporter assay in 3.5.1, whereby tracking information depends on the radioisotope half-life and the radiotracer efflux kinetics from the cells (see example in *(26)*). **Figure 5** shows hNIS-mGFP[+] HLCs that were intrahepatically administered and imaged after 24h following systemic injection of the hNIS radiotracer [^{99m}Tc]TcO$_4^-$; corresponding protocol steps are 3.6.1, 3.6.2 and 3.6.3. A sham

mouse which has not been transplanted with transgenic HLCs is used as a control to indicate organs with endogenous radiotracer uptake, and demonstrating that background-free liver imaging is feasible.

3.6.1 Intraliver HLC transplantation

The surgical procedures detailed here involve HLC injection directly into the left liver lobe following its externalisation from the abdominal cavity; here, this choice was based on similar procedures from the liver cancer arena in which this procedures is one option to establish tumours preclinically (*e.g.* see *(44)*). This model is advantageous for downstream analyses as in addition to examining the whole liver to corroborate *in vivo* imaging results by *ex vivo* biodistribution analyses (3.6.4), the injected lobe can be easily examined histologically for cell survival in the first instance. Many other procedures for hepatocyte/HLC transplantation are also appropriate *e.g.* transplantation: on the surface of the liver/under the liver capsule *(45, 46)*, via intrasplenic *(47, 48)* or intraportal injection *(49, 50)* or ectopically such as under the kidney capsule or at mesenteric sites *(51)*. Selecting the appropriate transplantation route will depend on the strain of the mouse, whether liver injury has been induced prior to cell transplant and the tracking goals and experimental expectations with respect to cell survival/engraftment/expansion.

3.6.1.1 Preparation

1. Autoclave the surgery kit and have sterile PBS or saline prepared.

2. Set up the bio-safety class I cabinet in readiness for performing surgery by sterilising all surfaces with 70% ethanol and arranging the surgical area with the heat pad and anaesthetic tubing/nose cone orientated in a comfortable position. All surgical tools should be placed on sterilised surfaces (*e.g.* autoclaved metal trays). Perform all

procedures within the cabinet, especially where immunocompromised mice such as NSGs are used.

3. Weigh mice prior to the surgery. Those weighing ≥20g and at least 12 weeks old are suitable and will usually recover well from the prolonged period of anaesthesia required for the combined processes of surgery and *in vivo* radionlucide imaging.

4. Closely shave the mouse abdominal region with clippers removing all hair from the region to undergo surgery (ideally perform this step away from the surgical area).

5. Transfer the shaved mouse to an induction chamber and anaesthetise with isoflurane in O_2 initially at 3% (v/v) with a flow rate of 1.0-1.5 L/min before reducing to 1.5-2.0% once the mouse is induced.

6. Monitor for pedal reflex absence to confirm sufficient anaesthesia and adjust if necessary.

7. Transfer the anaesthetised mouse to the surgery heating pad within the biosafety cabinet, maintaining anaesthesia with a nose cone supply and position the mouse so the ventral portion is facing up.

8. Apply Lacrilube liberally to animal eyes to prevent damage or dryness during anaesthesia.

9. Sterilise the exposed abdominal skin by wiping with 70% ethanol-soaked sterile cotton swabs and disinfect with an antimicrobial surgical scrub.

10. Inject an appropriate analgesic (*e.g.* carprofen) subcutaneously.

11. Position surgical drapes across the operating region of the abdomen and cut an appropriate window to expose the surgical area.

12. If necessary, resuspend your cell suspension at this point to your desired concentration in 1.5mL tubes, ensuring the final volume of cells and PBS is <50µl and draw entire volume into a 29-gauge insulin syringe (see **Note 26**).

3.6.1.2 Surgery and cell injection

1. Grasp and elevate the skin just below the sternum and parallel to the rib cage with forceps, then using straight surgical scissors cut a transverse incision of roughly 1cm.

2. In the incised region carefully separate the skin from the abdominal muscle layer with blunt-ended scissors. If necessary, moisten the exposed intradermal region with sterile cotton buds soaked in PBS to avoid tissues becoming dry.

3. Using tissue forceps, elevate the exposed peritoneal layer and just below the xiphoid make a transverse incision through the peritoneal layer.

4. To exteriorise the left liver lobe, use two PBS moistened cotton buds placed at the abdominal and diaphragm sides of the incision and gently apply sliding pressure to lift the lobe out through the incision (see **Note 27**).

5. Insert the needle of the syringe into the lobe and slowly depress the piston of the syringe, gradually injecting the entire cell suspension volume into the exposed liver lobe whilst keeping the syringe stable throughout.

6. Gently press sterile PBS-moistened cotton buds to the site of injection and remove the needle slowly from the liver lobe to avoid bleeding or cell loss, whilst maintaining the pressure from the buds throughout. If bleeding occurs apply a small tab of absorbable haemostatic gauze to the site of bleeding and continue to apply pressure with the cotton buds until the bleeding ceases.

