Directed differentiation of Dental Pulp Pluripotent-like Stem Cells into Hepatocyte-like Cells

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Running Title: Hepatocyte generation from DPPSCs

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Abstract: Liver diseases are a major cause of morbidity and mortality. Dental pulp pluripotent- like stem cells (DPPSC) are a considerable promise in tissue engineering and regenerative medicine as a source of tissue-specific cells, therefore this study aims to demonstrate their ability to generate functional hepatocyte-like cells *in vitro*. Cells were differentiated on a collagen scaffolds in serum free media supplemented with growth factors and cytokines to recapitulate liver development. At day 5, the differentiated DPPSC cells expressed the endodermal markers FOXA1 and FOXA2. Then, the cells were derived into the hepatic lineage generating hepatocyte-like cells. In addition to the associated morphological changes, the cells expressed the hepatic genes HNF6, AFP. The terminally differentiated hepatocyte-like cells expressed the liver functional proteins albumin and CYP3A4. In this study, we report an efficient serum-free protocol to differentiate DPPSCs into functional hepatocyte-like cells. Our approach promotes the use of DPPSCs as a new source of adult stem cells for prospective use in liver regenerative medicine.

Keywords: hepatocyte differentiation; adult stem cells; dental pulp pluripotent stem cells; DPPSCs

1. Introduction

The liver is the largest internal organ providing essential metabolic, exocrine, and endocrine metabolites. Hepatocytes and cholangiocytes, the bile duct epithelial cells, are parenchymal cells which comprise approximately 70% of the adult liver mass. Both cell types are derived from the embryonic definitive endoderm [1]. The liver is the main body homeostasis regulator; therefore, liver diseases cause high morbidity and mortality rates. The incidence of end-stage liver disease (ESLD) is increasing worldwide [2] due to alcohol induced liver disease, nonalcoholic fatty liver disease, or hepatitis. Orthotopic liver transplantation (OLT) is the current optimal treatment with more than 70% of the overall 5-year survival rate for certain ESLD cases [3]. OLT obstacles include donor availability, surgical risk, high costs, and the requirement for life-long immunosuppressors [4; 5; 6]. Alternatively, hepatocyte transplantation (HT) is a promising procedure, less invasive, can be performed repeatedly, used mainly to replace an inborn deficient metabolic function [7] [8]. Yet, HT hurtles include donor availability, low cell engraftment, and longevity [9]. Therefore, the scientific community has put an effort to improve cell-based hepatocyte generation.

Dental pulp stem cells (DPSCs) are a multipotent heterogeneous cell population embedded within the pulp cavity of impacted third molars. DPSCs were initially isolated and characterized by Gronthos *et. al.* [10]. Subsequently, several investigators reported DPSCs' isolation, characterization, differentiation, and banking [11; 12]. In comparison with embryonic and other adult stem cells, DPSCs are obtained from disposable dental pulp after occlusion management, which is considered as a medical waste, noninvasive isolation procedure, not associated with ethical constraints, and no risk of teratoma formation. Furthermore, DPSCs do not compromise their stemness, viability, proliferation, or differentiating capabilities after cryopreservation [13; 14]. Therefore, DPSCs have the potential to be a promising personalized pa-

tient-specific stem cell source for regenerative therapy. Nevertheless, a major concern is that DPSCs comprise progenitor cell populations that are marked by diverse characteristics, such as clonal heterogeneity, multilineage differentiation, self-renewal capacity and phenotypic complexity.

In the context of cell-based hepatogenesis, DPSCs-mediated hepatocyte-like generations has advanced rapidly because of the previously accumulated differentiation protocols applied for human embryonic stem cells (hESCs) and bone marrow mesenchymal cells (BM-MSCs) as prospective sources for regenerative hepatocytes [15; 16; 17; 18; 19; 20]. Implementing defined, serum-free and stepwise differentiation protocols that mimic hepatocytes' developmental stages during embryogenesis are sufficient for inducing hepatogenesis. Using this approach, Ishkitiev *et. al.* were the pioneers to demonstrate the hepatogenic differentiation potential of DPSCs. Initially, Ishkitiev *et. al.* developed two staged conditioned media containing a low percentage of fetal bovine serum (FBS) but enriched with essential hepatogenic inducers [21]. Later, they used serum-free conditioned media to generate hepatocyte-like cells from $CD117^{+}$ DPSCs subpopulation [22]. The latter hepatogenesis protocol utilized three developmental stages, i.e., cell specification, differentiation, and maturation, to generate cells with morphological, phenotypical, and functional characteristics like hepatocytes. This approach was further improved and implemented in later studies [11; 23]. Nevertheless, the Heterogeneity nature of the DPSCs influences their differentiation capacity and yield, therefore, we focused our efforts to identify and characterize the differentiation potential of a defined subpopulation of DPSCs with pluripotent-like phenotype [24; 25; 26; 27; 28].

Dental pulp pluripotent stem cells (DPPSCs) are a unique sub-population of DPSCs [25], expressing the pluripotency markers Oct4A, NANOG, and SOX2 [24; 29; 30]. DPPSCs exhibits a normal human karyotype with no aneuploidy, polyploidy, or any chromosomal abnormality

during the metaphase stage even after more than 65 passages [24; 31]. Unlike other adult stem cells, DPPSCs form teratoma-like structures when injected subcutaneously in immunodeficient mice, and generate embryonic bodies like in *in vitro* cultures, which are exclusive phenotypes of hESCs and induced pluripotent stem cells (iPSCs) iPSCs [24; 32; 33; 34]. Furthermore, DPPSCs can be differentiated into cells from each of the three germ-embryonic layers, including osteocytes and bone- [25; 26], endothelial- and neural- like cells [25; 26], smooth and skeletal muscles [27].

In this study, we evaluated the adult DPPSCs- mediated hepatogenic capacity using a novel serum free- directed- step wide differentiation protocol and collagen scaffolds to induce 3-dimentaial (3D) culture conditions. DPPSCs were first differentiated into definitive endoderm (DE), which were then directed to the hepatogenic lineage. We observed that treatment of DPPSCs, seeded on scaffolds with Activin A (Act A), Wnt Family Member 3A (Wnt3A), and knockout serum replacement (KOSR) conditioned media, enhances their commitment capacity toward DE- lineage. Subsequently, treatment with fibroblast growth factor 4 (FGF4) and hepatocyte growth factor (HGF) induced gut tube formation, early hepatogenic markers, and finally, functional hepatocyte-like cells were generated in conditional differentiation medium supplemented with HGF, FGF4, dexamethasone (Dex) and oncostatin M (OSM). Preliminary results were previously published in a preprint [35].

2. Materials and methods

2.1. Ethics Approval and Statement

All research in the present study was conducted in accordance with the code of ethics of the World Medical Association for experiments involving humans (Helsinki Declaration of 1975) and the guidelines on human stem cell research issued by the Committee of Bioethics at the Universitat International de Catalunya, Spain.

