

Photochemically Activated Notch Signaling Hydrogel Preferentially Differentiates Human Derived Hepatoblasts to Cholangiocytes

Muhammad Rizwan, Ana Fokina, Tove Kivijärvi, Mina Ogawa, Markus Kufleitner, Onofrio Laselva, Laura J. Smith, Christine E. Bear, Shinichiro Ogawa, Gordon Keller, and Molly S. Shoichet*

Cholangiocytes form an intricate network of bile ducts to enable proper liver function; yet, recapitulating human stem cell differentiation to cholangiocytes *in vitro* requires Notch signaling and soluble ligands do not activate the Notch pathway. To overcome these limitations, jagged1 is immobilized on a chemically defined hyaluronan to specifically differentiate human embryonic stem cell-derived hepatoblasts to cholangiocytes. Hepatoblasts cultured on the jagged1-hydrogels upregulate Notch target genes and express key cholangiocyte markers including cystic fibrosis transmembrane conductance regulator. Moreover, cholangiocytes adopt morphological changes that resemble liver biliary structures. To emulate natural biliary system development, a new strategy is developed to achieve spatiotemporal control over the Jagged1–Notch2 interaction: jagged1 is first caged with a photocleavable streptavidin and then it is uncaged photochemically to restore the biological function of Jagged1, which is confirmed with Notch2 activation in a fluorescent reporter cell line. Moreover, the differentiation of human embryonic stem cell-derived hepatoblasts to cholangiocytes is temporally controlled with photochemical uncaging of this streptavidin–Jagged1-immobilized hyaluronan hydrogel. This strategy defines a framework to control protein signaling in time and space and specifically for Notch signaling for ultimate use in regenerative medicine strategies of the liver.

1. Introduction

Bioengineered hepato-biliary livers rely on human cholangiocytes; however, access to primary cholangiocytes is limited.^[1] Cholangiocytes differentiated from human pluripotent stem cells can be used to develop treatment modalities for critically ill liver patients; however, current methods complicate cholangiocyte use for transplantation due to risks of pathogen transfer and irreproducibility.^[1–6] To overcome these challenges, we aimed to both develop a chemically defined hydrogel capable of inducing Notch signaling to direct cholangiocyte differentiation and mimic the spatial and temporal Notch signaling in liver development with a photochemically induced system.

Hepatoblasts differentiate toward hepatocytes and cholangiocytes, the latter of which is controlled by Notch signaling—a key endogenous signaling pathway that

Dr. M. Rizwan, Dr. A. Fokina, L. J. Smith, Dr. M. S. Shoichet
Department of Chemical Engineering and Applied Chemistry
University of Toronto
Toronto, ON M5S 3E5, Canada
E-mail: molly.shoichet@utoronto.ca

Dr. M. Rizwan, Dr. A. Fokina, L. J. Smith, Dr. M. S. Shoichet
Institute of Biomedical Engineering
University of Toronto
Toronto, ON M5S 3G9, Canada

Dr. M. Rizwan, Dr. A. Fokina, L. J. Smith, Dr. M. S. Shoichet
The Terrence Donnelly Centre for Cellular and Biomolecular Research
University of Toronto
Toronto, ON M5S 3E1, Canada

T. Kivijärvi
Department of Fibre and Polymer Technology
KTH Royal Institute of Technology
Stockholm SE-100 44, Sweden

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/adfm.202006116>.

Dr. M. Ogawa, Dr. S. Ogawa, Dr. G. Keller
McEwen Stem Cell Institute
University Health Network
Toronto, ON M5G 1L7, Canada

M. Kufleitner
Department of Chemistry
University of Konstanz
Konstanz D-78457, Germany

Dr. O. Laselva, Dr. C. E. Bear
Molecular Medicine Programme
Hospital for Sick Children
Toronto, ON M5G 1X8, Canada

Dr. O. Laselva, Dr. C. E. Bear
Department of Physiology
University of Toronto
Toronto, ON M5S 1A8, Canada

Dr. C. E. Bear
Department of Biochemistry
University of Toronto
Toronto, ON M5G 0A4, Canada

DOI: 10.1002/adfm.202006116

controls several steps in biliary morphogenesis.^[7–10] During liver development, portal mesenchyme cells directly activate Notch signaling in neighboring hepatoblasts in a spatiotemporal manner.^[7,8] Portal vein endothelial cells in mice express Jagged1 (Jag1—a Notch signaling ligand) in restricted regions in the liver (portal mesenchyme) and in a temporal manner (at approximately embryonic day 12.5 in mice and embryonic week 14 in humans).^[11] Only the cells adjacent to the endothelium express Hes1, a Notch pathway activation marker, two days later at E14.5.^[10] This underlines that Notch signaling is spatiotemporally controlled in development and restricts the differentiation of hepatoblasts to cholangiocytes to periportal regions.^[8,10] Interestingly, inhibition of Notch signaling is required for the maturation of stem cell-derived hepatocytes, which constitute the bulk of the liver cellular mass, reflecting the importance of spatiotemporal control of Notch signaling.^[12]

Hyaluronan (HA), a primary ligand of the CD44 receptor, is ubiquitous in the extracellular matrix of various tissues including the liver.^[13,14] HA hydrogels are frequently used as scaffolds for tissue engineering and to stimulate endogenous regeneration.^[15–18] HA is found in the submucosal space of neonatal and adult extrahepatic bile ducts, and both hepatoblasts and cholangiocytes express CD44 receptors.^[19,20] HA localization in the vicinity of liver bile ducts coupled with the presence of the CD44 receptor in cholangiocytes, and the lack of CD44 in hepatocytes,^[21] suggests that HA regulates cholangiocyte function through HA-CD44 interactions.^[22] HA also binds to toll like receptor 4 (TLR4), which is expressed by cholangiocytes.^[23,24] Thus, HA based hydrogels should provide a conducive extracellular matrix-mimetic environment for the differentiation of hepatoblasts to cholangiocytes.

HA must be crosslinked to produce stable hydrogels for long-term cell culture. While several strategies have been described to crosslink HA,^[25–27] we chose the aqueous-based, Diels–Alder click chemistry to crosslink HA because of its bio-orthogonality, ease of use, and lack of byproducts.^[15] Using HA-methylfuran and a bismaleimide-terminated peptide, HA hydrogels can be synthesized under physiological conditions.^[28,29] We used the GPQGIWGQ-peptide crosslinker to enable cells to remodel the crosslinked HA matrix.^[29]

Cells exert a pulling force on jagged ligands, which enables the unmasking of metalloprotease cleavage sites on the Notch receptor, thereby activating the Notch pathway.^[30–32] Since soluble Notch ligands are ineffective at initiating the signaling cascade, Jag1 must be immobilized to activate Notch signaling.^[33–35] We hypothesized that Jag1 ligands immobilized on crosslinked HA hydrogels would stimulate human embryonic stem cell (hESC)-derived hepatoblasts to differentiate toward cholangiocytes (**Figure 1**). To test this hypothesis, we took advantage of the Fc-protein G interaction^[36–38] to immobilize bioactive Fc-Jag1 ligands to protein G-functionalized HA hydrogels. We examined the differentiation of hESC-derived hepatoblasts toward cholangiocytes by the expression of a series of cholangiocyte markers, including Sox9 and cystic fibrosis transmembrane regulator (CFTR).^[39] Moreover, we hypothesized that our Jag1-HA hydrogels could be engineered to provide a dynamic system where Notch signaling could be spatiotemporally controlled, thereby mimicking liver morphogenesis.^[8] Using a photocleavable caging system, we examined hESC-derived hepatoblasts differentiation to cholangiocytes with temporally controlled exposure of Jag1. To demonstrate the broader applicability of this platform in directing Notch signaling, we also photochemically controlled another Notch ligand, DLL4, and demonstrated subsequent Notch activation.

2. Results

2.1. Jag1 Immobilized on the HA Hydrogel Activates Notch Signaling

In order to immobilize Jag1 on HA hydrogels, we first synthesized HA-methylfuran and reacted it with a collagen-derived bismaleimide peptide crosslinker (KKGGPQGIWGQKG-PQGIWGQKKS) using Diels–Alder chemistry to obtain a crosslinked HA hydrogel (**Figure 2a**). We then immobilized protein G–maleimide (ProGmal; **Figure S1**, Supporting Information) by reacting it with unreacted HA-methylfuran. This ProGmal-HA hydrogel was then modified with recombinant human Fc-Jagged1 (Jag1) by leveraging the binding affinity between ProG and Fc, resulting in Jag1-ProGmal-HA (or simply Jag1-HA) hydrogels. To quantify the immobilized Jag1, we reacted Jag1-cysteines with Alexa Fluor-647–maleimide (AF647mal) by Michael-type addition and measured fluorescence relative to a series of controls. We observed significantly more fluorescence on Jag1-HA hydrogels than all controls, demonstrating the successful immobilization of Jag1 on HA via the ProG-Fc-Jag1 affinity reaction (**Figure 2b,c**). The following controlled for: 1) the binding of ProGmal to HA-methylfuran by reacting Fc-Jag1 with HA hydrogels synthesized in the presence of ProG (but without the maleimide group): Jag1 + ProG + HA; 2) the binding of Fc-Jag1 to ProGmal-HA by measuring the fluorescence of ProGmal-HA gels in the absence of Jag1: ProGmal-HA; 3) the binding of Fc-Jag1 to ProGmal-modified HA by measuring the fluorescence of Jag1 + HA (in the absence of ProGmal): Jag1 + HA. Some fluorescence was observed on all controls, reflecting the binding of the Alexafluor647-maleimide to HA-methylfuran. To measure the

Dr. S. Ogawa
Soham & Shalia Ajmera Family Transplant Centre
Toronto General Research Institute
University Health Network
Toronto, ON M5G 2C4, Canada

Dr. S. Ogawa
Department of Laboratory Medicine and Pathobiology
University of Toronto
Toronto, ON M5S 1A8, Canada

Dr. G. Keller
Princess Margaret Cancer Centre
University Health Network
Toronto, ON M5G 2C1, Canada

Dr. G. Keller
Department of Medical Biophysics
University of Toronto
Toronto, ON M5G 1L7, Canada

