Stem Cell Reports, Volume *5*

Supplemental Information

Amelioration of Hyperbilirubinemia in Gunn Rats after Transplantation of Human Induced Pluripotent Stem Cell-Derived Hepatocytes

Yong Chen, Yanfeng Li, Xia Wang, Wei Zhang, Vanessa Sauer, Chan-Jung Chang, Bing Han, Tatyana Tchaikovskaya, Yesim Avsar, Edgar Tafaleng, Sanal Madhusudana Girija, Krisztina Tar, Zsuzsanna Polgar, Stephen Strom, Eric E. Bouhassira, Chandan Guha, Ira J. Fox, Jayanta Roy-Chowdhury, and Namita Roy-Chowdhury

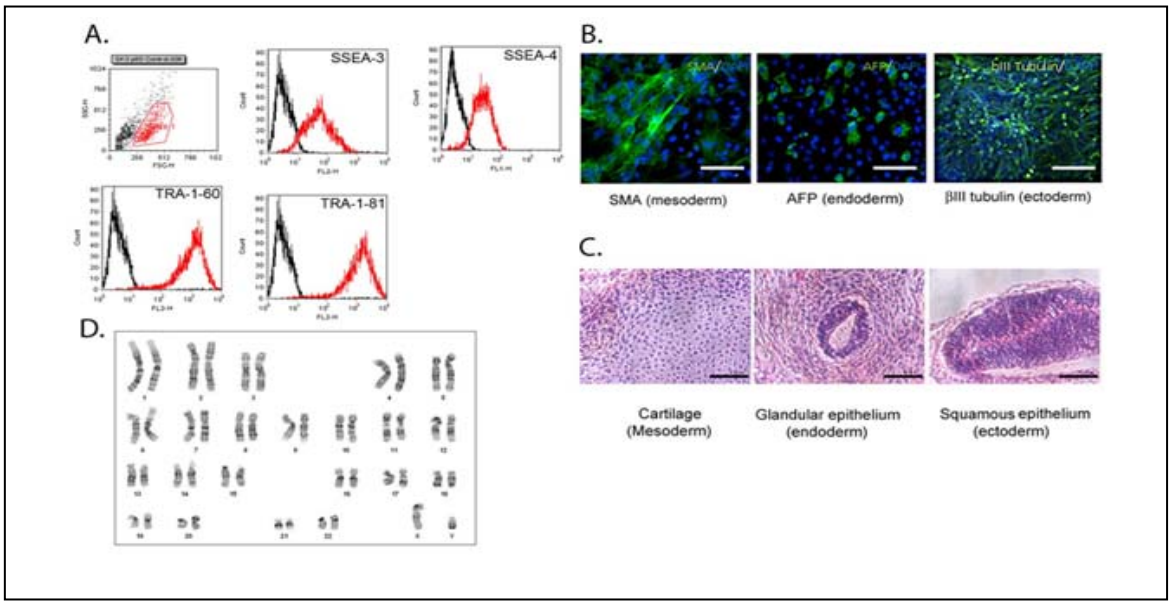

Figure S1. ***Characterization of induced pluripotent stem cells:Related to hiPSCs shown in Figure 1.*** **A.** Flow cytometric analysis of surface markers (SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) of skin fibroblast-derived iPS cells. Primary mouse monoclonal antibodies specific for the respective surface proteins (red) or control non-immune IgG of the same isotype (black) were used, along with FITC-labeled anti-mouse IgG secondary antibodies. **B.** In vitro differentiation of human iPS cells. Embryoid bodies from iPS cells were plated on gelatin-coated chamber slides, and cultured in ES cell medium for seven days. Staining with anti-smooth muscle (SMA), AFP and anti-β-III tubulin antibodies indicated that the iPS cells could differentiate into cells derived from mesoderm, endoderm and ectoderm, respectively. Magnification 40x. **C.** Teratoma formation: iPS cells were injected intramuscularly in SCID mice. Teratomas were dissected after six weeks. Hematoxylin/eosin staining of teratoma sections show differentiation of iPS cells to tissues derived from mesoderm (cartilage), endoderm (glandular epithelium) and ectoderm (squamous epithelium). Bars = 100 microns. **D.** Karyotyping of iPS cells, performed as described in the Methods section, showed a 44 + X,Y pattern.