7. Using sterile PBS-moistened cotton buds, press the liver lobe back into the peritoneal cavity.

8. Close the peritoneal incision using continuous 5-0 sutures and moisten the peritoneal layer with PBS.

9. Close the skin wound using interrupted 5-0 sutures (see **Note 28**).

10. Administer 500µl of sterile PBS by subcutaneous injection to prevent dehydration.

3.6.2 Animal preparation for radionuclide imaging and radiotracer administration

In instances of repeat imaging (*i.e.* where not imaging immediately following surgery as outlined in 3.6.1) first perform steps 1-5. Where the mouse is already anaesthetised and you are imaging/administering radiotracer directly following surgery and cell transplantation skip to step 6.

1. Prewarm mice in a heating chamber for 20 minutes.

2. Transfer mice to an induction chamber and anaesthetise with isoflurane in O_2 at 1.5-2.0 % (v/v) with a flow rate of 1.0-1.5 L/min.

3. Monitor for pedal reflex absence to confirm sufficient anaesthesia.

4. Transfer the anaesthetised mouse to a heating pad, maintaining anaesthesia with a nose cone supply (see **Note 29**).

5. Apply Lacrilube liberally to animal eyes to prevent dryness during anaesthesia.

6. Dilute $[^{99m}Tc]TcO_4^-$ in sterile saline to produce a stock with 30MBq/100µl.

7. Draw 100µL of the $[^{99m}Tc]TcO_4^-$ solution into a 29-31 gauge syringe, being careful to ensure there are no air bubbles.

8. Measure the radioactivity in the syringe using a γ-counter, record the activity and time.

9. Administer all 100µl of the $[^{99m}Tc]TcO_4^-$ solution into the pre-warmed tail vein.

10. Measure residual radioactivity in the syringe using the γ-counter, record the activity and time. The difference between the syringe radioactivity values before and after intravenous injection is the injected dose (ID).

11. Set a timer for 45-minutes (see **Note 30**).

3.6.3 Imaging by nanoSPECT/CT

1. Ensure nanoSPECT/CT scanner is equipped with 1mm collimators and has been recently calibrated.

2. Pre-warm scanner mouse bed and switch on anaesthetic and O_2 supply.

3. Transfer the mouse to the nanoSPECT/CT bed, confirm anaesthetic supply and maintained depth by absence of pedal reflex.

4. Position the mouse prone and setup animal monitoring devices (*e.g.* temperature and breathing probes).

5. 30-minutes after tracer administration perform CT scan using image acquisition settings of 55 kVp tube voltage, 1200 ms exposure time in 360 projections, ensuring the whole body of the mouse is captured within the imaging window.

6. 45-mins after tracer administration commence SPECT scan with a 30min acquisition time (see **Note 31**).

7. Following image acquisition and reconstruction either a) Transfer animals back to the warming chamber with water and food available *ad libitum* and monitor to ensure the animal regains full consciousness and recovers fully from anaesthesia. Subsequently transfer them to a maintenance unit until future imaging sessions, or b) if this is the terminal imaging session, note the time and proceed to animal euthanasia and dissection for *ex vivo* biodistribution analyses (see 3.6.4).

3.6.4 *Ex vivo* confirmation of *in vivo* tracking data

1. Transfer the euthanised mouse to a 50mL tube and measure total activity in the carcass using a γ-counter. Record the value and time.

2. Perform cardiac puncture and collect blood into a pre-weighed 1.5mL tube.

3. Perform dissection collecting the following organs into pre-weighed tubes: liver, stomach, spleen, kidneys, lungs, heart, small and large intestines as well as thyroid and salivary glands combined.

4. Transfer remaining carcass back to the 50mL tube, remove the tail and measure the remaining activity using the γ-counter. Record the value and time.

5. Measure the radioactivity in the tail using the γ-counter. Record the value and time (see **Note 32**).

6. Weigh all collected organs and blood and note details in a spreadsheet/table.

7. Immerse organs in fixative of choice for downstream histological analyses (*e.g.* optimal cutting temperature medium for cryo-cutting, or formalin for subsequent paraffin-embedding etc).

8. Prepare radiotracer calibration standards of 0, 1, 3, 10, 30 and 70kBq from residual radiotracer stock.

9. Use a γ-counter to measure the radioactivity of harvested organs alongside the calibration standards. Record the time of measurement (see **Note 33**).

10. Present data as standard uptake value (SUV) or %ID/g (see **Note 34**).

11. Dispense with all collected organs not required for downstream analyses following local radioactive waste management rules.

4. Notes

1. Any radionuclide reporter gene requires a matching radiotracer. For hNIS-mGFP, suitable matching radiotracers include Tc-99m-pertechnetate ($[^{99m}Tc]TcO_4^-$) for

SPECT/CT imaging or $[^{18}F]BF_4^-$ for PET/CT imaging. $[^{99m}Tc]TcO_4^-$ is usually generator-produced *(52)* and frequently available from hospital radiopharmacies. $[^{18}F]BF_4^-$ requires cyclotron-produced ^{18}F and can be synthesized at specific activity by the fluorine exchange method *(35, 53)*. Radiotracers should ideally be used within two half-lives.