Dr. M. S. Shoichet
Department of Chemistry
University of Toronto
Toronto, ON M5S 3H6, Canada

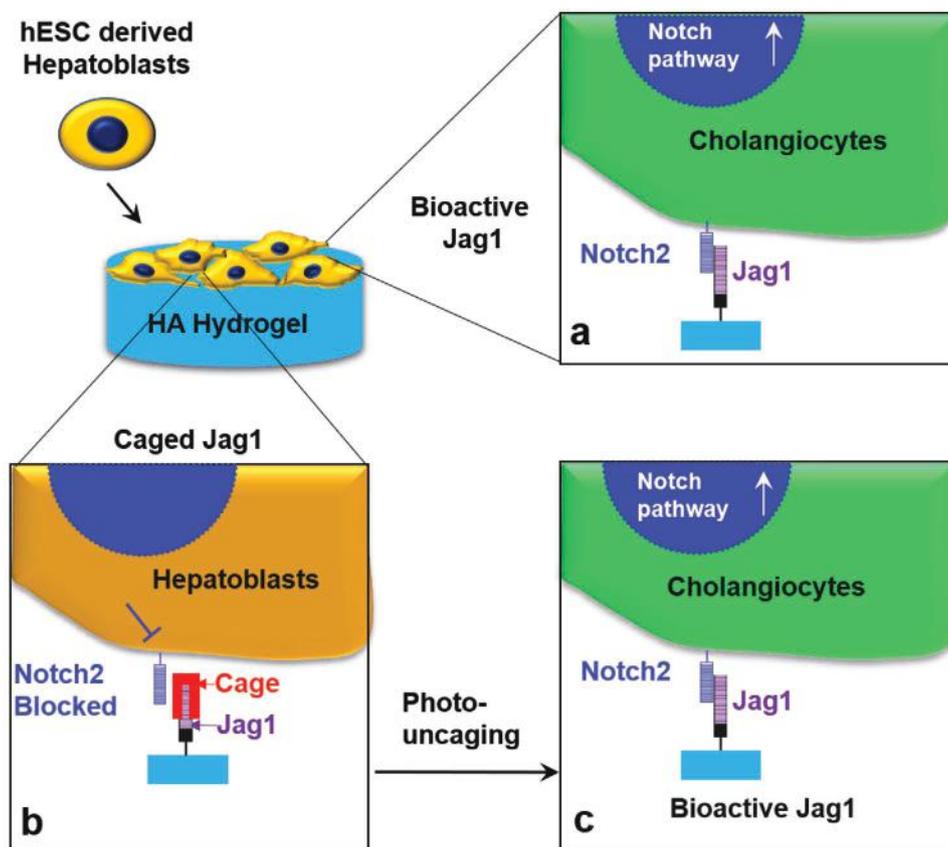


Figure 1. Engineered Notch signaling hydrogel for cholangiocyte differentiation. a) Hyaluronan (HA) hydrogels with immobilized jagged1 (Jag1) binds to the Notch2 receptor in hepatoblasts, activates Notch signaling, and differentiates hepatoblasts to cholangiocytes. b) HA hydrogels with caged Jag1 inhibits Notch2 binding and activation. Upon photochemical uncaging, c) the Notch2 pathway is activated, resulting in cholangiocyte differentiation.

amount of ProG and Jag1 immobilized per gel, we used the micro BCA protein assay. We found, on average, 53 ng ProG in ProG-HA gels, 245 ng Jag1 + ProG in Jag1-HA gels and 223 ng biotin-PL-Jag1 + ProG in biotin-PL-Jag1-HA gels per 15 μ L HA gel (Figure S2, Supporting Information). We also measured the compressive modulus of the HA and Jag1-HA gels using micromechanical testing, which showed that the immobilization of Jag1 did not significantly alter the modulus of the HA hydrogel, thus allowing us to study the role of Jag1 on Notch activation independently from the gel's mechanical properties (Figure S3, Supporting Information).

To determine whether the immobilized Jag1 is bioactive, we cultured fluorescent Notch2-CHO (N2-CHO) reporter cells that express citrine in cell nuclei upon Notch2 activation, on Jag1-HA hydrogels, which were further modified with fibronectin by mixing it with prepolymer solution to enhance cell adhesion and then used these hydrogels for all subsequent cell culture experiments. N2-CHO cells expressed more citrine (green channel) when cultured on Jag1-HA gels than on unmodified, crosslinked HA control gels at 14, 22, and 48 h after seeding (Figure 2d). Merged channels [nuclei (blue) and Notch2 (green)] confirmed that the citrine was present in cells' nuclei (Figure S4a, Supporting Information). The quantification of Notch2 fluorescence showed significantly higher Notch2 expression on Jag1-HA than HA control gel at all time

points (Figure S4b, Supporting Information), which clearly demonstrates that the immobilized Jag1 is bioactive. Importantly, soluble Jag1, added to the cell culture media, did not stimulate Notch2 expression (Figure 2e). The Notch2 fluorescence was significantly lower in the cells cultured on HA gels in the presence of soluble Jag1 versus cells cultured on Jag1-HA, demonstrating that the Jag1 needs to be immobilized in order to activate Notch signaling (Figure S5a,b, Supporting Information).

To confirm the reproducibility and broad applicability of this strategy, we immobilized Fc-DLL4 to ProGmal-HA hydrogels. These DLL4-HA hydrogels also activate Notch2 expression of N2-CHO reporter cells significantly more than the cells cultured on unmodified, HA control gels at 24 h (Figure S6, Supporting Information).

2.2. Human ESC-Derived Hepatoblasts Cultured on Jag1-HA Express Cholangiocyte Genes

We wondered if we could induce Notch2 signaling in hESC-derived hepatoblasts given that the Jag1-HA gels stimulate Notch signaling in the reporter cell line. Human ESCs were differentiated over the course of 27 days to hepatoblasts, as confirmed with alpha-fetoprotein (AFP) and albumin (ALB) expression

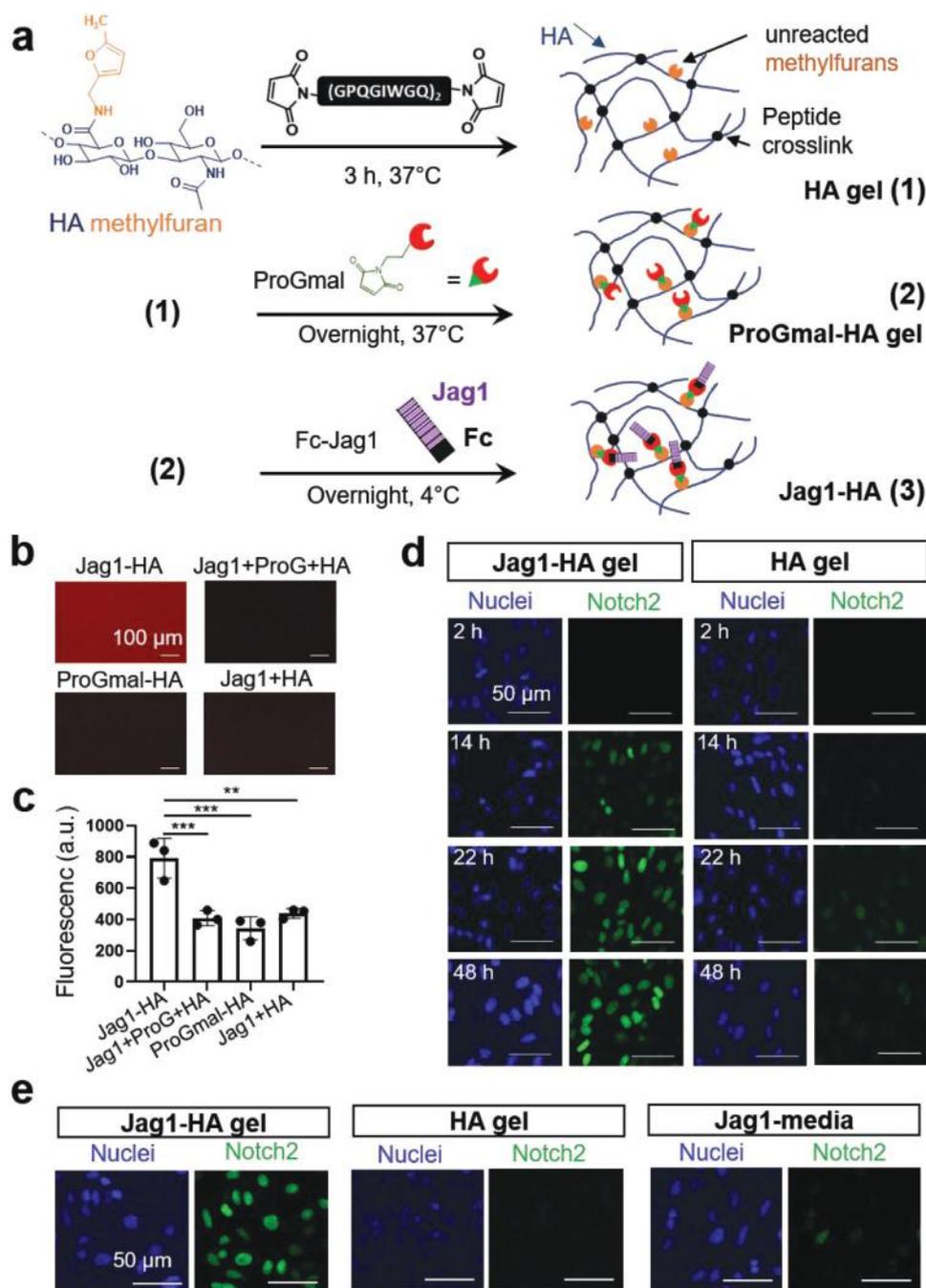


Figure 2. Jag1 immobilized on HA hydrogel activates Notch signaling. a) Schematic of the synthesis of Jag1-HA gels: 1) HA-methylfuran is first crosslinked with bismaleimide GPQGIWGQ-peptide to produce crosslinked HA hydrogels. 2) HA-methylfuran groups are then modified with ProGmaleimide, yielding ProGmal-HA hydrogels. 3) The latter are then modified with Fc-Jag1 to yield Jag1-HA hydrogels. b,c) Characterization of the protein G maleimide (ProGmal) binding to HA hydrogels and Jag1 binding to ProGmal using Alexa Fluor647-maleimide which preferentially reacts with Jag1-cysteines. The following controls were used: Jag1 + ProG + HA (absence of maleimides on ProG); ProGmal-HA (absence of Jag1); Jag1 + HA (absence of ProGmal) ($n = 3$ independent experiments, mean \pm S.D., one-way ANOVA and Dunnett's post hoc, ** $p < 0.01$; *** $p < 0.001$). d) Jag1-HA induced activation of Notch signaling using the Notch2-CHO (N2-CHO) fluorescent reporter cell line whereas unmodified, crosslinked HA control gels did not. Activation of the Notch signaling pathway is visualized through the expression of Notch2 in cell nuclei (green fluorescence) as a function of time. e) Notch2 expression was induced only when N2-CHO cells were cultured overnight on Jag1-HA but neither on unmodified HA control gels nor on HA hydrogels with soluble Jag1 ($100 \mu\text{g mL}^{-1}$) added to the cell culture media (Jag1-media).

