In Vivo Tumorigenicity of the 20q11.21 Amplicon in an Engraftment Model of hPSCs and Differentiated Liver Cells

Chris S. Pridgeon†, Shiva Seyed Forootan†, Fang Zhang†, Nicholas Harper†, Daniel Palmer†, Richard Weightmann†, Sian Gregory†, Zoe Hewitt†, Duncan Baker†4, Jason Halliwell†, Harry Moore†, Emanuele Ricci†, Peter W. Andrews†, Harish Poptani†, David C. Hay†, B. Kevin Park† and Chris E. P. Goldring†5

Abstract

Human pluripotent stem cells (hPSCs) are a promising source of somatic cells for clinical applications and disease modelling. However, during culture they accumulate genetic aberrations such as amplification of 20q11.21 which occurs in approximately 20% of extensively cultured hPSC lines and confers a BCL2L1-mediated survival advantage. During the production of the large number of cells required for transplantation and therapy these aberrations may become unavoidable which has important safety implications for therapies and may also impact upon disease modelling. Presently, these risks are poorly understood; whilst it is apparent that large-scale genetic aberrations can pose an oncogenic risk, the risks associated with smaller, more insidious changes have not been fully explored.

In this report, the effects of engraftment of human embryonic stem cells (hESCs) and hESC-derived hepatocyte-like cells (HLCs) with and without amplification of the 20q11.21 minimal amplicon and isochromosome 20q (i20q) in SCID-beige mice are presented. The cells were tracked in vivo using a luminescent reporter over a period of approximately four months. Intrasplenic injection of hESCs showed greater in vivo tumorigenicity of the 20q11.21 compared with i20q and wild type. HLCs with 20q11.21 engrafted more successfully and formed more severely disruptive lesions than wild-type cells or cells with i20q. These results reinforce the notion that karyotyping of therapeutic hPSC is required for transplant, and suggest that screening for known common aberrations is necessary. Further work to identify commonly arising genetic aberrations should be performed and routine screening for hPSCs intended for therapeutic use should be used.

Keywords: Stem cell; Tumorigenicity; Amplicon 20q11.21

Introduction

Demand for donor organs outstrips supply, consequently, there have been recent efforts to find alternate sources of cells and tissue for transplant. Human pluripotent stem cells (hPSCs), both embryonic (ESCs) and induced (iPSCs), offer a potentially unlimited source of cells for this purpose. However, the risks associated with the use of hPSCs for transplant are incompletely understood. There are concerns regarding the tumorigenic potential of hPSC-derived therapies that could contain residual undifferentiated cells, which have the inherent ability to form teratomas in vivo, but also regarding the more insidious genetic changes, which occur during isolation and prolonged culture that these cells experience.

During culture, hPSCs are maintained in a highly proliferative state and subjected to selective forces which drive culture adaptation. For example, frequent passaging, growth in typically high-glucose medium and so on [1]. As genetic mutations accumulate randomly during culture, advantageous adaptations, for example those that increase the rate of proliferation, will naturally become selected. For example, trisomy 12 occurs commonly in hPSCs, and confers a survival advantage by increasing proliferation rate, and changing the cellular expression profile to more like a germ cell cancer [2,3]. In addition, amplification of 20q11.21 is present in up to 20% of hPSC lines after prolonged culture and confers a survival advantage [3]. Recurrent amplification in the 20q region has been observed for over 15 years in hESCs [4-5] and longer in pancreatic cancer [6] and is associated with an increase in the rate of proliferation. HESCs containing amplifications in 20q showed increased capacity to initiate teratomas in vivo without increased tendency to produce malignant teratocarcinomas [7]. The 20q11.21 minimal amplicon is approximately 0.55Mb long and encodes HM13, ID1, BCL2L1, KIF3B and an immature form of miR-1825. Although ID1 is also a potential oncogene, it is BCL2L1 that confers a survival advantage in carrier cells [8-9]. BCL2L1 encodes BCL-XL and BCL-XX as splice variants, however, BCL-BS is undetectable at the protein level in ESCs [8]. BCL-XL exerts an anti-apoptotic effect by regulating mitochondrial release of cytochrome c. Although BCL-XL overexpression, as a consequence of amplification of 20q11.21, confers resistance to cell death associated with single-cell passaging in vitro [8], the oncogenic risk in vivo remains to be established. Therefore, in vivo modelling is required.

The functional engraftment of hPSC-derives hepatocyte-like cells (HLCs) in murine liver has been demonstrated previously [10]. Additionally, undifferentiated hPSCs with known aberrations have...
been used in vivo to demonstrate enhanced tumorigenicity [1]. The aim of the study was to combine these approaches, luminescent hESCs both with and without 20q variants were differentiated into HLCs and injected intrasplenically into SCID-beige mice whose livers had been acutely damaged with carbon tetrachloride (CCl₄) to facilitate engraftment. The engraftment of these cells into the liver was tracked using in vivo luminescence imaging for approximately 4 months and the formation of malignancies was assessed.