2. The volume of the plasmid solution must not exceed 1/10 of the volume of the bacteria suspension.

3. We recommend 45-60min for vectors carrying the kanamycin resistance marker, while 15-30min is sufficient for those with the ampicillin resistance marker.

4. The volume to add to the plate depends on expected efficiency of transformation, the goal is to have well isolated colonies the next day (rather than a 'lawn' of bacteria or very few rare colonies) that can be easily picked. If one is uncertain, multiple plates can be used and different volumes of the incubated media spread on each plate.

5. For plasmids with only a few colonies successfully grown a 'starter culture' can be performed ahead of this step to increase the chances of successful liquid culture growth. Transfer the picked colony to 5-10mL of antibiotic-containing LB in a 50mL falcon tube and shake for 8-16 hours at 225rpm, 37°C with the lid of the tube loosened to enable sufficient aeration. Once this 'day culture' becomes cloudy indicating successful bacterial growth, transfer an aliquot to a larger culture vessel containing more fresh selection media and incubate overnight ('overnight culture'; max dilution 1/300 recommended).

6. Ensure to not overload the columns for DNA isolation by adhering to the manufacturers' recommendations, whereby 100mL of a culture with OD_{600}~3 is a good starting point.

7. In general, aim for between $^1/_4$ to $^1/_3$ confluence of the 293T cells at the point of plasmid transfection to allow sufficient space for expansion. Do not use HEK293T cells beyond passage 15 and avoid growing to confluency during general maintenance.

8. Batch-to-batch variation when preparing the PEI working stock can occur. When the transgene plasmid encodes a fluorescent protein one can optimise the ratio of DNA:PEI by transfecting HEK293T cells with a range of ratios and monitoring the percentage of fluorescent positive cells 48-hours post-transfection by fluorescence microscopy or flow cytometry.

9. HEK293T transfection using calcium phosphate ($CaPO_3$) is another common approach for production of lentiviral particles, however direct comparisons in our hands demonstrated linear PEI yields higher titres and it is a simpler method for lentivirus production.

10. The medium for transfection or when the media is changed 24-hours post-transfection must not be cold as this will cause thermal shock and the HEK293T cells will shrink and detach. Medium must also be added slowly and gently. Care should be taken to ensure that the flask is sitting perfectly flat in the incubator with a layer of media across each of the cell layers.

11. Whilst it may seem counterintuitive to change the media after 24h, as some viral particles could have already been produced by the HEK293T cells in this time, as the cells are still proliferating this media change supports their continued growth, minimises the number of dead cells, and cell waste products in the media which ultimately leads to a higher titre.

12. Depending on the centrifuges available, either autoclaved 450mL centrifuge buckets for large batches of lentivirus or 50mL ultracentrifuge tubes can be used, ideally with a swinging bucket rotor for maximal particle recovery.

13. Unlike with centrifugation of bacteria or mammalian cells, a pellet of lentiviral particles may not be (easily) visible by eye, especially if producing small batches. If a pellet is not observed, as long as the medium was removed carefully, resuspend as planned and test the obtained concentrated particles by transduction of cells.

14. Viral titre is commonly estimated by transducing HEK293T cells (or other easy to transduce cells such as HT1080) with serial dilutions of viral stock. However, HEK293T cells are one of the easiest immortalised cell lines to transduce and therefore often this practice is not a good estimate when comparing to intended target cells. Here, we recommend using the hepatic cell line HepG2 for titration of viral stocks intended for HLC transduction as this gives a more representative titre without the costly process of differentiating HLCs solely for the purposes of titration. The most important factor when selecting a cell type for titrating virus is to be consistent so that downstream experiments remain comparable. It is noteworthy, that alternative methods exist, which do not rely on actual infection of target cells but instead count viral particles or antigens expressed on viral particles (*e.g.* HIV-1 p24 determination by ELISA). The latter methods are not recommended for the purpose of this protocol.

15. At this point individual cells within colonies may be more easily discerned under a microscope and the edges of the colonies have begun to shrink or lift from the well surface. Some cells may be aspirated with the gentle cell solution during this step, but these will be differentiated cells from colony edges that release easily.

16. All colonies should have been lifted from the well by this point, if one finds colonies are hard to lift during this step an additional PBS wash step can be added before

addition of gentle cell solution. Incubation time with gentle cell solution can be increased, by up to 30 seconds to 1 minute, too.

17. Once a week a double feed can be performed (where twice the usual volume of E8 media is added and no media change occurs the next day), however, this should be done within a day or two of passaging before the colonies have expanded too much as the waste products in the media are likely to cause spontaneous differentiation, and more of these will be released by a greater number of cells as the colonies begin to expand. For best results daily medium replenishment is advised.

18. hiPSCs should be passaged as whole colonies approximately every 4 days (based on colony size and morphology). Aim to passage before cells begin to differentiate once they begin to lose their tightly packed morphology or when colonies start to merge and fill the whole well. Thawed hiPSCs should be maintained for at least 2 weeks and undergo 3 passages before differentiation to produce the most mature HLCs.