(Figure S7, Supporting Information), and then cultured on Jag1-HA hydrogels for an additional 7 days for cholangiocyte differentiation (Figure 3a). Hepatoblasts formed a monolayer on crosslinked Jag1-HA gels, but not on tissue culture polystyrene

where they were relatively elongated (Figure S8a,b, Supporting Information). Hepatoblasts also displayed polygonal morphology on matrigel coated polystyrene, which resembled cells on Jag1-HA hydrogel (Figure S8c, Supporting Information).

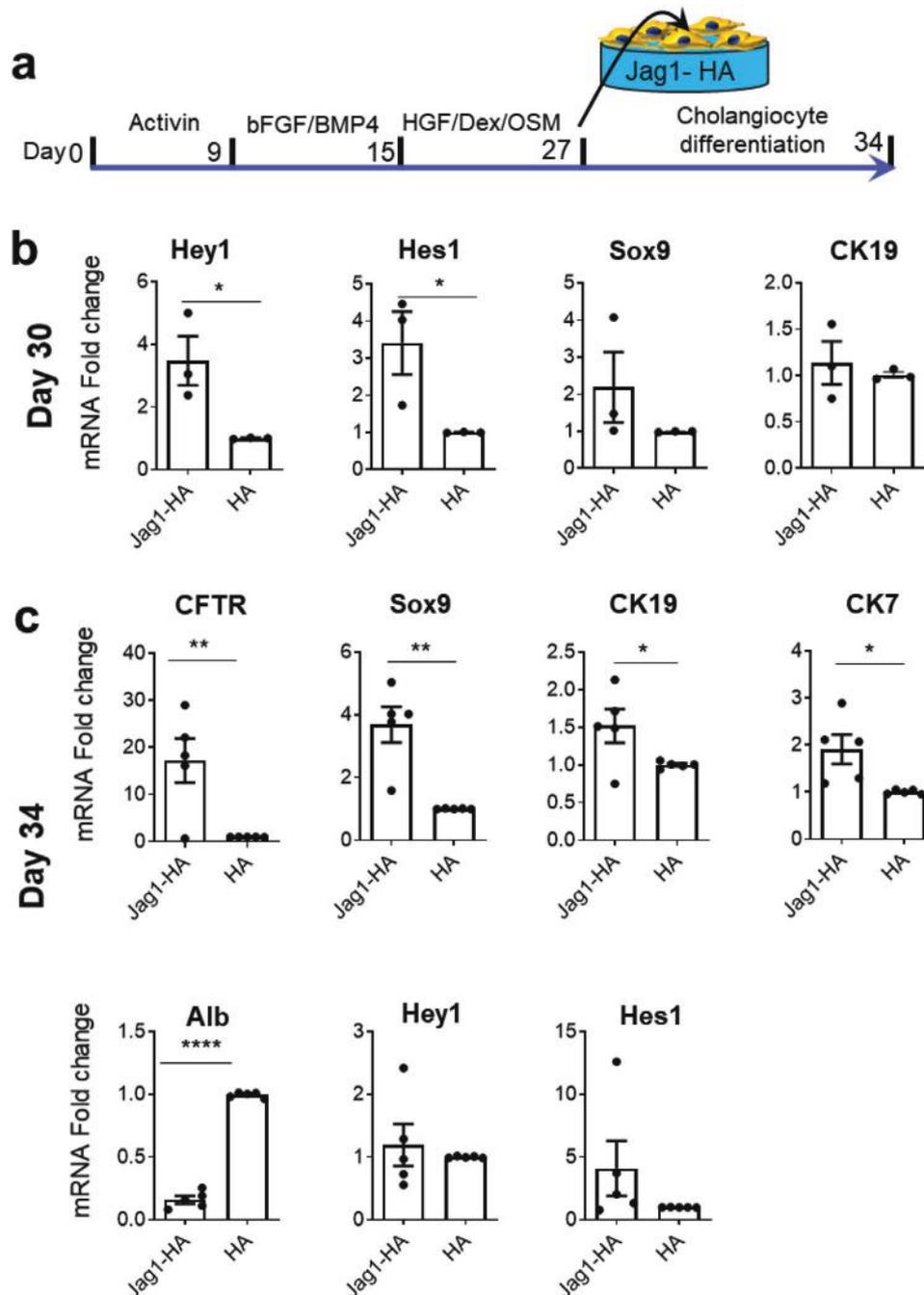


Figure 3. Human embryonic stem (hES) cell-derived hepatoblasts cultured on Jag1-HA hydrogel upregulates cholangiocyte genes. a) Protocol for the generation of hES cell-derived hepatoblasts for 27 days, and then generation of cholangiocytes by culturing on Jag1-HA gels for an additional 7 days. b) Analysis of Notch activation in hepatoblasts after 3 days of culture on Jag1-HA versus control HA gels (or day 30 total culture time). Significant upregulation of Notch target genes (Hey1 and Hes1) were observed in cells cultured on Jag1-HA compared to cells cultured on control HA gels. However, expression of cholangiocyte markers Sox9 and CK19 were not significantly different ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, $*p < 0.05$). c) Analysis of the differentiation of hepatoblasts toward the cholangiocyte lineage after 7 days of culture on Jag1-HA versus control HA gels (or day 34 total culture time). Expression of cholangiocyte markers (CFTR, Sox9, CK19, CK7) was significantly higher on Jag1-HA than HA gels. Albumin (Alb), a marker of hepatocyte differentiation, was significantly downregulated. However, Hey1 and Hes1, were not significantly different at this time point ($n = 5$ independent experiments, mean \pm S.D., Student's t -test, $*p < 0.05$; $**p < 0.01$; $***p < 0.0001$).

To investigate the activation of Notch signaling in hepatoblasts, we analyzed the expression of target genes of the Notch signaling pathway, Hey1 and Hes1, using quantitative polymerase chain reaction (qPCR). After 3 days of culture

on Jag1-HA gels (or 30 days of total culture time), Hey1 and Hes1 expression significantly increased compared to cells cultured on HA control gels ($p < 0.05$, Figure 3b), indicating that the Notch signaling pathway was active, even though

Sox9 and CK19 markers were not significantly changed. With Notch signaling, we expected that other cholangiocyte-specific markers would be upregulated. After 7 days of culture on Jag1-HA gels versus HA control gels (or 34 days of total culture time), we observed significant increases in the gene expression of CFTR ($p < 0.01$), Sox9 ($p < 0.01$), CK19 ($p < 0.05$), and CK7 ($p < 0.05$), confirming hepatoblast differentiation to cholangiocyte-like cells (Figure 3c). Moreover, albumin, which is a marker of hepatocyte differentiation, was significantly downregulated ($p < 0.0001$), which further corroborated the differentiation of hepatoblasts toward the cholangiocyte lineage on Jag1-HA gels. Interestingly, Hey1 and Hes1 were significantly upregulated at day 3 (30 days total culture time), but not at day 7 (34 days total culture time) on Jag1-HA versus HA control gels, reflecting the transient nature of gene expression in the Notch pathway.

2.3. Human ESC-Derived Hepatoblasts Cultured on Jag1-HA Express Cholangiocyte Proteins

We immunostained the differentiated hESC-derived hepatoblasts to cholangiocyte-like cells to detect cholangiocyte-specific protein expression. Cells cultured on Jag1-HA expressed both Sox9 and CK19 whereas those cultured on HA control gels only weakly expressed these markers (Figure 4a). Conversely, cells cultured on Jag1-HA did not express albumin whereas those cultured on HA gel weakly expressed albumin. Analysis of the normalized fluorescence levels revealed significant upregulation of Sox9 (Figure 4b, $p < 0.05$), CK19 (Figure 4c, $p < 0.01$), and significant downregulation of albumin (Figure 4d, $p < 0.05$) in cells cultured on Jag1-HA versus those cultured on HA control gels. Bipotent hepatoblasts are known to express weak levels of CK19,^[1] which may explain the low expression in cells cultured