Materials and methods

Production of the pCAG-LUC plasmid

pCAG Firefly Luciferase-2A-Puro was constructed by removing the EGFP-IRE-3-pur-2A cassette from pCAG-EGFP using XhoI and NsiI restriction enzymes. Firefly luciferase linked to puromycin by a 2A motif was amplified from pEF6 firefly Luciferase-2A-Puro and ligated into pCAG (Figure 1A). The plasmid was sequenced prior to use (Source Bioscience, Rochdale UK).

Derivation of hESCs

Derivation of the MS7 and MS8 cell lines was performed as described previously[11,12]. Briefly, derivation was performed at the Stem Cell Derivation Facility at the Centre for Stem Cell Biology, University of Sheffield, under HFEA licence R0115-8-A (Centre 0191) and HTA licence 22510, in a clean room setting, following standard operating procedures. The embryos used to derive MS7 (frozen) and MS8 (fresh) were donated from different Assisted Conception Units, following informed consent, with no financial benefit to the donors, and were surplus or unsuitable for their IVF treatment. Briefly, the embryos, were cultured using standard IVF culture media (Medicult), to the blastocyst stage. Following removal of the trophectoderm using a dissection laser the embryos were explanted whole onto mitotically inactivated human neonatal fibroblasts (human feeders) in KOSR/KODMEM medium (Life Technologies). Cell lines were initially maintained at 37°C under 5% O₂/5% CO₂, until the lines were established, after which maintenance switched 5% CO₂ in air at 37°C. Cultures were passaged by cutting selected colonies under a dissection microscope at an average split ratio of 1:2 every 7 days. Both cell lines have been deposited at the UK Stem Cell bank (https://www.nibsc.org/ukstemcellbank) as per HFEA licence as accordance with the HFEA licence. From these clinically derived lines, four research grade cultures were generated: MS7 (~20q11.21), a clonal cell line, karyotyped as 46XY and diploid for BCL2L1 by FISH. MS7 CNV (MS7[20q11.21+>20q11.21]), was also a clonal cell line from the same heterogeneous starting population of MS7 QC6.2 (a research bank) and shown by FISH to have triplicate copies of BCL2L1, but otherwise karyotyped as 46XY. The breakpoint of the CNV was shown to be after the XKR7 (31,968,002) gene by qPCR using the method of Laing et. al.[13].

Cloning was performed by single cell deposition of a heterogeneous starting population of MS7 (QC6.2) at P5+8+2 using a FACSSelecta flow cytometer (Becton Dickinson) into 96 well plates with human feeder cells, Nutristem hPSC XF medium (Biological Industries) and Y-27632 [14]. After 24 hours the medium was changed to remove Y-27632 and the clones were expanded for 2 weeks before manual picking and culture. Seed banks were frozen from each clone at P5+8+5 (a +/+ in the passage number refers to a thaw from a cell bank), and material taken for detection of variants by qPCR[15], karyotyping and FISH. Once the genotypes were confirmed the clonal lines were thawed and were transferred to the University of Liverpool at passage P5+8+5+5+8 (MS7) and +9 (MS7[20q11.21+]).

MS8 (~20q11.21) was a non-clonal culture originating from a research bank of MS8 (QC3.2) frozen at passage P2+9+5, and karyotyped as 46XY (normal), and were thawed and passaged a further 5 times (P2+9+5+5) before transfer to the University of Liverpool. MS8[20q] (isochromosome 20q, i20q) originated from a different research bank (MS8 QC2), which was frozen at P2+9+5+9 and was shown by karyotype to be 46XY. This was confirmed shortly after thaw at P2+9+5+9+4 but following further culture at P2+9+5+9+13 (17 passages later) it developed a spontaneous i20q mutation in ~90% of the culture. These cells were frozen as a research cell bank (QC2.1 Abnormal) at passage P2+9+5+9+19 and then thawed and transferred to the University of Liverpool three passages later (P2+9+5+9+19+3).

hESC culture

hESCs were cultured in Nutristem hPSC XF medium on laminin-521 (In-521) and passaged approximately every 5-6 days or when approaching confluence. Cells were passaged non-enzymatically using EDTA. Culture medium was removed, and cells were washed twice with phosphate buffered saline without calcium or magnesium (PBS). Cells were incubated in prewarmed 0.5 mM EDTA at 37°C until detachment was observed, typically within 5 minutes. Once detachment was observed, EDTA was removed, and cells were washed into culture medium and agitated by pipetting to disrupt large clusters before plating onto In-521 coated tissue culture plates.