127 *2.2. DPPSCs Culture and Maintenance*

128 DPPSCs clones were previously isolated and characterized by our group as previously de-
129 scribed [24; 31]. In Brief, immediately after extraction, the third molars were vigorously
130 washed with 70% ethanol and sterile distilled water. The molar pulp tissues were extracted,
131 and cells were isolated by digesting the pulp tissue with collagenase type I (3 mg/ml, Sig-
132 ma-Aldrich, Germany) for 60 minutes at 37°C. Then, cells were separated mechanically with
133 an insulin syringe and centrifuged for 10 minutes at 1800 rpm. Primary cell lines were estab-
134 lished, the medium was changed every 4 days, and cell density was maintained at a low den-
135 sity of 80-100 cells/cm^2. DPPSCs were cultured in precoated flasks with 100 ng/ml fibronectin
136 (Life Technologies, Waltham, MA, USA) in a medium consisting of 60% DMEM-low glucose
137 (Life Technologies) and 40% MCDB-201 (Sigma-Aldrich); supplemented with 1X SITE Liquid
138 Media Supplement (Sigma-Aldrich); 1X linoleic acid-bovine serum albumin (LA-BSA, Sig-
139 ma-Aldrich); 10^{-4} M L-ascorbic acid 2-phosphate (Sigma-Aldrich); 1X Penicillin-Streptomycin
140 (Life Technologies); 2% fetal bovine serum (FBS, Sigma-Aldrich); 10 ng/ml hPDGF-BB (Abcam,
141 Waltham, MA, USA); 10 ng/ml EGF (Sigma-Aldrich); 1000 U/ml LIF (Millipore, USA); chemically
142 defined Lipid Concentrate (Life Technologies); 0.8 mg/ml BSA (Sigma-Aldrich) and 55 µM
143 β-mercaptoethanol (Sigma-Aldrich).

144 *2.3. Definitive Endoderm Induction using Growth Factors*

145 For the DE- induction, 5x10^4 cells/cm^2 were seeded on 6-well plates (Cell Coat, UK) and
146 cultured in RPMI medium (Mediatech, Los Altos, CA, USA) containing GlutaMAX (Life Tech-
147 nologies), penicillin/streptomycin, 0.5% defined fetal bovine serum (FBS, HyClone, Cytiva Eu-
148 rope GmbH, Spain) and supplemented with each one of the following combinations of growth
149 factors: (i) 100 ng/ml Act A (R&D Systems, UK), (ii) 100 ng/ml Act A + 50 ng/ml Wnt3A (R&D
150 Systems), (iii) 100 ng/ml Act A + 50 ng/ml bone morphogenetic protein-4 (BMP4, R&D Sys-

151 tems), (iv) 100 ng/ml Act A + 50 ng/ml Wnt3A + 50 ng/ml BMP4, (v) 100 ng/ml Act A + 10
152 ng/ml FGF4 (R&D Systems) or (vi) 100 ng/ml Act A + 10 ng/ml basic-FGF (bFGF, Life Technolo-
153 gies). Three days post-induction, the medium was changed to the same RPMI-based medium
154 and supplements except for the replacement of FBS with 2% KOSR (Life Technologies) and the
155 incubation was maintained for another 2 days.

156 *2.4. Definitive Endoderm Induction Using Different Biomaterials*

157 Application of biomaterials to cell culture has been shown to enhance cellular program-
158 ming and differentiation. In this study, we used collagen I or fibronectin, the most abundant
159 components of the extracellular matrix that provide a structural network for the cells micro-
160 environment and were reported to enhance DE- differentiation *in vitro* [36; 37]. Using the
161 sample induction media described above, cells were seeded on plates coated with collagen
162 type I, 100 ng/ml fibronectin, or were co-cultured with HepG2 cells (20,000 cell/cm^2), in addi-
163 tion to the controls. 2.5. Hepatogenic differentiation

164 After setting the optimal conditions for DE-induction; we aimed to generate stepwise
165 functional hepatocyte-like cell. The gut tube and hepatocyte specifications were initiated us-
166 ing RPMI medium supplemented with 2% KOSR, 10 ng/ml FGF4, and 10 ng/ml HGF (R&D Sys-
167 tems). Three days later, RPMI was replaced with the enriched minimum essential medium
168 (MEM, Sigma-Aldrich) supplemented with 1% BSA, 10 ng/ml FGF4, and 10 ng/ml HGF. Three
169 days later, hepatocyte maturation was induced using complete hepatocyte culture medium
170 (HCM, Single-quote, Lonza, USA) with all supplements according to the manufacturer recom-
171 mendations; containing 10 ng/ml FGF4, 10 ng/ml HGF, 10 ng/ml OSM and 10^{-7} M Dex (Sig-
172 ma-Aldrich). The hepatocyte maturation was proceeded for nine days as previously reported
173 by Ishkitiev *et. al.* [21; 22]. The differentiation protocol was performed for a total of 22 days,
174 and the media were refreshed every 2-3 days.

175 *2.6. Immunofluorescence microscope imaging*

176 Immunofluorescence was performed as described previously [38; 39]. Briefly, cells were
177 cultured overnight on glass coverslips coated with collagen I, fixed with 4% paraformaldehyde
178 for 15 min, and permeabilized with 10% Triton X-100 for 30 min. Then, cells were blocked
179 with 5% BSA in PBS for 30 min and incubated for one hour with the primary antibodies: al-
180 pha-1 antitrypsin (AAT; Abcam) or albumin (Alb; Abcam). Followed by one-hour incubation
181 with prospective secondary antibodies (Abcam). Between each step, cells were washed with
182 1% BSA in PBS. Fluorescent images were captured using a fluorescence microscopy Olympus
183 AX70 (Olympus Optical, Tokyo, Japan) as previously described [40; 41].

184 *2.7. Immunocytochemistry*

185 Cells were fixed with Thin Prep-CytoLyt solution for 30 min at room temperature and
186 centrifuged at 5,000 rpm for 5 min. Cell's pellet was solubilized in Thin prep-CytoLyt solution
187 and then resuspended in PreservCyt solution (Thinprep) for 15 min and processed on a
188 ThinPrep 2000 processor. The microscope slides harboring the processed cells were fixed with
189 96% ethanol, washed twice with distilled water, and blocked in 0.5% hydrogen perox-
190 ide/methanol for 10 min. Immunostainings were performed using a Leica Bond-MAX auto-
191 mated IHC and ISH *Stainer* in accordance with the manufacturer's instructions. Briefly, the
192 slides were washed with bond wash solution (Biosystems, USA) and the antigens retrieval
193 procedure was performed in accordance with Bond heat standard protocol (ER1) using citric
194 buffer, pH 6, for 30 min at 95°C. Slides are washed and treated with post-primary solution for
195 8 min. Next, the slides were incubated in Polymer AP for 8 min and washed. Then, the mixed
196 Diaminobenzidine Refine solution was applied for 10 min. Samples were counterstained with
197 water hematoxylin for 5 min. Non-immune immunoglobulins of the same isotype as the pri-
198 mary antibodies were used as a control for each experiment.