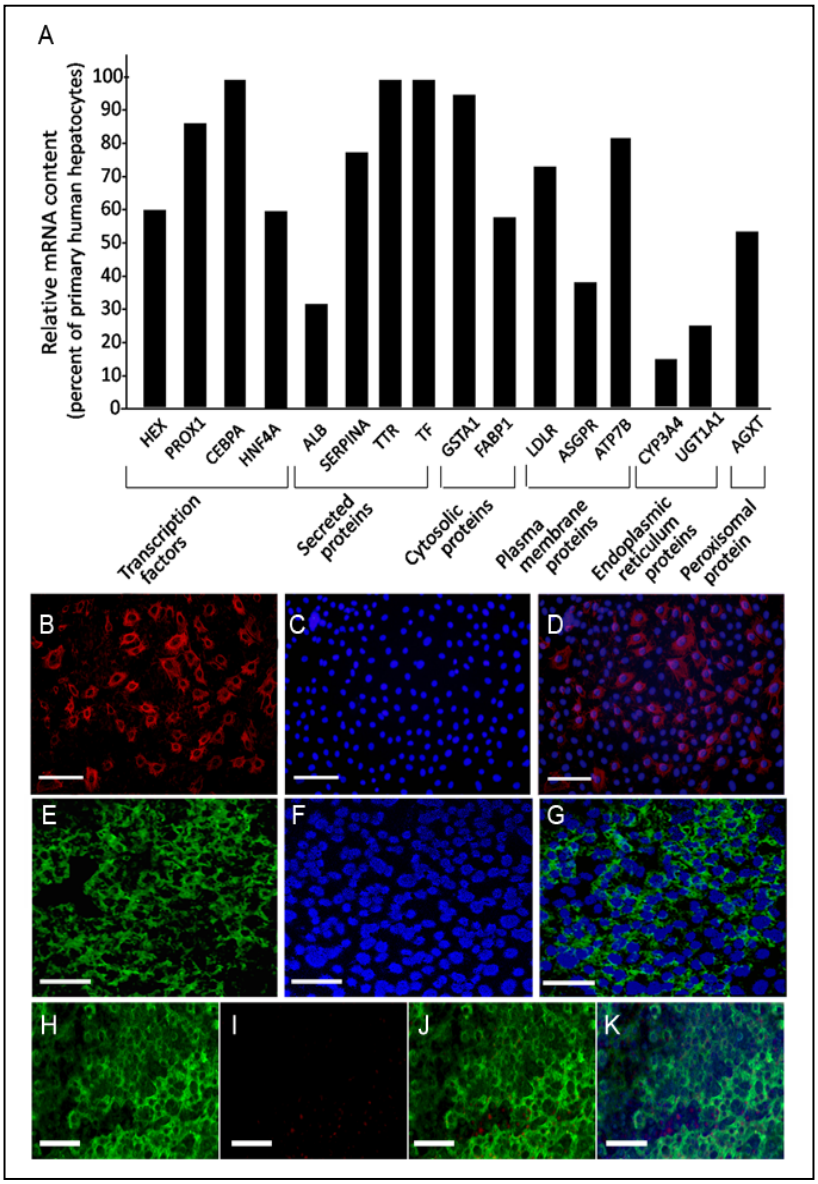

Figure S2. ***mRNA profile of iHep cells, and SOX17, HSA ASGPR expression.*** **Related to Figure 1 and Figure 2.** A. mRNA content of an iHep preparation was compared with that of isolated human hepatocytes that were cryopreserved and thawed before use, using a quantitative RT-PCR array. N-Acetyltransferase 1 (NAT1) mRNA was used as an invariant internal reference. Relative abundances are shown (means of three determinations). The genes are arranged according to their function or intracellular localization of their products. B,

immunofluorescent staining for ASGPR in iHep cells attached to glass slides by cytospin and immunostained without permeabilization, using a human anti-ASGPR1 monoclonal antibody; C, DAPI-stained nuclei of the same field as B; D, overlay of B and C. E, immunofluorescent staining for HSA; F, DAPI-stained nuclei; G, overlay. H-K, Double immunofluorescent staining for HSA and SOX17. H, HSA; I, SOX17; J, HSA/SOX17 overlay; panel J overlaid with DAPI-stained nuclei. Bars=100μm.