Transfection of hESC with pCAG-LUC plasmid

Plasmid DNA was prepared using the Qiangen Plasmid Maxi kit (Qiagen, UK) following the manufacturer’s protocol. Prior to transfection, hESCs were detached in the same manner as described for passaging and 2.5 × 10⁵ cells were aliquoted to each transfection or control. The cells were pelleted by centrifugation and resuspended in 100 μL of nucleofector solution 2 (Lonza, UK) and 6μg DNA. The suspension was transferred to an electroporation cuvette and single pulse at 1600 V, 20 ms was applied using an Amaxa Nucleofector II. The suspension was transferred to In-521-coated 6-well plates and incubated under standard tissue culture conditions.

After 24h, the medium was removed and cells were washed with PBS and incubated with luciferin (150 μg/mL in PBS) in darkness for 5 minutes and bioluminescence was measured using an IVIS Spectrum (Perkin Elmer, UK). Cells were washed with PBS and Nutristem hPSC XF medium was added. Puromycin was added to the transfected cells 48 hours after transfection with the concentration of 0.7, 0.5, 0.1 and 0.4 μg/mL for MS8, MS8[20q], MS7 and MS7[20q11.21], respectively (based on a killing curve). Day 12 post-transfection, single clones were selected and transferred to In-521-coated 96-well plates. Thirty clones were selected from each cell line and the ten with the greatest bioluminescence signal were expanded and cryopreserved. Clones were tested for amplicon 20q11.21 and karyotyped to check for chromosomal abnormalities. Next, the two clones with the strongest luminescent signal were selected and used for all subsequent experiments. Luminescence was periodically assessed after transfection to ensure that it was stably maintained, this was performed as described above.

Hepatocyte-like cell differentiation

hESCs were differentiated as previously described [16] and adapted according to protocol described by Siller et al.[17]. Prior to differentiation, hESCs were grown to 30-60% confluency on In-521-coated plates in NutriStem hPSC XF medium. On day 1, medium was changed to RPMI-1640 medium supplemented with 100 ng/mL activin A (Peprotech, UK) and 50 ng/mL Wnt3a (R&D Systems, UK) and changed daily for 3 days. On day 4, medium was changed to Knockout DMEM supplemented with 20% (v/v) KnockOut Serum Replacement, 1% (v/v) GlutaMAX, 100 μM MEM Non-Essential Amino Acids, 200 μM 2-mercaptoethanol (all Thermo Fisher Scientific), and 1% (v/v) DMSO (Sigma Aldrich) and changed

---

The text above contains information on the derivation and culture of hESC cell lines, their differentiation into hepatocyte-like cells, and the methods used for monitoring their tumorigenicity and differentiation in vivo.
every other day for 5 days. On day 9, the medium was changed to Leibovitz’s L-15 supplemented with 8.3% (v/v) tryptophan phosphate broth, 8.3% (v/v) embryonic stem-cell FBS, 1% (v/v) GlutaMAX (all Thermo Fisher Scientific), 50 ng/mL sodium-L-ascorbate, 10 μM hydrocortisone 21-hemisuccinate, 1 μM insulin (bovine pancreas), 100 nM dexamethasone (all Sigma Aldrich), 20 ng/mL oncostatin M and 10 ng/mL hepatocyte growth factor (both Peprotech) and changed daily until day 12. All growth factors and other heat labile components were added immediately prior to use.

HepG2 cells were included as a positive control of an aggressively engrafting liver cancer cell type. They were cultured under standard tissue culture conditions in DMEM, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% GlutaMAX. HepG2 cells were treated identically to HLCs at later stages with regard to cell number and injection method.

**Flow cytometry**

To assess differentiation efficiency of hESCs, flow cytometry was performed on cells at day 8 during the differentiation protocol. Cells were washed with PBS and detached using pre-warmed Accutase (STEMCELL Technologies, UK). Accutase was removed by centrifugation (1000 g, 10 minutes) and cells were washed twice in PBS. Cells were fixed in 2% PFA (Sigma Aldrich) in PBS at room temperature (RT) for 30 minutes, washed with PBS and permeabilised in 0.1% (v/v) triton X-100 (Sigma Aldrich) at RT for 20 minutes. Cells were washed in PBS and blocked in 2.5% (v/v) normal donkey serum (NDS) (Abcam, UK) for 30 minutes at RT. Cells were washed again in PBS and resuspended in antibody diluent (0.5% (v/v) NDS, 0.05% sodium azide in PBS) with rabbit anti-human HNF4a (Santa Cruz) 1:50 overnight at 4°C. After primary incubation cells were washed 3 times in PBS and resuspended in anti-donkey AlexaFluor 488 (Life Technologies) 1:750 in antibody diluent and incubated for 2 hours at RT in darkness. Cells were washed once in PBS before resuspension in 1mL PBS with 1μM DAPI and subjected to flow cytometric analysis using a FACS Canto II (BD Biosciences).

**Animal experiments**

All animal experiments were conducted according to the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Welfare Committee. 8-10-week-old SCID-beige mice (Charles River Laboratories, UK) were housed in a 12 h dark/light cycle in a temperature and humidity controlled, specific pathogen-free environment. Mice were fed CRM (P) diet (Special Diets Services) ad-libitum. Mice were acclimatised for seven days upon arrival.