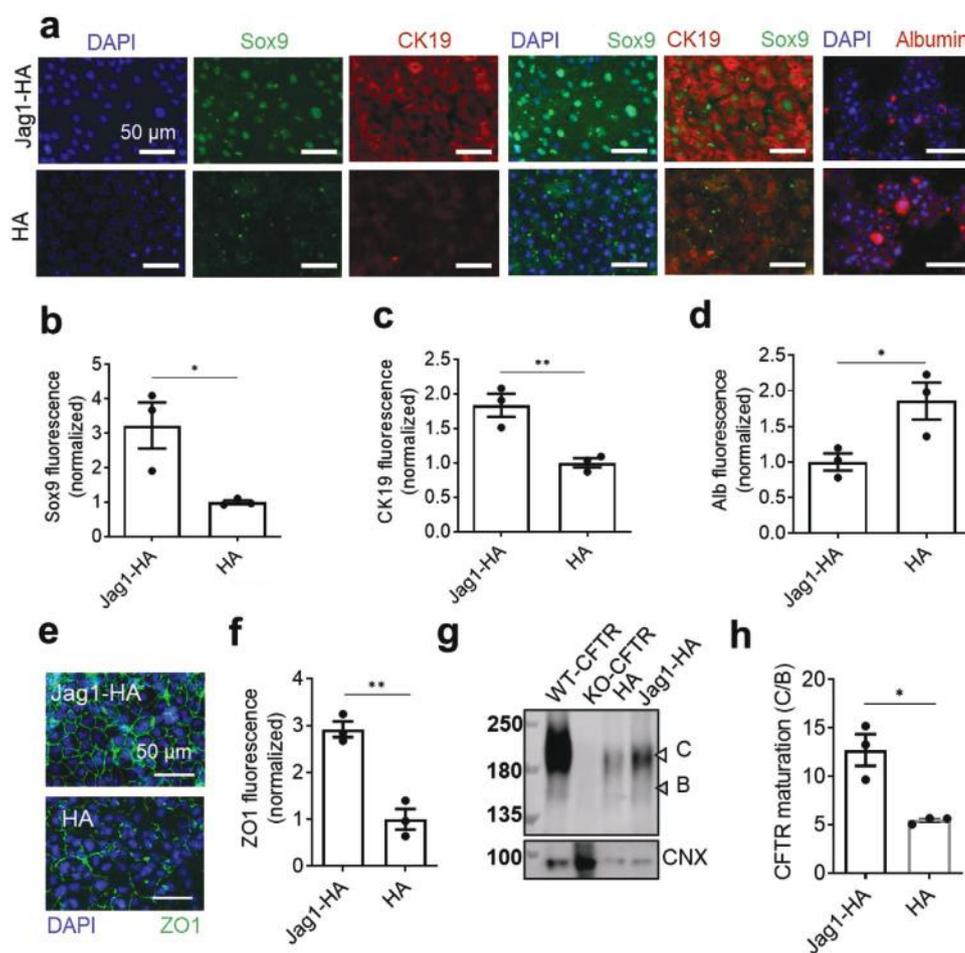


Figure 4. Human ES cell-derived hepatoblasts cultured on Jag1-HA express cholangiocyte proteins. a) Sox9, CK19, and albumin protein expression following 7 day culture on Jag1-HA versus control HA hydrogels. Cells on Jag1-HA hydrogels expressed Sox9 and CK19, which are markers of cholangiocyte differentiation, and did not express albumin, which is a marker of hepatocytes. Analysis of the normalized fluorescence levels revealed significant upregulation of b) Sox9, c) CK19, and downregulation of d) albumin in cells cultured on Jag1-HA versus those cultured on HA control gels ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, $*p < 0.05$; $**p < 0.01$). e) Cells on Jag1-HA formed a monolayer and f) expressed significantly more zonula occludens-1 (ZO1) at cell-cell junctions compared to cells on HA hydrogels ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, $**p < 0.01$). g) Western blotting showed higher maturation of CFTR in cells cultured on Jag1-HA gels than those cultured on HA control gels (WT, wild type; KO, knockout; band C, mature complex-glycosylated CFTR; band B, immature core-glycosylated CFTR; CNX, calnexin loading control). h) CFTR maturation measured as ratio of bands C/B was significantly higher in cells cultured on Jag1-HA compared to those on HA gels ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, $*p < 0.05$).

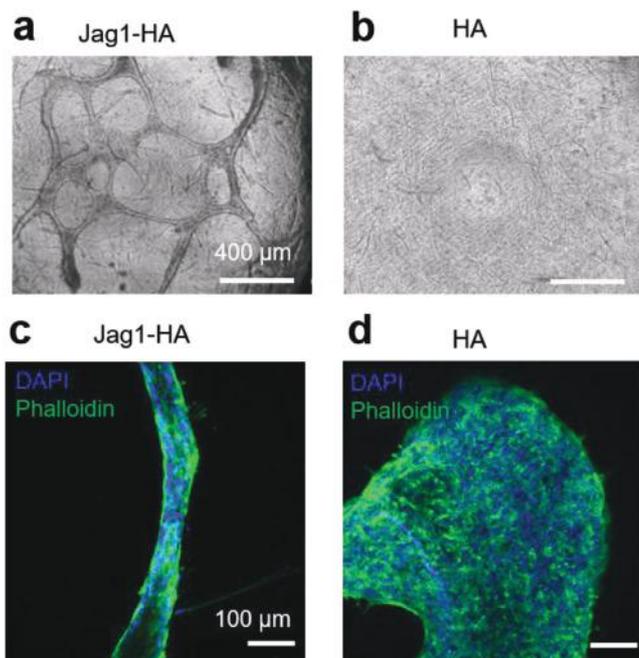


Figure 5. Differentiated cholangiocyte-like cells self-organize into duct-like structures at day 11. Brightfield images of a) cells on Jag1-HA versus b) cells on HA controls; and fluorescent images of c) cells on Jag1-HA versus d) cells on HA controls show the morphological changes in cells cultured on Jag1-HA versus HA control gels.

on HA control gels. We found that our hES-hepatoblast derived ductal epithelial, cholangiocyte-like cells expressed significantly more ZO1 ($p < 0.01$) when cultured on Jag1-HA gels than HA control gels, demonstrating strong tight junctions (Figure 4e,f). Importantly, by western blot analysis, we found that the higher molecular weight, mature form of CFTR (that is the complex glycosylated form, band C) was more significantly expressed in cells cultured on Jag1-HA than those cultured on HA control gels (Figure 4g,h, $p < 0.05$), further corroborating successful cholangiocyte differentiation. Sox9, CK19, albumin, and CFTR protein expression substantiated the qPCR data. Strikingly, the hESC-derived cholangiocyte-like cells demonstrated substantial morphological changes at day 11 on Jag1-HA hydrogels, without the use of feeder cells, that resembled ductal-like structures, which did not occur on HA control gels (Figure 5).

2.4. Photoresponsive Protein Caging and Uncaging of Jag1

In a quest for a dynamic system that would mimic the spatiotemporal presentation of Notch signaling in vivo, we immobilized a photolabile, streptavidin-caged Jag1 to HA. First, we synthesized an o-nitrobenzyl (oNB) based photocleavable linker, compound 2 (Scheme S1, Supporting Information) where the terminal biotin enables streptavidin binding and the terminal NHS ester allows Jag1-Fc binding. The synthesis was verified by using ^1H NMR (Figure S9, Supporting Information). Lysine residues of Jag1-Fc reacted with the NHS-activated terminus of the biotinylated photolinker (biotin-PL) to produce biotin-PL-Jag1 (Figure 6a). The conjugation was confirmed by reacting biotins

of biotin-PL-Jag1 with 4'-hydroxyazobenzene-2-carboxylic acid (HABA) (Figure 6b). These data show that 31% (or 20 of the 65) lysines in Jag1-Fc were biotinylated, forming biotin-PL-Jag1. To ensure that the photolinker could be removed, biotin-PL-Jag1 was UV-irradiated at 365 nm for 15 min, resulting in 55% yield and just 14% (or 9 of the 65) biotinylated lysines remaining (biotin-PL-Jag1 + UV).

To assess the caging and uncaging potential of streptavidin-Jag1, we used a fluorescently labeled AlexaFluor 647 streptavidin conjugate (strepAF647). After binding biotin-PL-Jag1 to protein G coated well-plates, we added strepAF647 for 2 h at 37 °C, resulting in strepAF647-biotin-PL-Jag1. When Jag1-Fc was simply mixed with strepAF647, the fluorescence was only barely detectable, demonstrating that strepAF647 specifically binds to the biotin-PL modified Jag1 (Figure 6c). When immobilized strepAF647-biotin-PL-Jag1 was UV irradiated at 365 nm for 15 min, the fluorescence of "strepAF647-biotin-PL-Jag1 + UV" decreased by 75%, demonstrating the photocaging strategy (Figure 6c,d). To demonstrate broad applicability, DLL4 was similarly modified with streptavidin, as confirmed with biotin quantification before and after UV exposure (Figure S10, Supporting Information). We tested the spatial caging and uncaging of streptavidin-Jag1 by immobilizing fluorescent strepAF647-biotin-PL-Jag1 on HA hydrogels, as previously described using Fc-Protein G affinity binding. We then exposed it to UV light through a striped photomask patterned with alternating 100 μm wide black lines and 300 μm spacing. Following washing, HA hydrogels revealed $\approx 91 \mu\text{m}$ wide fluorescent lines, which corresponded to the masked regions on the photomask (Figure S11, Supporting Information). The exposed area of the gel became drastically darker, which reflected cleavage of StrepAF647, leading to the uncaging of Jag1. This demonstrates that we can spatially pattern Jag1 with sub-100 μm resolution.

2.5. Jag1-Notch2 Interaction is Spatiotemporally Controlled on HA Hydrogel

We immobilized photocaged, biotin-PL-Jag1 on ProGmal-HA hydrogels and verified the immobilization using micro BCA assay (Figure S2, Supporting Information). The data showed no significant difference in protein concentration between Jag1-HA gel and biotin-PL-Jag1-HA gel. Subsequently, we caged Jag1 with streptavidin to produce Jag1 OFF HA gels (Figure 7a). We then cultured N2-CHO cells on caged hydrogels that were exposed to UV light for 0 to 25 min to uncage Jag1. We found no further Notch2 fluorescence activation with longer than 15 min UV exposure to uncage Jag1, and thus pursued this time (Figure S12, Supporting Information). To test whether Notch can be activated in a temporal manner in our engineered hydrogels, we photocaged Jag1 OFF hydrogels by UV irradiation to remove strep-biotin-PL either before (day 0) or 3 days after seeding N2-CHO reporter cells, and then cultured these cells for an additional day prior to examining Notch activation (Figure 7b). Notch2 expression was significantly higher on Jag1 ON gels compared to Jag1 OFF gels after both 1 and 4 days of culture ($p < 0.05$ and $p < 0.01$, respectively, Figure 7c). Images of merged channels showed the presence of Notch2-induced fluorescence signal in the cell nuclei (Figure S13, Supporting