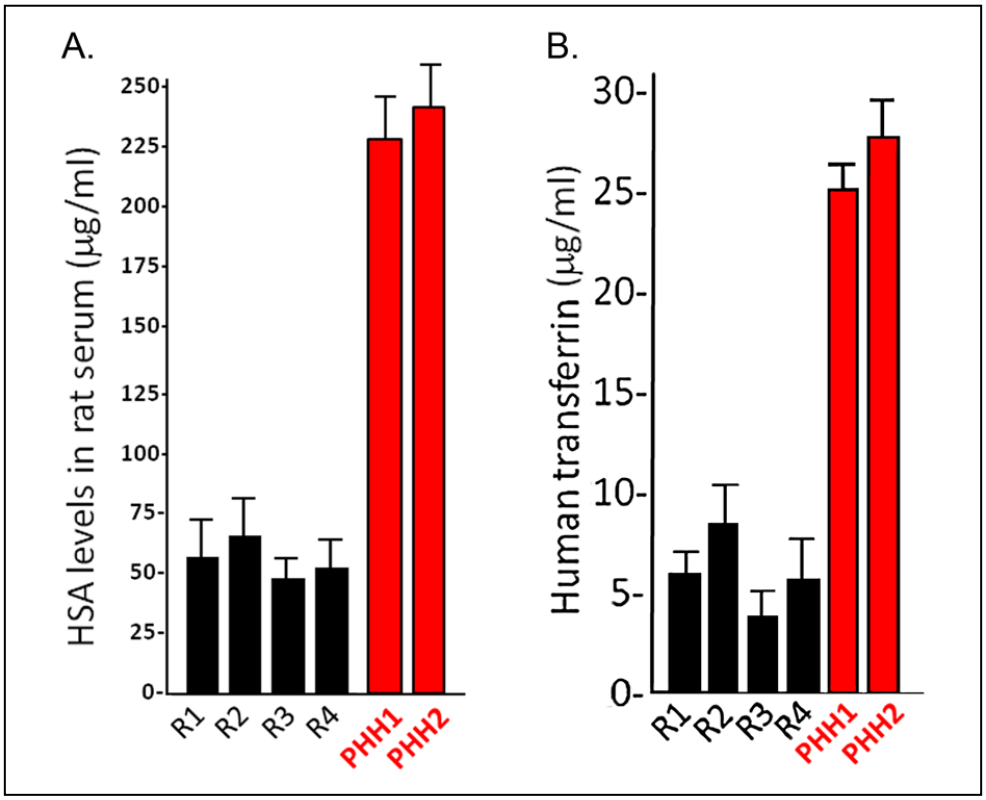

Figure S3. Human serum proteins in the rat plasma. Related to Figure 3 and Figure 4.

Plasma levels of HSA (panel A) and human transferrin (panel B) in four Gunn rats (R1, R2, R3 and R4) that received transplantation of iHep cells (black bars) and two that received primary human hepatocytes (HepCure®, Yecuris) (PHH1 and PHH2: red bars) were determined by ELISA 4 months after transplantation. Each bar represents one recipient Gunn rat, means ± SD of 4 technical replicates of each serum sample are shown.

Supplemental Experimental Procedures

Animals: Inbred Gunn rats were bred and maintained in our colony at Albert Einstein College of Medicine on standard laboratory chow in a climate-conditioned environment and a 12-h light/dark cycle. Both male and female Gunn rats weighing 150-200 g were used. All experiments were performed under humane conditions as approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Generation of iPSCs from human skin fibroblasts: Skin biopsy was obtained from an adult human volunteer after obtaining informed consent in accordance with the Declaration of Helsinki and following the approval of the institutional review board and established in culture. The fibroblasts were reprogrammed to iPSCs by transduction with four retroviruses expressing *OCT-4, SOX2, KLF4,* and *c-MYC* (Takahashi and Yamanaka, 2006) (kindly provided by Dr. S. Yamanaka). The transduced cells were cultured and passaged on Matrigel-coated plates in serum-free hESC culture medium, consisting of DMEM/F12 medium (GIBCO) containing 20% knockout serum replacement supplement (KOSR, GIBCO), 1% insulin-transferrin-selenite (ITS, GIBCO), 2.0 mM L-glutamine (GIBCO), 0.1 mM β-mercaptoethanol (Sigma), 1% non-essential amino acids (GIBCO), 1% penicillin and streptomycin (GIBCO) and 100 ng/ml basic fibroblast growth factor (bFGF, Royan Institute). Three weeks later, hiPSC colonies with hESC-like morphology were released by incubating with 1 mg/ml DMEM/F12 containing collagenase IV (GIBCO), and cultured and passaged on Matrigel-coated plates in the hESC medium (Takahashi and Yamanaka, 2006).