**CCl4 dose determination**

The dose for CCl4 in SCID-beige mice has not previously been reported and was determined experimentally. A single dose of 1 mL/kg (10.33 mmol/kg), 0.4 mL/kg (4.14 mmol/kg) and 0.2 mL/kg (2.07 mmol/kg) of CCl4 was administered intraperitoneally and animals were monitored for recovery. Animals were sacrificed 24 and 72 hours later and liver damage was examined by H&E staining of liver tissue and ALT measurement as described below.

**Cell engraftment**

Animals received 0.2 mL/kg single dose of CCl4 diluted in olive oil (Sigma Aldrich) intraperitoneally (IP) to produce acute liver injury. After 24 hours, 75 μL of blood was collected by tail bleed to confirm the presence of liver injury. After 72 hours, animals were anaesthetised, and a laparotomy was performed to inject 1 x 10⁶ of either normal or abnormal undifferentiated hESCs (n=4 per cell line), HLCs (n=7 per cell line) or HepG2 cells (n=7) into the spleen [18]. For undifferentiated hESCs, animals were kept for 4 weeks and imaged weekly or fortnightly. The weights of the animals were monitored 3 times a week. Animals that received hepatocyte-like cells, were kept for up to 4 months and were imaged weekly or fortnightly. Animals receiving HepG2 cells were kept for up to 7 weeks before sacrifice and were imaged weekly. Twenty out of 74 (20/74) animals were excluded from the study for health reasons.

At the end of the experiment, following bioluminescence imaging (see below), animals were culled via exposure to rising CO2 concentration. Livers and spleens were imaged ex vivo. For each animal, the right half of the liver median lobe and kidneys were flash frozen. The other half of median lobe was fixed in 4% PFA for histological examination. Blood was collected via cardiac puncture and allowed to clot for 30 min at room temperature before serum was isolated via centrifugation.

These experiments were planned and carried out in compliance with the ARRIVE guidelines.

**Alanine aminotransferase assay**

Serum ALT levels were measured using Infinity ALT Liquid Stable Reagent (Thermo Fisher Scientific), using the manufacturer’s protocol.

**Bioluminescence imaging**

SCID-beige mice received luciferase-labelled cells and were imaged using an IVIS Spectrum under anaesthesia with isoflurane. Prior to imaging, animals were injected IP with 150 mg/kg D-luciferin (Promega). Data were collected and analysed using Living Image software (Xenogen) according to the manufacturer’s instructions. Quantification of luminescence signals was achieved using the Region of Interest (ROI) function within Living Image software. Within a single experiment, an area was drawn around the broadest signal and used for all other animals in that experiment. Total flux within the ROI was considered as the signal intensity. For ex vivo imaging, mice were injected a second time with luciferin, culled, tissues excised and analysed using the IVIS platform. For quantification of ex vivo luminescence signals, an area was drawn around the dish containing the tissue.

**qPCR for the presence of amplicon 20q11.21**

The presence or absence of the 20q amplicon was checked regularly. Quantitative real-time PCR was performed for BCL2L2 with RELL1 as a reference gene following the protocol published by Baker et al. [15].

**Human Albumin ELISA**

Human albumin was quantified in serum taken from mice by cardiac puncture after sacrifice using a Human 72 Albumin Quantitation Set (Bethyl Laboratories, TX, USA) following the manufacturer’s protocol.