19. Media should be prepared fresh. Thawed growth factors and small molecules can be stored at 4°C for up to one month but for best results aliquots that can be used within 1 to 2 weeks after thawing are advised.

20. Tissue hypoxia plays an important role in the regulation of (normal) mammalian embryogenesis and stem cell differentiation and studies in vertebrates have shown that the developing liver bud expresses hypoxia inducible factors prior to foetal circulation initiation *(54)*, suggesting transient exposure to a hypoxic environment is important in early hepatoblast expansion and delamination. Hypoxic culture conditions have been shown to enhance expression of endodermal and hepatic differentiation from PSCs *in vitro* leading to an improved hepatic phenotype and function*(55, 56)*. It may not be critical to maintain differentiation in a hypoxic incubator beyond the progenitor cell stage, but this should be individually tested for

any hiPSC lines; in particular, whether transfer to normoxia impacts subsequent HLC maturity.

21. Cells will be undergoing massive proliferation during this stage of differentiation and should expand to fill the entire culture dish with a confluent layer of cells (see **Figure 3**). It is normal due to the high levels of proliferation during these steps to observe under the microscope and aspirate many dead/floating cells with each media change.

22. Speed of differentiation and morphologies may vary depending on the hiPSC line used. Some hiPSC lines are inherently better at differentiating towards the hepatic lineage and thus a more pronounced polyhedral morphology will be seen in the culture dish *(57, 58)*.

23. As a guide, with the line in this protocol, an average of 2×10^6 cells per 10cm diameter dish can be expected.

24. Optimal cell seeding density per well and the day of peak HLC maturity may vary between hiPSC lines and should be optimised for any new lines.

25. If multiple 10cm diameter dishes have been labelled, the liquid volume of all washout/passaging steps may be quite large and suitable vessels for the γ-counter may not exist; instead triplicate sample volumes (500μL or 1mL recommended) from each step can be aliquotted and measured in 1.5mL tubes, an average taken from the triplicates and the counts per minute (CPM) from the average value multiplied to reflect the total liquid volume.

26. Ideally you want the HLCs in the syringe for the minimum amount of time prior to transplant to reduce the risk of cells sticking to the walls of the syringe, therefore do not draw the suspension up into the syringe until you are almost ready to transplant. Store on wet ice in 1.5mL tubes in the interim. If surgical complications delay

injection after preparation of the syringe gently continuously rotate the syringe between your thumb and forefinger to reduce cells sticking to the syringe walls until injection can be performed.

27. If your incision is too small to exteriorise the lobe you can stretch it slightly with blunt ended scissors, but try to keep it as small as is practical so that the incision holds the exteriorised lobe in place for cell injection. Similarly, if the incision is too wide suture it slightly closed before continuing, to ensure the lobe can be held exteriorised unaided.

28. Some groups prefer to use metal wound clips to close the skin given this is a speedier and easier method, however these clips will be present in the preclinical CT scan adjacent to the region of interest, thus for the purposes of obtaining clean images it is worth taking the additional time to close the skin layer by suturing.

29. If vessels in the mouse tail are not sufficiently dilated to enable easy intravenous injection, direct an infrared light lamp from safe distance at the mouse to warm the animal tail further and encourage dilation.

30. This period for radiotracer tissue uptake is based on previous studies that have indicated good levels of tracer clearance from circulation within this time frame *(35)*.

31. Longer acquisition times might improve signal-to-background. However, keeping imaging times and therein the times animals are under anaesthesia shorter is preferable; ultimately this is guided by animal welfare considerations and regulations.

32. Residual radioactivity detected in the tail indicates misinjection of radiotracer. It does not contribute to the injected dose and can be subtracted during later analyses. However, misinjections should be avoided.