Flow cytometric analysis of hiPSCs and iHep cells: The pluripotency surface markers, SSEA-3, SSEA-4, TRA 1-60, TRA 1-81 that are highly expressed in hESC, and SSEA-1 which is not expressed in hESCs were tested by flow cytometry. iHeps were analyzed for HSA and ASGPR.

Single cell suspension of the hiPSCs or iHeps were prepared by washing the cells twice with CMF-PBS (Ca^{2+}, Mg^{2+}-free PBS) and dissociating with trypLE (10 minutes at 37^{o}C). After washing with CMF-PBS and staining with human-specific antibodies (for HSA, DAKO; for other antigens, BD biosciences) for 30 minutes at 4^{o}C, the cells were washed twice with CMF-PBS + 1% FBS and then analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). Negative controls included isotype-specific IgGs.

Spontaneous differentiation of hiPSCs in vitro: To evaluate whether the iPSCs could give rise to cells of all germ layers in vitro, we generated embryoid bodies (EBs) by plating collagenase-dispersed cells on low-attachment dishes for 48 hours in DMEM/F12 supplemented with 15% knockout serum replacement (KSR) containing 1 mmol/L nonessential amino acids, and L-glutamine. EBs were then transferred to gelatin-coated chamber slides and cultured for seven more days. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Saponin (Sigma) and stained with antibodies against α-fetoprotein, β-III-Tubulin and smooth muscle actin (SMA) (R&D system), using FITC-conjugated secondary antibodies. Human OCT3/4 was stained with a PE-conjugated antibody a (BD Pharmingen). Labeled slides were mounted in Prolong Gold antifade and visualized with a fluorescence microscope (ZEISS, AxioVert 200M).

In vitro differentiation of iPSCs to iHep cells: The iPSCs were differentiated to hepatocyte-like cells as previously described for human ESCs with some modifications (*Basma et al., 2009*). Briefly, the dispersed iPS cells were plated on 5% Growth Factor Reduced Matrigel (R&D Systems, Minneapolis, MN), and cultured for 3 days in DMEM/F12 media containing 100 ng/mL recombinant activin-A (R&D Systems) and 100 ng/mL fibroblast growth factor-2 (Invitrogen). The KSR concentration was 0% for the first 24 hours, 0.2% for the second 24 hours, and 2.0% for the last 24 hours. For the following 8 days, the cells were cultured in

DMEM/F12 containing 10% KSR, 1 mmol/L nonessential amino acids, L-glutamine, 1% dimethyl sulfoxide (Sigma–Aldrich), and 100 ng/mL HGF (R&D Systems Inc), followed by culture for 3 additional days in DMEM/F12 containing 10% KSR, 1 mmol/L nonessential amino acids, L-glutamine, and 10^{-7} mol/L dexamethasone (Sigma–Aldrich).

Karyotype determination: Human iPS cells, grown in Matrigel-coated T25 flasks were processed for Karyotype analysis by Cell Line Genetics (Madison, Wisconsin). Twenty metaphase preparations were counted per karyotype.

Teratoma formation by iPS cells: The hiPSCs were dispersed by collagenase IV treatment, resuspended in ES cell medium, mixed with one-third the volume of matrigel (BD Bioscience) and 10^6 cells were injected into the leg muscles of 6-8 week old SCID mice. After 8 weeks, the visible tumors were dissected, fixed in 10% formalin and paraffin embedded sections were examined after hematoxylin/eosin staining.

Immunofluorescence staining: The cells at different stages of differentiation were fixed with 4% paraformaldehyde in PBS for 20 minutes and then blocked with PBS containing 10% normal donkey serum, 0.1% Triton® X-100 and 1% BSA for 45 minutes at room temperature. After blocking, cells were incubated overnight at 4°C with primary antibodies at dilutions indicated: hOCT3/4 (R&D system) and FOXA2 (R&D system) 10μg/ml, AFP (Dako) and serum albumin (hALB, Dako) 1:100, cytokeratin 18 (hCK18, Santa Cruz) and asialoglycoprotein receptor (hASGPR Santa Cruz) 1:200. After this the cells were incubated with secondary antibodies conjugated with FITC or Rhodamine at room temperature in the dark for one hour. Between each step, cells were washed with PBS containing 0.1% BSA.