**Histology and immunohistochemistry**

Liver tissues were fixed in PFA for 24-48 hours, were trimmed and routinely embedded in paraffin wax. Consecutive 4 μm thick sections were prepared and routinely stained with haematoxylin and eosin (H&E). Individual selected cases were further examined with both histochemistry (PAS and Alcian-PAS) or subjected to immunohistochemical labelling. For the latter technique, a Dako
Figure 1. Transfection of undifferentiated hESCs with a luciferase construct and identification of appropriate dosage of CCl₄ for consistent induction of acute liver injury in SCID-beige mice. (A) pCAG Firefly Luciferase-2A-Puro map (B) Haematoxylin and eosin staining of the liver of SCID-beige mice 24 or 72 hours after a single dose of CCl₄ (0.2 or 0.4 mL/kg) or olive oil. Both doses of CCl₄ induced severe coagulative necrosis of the centrilobular hepatocytes with or without the presence of acute haemorrhages (black arrows). After 72 hours, both groups showed a decreased degree of damage and a narrowing of the area of coagulative necrosis around the central vein with a continuous rim of hyper-basophilic regenerating hepatocytes and an increased number of mitotically active hepatocytes within the intervening healthy parenchyma. Olive oil did not elicit appreciable morphological degeneration of the hepatic parenchyma. (C) Mean hepatocellular damage score according to Thoolen et al. [19]. n=4 per condition (D) ALT activation in SCID-beige mice 24 and 72 hours after injection with CCl₄. Data are displayed as U/L and show the mean, error bars show standard deviation. Significance was assessed using a paired two-tailed t-test, asterisks denote significance (*** = p≤0.001).
Figure 2. Undifferentiated hESCs with 20q11.21 amplicon induce extensive immature teratoma and teratocarcinoma in the murine liver. SCID-beige mice were injected with 1×10^6 of undifferentiated MS7N, MS7^{20q11.21}, MS8N or MS8^{20q} into the spleen and sacrificed after 28 days. (A) In vivo and ex vivo (liver and spleen) bioluminescence imaging of representative mice from each group after 28 days. See Supplementary Figure 1 for data from all mice. (B) Bioluminescence over time in vivo grouped by cell line. Values are plotted as mean total flux, error bars show SEM. (C) Mean bioluminescence of different cell lines imaged in livers and spleens ex vivo. Values are plotted as mean total flux, error bars show SEM. Differences were assessed using a two-tailed unpaired student’s t-test, asterisks denote the p-value (* = p ≤ 0.05, *** = p ≤ 0.001). Where no asterisks are shown no statistically significant difference was observed. (D) Haematoxylin and eosin staining of a representative liver tissue after injection with undifferentiated hESCs. Black arrows indicate immature teratoma, white arrows denote carcinomatous features (E) Grading of liver and spleen tissue from animals with intrasplenic injection of undifferentiated cells and grading system used to score liver and spleen tissues. Scoring was performed by a veterinary histopathologist according to Thoolen et al.[19]. (F) Representative IHC staining of liver and spleen tissue for vimentin and pan-cytokeratin to confirm the presence of teratoma.
Autostainer Link 48 was used. Following paraffin removal and rehydration, EnVision™ FLEX Peroxidase Blocking Reagent was used to quench endogenous peroxides. Before addition of the primary antibodies [against Vimentin (clone V9, Dako, 1:500), Pan-Cytokeratin (clones AE1/AE3, Dako, 1:200) and GFAP (Dako, Z 0334, 1:1000)], goat serum and Fab Fragment (unconjugated affinity purified Fab fragments goat anti-mouse IgG, Stratech Scientific) blocking steps were serially applied. Following this, slides were incubated with the EnVision™ anti-mouse FLEX/HRP labelled polymer for 20 minutes, rinsed and then incubated with the EnVision™ FLEX DAB + Substrate Chromogen System. Slides were counterstained with EnVision™ FLEX Haematoxylin, then rehydrated and mounted as per routine histology. All histological and immunohistochemical specimens were examined by a veterinary pathologist (ER) who was blinded to the treatment of the animals. Samples were scored according to Thoolen et al. [19], these scores are a standard nomenclature to classify proliferative and non-proliferative lesions of the hepatobiliary system in rats, and were applied to mouse tissue in this case.

Statistical analysis

Statistical analyses were performed using several software packages including R, GraphPad and Microsoft Excel. Histology scores were tested using the Kruskal-Wallis rank sum test, all other statistical tests were two-tailed, unpaired student’s t-tests.

**Figure 3. Differentiation protocol and differentiation efficiency in MasterShef ESCs.** (A) Overview of the differentiation protocol used, adapted from published protocols [16][17]. The top bar represents the day of the differentiation procedure and the medium and supplements used are shown underneath. Full details of the differentiation protocol can be found in the methods section. (B) Differentiation efficiency towards hepatocyte-like cells shown by HNF4α-positivity using flow cytometry. Cells were stained for HNF4a as a marker of differentiation and DAPI was used as a nuclear stain. Differentiation proportion is shown in white for each line.
Figure 4. MasterShef Fluc HLCs sporadically luminesce in vivo and increase liver tissue histology scores. (A) Representative luminescence images of SCID beige mice 6 weeks after intrasplenic injection with MS HLCs or HepG2 cells. Luminescence is displayed in the coloured overlay where warmer colours indicate higher luminescence intensity. (B) Luminescence over time for each MS HLC line or HepG2 cells. Values are plotted as mean total flux and error bars show SEM. (C) Mean histology scores for liver and spleen tissues separated by tissue and amplicon status. Tissues were scored by a veterinary histopathologist according to [19]. Horizontal black bars indicate the mean and SEM. Statistical analysis (Kruskal-Wallis rank sum test) showed no significant differences in histology score in liver (p= 0.06865) and spleen (p=1) based on amplicon 20q11.21 status. (D) Haematoxylin and eosin staining of mouse liver after injection with MS HLCs. The line injected and the grade assigned to the imaged tissue is noted. Moving left to right, beginning with the top row, the images show 1) distended central veins in normal tissue, 2) large single nodular mass made of stellate mesenchymal cells suspended in a light eosinophilic matrix 3) normal tissue, mild central vein distension 4) moderate leukocyte infiltration in portal space and around biliary ducts and 5) a large neoplastic mass with scattered cyst-like areas (both acinus-like and distended vascular spaces). All images are 20× except for HepG2 which is 10×. Scale bars are 100 μm.
Results

SCID-beige mice are highly sensitive to acute carbon tetrachloride toxicity

With 10.33 and 4.14 mmol/kg CCl₄, animals exceeded severity limits before reaching time points. Using 2.07 mmol/kg of CCl₄ demonstrated a substantial degree of liver damage (Figure 1B, C) and ALT elevation (Figure 1D) at 24 and 72 hours after injection. Therefore, for subsequent experiments 2.07 mmol/kg was used.