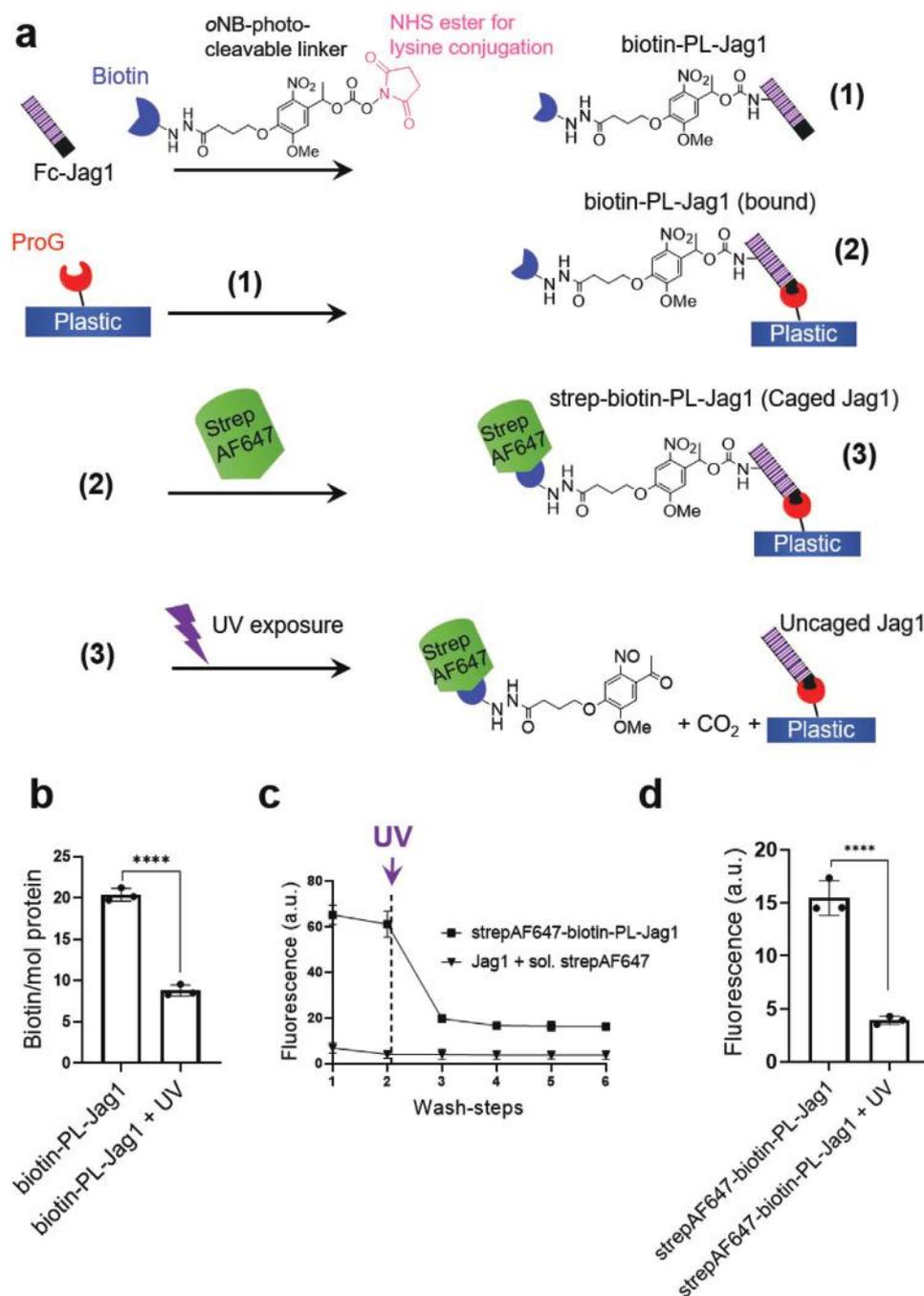


Figure 6. Synthesis of caged and photouncaged Jag1: a) Jag1 is modified with a biotinylated oNB photocleavable linker (biotin-PL) to produce biotin-PL-Jag1. Biotin-PL-Jag1 is then immobilized on protein G coated wells and coupled to streptavidin to provide caged Jag1 (strep-biotin-PL-Jag1). Upon exposure to UV light, the streptavidin photocage is released, thereby activating Jag1. To demonstrate the removal of photocleavable groups, b) biotins on biotin-PL-Jag1 decreased after exposure to UV light, resulting in photouncaged Jag1 (biotin-PL-Jag1 + UV) ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, **** $p < 0.0001$) and c) fluorescence of streptavidin-Alexafluor647-modified Jag1 (strepAF647-biotin-PL-Jag1) decreased after UV exposure (strepAF647-biotin-PL-Jag1 + UV), demonstrating photocleavage of streptavidin from Jag1 ($n = 3$ independent experiments, mean \pm S.D., Student's t -test). d) To ensure that the streptavidin is coupled to Jag1, Jag1 was modified with photolinker and fluorescently (AlexaFluor647) tagged streptavidin (strepAF647) and compared to Jag1 mixed with strepAF647. The coupled Jag1-strepAF647 had a significantly higher fluorescence than the control and exposure to UV light resulted in a significant decrease in fluorescence of Jag1-AF647, demonstrating successful photouncaging ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, **** $p < 0.0001$).

Information). Importantly, there was no difference in Notch expression on Jag1 OFF gels on day 1 and day 4, demonstrating that our photocaging strategy can be used to efficiently and reversibly cage Jag1 for at least 3 days and can be subsequently

activated to induce Notch signaling. To confirm that the temporal control that we achieved with photocaged Jag1 was broadly applicable, we repeated this experiment with another Notch ligand, DLL4, which was similarly activated after photocleaving

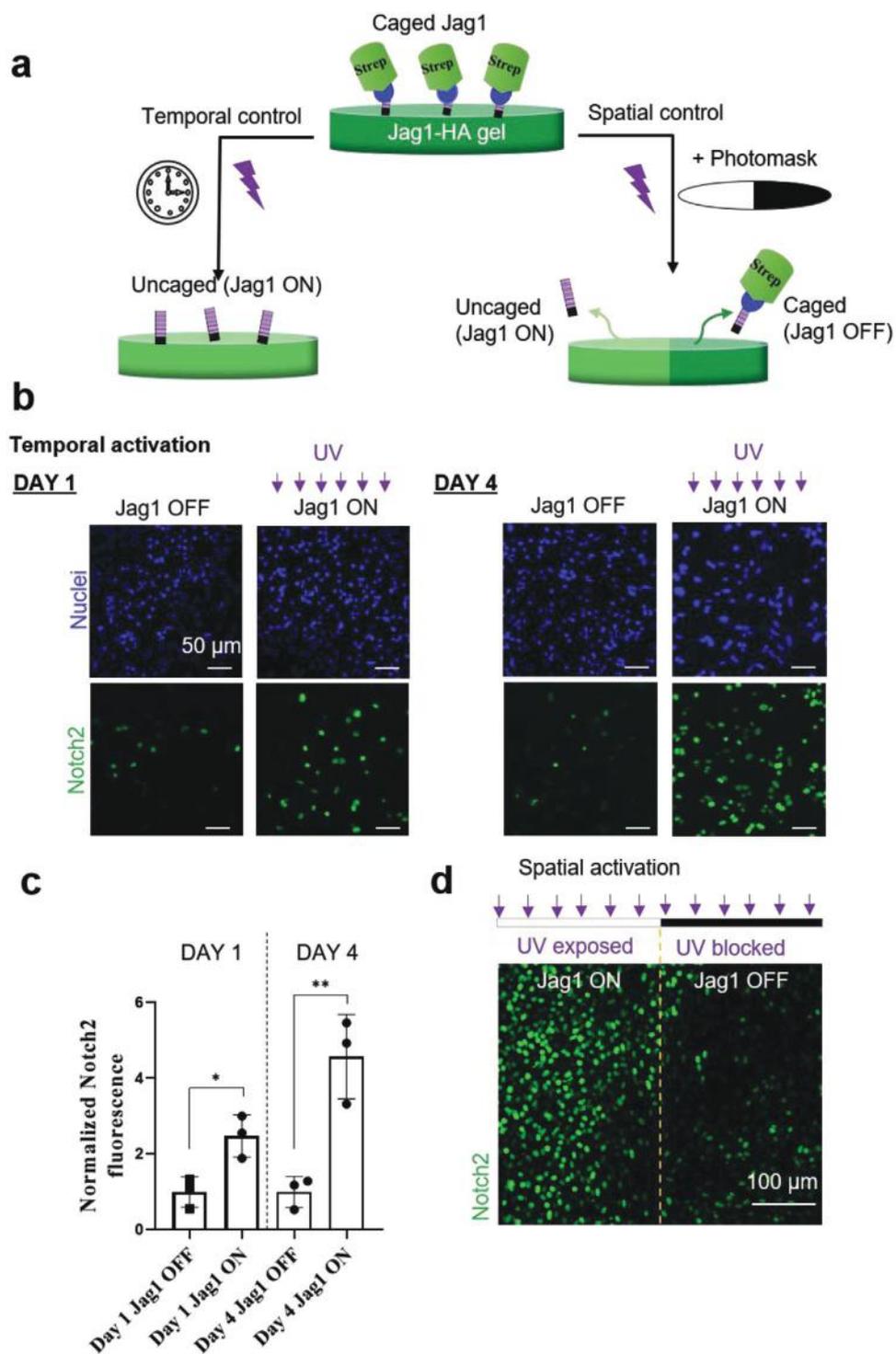


Figure 7. Temporally and spatially defined Notch signaling hydrogels: a) Schematic representation of spatiotemporal photouncaging of Jag1 hydrogels. b) Temporal control of Notch signaling characterized using the N2-CHO fluorescent reporter cell line. Strep-biotin-PL-Jag1-HA gels were UV-irradiated to yield Jag1 ON hydrogels on either day 0 or day 3 of culture and then incubated for an additional day and compared to strep-biotin-PL-Jag1-HA gels not exposed to UV light (Jag1 OFF). c) Analysis of the N2-CHO cells revealed that Notch2 expression was significantly higher on Jag1 ON gels compared to Jag1 OFF gels after both 1 and 4 days of culture ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, * $p < 0.05$; ** $p < 0.01$). d) Spatial control of Notch signaling was characterized using strep-biotin-PL-Jag1-HA gels by culturing the N2-CHO fluorescent reporter cell line. Cells on strep-biotin-PL-Jag1-HA gels were exposed to UV light through a photomask to turn on the Jag1 signaling only in exposed areas.