Western blot for human AFP, albumin and UGT1A1: Cell homogenates (25 μg protein) were subjected to Western blot analysis using specific antibodies against human ALB (Dako), AFP

(Dako) and a monoclonal antibody (WP1) that recognizes human UGT1A group of enzymes (Peters et al., 1987). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Pierce).

Cytochemical staining for glycogen: After the final differentiation step, the cells were stained with the periodic acid Schiff (PAS) reagent.

Indocyanine green and low-density lipoprotein (LDL) uptake: Uptake of the organic anion indocyanin green (ICG, Sigma) was studied as described (Yamada et al., 2002). LDL uptake was determined using DiI-LDL as described (Roy-Chowdhury et al., 1991).

UGT1A1 activity of primary human hepatocytes and iHep cells toward bilirubin: After dexamethasone treatment, the cells were scraped and homogenized. UGT1A1 was activated by incubation with 5mg/ml digitonin suspension, and bilirubin glucuronidating activity was measured as described (Roy-Chowdhury et al., 1982). For this purpose, fresh frozen primary human hepatocytes were purchased from Triangle Research Labs, Research Triangle Park, NC.

Secretion of human proteins and urea in the media: iHeps and primary hepatocytes isolated from an explanted liver were plated in Matrigel-coated 6-well plates at 80% confluency and cultured in serum-free media for 16 hours. Human proteins secreted in the media were determined by ELISA using antibodies against human ALB and human α1-antitrypsin (Ding et al., 2011) (AAT, Bethyl Laboratories, Montgomery, TX). Urea secreted in the media was assayed colorimetrically using a kit (Thermo, MA).

Transplantation of iHep cells or human hepatocytes into Gunn rats: As Gunn rats are immune competent, we developed a special immunosuppression regimen to prevent xenograft rejection. Recipient Gunn rats were injected with tacrolimus 2 mg/kg, subcutaneously daily, beginning 7 days before transplantation (Basma et al., 2009). To provide a proliferative advantage to the

transplanted cells, we used hepatic irradiation (HIR) (50 Gy), which reduces the mitotic capacity of the host hepatocytes without causing large scale cell death. For mitotic stimulation, an adenovector expressing human HGF (Ad-HGF) was injected ($1x10^{12}$ particles, intravenously) as described (Roy-Chowdhury et al., 1982, Jian et al., 2008). The mitotically competent transplanted hepatocytes proliferate in response to HGF, progressively replacing the host hepatocytes (Jian et al., 2008). Taking advantage of the region-specificity of X-irradiation, we limited HIR (50Gy) to the median liver lobe (~33% of the liver mass), leaving the remaining liver unaffected, as would be performed for safety in the clinical setting. We have observed previously that repopulation of a single Gunn rat liver lobe with wildtype rat hepatocytes is sufficient to normalize serum bilirubin levels (Zhou et al., 2012). One day after HIR, we transplanted iHep cells by intrasplenic injection (Guha et al., 2002). Based on a prior determination of cell viability and the percentage of cells positive for HSA, we transplanted $2x10^6$ HSA positive viable iHep cells. For comparison, we transplanted $2x10^6$ viable cryopreserved human hepatocytes isolated by collagenase perfusion of the livers of fumarylacetoacetate hydrolase (Fah) knockout, and the Rag2 and Il2rγ deficient mice (FRG) repopulated with human hepatocytes (HepCure, Yecuris, Tualtin, OR). Two control groups were studied also: (1) six age-matched Gunn rats receiving tacrolimus and Ad-HGF, but no other treatment; (2) four sham operated controls that received HIR to the median lobe, but no transplantation.

Immunocytochemistry and immunofluorescence staining of transplanted iHeps: After 4-6 months of transplantation liver tissues were fixed, cut and stained immunohistochemically using the monoclonal antibody WP1 against human UGT1A isoforms (Peters et al., 1987). Livers of untreated Gunn rats were used as control.