199 *2.8. RNA Extraction, cDNA Synthesis and qRT-PCR Reactions*

200 Total RNA was extracted from cells using the total RNA purification Trizol Reagent (Life
201 Technologies) in accordance with the manufacturer's protocol. RNA was quantified using a
202 NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA
203 integrity was evaluated by 2% agarose gel electrophoresis [42]. First strand cDNA was synthe-
204 sized from 200 ng RNA by reverse transcription using QuantiTect Reverse Transcription Kit
205 (Qiagen Inc., Germantown, MD, USA). qRT-PCR reactions were performed as previously de-
206 scribed [43; 44]. Primer pairs with equivalent efficiencies (Table 1) were selected from Primer
207 Bank [45] or designed using primer-BLAST tools
208 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) [46]. qRT-PCR was performed on the
209 ABI7900 system (Applied Biosystems, USA) using SDS software. Relative gene expression was
210 calculated using a comparative Ct method as previously described [47; 48]. Results were
211 normalized to GAPDH CT-values, and the relative expressions were determined relative to
212 control or Day 0 undifferentiated cells. Data show Means ± standard deviation. Total liver
213 RNA (Life Technologies) was used as a positive control.

214 *2.9. Hepatic Biochemical Analysis of Supernatants*

215 Hepatic enzymatic profiles for aspartate transaminase (AST), alanine aminotransferase
216 (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were analyzed from
217 supernatant extracts of the different samples. The activity was measured by specific colori-
218 metric detection kit (Linear Chemicals, Spain) at 25°C in accordance with the manufacturer's
219 instructions. The specific compounds used for the following kinetics of the reaction were as
220 the following: for AST and ALT, the oxidation of NADH was measured at
221 light *absorbance* at 340 nm; for ALP, the formation of 4-Nitrophenol was measured at

222 light *absorbance* at 405 nm; for GGT, the catalysis of γ-glutamyl-3-carboxy-4-nitroanilide was
223 measured at light *absorbance* at 410 nm using spectrophotometer (Thermo Fisher Scientific).

224 *2.10. Cytochrome P450 3A4 Metabolic Activity Assay*

225 Cytochrome P450 3A4 (CYP3A4) enzyme activity assays were assessed by measurement
226 of luciferase activity using P450-Glo CYP3A4 assay kit (Promega, Madison, WI, USA) in ac-
227 cordance with the manufacturer's instructions. Differentiated and undifferentiated control
228 DPPSC cells were treated with 25 µM Rifampicin for 48 hrs. Then, treated cells were incubat-
229 ed with a fresh serum-free medium containing 50 µM Luciferin PFBE with or without 5 µM
230 Erythromycin (an antagonist) for 30 min at 37°C. Then, 50 µL of the medium was transferred
231 into a 96-well plate and mixed with 50 µL luciferin detection reagent to initiate the lumines-
232 cent reaction. After 20 min incubation at room temperature, the luminescence was measured
233 with a luminometer (biotek, USA).

234 *2.11. Albumin Assay*

235 Alb secretion was measured by using an Albumin Fluorescence Assay Kit (Fluka, USA) in
236 accordance with the manufacturer's instructions. Briefly, a calibration curve was generated
237 using the kit- supplied standard human Alb concentrations. These calibration samples were
238 mixed with albumin Blue 580 reagent pre-diluted in buffer solution. The fluorescence was
239 measured in a spectrophotometer (absorbance wavelength peal, λex = 600 nm, and λem =
240 630 nm. Differentiated and undifferentiated control DPPSCs were mixed with the same rea-
241 gents and the fluorescence was measured under the same conditions. Results are extrapo-
242 lated to the calibration curve. Conditioned medium was collected from equivalent numbers of
243 cells.

244 *2.12. Periodic acid–Schiff staining for glycogen accumulation*

245 At differentiation day 22, differentiated and undifferentiated control DPPSCs were fixed
246 in 4% formaldehyde for 15 min at room temperature. After two washing steps with PBS, cells
247 were incubated in 1% periodic acid for 5 min, then washed with distilled water. The treated
248 cells were incubated with Schiff's reagent (Sigma-Aldrich) for 15 min. After a 10 min wash in
249 distilled water, hematoxylin counterstain solution was applied for 90 sec and washed with
250 distilled water.

251 *2.13. Statistical analysis*

252 Results are reported as mean ± standard deviation (see Figure legend for specific details
253 regarding the number of biological replicates, independent experiments, and technical repli-
254 cates). Statistics were performed using the Statgraphics XVI software. The methods used were
255 two-tailed Student's t-test and ANOVA for multiple factors. Values with $p<0.05$ were consid-
256 ered statistically significant.

257 **3. Results**

258 *3.1. Definitive Endoderm Induction*

259 Using hESCs, iPSCs, and human umbilical cord Wharton's jelly mesenchymal stem cells,
260 studies have shown that Act A is the main DE induction factor [49; 50; 51; 52]. Therefore, we
261 tested this concept on DPPSCs using Act A with or without other signaling factors to delineate
262 the optimal conditional media sufficient to mediate DE- differentiation. Quantitative real-time
263 PCR (qRT-PCR) analyses demonstrated a differential induction of the DE genes Forkhead Box-
264 es A1 and A2 (FoxA1 and FoxA2) at day 5. FoxA1 transcripts elevated significantly in condi-
265 tional media supplemented with Act A and Wnt3A, 14-fold relative to undifferentiated cells
266 (Fig 1A). Similar induction levels were also observed in cells treated with Act A alone or Act A
267 and BMP4, but albeit to a lower degree (6- to 8-fold) and notable variations between differ-

268 ent experiments. Alternatively, FoxA2 expression levels were comparable in all tested condi-
269 tional media, with a statistically significant 20-fold induction in media supplemented with Act
270 A alone, and with Wnt3A or BMP4. Remarkably, conditional media supplemented with Act A
271 and bFGF or FGF4 failed to significantly upregulate the studied DE-genes. Moreover, qRT-PCR
272 analyses indicated an elevation in the transcript levels of GATA4, with a 40 to 60-fold increase
273 relative to undifferentiated cells, post-treatment with Act A alone or in the presence of
274 Wnt3A or BMP4 (Fig. 1A). In contrary, no GATA4 transcripts were detected in conditional me-
275 dia containing the three signaling factors together (Fig. 1A). Noticeably, Act A failed to induce
276 GATA4 in media supplemented with either bFGF or FGF4 (Fig. 1A).

277

Worthy to note that at day 5, no significant upregulation of visceral endoderm and the early hepatogenic marker alpha-fetoprotein (AFP) in cells treated with the studied conditional media, suggesting that Act A and other signaling molecules do not support visceral endoderm lineage differentiation and are not sufficient for hepatogenic specification.