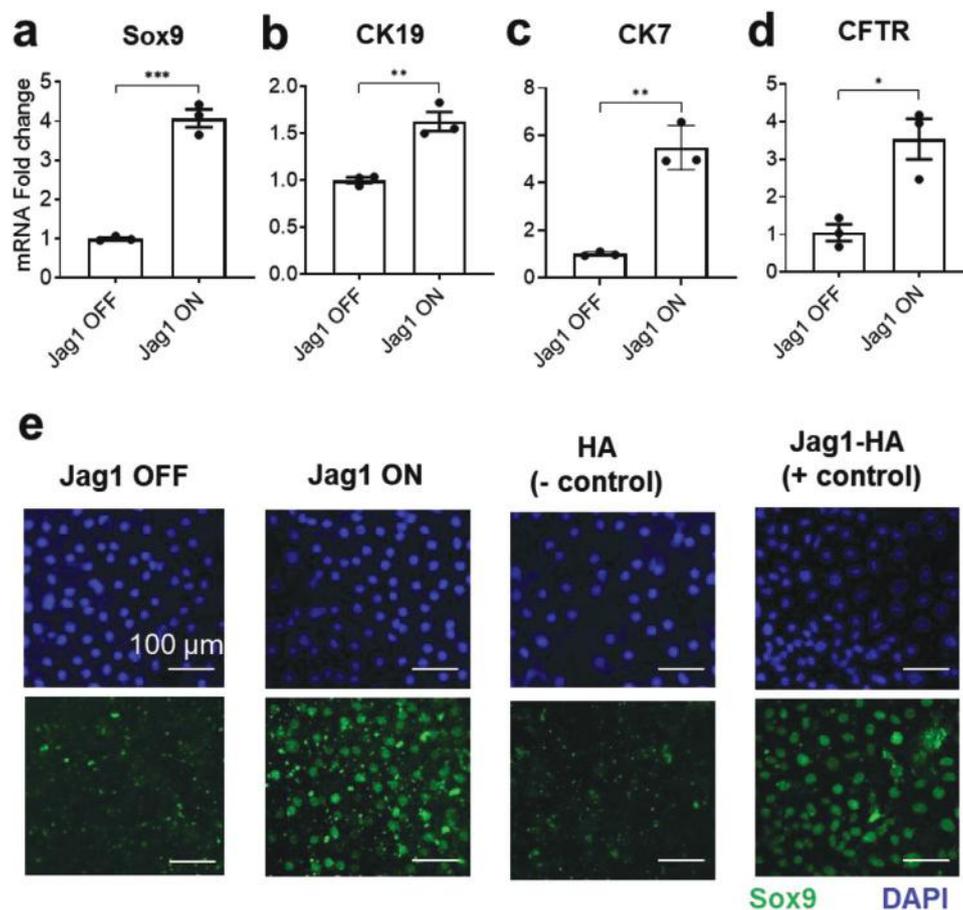


Figure 8. Control of cholangiocyte differentiation using photochemically triggered Jag1 signaling. Primary human ES cell-derived hepatoblasts, when differentiated for 5 d, showed significantly greater cholangiocyte gene expression by qPCR on Jag1 ON versus Jag1 OFF gels for a) Sox9, b) CK19, c) CK7, and d) CFTR ($n = 3$ independent experiments, mean \pm S.D., Student's t -test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). e) Sox9 protein was expressed when cells were cultured on Jag1 ON gels versus Jag1 OFF gels for 5 days. Similarly, cholangiocytes expressed Sox9 on positive Jag1-HA controls and none on negative HA controls.

streptavidin protecting groups (Figure S14, Supporting Information). Significantly more cells expressed Notch2 when cultured on photouncaged DLL4 ON gels than photocaged DLL4 OFF gels after both 1 and 4 days of culture (Figure S14a,b, Supporting Information). Thus, Notch signaling can be turned ON by a selective and temporal photoinduced triggering system, which restores the biological function of the protein.

To test whether Notch could be spatially activated, N2-CHO reporter cells were cultured on caged Jag1 OFF gels for 1 day, exposed to UV light through a photomask and then cultured for an additional day (Figure S15a, Supporting Information). Notch2 expression was considerably stronger in areas which were exposed to UV irradiation (Figure 7d), demonstrating that the Jag1 signaling is spatially controlled. Uniform cell distribution was confirmed with nuclei imaging (Figure S15b, Supporting Information) in UV exposed and unexposed areas, which ruled out the effect of cell density on Notch2 activation.

To extend the photocaged control of Notch signaling to cholangiocytes, we cultured primary hepatoblasts (after 27 d of differentiation) on either caged strep-biotin-PL-Jag1-HA (Jag1 OFF, not exposed to UV light) gels or photocaged Jag1 (Jag1 ON, pre-exposed to UV light to activate Jag1) gels. The hepatoblasts were then seeded on either Jag1 OFF gels

or Jag1 ON gels, cultured for 5 days, and gene expression was measured by qPCR (Figure 8a–c). Significantly higher expressions of Sox9 ($p < 0.001$), CK19 ($p < 0.01$), CK7 ($p < 0.01$), and CFTR ($p < 0.05$) were detected in cells cultured on Jag1 ON gels than on Jag1 OFF gels, demonstrating successful differentiation of the cholangiocyte-like cells using photoinduced Notch activation. The cholangiocyte-like differentiated cells cultured on photouncaged, Jag1 ON gels expressed Sox9 proteins at levels similar to the positive Jag1-HA controls whereas those cholangiocyte-like cells cultured on photocaged Jag1 OFF gels expressed Sox9 at levels similar to negative HA controls (Figure 8d). Thus, Notch signaling and subsequently cholangiocyte differentiation can be turned ON by a selective photoinduced triggering system, which restores the biological function of the Jag1. Surprisingly, these data also demonstrate that the 5-day culture was sufficient to stimulate significant upregulation of cholangiocyte marker mRNA and expression of Sox9 protein.

3. Discussion

We engineered a chemically defined Jag1-modified hyaluronan hydrogel (Jag1-HA) that stimulates Notch signaling in

human embryonic stem cell derived hepatoblasts to differentiate toward cholangiocyte-like cells, thereby obviating the use of both animal feeder cell coculture^[1] and Matrigel for cell differentiation. By immobilizing the Jag1-Fc on HA-protein G by affinity binding, we were able to activate the Jag1-Notch interaction, which regulates cholangiocyte differentiation.^[1,40–42] Following 7 days of culture on Jag1-HA gels, our differentiated cells both significantly upregulated expression of Sox9, CK19, and CFTR, which are well-established markers of cholangiocytes and downregulated the expression of albumin, a marker of hepatocytes. Moreover, we demonstrated, for the first time, spatiotemporal control over the activation of Notch signaling in HA hydrogels by photocontrolled uncaging of immobilized Jag1. We recapitulated key aspects of the spatiotemporal control observed during hepatoblast differentiation to cholangiocytes by on-demand activation of Notch signaling in our HA hydrogels.^[10]

Our Jag1-HA hydrogel stimulated Notch signaling within 14 h, as evidenced by the increased fluorescence in Notch2 reporter cells, which is consistent with the literature.^[43] After 3 days of culture of bipotent hepatoblasts on Jag1-HA, Notch was activated; yet Sox9, an early biliary differentiation marker, was not significantly upregulated. These data are consistent with the literature: Notch signaling precedes the differentiation of ductal plate cells (i.e., hepatoblasts).^[40] When cells were cultured for an additional 4–7 d, both markers of mature cholangiocyte differentiation, CK19 and CFTR, were expressed at significantly higher levels than cells on control HA gels. CFTR is glycosylated in the endoplasmic reticulum (ER) to produce the core-glycosylated (immature) form^[44] and then processed by the Golgi glycosyltransferases to produce a complex-glycosylated (mature) form.^[45] Importantly, we detected the majority of CFTR as the mature form of the protein, which shows efficient processing of CFTR in our cells. Thus, our hydrogel system is a robust, xeno-free platform for cholangiocyte differentiation. Perhaps surprisingly, Hey1 and Hes1 expression were higher, but not significantly, on day 7 relative to day 3, suggesting that Notch signaling is transient in nature, and its consistent upregulation may not be required for differentiation. This is consistent with the findings of Dishowitz et al., albeit with the differentiation of a different stem cell population.^[46] HA has been shown to stimulate Notch1 and its associated gene, Hes1, in hepatic stellate cells through CD44 receptor activation.^[47] In our system, HA alone was insufficient for hepatoblasts to differentiate to cholangiocytes. Previous studies showed that Notch2, and not Notch1, is required for hepatoblast differentiation and bile duct development.^[48] Therefore, based on previous studies, we speculate that our Jag1-HA gel engages with the Notch2 pathway to achieve cholangiocyte differentiation. It is important to realize that our newly developed Jag1-HA hydrogel obviated the use of Matrigel and animal cell coculture during the differentiation phase of hepatoblasts to cholangiocytes; however, more work is needed to identify a suitable chemically defined platform that can be used to differentiate PSCs to hepatoblasts, which still relies on Matrigel coating.

Notch signaling is also known to play a critical role in cholangiocyte self-assembly and biliary morphogenesis.^[49] Cells cultured on Jag1-HA demonstrated higher levels of ZO1 expression, tight junctions and self-assembly than those cultured on

HA control gels. ZO1 induces cell-cell tight junctions, which are important in epithelial cell polarization,^[50–52] and, in turn, lead to self-assembly and morphogenesis of epithelial structures.^[53] The expression of ZO1 reflects a key property of cholangiocyte monolayers,^[54] and may account for the cellular self-assembly observed on Jag1-HA. Interestingly, hepatoblasts cultured on Jag1-HA showed substantial morphological changes in the presence of Notch signaling without additional cytokines, which were required when these cells were cocultured with OP9 cells on tissue culture polystyrene.^[1,55] We hypothesize that Notch signaling coupled with the soft microenvironment of the HA hydrogel induced the morphological changes of the cholangiocytes without additional cytokines.^[28] However, further research is required to optimize and characterize the self-assembly of cholangiocyte-like cells to form 3D bile duct-like structures as well as to study the functional maturity of the differentiated cholangiocyte-like cells.