33. If samples are too hot for accurate detection by the γ-counter, wait for 1-2 half-lives.

34. SUV = [activity (organ)/mass (organ)] ÷ [activity (whole mouse)/mass (whole mouse].

Acknowledgements

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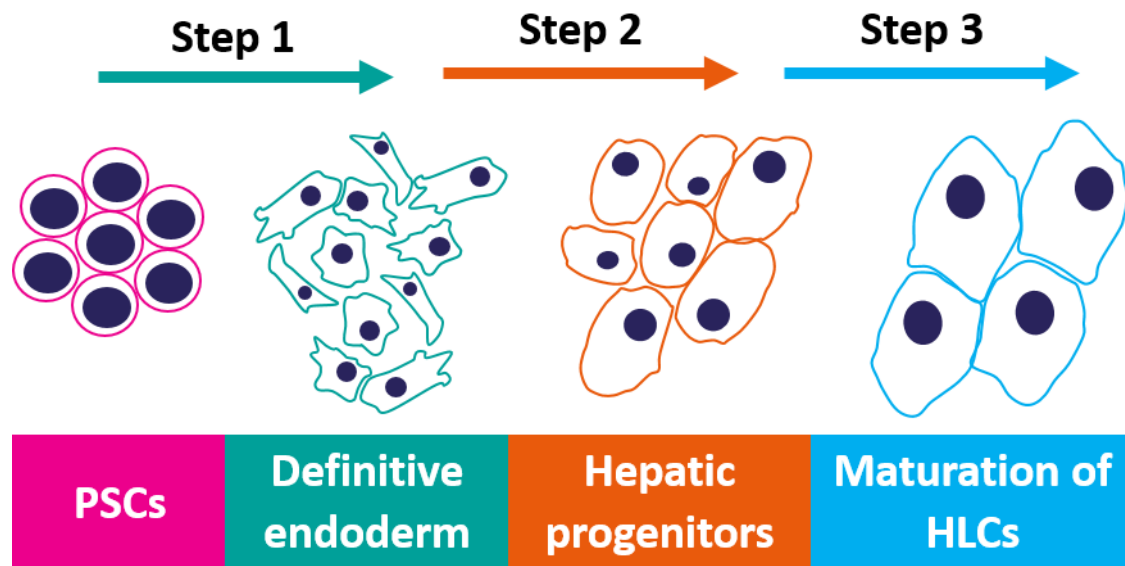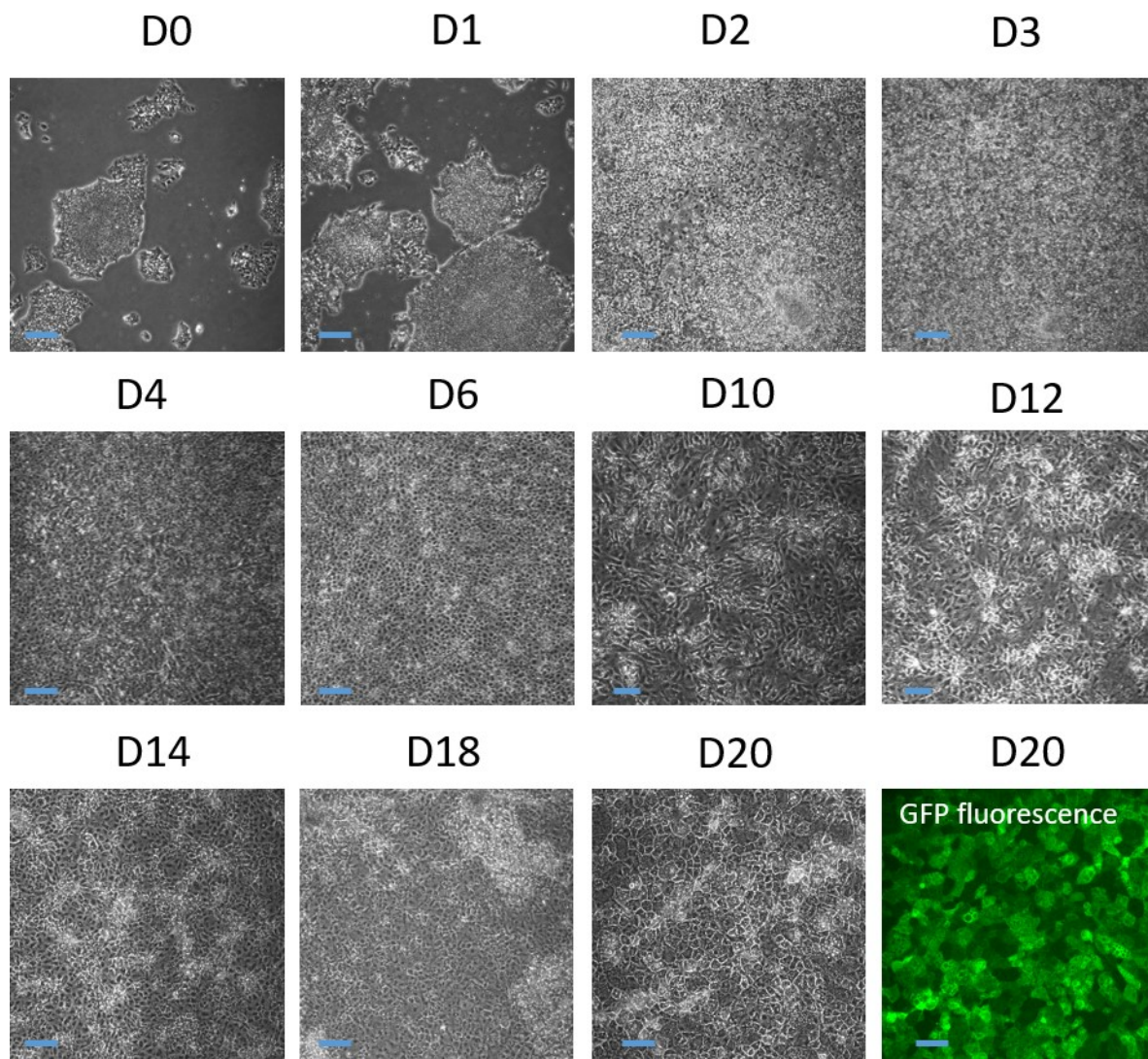
Figures