Taking together, the increase in expression levels of bona-fide DE- markers in conditional media supplemented with Act A alone or with Wnt3A suggests that our approaches using chemically defined- differentiation conditions has successfully enriched the DE- lineage of DPPSCs. Due to the experimental variations in the transcript levels of the DE- genes in cells treated with conditional media supplemented with containing Act A alone, therefore, the following experiments were carried out using Act A and WNT3A as the main induction cytokines.

It is well documented that of biomaterials in cell cultures supports viability, differentiation, maturation, and long-term function [36; 53]. To further improve the differentiation protocol and study the influence of biomaterials on DPPSCs mediated DE- indication, we perused DE- differentiation under three different conditions: cells were cultured on plates precoated with collagen I, fibronectin, or co-cultured with HepG2 cells for 5 days.

Interestingly, the DE-marker gene expression was significantly elevated in DPPSCs differentiated on the scaffolds (Fig. 1B) versus on no scaffolds (Fig. 1A). Relative to DPPSCs differentiated on plain plates (Fig. 1A), FOXA1 transcript levels in cells differentiated on collagen I or fibronectin were 3- to 4-folds higher, respectively. Similarly, the expression levels of FOXA2 mRNA were elevated by 1.5- to 3-fold when cells were differentiated on the scaffolds (Fig. 1B). qRT-PCR analyses of GATA4 transcripts were significantly enriched in cells differentiated with collagen I or fibronectin, 120-fold increase relative to undifferentiation cells, at day 5 (Fig 1B). No DE-marker gene expressions were detected in DPPSCs co-cultured with HepG2, suggesting a differentiation suppression effect of hepatic cells (Fig. 1B). Furthermore, the used

302 biomaterials did not facilitate prospective generation of visceral endoderm cells and did not
303 support early hepatogenic specification, since AFP transcripts were not detected. In summary,
304 biomaterial usage enhanced DPPSCs- mediated DE-differentiation. Since collagen I is an af-
305 fordable, inexpensive substrate, and commonly used in stem cell studies, we use it as the
306 scaffold to induce hepatogenic differentiation.

307 *3.2. Hepatic Specification and Maturation.*

308 We investigated the developmental progress in the hepatogenic differentiation potential
309 of DPPSCs using two-step protocol, which is schematically illustrated and presented in Fig. 2.
310 At day 5 post DE-induction, the conditional media were supplemented with FGF4 and HGF, for
311 8 days, to enrich for hepatic cells specification, followed by 8 days culture in HCM supple-
312 mented with hepatogenic inducers such as OSM and Dex in addition to the growth factors
313 (Fig. 2).

314 As we have previously reported, DPPSCs grow as a flat monolayer and exhibit a small
315 spindle-shaped fibroblast-like morphology when cultured on polystyrene tissue culture plates
316 or scaffolds (Fig. 3A) [25]. During the differentiation induction period, DPPSCs morphology
317 further changed to a round or epithelioid shape, a characteristic of DE- cells Fig 3 B) [54]. At
318 day 7, the cells became flat and tightly packed; and by day 22, they adopted a hepatocyte-like
319 morphology (Fig 3 C-E). In contrast, undifferentiated cells cultured in regular growth medium
320 containing FBS, leaned toward an elongated spindle-shaped fibroblastic morphology (Fig. 3F).

321 At the molecular level, gene expression studies revealed a distinct early posterior foregut
322 signature at day 7 of DPPSCs differentiation. The transcript levels of hepatic nuclear factor 6
323 (HNF6) and cytokeratin 19 (CK19) were notably elevated at day 7, relative to undifferenced
324 cells (Fig. 4), indicating a significant commitment toward the foregut tube. Although the tran-
325 scripts of GATA4 were upregulated at day 5 (Fig. 1B), simultaneously with the DE- markers, its

326 elevated expression levels were maintained until day 7. Unlike, FoxA1 mRNA levels which
327 were significantly reduced at day 7. This observation is likely due to a possible commitment of
328 a small cell fraction to DE- lineage and/or a significant profile of these genes in the early pos-
329 terior foregut (Fig. 4) [52; 55].

330 Next, the developmental progress of differentiated DPPSCs toward hepatogenic specifi-
331 cation was investigated at day 14 of treatment with conditional media. A notable reduction in
332 the expression levels of the foregut tube markers was observed, concomitant with a signifi-
333 cant increase in AFP transcripts (Fig. 4), which was not observed in earlier stages of differen-
334 tiation, indicating a progression toward early hepatic specification. In addition, moderate lev-
335 els of hepatic markers' transcripts were noticed at day 14 (Fig. 4), including hepatic nuclear
336 factor 4α (HNF4α), glucose 6-phosphate (G6P), and Cholesterol 7 alpha-hydroxylase (CYP7A1).

337 Simultaneously, eight days treatment of these committed DPSSCs with HCM supple-
338 mented hepatic induction molecules resulted in a significantly exaggeration in the expression
339 levels of the hepatic markers at differentiation day 22. Relative to undifferentiated DPPSCs,
340 the HNF4α and Alb transcripts were 40-and 15-fold higher, AAT and CYP7A1 transcripts were
341 20-fold higher, and G6P mRNA levels were 100-fold higher at day 22 (Fig. 4). Taking together,
342 we implemented an optimized 3-stage protocol that directed a stepwise differentiation of
343 DPPSCs to the hepatocyte-like cell populations.

344 *3.3. DPPSCs-derived hepatocyte-like cells demonstrate hepatic function:*

345 Next, to corroborate the qRT-PCR analysis and evaluate the differentiation efficiency, we
346 ascertained the protein expression of the hepatogenic markers by immuno-localization and
347 functional analysis. Immunofluorescence with specific antibodies directed against AAT and
348 Alb proteins revealed a significant expression of these hepatic markers at day 22 of DPPSCs
349 differentiation (Fig. 5A-B).

350 Mature hepatocytes are characterized by their ability to store glycogen [56]. Interesting-
351 ly, most of the differentiated DPPSCs showed a significant positive periodic acid Schiff (PAS)
352 staining in the cytoplasm, albeit to a different degree, at Day 22 (Fig. 5C), which is indicative
353 for glycogen storage, and the generation of mature hepatocyte-like cells. Alternatively, no
354 PAS staining was detected in undifferentiated cells (Fig. 5D).

355 Then, we investigated the activity of hepatic metabolic enzymes. Time course studies
356 performed on protein extracts from differentiated DPPSCs for GGT, AST, ALP, and ALT activi-
357 ties revealed a time-dependent upregulation of liver metabolic enzymes which beaked at day
358 18 of differentiation (Fig. 6A-D, respectively). Furthermore, the enzymatic activity of CYP3A4
359 was significantly induced in differentiated DPPSCs post treatment with rifampicin, 5-fold rela-
360 tive to untreated cells. Co-treatment with erythromycin, a CYP3A4 inhibitor, significantly
361 down-regulated the observed enzymatic action against rifampicin (Fig. 6E).