Quantification of liver repopulation by iPSC-Heps by genomic DNA PCR: To determine the proportion of iHeps or primary human hepatocytes in the recipient liver, a standard curve was prepared by mixing human liver homogenates into Gunn rat liver homogenates (10 ml/gm liver wet weight) at various ratios from 0% (i.e. only Gunn rat liver homogenate) to 100% (i.e. only human liver homogenate). Genomic DNA was extracted from homogenates of the preconditioned and other liver lobes. The human *HLA-A54* gene was quantified by DNA PCR using the following amplimers: Forward: 5'-GCTCAGTTCCAGTTGCTTG-3'; Reverse: 5'-GCAGTGAGCCAAGATTGCAC-3'. The PCR mixture contained Express SYBR GreenER Super Mix with Premixed ROX) 12.5 μl, genomic DNA template 100 ng in 1 μl, forward and reverse primer 10 μmoles and double distilled water 10 μl. PCR was performed up to 40 cycles using the following program: 50°C 2 min and 95°C 10 min, followed by 95°C 15 sec and 60°C. As an internal standard, we amplified the *36B4* gene using amplimers that yield amplicons from human and rat genomic DNA with equal efficiency. The primers were as follows: Forward: 5'-GCGACCTGGAAGTCCAACTAC-3'; reverse: 5'-ATCTGCTGCATCTGCTTGG-3'. The extent of liver repopulation was calculated by fitting the results of qPCR on the DNA extracted from liver lobes of the recipient rat to the standard curve generated from the mixtures of Gunn rat and human liver homogenates.

Measurement of serum bilirubin: Serum samples at various time points before and after transplantation were measured (Jendrassik and Grof, 1938). Serum bilirubin levels of age-matched control Gunn rats were determined for comparison.

Assay of human proteins in recipient rat sera: Human serum albumin and alpha-1 antitrypsin concentrations were determined in the sera of recipient Gunn rats by ELISA, employing human-specific antibodies as described (Bumgardner et al., 1998)).

UGT1A1 activity in the liver of the transplant recipient Gunn rats: UGT1A1 activity toward bilirubin was assayed in the liver homogenates of the transplant recipient Gunn rats and the untreated controls as described (Roy-Chowdhury et.al.,1981).

Analysis of pigments excreted in bile: Bile samples were collected by cannulating the bile ducts with PE10 catheters and the bilirubin species excreted in bile were analyzed by High Pressure Liquid Chromatography as described (Roy-Chowdhury et al., 1982). Bile samples from untreated Gunn rats and congeneic wildtype Wistar-RHA rats were used as controls.

Supplemental References

Bumgardner, GL., Heininger, M., Li, J., Dongyuan, X ., Parker-Thornburg, J., Ferguson, RM.and Orosz, CG. (1998) A functional model of hepatocytes transplantation for in vivo immunologic studies.Transplantation, **65**, 53-61.

Ding, J., Yannam, GR., Roy-Chowdhury, N., Hidvegi, TH., Basma, H., Rennard, SI., Wong, RJ., Avsar, Y., Guha, C., Perlmutter, DH., Fox, IJ., and Roy-Chowdhury, J. (2011) Spontaneous hepatic repopulation in transgenic mice expressing mutant human alpha 1-anti-trypsin by wild-type donor hepatocytes. J. Clin. Invest. **121**,1930-1934.

Jendrassik, L., and Gróf, P. (1938) Vereinfachte photometrische methoden zur bestimmung des blut-bilirubins. *Biochem. Zeitschrift.* **297**: 82-89.

Jian, J., Salido, EC., Guha, C., Wang X, Moitra, R., Liu, L., J. Roy-Chowdhury, J., and Roy-Chowdhury, N. (2008) Correction of Hyperoxaluria by liver repopulation with hepatocytes in a mouse model of primary hyperoxaluria type -1. Transplantation, **85,** 1253-1260.

Peters, W., Allebes, WA.,Jansen, PLM., Poels, LG., Capel, PJA. (1987) Characterization and tissue specificity of a monoclonal antibody against human uridine 5'-diphosphate glucuronosyltransferase. Gastroenterology **93**, 162-169.

Roy-Chowdhury, J., Grossman, M., , S., Roy-Chowdhury, N., Baker, JR., Wilson, JM. (1991) Long term improvement of hypercholesterolemia after *ex vivo* gene therapy in LDL-receptor deficient rabbits. Science **254**, 1802-1805.

Roy-Chowdhury, J., Roy-Chowdhury, N., Gartner, U., Wolkoff, AW., and Arias, IM. (1982) Bilirubin diglucuronide formation in intact rats and in isolated Gunn rat livers. J. Clin. Invest. **699**: 595-603.