Effects of variant 20q on teratoma formation

Luminescent cell lines were produced and each hESCs line showed similar luminescence in vitro (Supplementary Figure 2B). Undifferentiated hESCs (MS7 1, MS8 1, MS720q11.21 1 and MS820q 1) were injected intrasplenically into SCID-beige mice (n = 4/line). In the first week, 14/15 animals showed a bioluminescent signal which increased over time (Figure 2A & B, Supplementary Figure 1). Luminescence intensity was significantly higher in MS720q11.21 compared to all other lines in both liver and spleen when imaged ex vivo (MS7 versus MS720q11.21, p = 0.0152). In addition, there was no significant difference in the luminescence intensity between MS8 and MS820q in either tissue (MS8 versus MS820q, p = 0.1482) (Figure 2C). During ex vivo imaging, 14/15 livers and 4/15 spleens exhibited a luminescent signal in addition to macroscopically observable cystic lesions.

In H&E staining, all cell lines demonstrated immature teratoma in at least one animal (Figure 2D, black arrows) with frequent observation of neuroepithelial tubules and carcinomatous features in MS820q (Figure 2D, white arrow). The frequency and severity of immature teratoma was greater in animals injected with MS720q11.21 compared with other lines, animals injected with MS720q11.21 were grade 5 in liver and spleen. All other lines were grade 0 in spleen and grade 0-3 in liver. A summary of the grading system is also given (Figure 2E) [19] and high-magnification H&E images are presented in Supplementary Figure 2A. Vimentin and pan-cytokeratin were expressed in liver and spleen of neoplastic tissues (Figure 2F).

Effects of variant 20q on tumorigenicity of HLCs

Differentiation efficiency of hESCs to HLCs using the protocol described in Figure 3A was assessed. After 8 days of differentiation, the percentage of HNF4α-positive cells was 90.7% (MS7 1), 87.6% (MS720q11.21), 96.3% (MS8) and 80.4% (MS820q) (Figure 3B).

One million hepatocyte-like cells from each line were intrasplenically injected into SCID-beige mice 72 hours after CCl₄ injection and imaged as described in the methods section (n=7/line). Several animals showed poor health after injection of HLCs and were sacrificed early. The animals’ health deteriorated after approximately 4 months and the experiment was ended. SCID mice are prone to thymic lymphoma, indeed 6 thymus tumours were observed after the experiment was concluded and these animals were excluded from analysis. Animals injected with HepG2 cells were sacrificed after 7 weeks due to saturated luminescent signal and poor health.

In vivo luminescence was observed in the livers and spleen of 3 animals injected with MS820q 1 and MS820q 2 after injection of HLCs (Figure 4A & B, Supplementary Figure 4) and was measured over the course of the experiment in each line (Figure 4B). Luminescent signals were observed briefly or not at all after injection with HLCs from other lines (Supplementary Figures 5-7). Figure 4B shows an increase in mean luminescent signal over time from HLCs derived from MS820q 2 and from MS820q 1 to a lesser extent. The luminescent signal from other lines was low and did not increase over time in culture. Due to high inter-animal variation (supplementary figures 4-7), the luminescent signals are not significantly different when tested at peak luminescence (two-tailed, unpaired student’s t-test, p=0.17-0.25). HepG2 cells expressing the same luciferase reporter were used as a positive control of a hepatic cancer cell type. A strong luminescent signal developed rapidly and became saturated after 5 weeks (Figure 4B, Supplementary Figure 3).

Histological sections of the liver and spleen of each animal were scored by a veterinary pathologist according to Thoolen et al. [19] (Figure 4C). Scores are based on several factors including the size of necrotic lesions in the hepatic lobule and increased scores denote greater severity of the lesion (Figure 2E). Mean liver and spleen histology scores were not significantly changed under any condition but tended higher in animals treated with MS720q11.21 (mean liver histology score was 0.36 with MS820q 1.30 with MS720q11.21 and 0.19 in wild type, p = 0.069 using the Kruskal-Wallis rank sum test, mean spleen histology score was 0.01 with MS820q, 0.00 with MS720q11.21, 0.00 in wild type, p =1.00 using the Kruskal-Wallis rank sum test) (Figure 4C).