362 Alb secretion was assayed at the time course of the differentiation process. As shown in
363 Fig. 6F, at earlier stages of hepatogenesis, day 16 of differentiation, a limited amount of se-
364 creted Alb was detected in the media. However, at differentiation day 19 and 22, the amount
365 of secreted albumin was triplicated into 3.5 ug/mg protein (Fig. 6F). Taking these observations
366 together; the described differentiation protocol (Fig. 2) efficiently induced the generation of
367 DPPSCs mediated functional hepatocyte-like cells and indicates that DPPSCs are a useful cell
368 models for hepatogenesis.

369 **4. Discussion**

370 The current strategy in stem cell differentiation protocols is to mimic cellular signaling
371 events associated with the embryonic developmental process for the lineage of interest [8,
372 47, 48]. However, *in vitro* cell differentiation gives a broad range of outcome lineages, thus,
373 the development of a protocol that significantly improves a targeted cell type is of particular

374 interest [49]. Targeted differentiation requires culturing the cells in chemically well-defined
375 media, which regulate cell signaling pathways, resulting in the generation of cell type of in-
376 terest. This approach has been successfully applied to hESCs and iPSCs [51–53]; but has yet to
377 be applied to other pluripotent cell types, including adult DPPSCs.

378 In this study, we applied an optimized 3D- 3-stages protocol directed toward a stepwise
379 differentiation of DPPSCs to hepatocyte-like cells. For efficient differentiation, cells were
380 checked at each developmental stage using lineage-specific markers. Initially, DPPSCs were
381 cultured for 5 days in differentiation media supplemented with Act A, with or without Wnt3a,
382 BMP4, or the growth factors bFGF and FGF4. Interestingly, Act A alone was sufficient to in-
383 duce DE- differentiation. Our results are in accordance with the reported role of Act A induc-
384 ing DE from pluripotent stem cells [55; 57]. It is well known that nodal signaling is required for
385 endoderm specification during the gastrulation [58]. Furthermore, DE- induction depends on
386 the duration of exposure to the stimulation factors; Agarwal *et. al.* reported that 5-days incu-
387 bations are sufficient to induce DE [49].

388 Media supplemented with Act A and Wnt3A were superior in inducing DE- lineage [57;
389 59]. Using hESCs, Mathew *et. al.* observed an enhanced DE induction using Act A and Wnt3A
390 supplemented media compared to media contained Act A alone [51]. Like hESC, in this study,
391 we observed an improved induction of DE- markers in DPPSCs cultured with Act A and Wnt3A.
392 Alternatively, the growth factors bFGF or FGF4 suppressed Act A- mediated DE induction [60].
393 Noteworthy, FGF family members are multifunctional factors. They can maintain hESC
394 pluripotency [61] or DE- mediator [62], depending on the associated conditions.

395 During liver development, BMPs signaling is crucial; however, their functional role during
396 *in vitro*- adult stem cell differentiation is not clear. Cultural media supplemented with BMP2
397 and BMP4 fail to improve hepatogenesis from adipose tissue stem cell [63]. In accordance

398 with the other studies, our results confirmed BMP4 signaling has no functional role during
399 DPPSCs differentiation into DE reconciled by Act A induction. Otherwise, studies have shown
400 that a combination of Act A and BMP4 signaling induce mesoderm formation [64]. Notably,
401 DE induction was further enhanced using three-dimensional (3D) scaffolds. Treatment with
402 either collagen or fibronectin upregulated DE markers indicated that 3D enhances the cellular
403 programming potential, which is in accordance with previously reported observations using
404 ESCs, iPSCs and other mesenchymal stem cells [36; 37; 65].

405 The use of minimal amount of FBS in differentiation media has been previously reported
406 to inhibit PI3K signaling [66], which is necessary to enhance ACT A-mediated DE generation.
407 Using 0.5% FBS during the first 5 days of DE induction, D'Amour and her team observed an
408 enhancement in the expression of Sox17 or FoxA2 in hESC [57]. In our protocol, we used 0.5%
409 FBS-media for the first 3 days of differentiation. Then, it was replaced with 2% KOSR for an
410 extra 4 days, a serum that has been proven to be effective and suitable with defined compo-
411 nents [49].At differentiation days 7-22 , KOSR was removed and hepatogenic lineage induc-
412 tion was carried on with serum-free conditional culture media. As descripted in schematic Fig.
413 2, the growth factors FGF4 and HGF were used to induce hepatogenesis, followed by the us-
414 age of OSM with Dex for the hepatocytes' maturation. The role of FGF4 and HGF in hepato-
415 cyte specification has been previously reported for both Wharton's jelly- and bone marrow
416 mesenchymal stem cells [67; 68; 69]. In accordance, both FGF4 and HGF were required to
417 mediate hepatic specification in DPPSCs. We observed an improved expression of several he-
418 patic markers including immature and mature markers at day 13 of differentiation.

419 Studies have shown that the interleukine-6 family cytokine (OSM) is required for hepato-
420 cyte maturation in combination with glucocorticoids, such as Dex [70; 71]. Additionally, it is
421 well documented that HGF, EGF, and OSM have decisive effects on the maintenance of pri-

422 mary human hepatocytes *in vitro* [72]. The combination of HGF, OSM, and Dex is widely used
423 in protocols to differentiate stem cells into hepatocytes [49; 73]. In our protocol, we applied
424 the maturation factors OSM and Dex at day 13 posting commitment to the hepatic fate. Con-
425 sequently, we documented hepatocyte-like cell maturation using cellular and molecular tech-
426 niques. After 15 days exposure to media containing OSM and Dex, we observed changes in
427 cell morphology with hepatocyte- like structures, which was associated with Alb secretion,
428 and the activation of hepatic specific enzymes.

429 After the establishment of the most optimal protocol used for DPPSC, we performed the
430 tests that were available for us to prove the efficacy of the differentiation of DPPSC into
431 hepatocyte-like cells. At the molecular level, differentiated DPPSC expressed AAT, Alb, and
432 G6P transcripts, genes that are markers for mature hepatocytes. Moreover, the protein ex-
433 pression of these markers was also documented in our study. Cellular morphological changes
434 were also detected, and mature cells acquired polygonal-shape typical for hepatocytes [74].
435 In addition, DPPSCs mediated hepatocyte-like cells were functional. These cells stored glyco-
436 gen, secreted Alb, and other metabolic enzymes as efficient as other stem cell models used
437 for hepatogenesis including MSCs [39] and hESCs[75; 76]. Furthermore, differentiated DPPSCs
438 have shown an improved Cyp3A4 enzymatic activity, suggesting that the generated hepato-
439 cyte-like cells can be used as a model for drug metabolism, which would be useful for the
440 pharmaceutical industry [77].