We sought to mimic the *in vivo* like spatiotemporal control of Notch activation in our hydrogels, building on previous strategies for photoinduced protein and peptide activation.^[56–59] We discovered a powerful method to effectively cage Jag1 activity with streptavidin, and then photouncage it with spatiotemporal control to promote Jag1-Notch interaction and subsequent Notch pathway activation as confirmed with fluorescent Notch2 reporter cells both temporally and spatially. Temporally, we observed higher activation at day 4 compared to day 1 on Jag1 ON gels, which is intriguing. We speculate that this may be due to increased cell-cell contacts at day 4 as a result of greater cell spreading compared to that at day 1, which is known to affect Notch signaling.^[60] However further in-depth analysis is required to determine the exact mechanism of higher Notch activation at day 4.

Given the ability of proteins to modulate cellular behavior, several methods have been developed to photochemically pattern signaling proteins;^[61–65] however, most of these methods rely on swelling the hydrogel in a modified protein solution, followed by light exposure to trigger protein immobilization in regions of interest and finally washing away unbound proteins. This method, if performed in the presence of cells, exposes all of the cells to the proteins during the patterning process, which could then trigger cellular response in unwanted areas. Recently, a photolabile molecular glue carrying multiple guanidinium ion pendants was shown to inactivate hepatocyte growth factor (HGF) protein signaling, which could be spatiotemporally activated using photochemical removal of the glue, thereby activating HGF signaling.^[66] Our newly developed strategy has multiple benefits over other methods as it: 1) allows cells to be cultured for up to 5 days (and likely longer), unaffected by the presence of the caged protein, and then selectively activated by photouncaging; 2) does not require elaborate genetic engineering to cage the proteins; and 3) is highly versatile and can be applied to a wide range of proteins, as shown with both Jag1 and DLL4 herein. One potential limitation of our approach is the use of UV light to activate Jag1; however, a recent study showed that low dose UV light does not induce cell death or proteomic changes in cells,^[67] which is consistent with our results where we also did not observe cell death as a result of UV exposure. However, this may not be true for all cell types and the effect of UV exposure on different cells should be

characterized. In future studies, we will investigate photochemical Jag1 uncaging with multi-photon laser patterning to precisely control not only differentiation, but also the Jag1-induced self-assembly of encapsulated cells in 3D hydrogels.

Cholangiocytes rely on Notch signaling for differentiation and ductal morphogenesis whereas hepatocytes require Notch inhibition for maturation.^[12] As such, the spatiotemporally controllable Notch signaling designed herein will play an important role in the development of engineered liver tissue comprised of hepatocytes and cholangiocytes. Ultimately, small molecule-based Notch inhibition (such as with gamma secretase inhibitor) can be combined with our photoactivated Notch signaling hydrogels, thereby providing a way to both turn on and off Notch signaling.^[68] Selective activation of Notch signaling may be useful in answering fundamental biological questions on the role of Notch signaling in morphogenesis in liver and other tissues.

4. Conclusions

We designed a Notch signaling hydrogel (Jag1-HA) to differentiate cholangiocytes from pluripotent stem cells. Notch2 fluorescent reporter cells and stem cell derived hepatoblasts cultured on Jag1-HA gels demonstrated robust activation of the Notch signaling pathway. Hepatoblasts cultured on the Jag1-HA gels resulted in significant upregulation of cholangiocyte markers Sox9, CK19, and CFTR at both gene and protein levels. The differentiated cells expressed ZO1 and showed rudimentary morphogenesis. By leveraging the chemically defined nature of the system, we further engineered the Jag1-HA gels to achieve phototriggerable control over the activation of Notch signaling in a spatiotemporal manner. The robust Jag1–Notch2 axis deactivation and activation was demonstrated with Notch reporter cells as well as human embryonic stem cell derived hepatoblasts. This is the first demonstration of light activated Notch inducing gels for cholangiocyte differentiation. This platform enables spatio-temporal protein activation more generally.

5. Experimental Section

Materials: All materials were used as received unless otherwise indicated. Lyophilized sodium hyaluronate (HA; 242 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). 5-Methylfurfurylamine and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were purchased from TCI (Philadelphia, PA, USA). Pierce Biotin Quantification Kit, Sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC), VILO cDNA kit, Zeba Spin Desalting Columns, Alexa Fluor 647 maleimide (AF647mal), Pierce protein G coated wells, Alexa Fluor 647 Streptavidin conjugate were purchased from ThermoFisher Scientific (Waltham, MA, USA). Bis-maleimide-poly(ethylene glycol) [Mal₂-PEG] was purchased from RAPP Polymere GmbH (Tübingen, Germany). Sodium chloride and 2-(N-morpholino)-ethanesulfonic acid (MES) were purchased from BioShop Canada Inc (Burlington, ON, Canada). N-Succinimidyl carbonate, triethylamine, HoBt, Biotin hydrazide, 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy]butanoic acid, deuterium oxide (D₂O), Dulbecco's phosphate buffered saline (PBS) and bovine fibronectin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dialysis membranes were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Recombinant protein G was purchased from

Cedarlane Labs Inc (Burlington, ON, Canada). Antibodies were purchased from Abcam (Cambridge, United Kingdom). NucleoSpin RNA isolation kit was purchased from MACHEREY-NAGEL (Düren, Germany). SYBR green kit and DNase-free water was purchased from Roche (Basel, Switzerland). Fc-DLL4 and Fc-Jagged1 were purchased from Sino Biological (Beijing, China).

Modification of Protein G with Maleimide: 10 mg of protein G was dissolved in PBS at room temperature. Sulfo-SMCC (4 mg) was added to protein G solution, stirred for 15 min at room temperature (RT), and then overnight at 4 °C. Subsequently, the solution was purified with a 7 kDa spin desalting column (ThermoFisher Scientific; Waltham, MA, USA) followed by FPLC using Superdex75 column (GE Life Sciences; Boston, MA, USA) to obtain maleimide-functionalized protein G (ProGmal), which was characterized by using mass spectroscopy (ESI-MS).

Synthesis of HA Hydrogel and Immobilization of Jagged1: Hyaluronan-methylfuran (HAMf) hydrogels were synthesized as previously described.^[28] Briefly, 1% w/v HA was dissolved in MES buffer (0.1 M, pH 5.5). DMTMM was added to HA solution, followed by dropwise addition of 5-methylfurfurylamine. After the 48 h reaction at RT, the reaction mixture was dialyzed for 24 h against 0.1 M NaCl, followed by dialysis in distilled water for 2 d in 12–14 kDa MW cutoff dialysis tubes and then lyophilized. The degree of furan substitution was determined as previously described using ¹H NMR to be 60%.^[28] To synthesize hydrogels, 1.5% (w/v) HAMf was dissolved in 0.1 M MES (pH 5.5) at 4 °C, fibronectin (1 mg mL⁻¹) was added at 4% v/v of the final gel volume and then 0.8 molar equivalent (to furfuryl) of bis-maleimide terminated peptide (KKGGPQGIWQKGPQGIWQGGKS; synthesized as described previously^[29]) in 0.1 M MES (pH 5.5) reacted for 3 h with HA-methylfurans resulting in a crosslinked HA polymer. Following crosslinking, 20 µL ProGmal (60 µg mL⁻¹) was added to the HA gels and incubated overnight. Subsequently, the gels were washed with PBS (3 times, 45 min each) to remove unbound ProGmal and incubated with 30 µL of Jag1 (100 µg mL⁻¹) overnight at 4 °C followed by washing with PBS (3 times, 45 min each) to remove unbound Jag1 to produce Jag1-ProGmal-HA gels (or simply Jag1-HA gels). Control gels used ProG instead of ProGmal.

Characterization of Jagged1 Immobilization and Mechanical Testing: 15 µL of each of Jag1-HA and ProG-HA control gels were incubated overnight in 30 µL Alexafluor-647-maleimide, AF647mal (10 × 10⁻³ M). Unbound AF647mal was removed with repeated PBS washes (5 times, 45 min each), and then the gels were imaged with FV1000 confocal microscope (Olympus; Tokyo, Japan) and the fluorescence intensity analyzed using ImageJ.

To measure the concentration of tethered protein, HA gel (no proteins), ProG-HA, Jag1-HA and biotin-PL–Jag1–HA gels were prepared as described. Subsequently, the gels were analyzed using the Micro BCA protein assay and the amounts of immobilized protein were calculated by comparison to a calibration curve according to the manufacturer's protocol.

Compressive moduli of the HA and Jag1-HA hydrogels were measured as previously described.^[27] Briefly, 75 µL HA and Jag1-HA hydrogels were swelled overnight in 1× PBS and then analyzed using Mach-1 micromechanical tester (Biomomentum) by applying 10% strain. Compressive moduli were calculated using the slope of stress-strain curves.

CHO Cell Culture and Notch Signaling Analysis: Notch2 expressing polyclonal CHO-K1 fluorescent reporter cells (N2-CHO cells) were kindly donated by Elowitz lab.^[43] N2-CHO cells contain the UAS-H2B-Citrine reporter and express citrine upon Notch2 activation. N2-CHO cells used in this study also constitutively express H2B-Cerulean that was used to image cell nuclei. The cells were cultured in αMEM media supplemented with 10% FBS, penicillin/streptomycin, 2 × 10⁻³ M L-glutamine, and passaged when 80–90% confluent. To analyze the Notch signaling activation, N2-CHO cells were seeded on Jag1-HA gels at 1.5 × 10⁴ cells cm⁻², cultured for up to 3 d and live-imaged using the confocal microscope at various time points during the 3 d culture. HA hydrogels without Jag1 ligands were used as controls. The Notch2-induced citrine fluorescence was analyzed using green channel images

in image], normalized to the total number of cells, and then to the respective HA hydrogel control at each time point. To study the effect of soluble Jag1 ligands, Jag1 was added to the cell culture media at 100 $\mu\text{g mL}^{-1}$ concentration. N2-CHO cells were cultured in Jag1-media overnight followed by imaging and fluorescence intensity analysis, as described above.