Figure 1. Scheme depicting the typical 3-step process adopted in protocols differentiating pluripotent stem cells (PSCs) to HLCs. | Associated morphological changes illustrated.
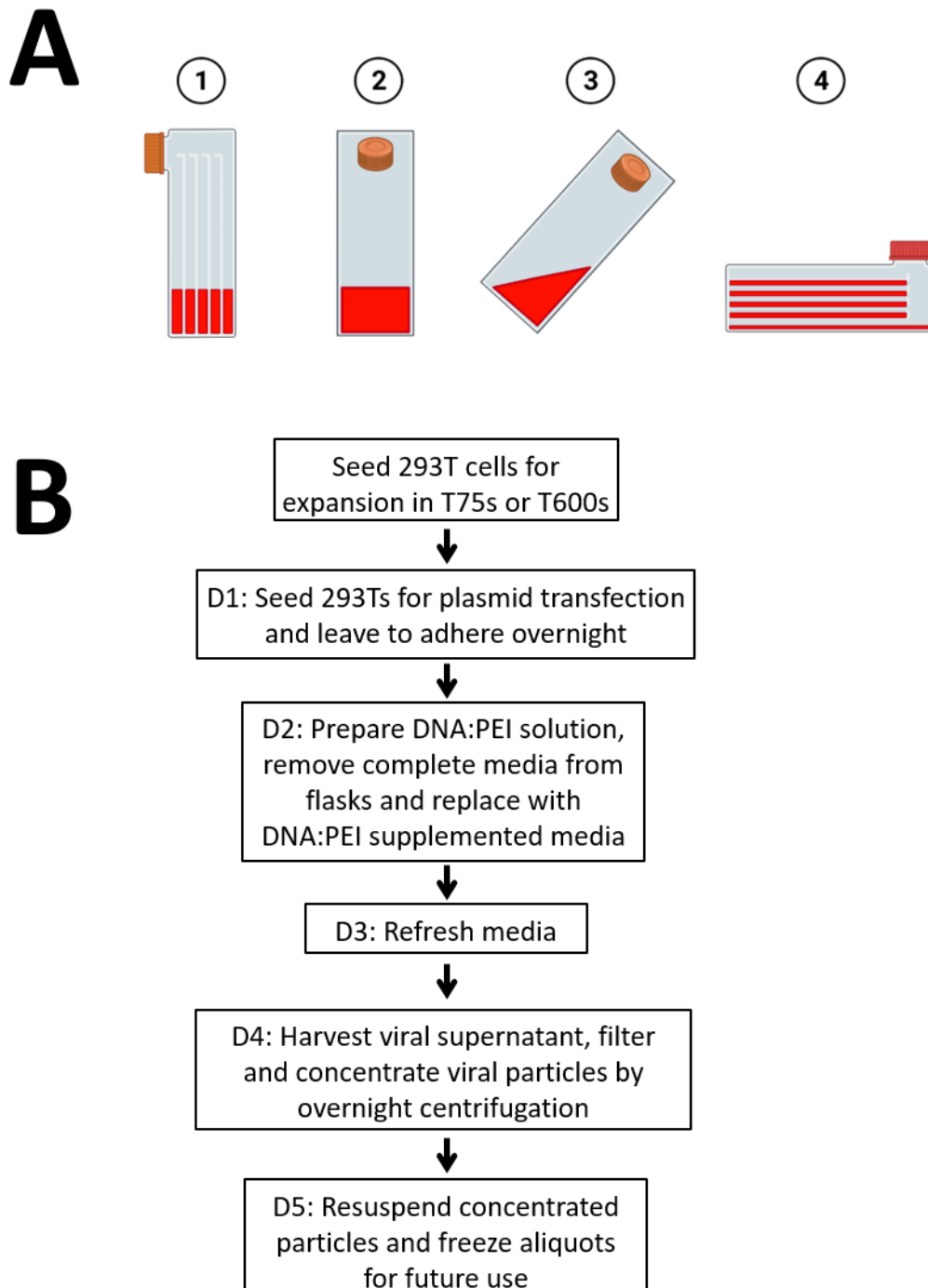
Figure 2. Lentiviral particle production schematics. | (A) Diagram demonstrating the steps (numbered 1 to 4) necessary to evenly distribute media and cell suspensions in multi-layered tissue culture flasks **(B)** Simplified flowchart demonstrating steps in the production of lentiviral particles. Parts of this Figure were produced using biorender.com

Figure 3. Micrographs depicting changes in cell morphology during differentiation of hiPSC colonies to HLCs. | hiPSC colonies are initially tightly packed, displaying a high nuclear-to-cytoplasm ratio and prominent nucleoli (day 0/D0). On D1 and D2, cells expand rapidly from the colonies following induction of endoderm differentiation and on D3 and D4 hepatic endoderm expands rapidly. The high levels of proliferation mean one will find many floating dead cells are removed with each media change; this hepatoblast population will ultimately create a confluent monolayer in the dish but cells will remain small and irregularly shaped at this stage. On D5-D8, hepatic progenitors continue to proliferate with the number of dead/floating cells being removed with each media change gradually reducing as cells

increase in size. On D9-D18 and following the switch to maturation media, proliferation