441

442 **5. Conclusions**

443 We designed a novel protocol to differentiate DPPSCs into functional hepatocyte-like
444 cells. Our protocol is a directed differentiation protocol mimicking the stepwise process ob-
445 served during embryonic liver development. Using DPPSCs as a pluripotent-like stem cell

model suggesting their potential usage for liver regenerative medicine and prospective treatments to be developed in the future. However, for clinical applications, there are still many studies that are needed to be improved, yet the generated functional DPPSCs-hepatocyte-like cells can be used as a model for drug screening, hepatic metabolism studies, and hepatic disease applications.

Data Availability: All data are available.

Conflicts of Interest: The authors declare no conflicts of interest.

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Abbreviations: AST: Aspartate aminotransferase, BAL: Bioartificial liver, CGH: Chromosome genomic, hybridization, DEX: Dexamethasone, DPMSC: Dental pulp mesenchymal stem cells, DPPSC: Dental pulp pluripotent stem cells, EB: Embryoid bodies, ECM: Extracellular matrix, ESDL: End-stage liver disease , GGT: Gammaglutamyl transferase, HB: Hepatoblasts, HCC:

468 Hepatocellular carcinoma, HT: Hepatocyte transplantation, MSC: Mesenchymal stromal cells,
469 OLT: Orthotopic liver transplantation , OSM: Oncostatin M

470 **Authors' contribution:** C.G.R., Collection and assembly of data and first manuscript draft;
471 S.M. and S.A.D., Data analysis and interpretation; M.A.A. and C.M., Statistic analysis; E.F.P.
472 and E.F.A, Provision of study patients and cells isolation; M.B., A.A.M and M.A., Conception
473 and design, collection and assembly of data, data analysis and Interpretation, manuscript
474 writing, and final approval of the Manuscript.

475

476 **Figures Legend:**

477 **Figure 1.** Molecular evaluation of different signaling factors and biomaterials on the genera-
478 tion of definitive endoderm lineage. qRT-PCR was performed for the indicated genes at day 5
479 of DPPSCs differentiation into DE lineage. A. Cells treated with differentiation conditional me-
480 dia supplemented with indicated signaling factors. B. Cells were seeded on precoated plates
481 with/without the described scaffolds or co-cultured with HepG2 cells. Results were normal-
482 ized to GAPDH and expressed relative to undifferentiated cells at day. Data are shown as
483 mean ± SD (n = 4). *p < 0.05.

484 **Figure 2. The experimental protocol for DPPSCs mediated- hepatocyte-like cell generation.**
485 Schematic representation of the differentiation protocol including the key manipulated sig-
486 naling pathways and the different molecular and functional studies performed.

487 **Figure 3. Changes of cellular morphological during the time course of DPPSCs- mediated**
488 **hepatocyte-like.** Phage contrast representative images of DPPSCs at different stages of dif-
489 ferentiation from day 0 to day 22 and undifferentiated cells at day 22. Scale A. 100X magnifi-
490 cation. B - F. 200X magnification.

491 **Figure 4. Molecular and cellular evaluation of DPPSCs- mediated hepatocyte-like cells**. Time
492 course qRT-PCR analysis for lineage-specific gene expression. The differentiation was per-
493 formed using the previously described experimental protocol. Total RNA was harvested from
494 the cells at the indicated days for gene expression. Results were normalized to GAPDH and
495 expressed relative to DPPSCs at day 0, *p < 0.05. # p < 0.05 relative to Day 7. Data are shown
496 as mean ± SD (n = 4).

497 **Figure 5. Protein expression and glycogen storage of DPPSCs- mediated hepatocyte-like**
498 **cells.** A. and B. Representative fluorescence microscopy images of DPPSCS mediated hepato-
499 cyte-like cells at day 22, immunoassayed with antibodies directed against A. AAT (AF594, red)
500 and B. ALB (FITC, green) proteins. Hoechst nuclear marker in blue. 200X magnification. C. and

D. Representative light microscope images of differentiated and undifferentiated DPPSCs at day 22, respectively, stained with periodic acid Schiff (PAS) for glycogen storage detection. 100X magnification.

Figure 6. Functional analysis of the generated DPPSCs- mediated hepatocyte-like cells. Time course hepatocyte enzymatic activity of A. GGT, B. AST, C. APF, D. ALT were performed for the differentiated DPPSCs. A notable induction was observed at day 18. E. CYP3A4 activity was measured by luminescence in DPPSCs at day 0 and day 22 of differentiation using rifampicin in the presence or absence of the antagonist erythromycin. F. Alb secretion assay was performed at a time course differentiated DPPSCs, elevated level of secreted Alb was detected at day 19 and later. All measurements are relative to DPPSCs at Day 0, * $p<0.05$. # $p < 0.05$ relative to rifampicin treatment alone. Data are shown as mean ± SD (n = 4).

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735 **Table 1.** Oligonucleotide sequences of primers utilized for real-time qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
FoxA1	GCCTACTCCTCCGTCCCGGT	CCGGGGTCATGTTGCCGCTC
FoxA2	GCGACCCCAAGACCTACAG	GGTTCTGCCGGTAGAAGGG
Gata4	TCCCTCTTCCCTCCTCAAAT	TTCCCCTAACCAGATTGTCG
AFP	AGGGTGTAGCGCTGCAAACGA	TGTTCCTGGCCTTGGCAGCAT
Oct4A	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
HNF6	CTTAGCAGCATGCAAAAGGA	TGCGTTCATGAAGAAGTTGC
CK19	GAGGAAATCAGTACGCTGAG	GTTTCTGCCAGTGTGTCTTC
HNF4α	ACTACATCAACGACCGCCAGT	ATCTGCTCGATCATCTGCCAG
Alb	CCTTGGTGTTGATTGCCTTTGCTC	CATCACATCAACCTCTGGTCTCACC
G6P	TCAGCTCAGGTGGTCCTCTT	CCTCCTTAGGCAGCCTTCTT
AAT	TCGCTACAGCCTTTGCAATG	TTGAGGGTACGGAGGAGTTCC
CYP7A1	AGGACGGTTCCTACAACATC	CGATCCAAAGGGCATGTAGT