Haematoxylin and eosin staining was performed for the liver and spleen of each animal after sacrifice and scored according to Thoolen et al. [19]. Representative images of liver tissue sections are shown in Figure 4D, and the effects of each line are shown, the grade of the tissue pictured is noted beneath each image. In MS8 2, apart from congestion distension of veins, occasional periportal small infiltrates of inflammatory cells and a single cluster of mineralized parenchyma, no further changes were detected. In MS820q 2, the parenchyma appeared diffusely more compact as an effect of slightly more basophilic cytoplasm with homogenous appearance. Numerous and variably sized well demarcated masses, at the edges of tissue sections, composed of stellate or fusiform cells interspersed within an abundant pale eosinophilic matrix were present (benign mesenchymal neoplasms). In MS7 1, apart from the presence of small and rare aggregates of neutrophils and pigment granulomata, no further changes were observed. Occasional patchy areas of hydropic degeneration are also present. In MS720q11.21 2, there were multiple foci of perivascular lymphoplasmacytic infiltration, sparse Kupffer cells containing granular gold-stained material and numerous small aggregates of round cells (extramedullary haematopoiensis). In HepG2, the liver was multifocally distended and distorted by the expansion of highly cellular, well-demarcated and expansile neoplastic masses composed of cells arranged in large acini, papillae and large solid coalescing nests in association with moderate vascular stroma. Cells had a cuboidal profile, moderate amount of light eosinophilic cytoplasm and central round nuclei with finely stippled chromatin and large eosinophilic nucleoli. The mitotic index is elevated (18 x 10HPFs) with bizarre mitosis. Cellular atypia is mild. Cystic cavities containing pale eosinophilic fluid and sloughed necrotic neoplastic cells were observed. The remaining parenchyma presents numerous areas of mineralization.

Human albumin was detected in MS820q 1 at extremely low concentration compared to animals injected with HepG2 cells and was not detected in animals injected with other cell lines (data not shown).

Discussion

A major concern over the use of hPSC-derived cell therapies is the perceived risk of tumorigenicity. This is highlighted by reports of tumour formation several years after stem cell transplantation [20,21]. For example, a tumour was detected four years after foetal neural stem cell transplantation for ataxia telangiectasia [20] and a mucosal-like mass was reported eight years after treatment of a spinal injury.

Copyright © Journal of Stem Cells and Regenerative Medicine. All rights reserved
with olfactory mucosal cells\textsuperscript{[21]}. Undifferentiated PSCs can cause benign teratomas or potentially malignant teratocarcinomas, indeed teratoma formation can be used to examine the pluripotency of iPSCs\textsuperscript{[22]}. The safety of cell therapies to their recipients is essential and the risks of teratoma from undifferentiated cells and the tumorigenic risk from genetic changes in transplanted cells must be managed\textsuperscript{[23]}. With current 2D cell culture techniques, the accumulation of genetic aberrations appears to be unavoidable and even though some culture conformations reduce the rate of mutations\textsuperscript{[24]}, they do not eliminate them completely. Therefore, more data is required on which common genetic aberrations predispose hPSCs and their progeny to tumorigenesis, and which are relatively benign. With this information, a pragmatic position on which genetic changes are acceptable in hPSCs intended for clinical applications could be adopted\textsuperscript{[25]}. This study may serve as a proof-of-concept that differences in in vivo tumorigenicity related to such abnormalities can be tested in this manner.

The 20q11.21 amplicon contains potential oncogenes including BCL2L2, ID1 and TPX2 and confers a survival advantage in vitro mediated by BCL2L2 overexpression\textsuperscript{[8,9]}. However, it was not clear if this advantage would operate in vivo via the same mechanism, or at all. We hypothesised that cells containing the 20q11.21 amplification or i20q would show greater tumour-forming potential in vivo than wild type cells which would manifest as increased luminescence in vivo.

To facilitate engraftment, a single dose of CCl\textsubscript{4} was used to damage liver and improve stem cell implantation based on the work described by Payne et al.\textsuperscript{[18]}. Although chronic treatment with CCl\textsubscript{4} can lead to hepatic malignancies, we are not aware of any report on development of such a malignancy following a single dose of CCl\textsubscript{4}.

In the injection of undifferentiated hESCs, MS7\textsubscript{20q11.21} engrafted more readily than other cell types and produced the greatest disruption to tissue with higher histology scores in both liver and spleen. In undifferentiated cells the detection of vimentin and pan-cytokeratin in liver and spleen is noteworthy. Vimentin is the first intermediate filament expressed in the cytoplasm of foetal cells during development, before being replaced by cytokeratins in epithelial tissues during increasing cell differentiation. Therefore, coupled with the biphasic pattern of morphological differentiation of the neoplastic cells (Figure 2F), the observation of specific cytokeratin labelling in structures resembling large acini supports the hypothesis that initial epithelial commitment of a proportion of the neoplastic cells is well-established. Therefore, both mesenchymal and epithelial differentiations are present within most of the neoplastic masses.