ceases and a polyhedral monolayer of immature HLCs with high cytoplasm-to-nuclear ratio is

gradually visible. On D20 and following lentiviral transduction, hNIS-mGFP reporter gene

expression can be confirmed by fluorescence microscopy while cells exhibit a polyhedral

morphology indicative of immature hepatocytes suitable for further maturation *in vitro* or *in*

vivo. Scale bars are 100µm (D0-D6 and D14-D20) and 250µm (D10 and 12).
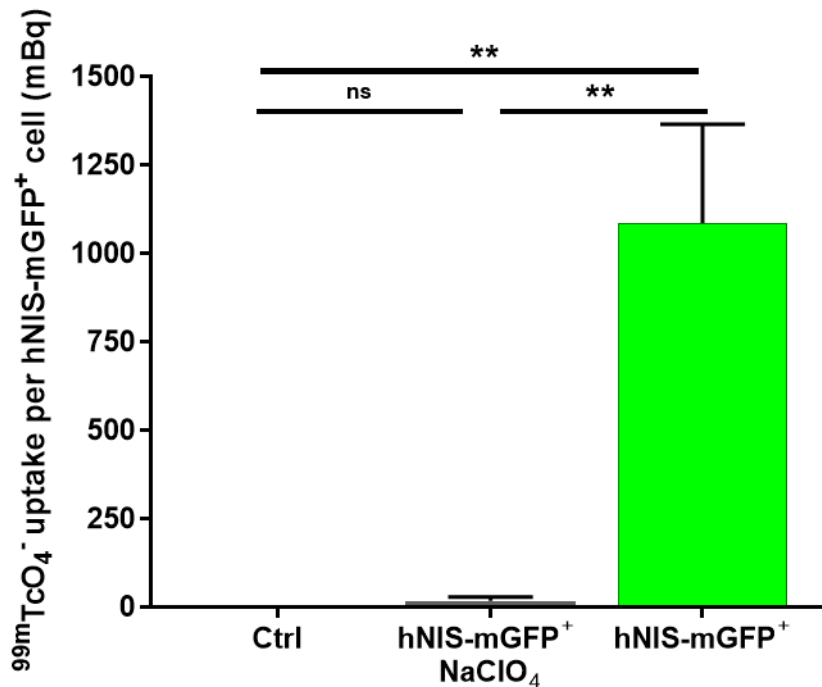
Figure 4. Verification of hNIS-mGFP function. | *In vitro* uptake of the NIS radiotracer

[^{99m}Tc]TcO$_4^-$ is indicative of correct hNIS-mGFP reporter function. HLCs transduced to

express hNIS-mGFP were passaged onto collagen-I coated plates and matured for a further

two weeks *in vitro*. Subsequently, cells were incubated with [^{99m}Tc]TcO$_4^-$ and cellular uptake

was measured by γ-counting of the dissociated cells. Results are expressed relative to

untransduced control HLCs. The hNIS co-substrate perchlorate served as a specificity

control. $N = 3$ biological replicates corresponding to independent differentiation and