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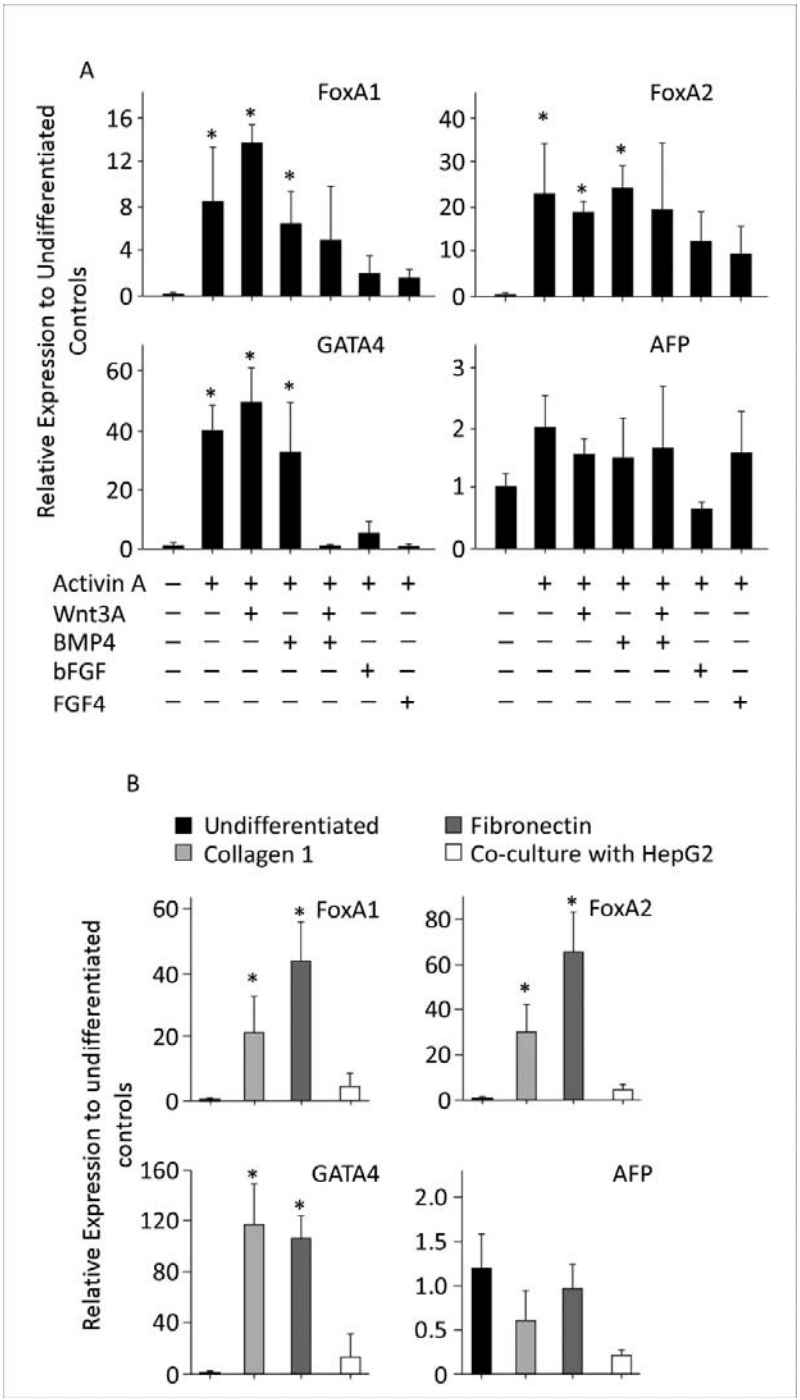
Figure 1


A
FoxA1
FoxA2
GATA4
AFP
Relative Expression to Undifferentiated Controls
Activin A
Wnt3A
BMP4
bFGF
FGF4
B
Undifferentiated
Collagen 1
Fibronectin
Co-culture with HepG2
FoxA1
FoxA2
GATA4
AFP
Relative Expression to undifferentiated controls

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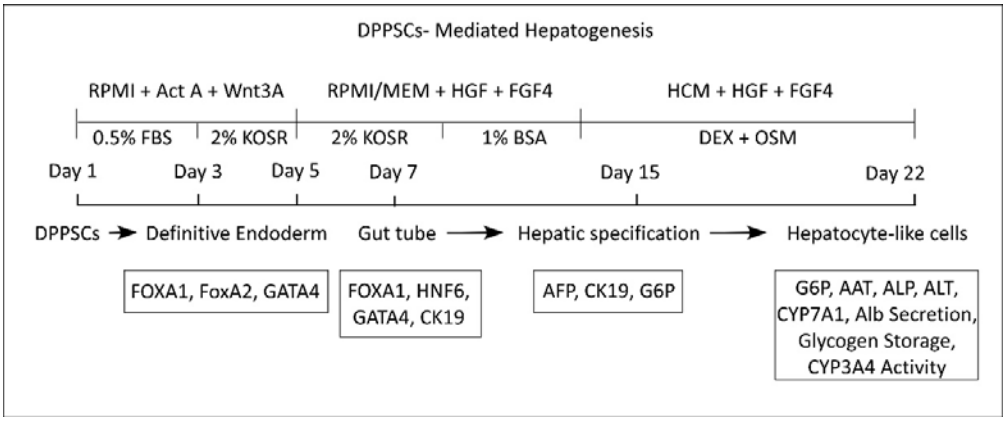
742 Figure 2



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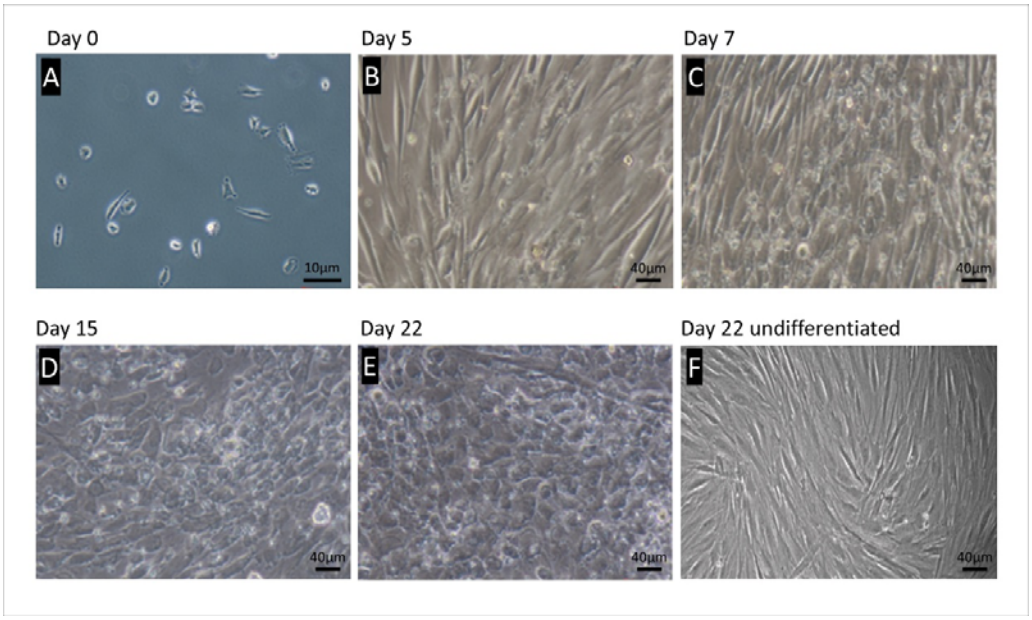
745 Figure 3