After differentiation, MS7\textsubscript{20q11.21}-derived HLCs tended to result in increased histology scoring, similar to their increased propensity to engraft aggressively when undifferentiated. MS8\textsubscript{20q} showed increased luminescent signal in vivo, but without substantially increased mean histology scores. In addition, MS8\textsubscript{20q} was the only cell type with detectable human albumin after 4 months, albeit at very low levels. The increased luminescence of MS8\textsubscript{20q} could be explained by the lower proportion of differentiated cells. Hepatic differentiation was assessed by HNF4a expression, an established marker for this purpose\textsuperscript{[20]}. It is not clear whether i20q itself hinders hepatic differentiation, the proportion of HNF4a-positive cells was lower than other lines but was deemed acceptable since the proportion of HNF4a-positive cells tends to increase after the timepoint measured in this study (unpublished observations) and ultimately did not result in teratoma formation in vivo.

In most animals, the engraftment of undifferentiated cells could be detected by a luminescent signal. However, in most animals injected with differentiated cells, a signal was observed only briefly or not at all. The loss of luminescent signal and lack of human albumin expression in most lines suggests a failure to engraft or survive in vivo. However, since the animal from which albumin was detected showed no luminescent signal it is also possible that the luminescent reporter was silenced, furthermore MS7\textsubscript{20q11.21}-derived HLCs tended to result in increased histology scoring without luminescence, suggesting long-term survival in vivo. It is also possible that the signals observed are derived from undifferentiated or poorly differentiated cells that have persisted through differentiation.

De-differentiation of cells after injection has been reported previously\textsuperscript{[19] and is possible in this case. However, other reports using similar protocols have observed long-term survival of engrafted cells in vivo\textsuperscript{[27]}. There were minor differences in the differentiation protocol, strain of mice and cell lines used, that may have prevented integration of HLCs in this study. In animals injected with HepG2 cells there were detectable quantities of human albumin indicating successful engraftment as demonstrated elsewhere\textsuperscript{[28]}. However, the animals injected with HepG2 cells were measured at an earlier time point than those injected with HLCs, potentially explaining the lack of human albumin expression in most lines.

It is surprising that HLCs derived from MS8\textsubscript{20q} behaved similarly to wild type given the survival advantage conferred by 20q amplification in vitro. Cells containing the amplicon are protected against cell death when reduced to a single cell suspension such as during single cell passaging and whilst an analogous process does not occur in vivo; the cells were injected in suspension and the same mechanism may have allowed a greater number of cells to survive and engraft after this process. However, why this advantage should not also apply to cells with i20q is unclear. Crucially, these results suggest that survival advantage in vitro does not necessarily translate to an increased propensity to form tumours in vivo and that the situation is more nuanced. Within the timeframe of this experiment, it is not possible to reject the notion that although no effects for i20q were observed after differentiation, the amplification may represent a ‘hit’ in the progression towards a cancerous phenotype.

In vivo imaging was used to track the fate of luminescent cells non-destructively allowing for the use of fewer animals than would otherwise have been necessary, in line with the key tenets of the 3R’s. Moreover, it was possible to track the progression of engraftment over time and to rapidly determine the success of engraftment based on the presence of luminescence. However, there were also limitations to this approach; imaging in this manner produces a 2D view of the luminescent signal, necessitating imaging from multiple angles to triangulate the source of the signal. Moreover, it is possible that weaker signals were attenuated by the animal’s body. Future studies may consider the use of emerging luminescence imaging techniques\textsuperscript{[29,30]} with increased tissue penetration and sensitivity. Also lack of long term sustainability of bioluminescence of engrafted stem cells is a known limitation\textsuperscript{[31]}. Using two step transcriptional amplification can boost bioluminescence signals in vivo\textsuperscript{[32]}. Future studies could consider the use of more complete humanisation models e.g. TK-NOG or fah\textsuperscript{(-/-)} mice, where survival of the animal is contingent upon engraftment, or a longer-lived immunocompromised mouse, such as a NOD-SCID gamma mouse, which would provide more certainty of the survival of the engrafting cells and their long-term effects, respectively.

In summary, these results show engraftment of differentiated cells into an animal model and demonstration of an improved engraftment or survival phenotype in vivo in differentiated cells with 20q11.21 amplification. However, the i20q minimal amplicon behaved similarly to wild-type cells once differentiated. Future studies should take place to screen lines known to harbour common amplicons.

Copyright © Journal of Stem Cells and Regenerative Medicine. All rights reserved
when they are being considered for use in transplant. These results suggest that the 20q11.21 minimal amplicon indeed poses a tumorogenic risk in vivo and that use of cells containing it should be excluded from future therapeutic and in vivo studies.

References


Funding

This work was funded by UK Regenerative Medicine Platform

Competing interests’ statement

The authors declare no conflicts of interest.

Corresponding Author

Chris E. P. Goldring, MRC Centre for Drug Safety Science, Department of Pharmacology and Therapeutics, Institute of Systems Molecular and Integrative Biology, University of Liverpool, L69 3GE, UK. Email: C.E.P.Goldring@liverpool.ac.uk