transductions (with triplicate wells assayed per biological sample). Results were analysed by one-way ANOVA with Tukey's multiple comparison correction ** $p<0.01$, ns $p> 0.05$, error bars are SD.
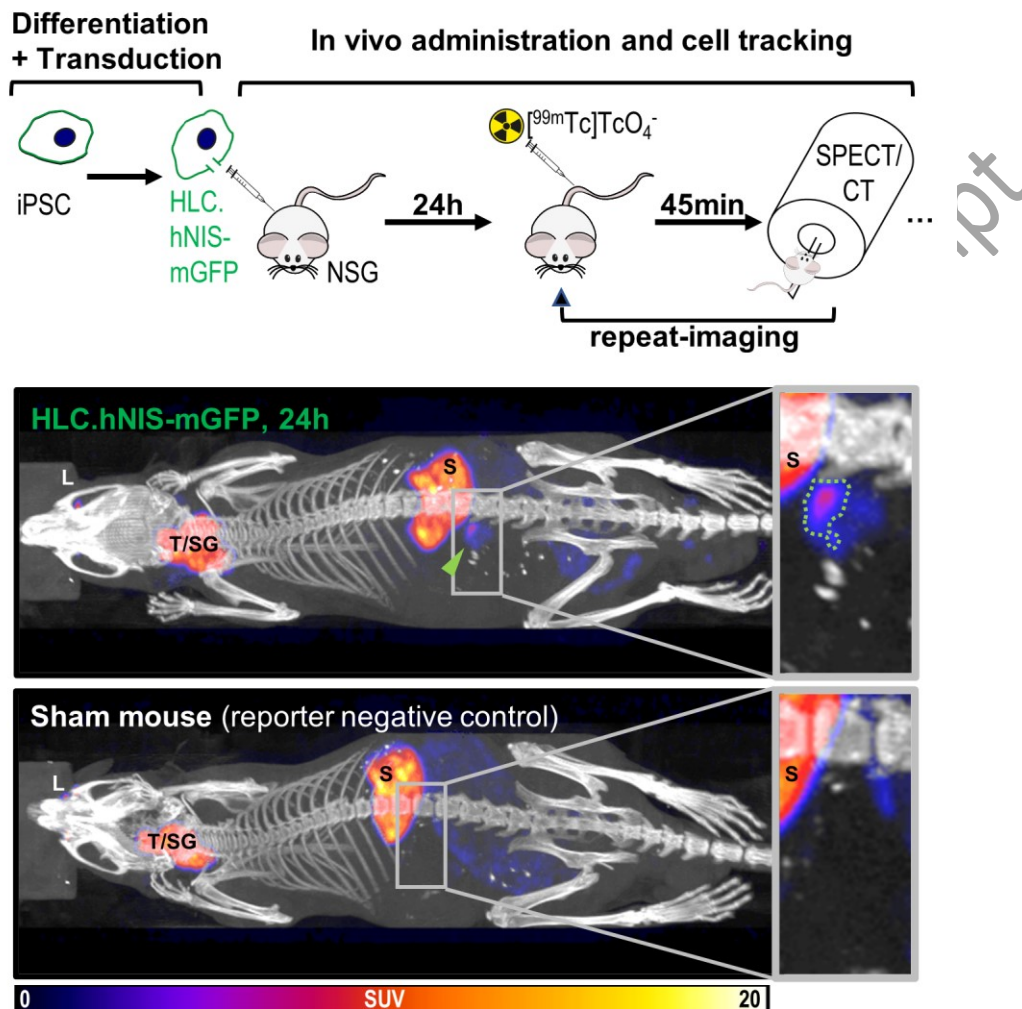
Figure 5. Non-invasive *in vivo* imaging of hNIS-mGFP$^+$ HLCs by SPECT/CT. | (Top) Experimental scheme. HLCs were transduced to express hNIS-mGFP and transplanted intrahepatically. 24 hours later 30MBq of [^{99m}Tc]TcO$_4^-$ was administered intravenously to anaesthetised mice. 45 min after radiotracer administration, SPECT imaging was performed to investigate HLC survival. This 45-minute period is necessary to obtain good contrast. During this time, CT scans were taken while animals were already under anaesthesia. **(Bottom)** SPECT maximum

intensity projections (MIP) are overlaid with CT images that provide anatomical context. A sham mouse, which underwent all procedures (but 'transplanted' with PBS alone) was imaged under the same conditions. Background radiotracer uptake in both mice due to organs with endogenous mouse NIS expression can be seen (*i.e.* thyroid/salivary glands (T/SG), stomach (S) and lacrimal glands (L)). The green arrow indicates the administered hNIS-mGFP[+] HLC located in the liver, which were administered 24h earlier. Enlarged areas to the right indicate a MIP of a volume of interest including the liver lobes in which HLCs/vehicle were injected. The encircled signals in the liver (green dotted line) indicate the hNIS-mGFP[+] HLCs. Notably, no signals stem from the liver lobes of the sham mouse under these conditions.

Table Captions

Table 1. Cell seeding number and culture volumes for production of lentiviral particles dependent on size of culture vessel

Table 2. Plasmid DNA and PEI concentrations for lentiviral particle production dependent on culture vessel size

Table 3. Basal culture media supplements for HLC differentiation defined by day of differentiation

Tables

Table 1

	Size of culture vessel				
	T25	T75	T175	T600	T1000
Number of cells to seed [x10^6]	1.5	4.5	10.5	36	60
Volume of culture media for seeding [mL]	5	20	45	150	250
Volume of complete culture media for transfection [mL]	4.5	13.5	31.5	108	180
Volume of serum free media for transfection [mL]	0.5	1.5	3.5	12	20

Table 2

		Size of culture vessel				
		T25	T75	T175	T600	T1000
Plasmid DNA [µg]	**hNIS-mGFP (transgene)**	3.8	11.3	26.3	90	150
	pΔ8.91	2.8	8.4	19.8	67.5	112
	pVSV-G	0.94	2.8	6.6	22.5	37.5
	Total	7.5	22.5	52.5	180	300
Volume of PEI (1µg/uL) needed [µL]		22.5	67.5	158	540	900

Table 3

Day of differentiation	Supplements/growth factors added (final concentration, source)
D1	100ng/mL Activin A, 10ng/mL BMP4, 3μM CHIR99021, 80ng/mL FGF2, 10μM LY294002.
D2	100ng/mL Activin A (), 10ng/mL BMP4, 80ng/mL FGF2, 10μM LY294002. This medium has the same composition as D1 except CHIR99021 is omitted.
D3	1% Penstrep, B27, 2% MEM non-essential amino acids (MEM NEAA), 100ng/mL Activin A, 80ng/mL FGF2.
D4-D8 (daily)	1% Penstrep, B27, 2% MEM NEAA, 50ng/mL Activin A.
Hepatocyte maturation media: D9 onwards (every other day)	1% Penstrep, 2mM L-Glutamine, 2% MEM NEAA, 2% Chemically Defined Lipid concentrate, 1% Insulin-Transferrin-Selenium (ITS), 10ng/mL Oncostatin-M, 50ng/mL HGF .