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750 Figure 4

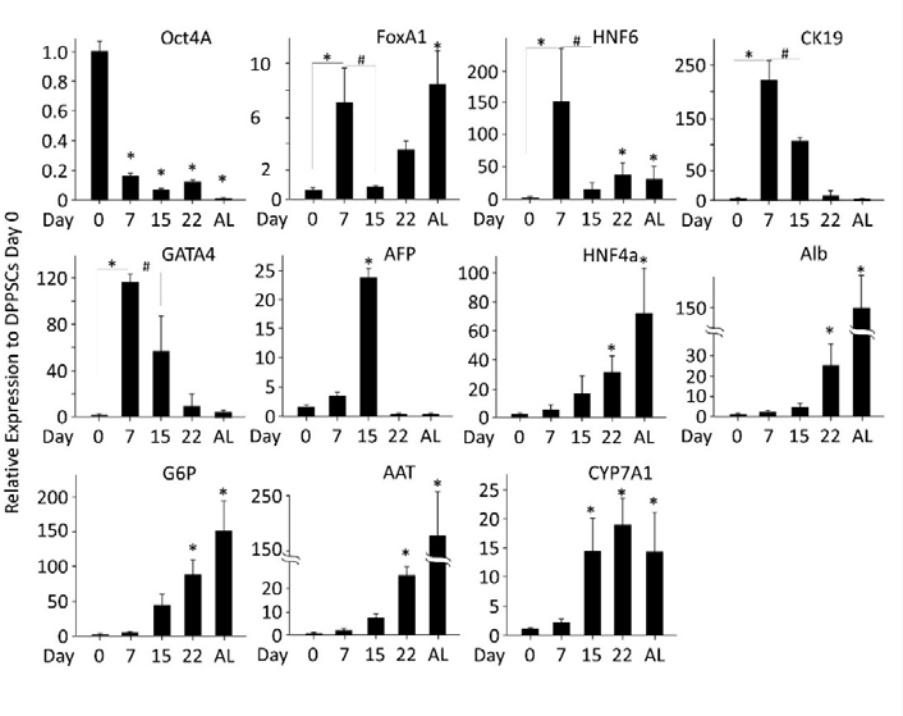

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Figure 5

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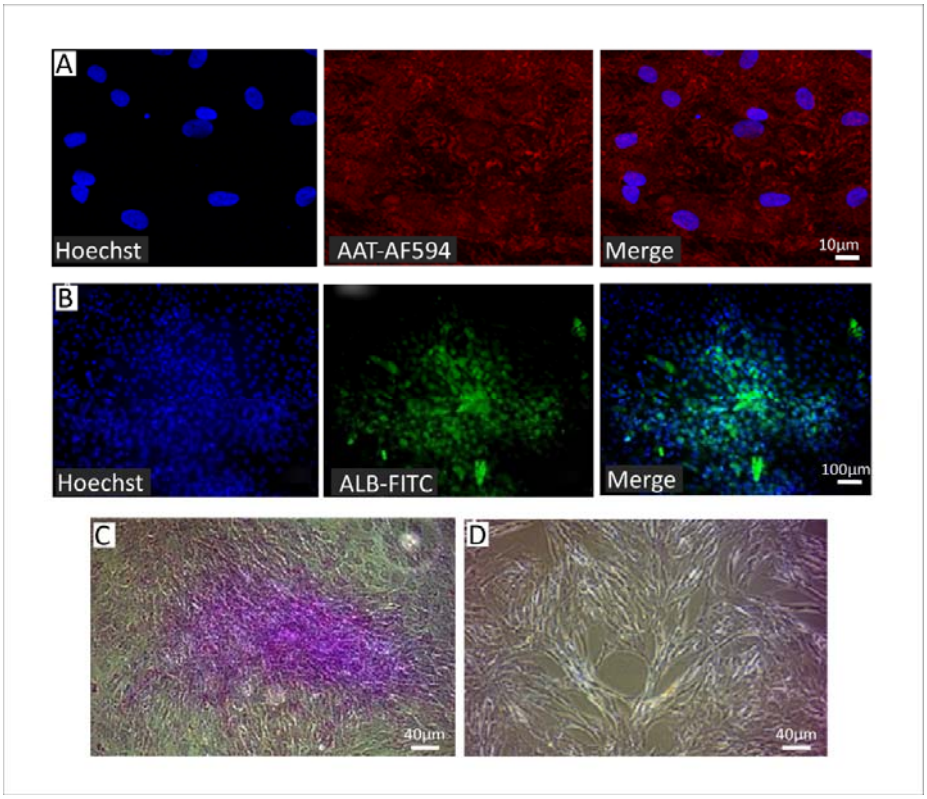

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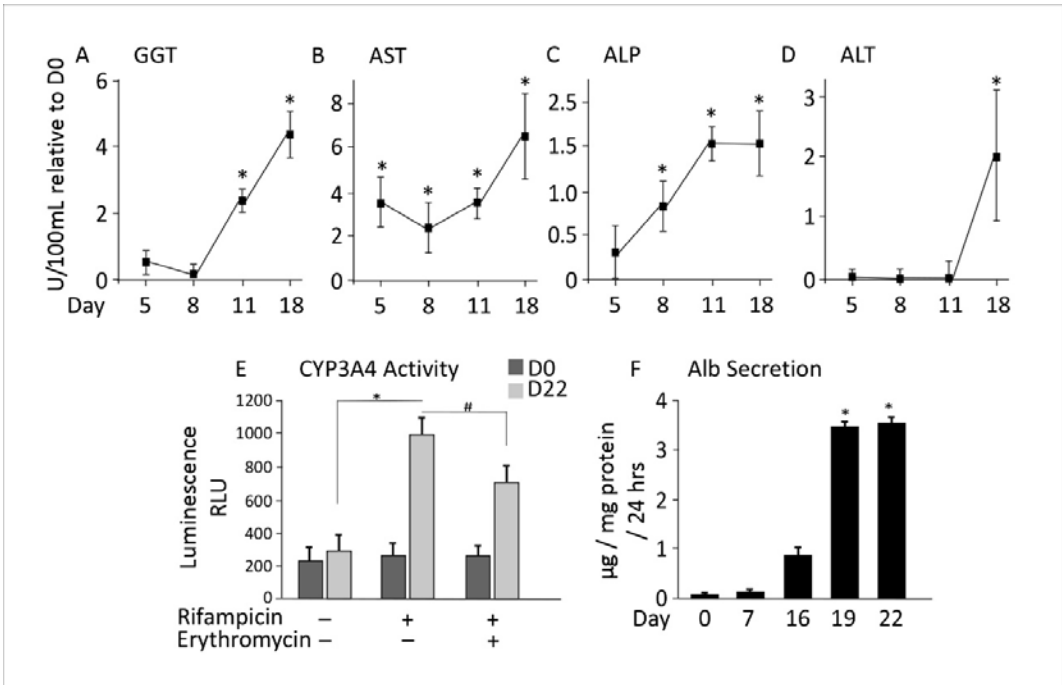
761 Figure 6



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