Derivation of Hepatoblasts from Human Embryonic PSCs: Human ES cells (H9: WA09) were differentiated to hepatoblasts as previously described^[1,69] with slight modifications. Briefly, the hepatic differentiation was carried out on 2.5% Matrigel coated wells (12 well plates) by step wise approach in the culture medium supplemented with flowing cytokines. For definitive endoderm with activin A (100 ng mL^{-1}) and CHIR99021 (2×10^{-6} M), for hepatic specification with BMP4 (50 ng mL^{-1}) and bFGF (40 ng mL^{-1}), and for hepatoblast differentiation with HGF (20 ng mL^{-1}), DEX (40 ng mL^{-1}), and Oncostatin M (OSM) (20 ng mL^{-1}). To quantify the hepatoblast differentiation, day 27 hepatoblasts were dissociated into single cell suspensions by TrypLE and fixed with 4% paraformaldehyde (PFA: Electron Microscopy Science, Hatfield, PA, USA) in PBS. The fixed cells were permeabilized with 90% ice-cold methanol for 20 min for ALB and AFP double staining as previously mentioned.^[1,69] The primary antibodies used were albumin (ALB) (Bethyl: A80-129A) and alpha-fetoprotein (AFP) (DAKO: A008). Cells were subsequently incubated with secondary antibodies (Alexa 647 for ALB and Alexa 555 for AFP: Invitrogen) for 30 min at room temperature. The stained cells were analyzed using LSR Fortessa Flow cytometer (BD).

Hepatoblasts Culture on Jag1-HA Hydrogels: Hepatoblasts that had been differentiated for 27 days on tissue culture polystyrene surfaces, were passaged and plated on Jag1-HA hydrogels at 3×10^4 cells per well (in a 384 well plate) and cultured for up to 7 d in maintenance media containing DMEM/F12 (1:1) supplemented with 0.1% BSA, 1% v/v B27 supplement, 2×10^{-3} M ascorbic acid (Sigma), 4×10^{-4} M monothioglycerol (MTG: Sigma), 2×10^{-3} M glutamine (Gibco-BRL) and 10×10^{-6} M Rock inhibitor Y-27632 in standard cell culture conditions. HA hydrogels without Jag1 ligands were used as controls.

Hepatoblasts Gene and Protein Expression Analysis: For gene expression analysis, cells were lysed, and RNA was isolated using the NucleoSpin RNA isolation kit as per the manufacturer's instructions. RNA was reverse transcribed to prepare cDNA using the superscript VILO cDNA kit. Quantitative polymerase chain reaction (qPCR) amplification was performed in an Applied Biosystems 7900HT instrument (40 cycles) using LightCycler 480 SYBR green with 5 ng RNA. Negative controls (wells without template RNA) were included to ensure accuracy of the data. The primer pairs used are given in Table S1 in the Supporting Information. The GAPDH was used as housekeeping gene.

For immunostaining following culture, the cells were fixed with 4% paraformaldehyde for 30 min. After three washes in PBS (10 min each), cells were permeabilized with a mixture of 100×10^{-3} M glycine and 0.5% triton X-100 for 15 min, washed with PBS containing 0.05% glycine, and blocked with 10% goat serum and 1% bovine serum albumin (BSA) for 1 h. Following blocking, cells were incubated overnight with primary antibodies, washed with PBS (5 times, 30 min each) containing 0.05% glycine, and incubated with secondary antibodies overnight. Subsequently, the cells were washed again with PBS (5 times, 30 min each) and counterstained with 20×10^{-3} M Hoechst 33 342 dye for nuclei staining. The primary antibodies used: mouse anti-sox9 antibody at 1:400 dilution in PBS; rabbit anti-cytokeratin 19 (CK19) at 1:400 dilution; goat anti-albumin at 1:200 dilution; rabbit anti-ZO1 antibody at 1:100 dilution in PBS. The following secondary antibodies were used (all at 1:500 dilution): AlexaFluor goat anti mouse 488; AlexaFluor goat anti rabbit 546; AlexaFluor goat anti rabbit 488; AlexaFluor donkey anti goat 555. Cells were imaged with an Olympus FV1000 confocal microscope. The relative fluorescence intensity of the proteins was analyzed using mean gray values (MGV) of the images determined by ImageJ. For CK19, albumin and ZO1, the MGV of the whole images were measured. For Sox9, the cell nuclei were manually outlined and the MGV was measured in cell nuclei only. Data were normalized to HA controls.

Immunoblotting: WT and KO-CFTR 16HBE14o- cells were grown as previously described.^[70] Following 7 d of culture on Jag1-HA versus HA hydrogels, the cells were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer (50×10^{-3} M Tris-HCl, 150×10^{-3} M NaCl, 1×10^{-3} M EDTA, pH 7.4, 0.2% SDS, and 0.1% triton X-100) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) for 10 min.^[71] Soluble fractions were analyzed by SDS-PAGE on 6% tris-glycine gels (Life Technologies). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and incubated in 5% milk. CFTR bands were detected with human CFTR-specific murine mAb 596 (UNC, North Carolina, USA) with 1:1000 dilution. The rAb Calnexin (CNX; Sigma) was used as a loading control with 1:10000 dilution. The blots were developed with ECL (Amersham) using the Li-Cor Odyssey Fc (LI-COR Biosciences, Lincoln, NE) in a linear range of exposure (2–45 min).^[72–73] Relative levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageStudioLite (LI-COR Biosciences, Lincoln, NE).

Synthesis of Biotinylated Photolinker NHS: Biotinylated photolinker NHS ester was synthesized as follows: 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (116 mg) was dissolved in 2 mL of dry dichloromethane (DCM) under nitrogen atmosphere. Biotin hydrazide (100 mg, 1 eq.) was dissolved in 2 mL dry dimethylformamide (DMF) and added to the above. The solution was cooled to 0 °C and stirred for 15 min. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (148 mg, 2 eq.) and HoBt (52 mg; 1 eq.) were dissolved in 4 mL DCM and added to the reaction flask. Subsequently, triethylamine (47 mg; 1.2 eq) was added to the reaction mixture, stirred for an additional 20 min at 0 °C, warmed to RT, and stirred overnight in the dark. Conversion of the reaction was monitored with thin layer chromatography (TLC). Solvent was evaporated under reduced pressure to yield Compound 1 (Scheme S1, Supporting Information) which was purified with flash chromatography (solvent 10% MeOH in DCM) (88% yield). Compound 1 (127 mg) and *N*-succinimidyl carbonate (150 mg, 2.5 eq.) were dissolved in 1 mL dry DMF under nitrogen. Subsequently, triethylamine (60 mg; 2.5 eq) was added and the reaction mixture was stirred overnight at RT in the dark. DMF was removed by rotary evaporation. The resulting viscous residue was dissolved in DCM followed by an aqueous work up. The organic phase was evaporated under reduced pressure and the residue purified by flash chromatography (10% MeOH in DCM) to obtain the final compound 2, biotinylated photolinker NHS ester (Scheme S1, Supporting Information). ¹H NMR spectrum of the biotinylated photolinker NHS ester was collected in 400 MHz Agilent VnmrS NMR Spectrometer using DMSO-d₆ solvent. ¹H NMR (400 MHz, DMSO-d₆) δ 9.75 (s, 1H), 9.65 (s, 1H), 7.61 (s, 1H), 7.18 (s, 1H), 6.40 – 6.27 (m, 3H), 4.33 – 4.29 (m, 1H), 4.19 – 4.07 (m, 3H), 3.99 (s, 3H), 3.12 – 3.07 (m, 1H), 2.85 – 2.73 (m, 7H), 2.59 – 2.56 (m, 1H), 2.32 (t, *J* = 7.5 Hz, 2H), 2.11 (t, *J* = 7.3 Hz, 2H), 2.02 – 1.98 (m, 2H), 1.74 (d, *J* = 6.2 Hz, 3H), 1.60 – 1.31 (m, 4H).

Photolinker Modification of Jag1 and Characterization: Conjugation of the biotin-photolinker-NHS (biotin-PL-NHS) onto Jag1 was performed by acylation of the lysines on Jag1. Jag1 was dissolved in sterile-filtered ddH₂O (500 $\mu\text{g mL}^{-1}$, 100 μL , 1 equiv.) to which 25 μL of 0.1 M NaHCO₃ pH 8.5 was added, followed by biotin-PL-NHS in DMSO (12.24 $\mu\text{g mL}^{-1}$, 5 μL , 250 equiv.) for 1 h at RT. The resulting biotin-PL-Jag1 was purified from unreacted small molecules by filtration through a spin desalting column (40 kg mol^{-1} MWCO, 0.5 mL). The biotin-PL-Jag1 concentration was determined using a Nanodrop spectrophotometer. The incorporation of the photolinker onto Jag1 was quantified by Pierce biotin quantification kit using the microplate format. Briefly, the absorbance of a HABA/Avidin Premix solution was measured at 500 nm. Then, a sample of biotin-PL-Jag1 in PBS was added to the HABA/Avidin Premix solution, and the absorbance measured at 500 nm. The moles of biotin per mole of Jag1 was calculated based on the change in absorbance at 500 nm and normalized to biotinylated horseradish peroxidase (HRP) used as positive control. To quantify the amount of photolinker left on Jag1 after UV-irradiation, biotin-PL-Jag1 was exposed to 365 nm UV (1.9 mW cm^{-2}) for 15 min (biotin-PL-Jag1 + UV), the solution was filtered through spin desalting columns to remove all the unbound biotinylated small molecules, and then the absorbance measured at 500 nm.

Characterization of Streptavidin Modification of Biotin–PL–Jag1 and Photoinduced Cleavage: Pierce Protein G coated wells were washed 3 times with 0.02% Tween-20 in PBS to prevent non-specific protein interactions. Jag1 or biotin–PL–Jag1 in PBS was added to the wells and incubated overnight at 4 °C. The wells were washed 3-times with PBS and incubated at 37 °C with AlexaFluor647 Streptavidin conjugate (strepAF647). After subsequent washing of the wells (3-times with PBS), the fluorescence was measured at an excitation maximum of 640 nm and emission maximum 678 nm (strepAF647–biotin–PL–Jag1). The wells were then exposed to UV irradiation at 365 nm (1.9 mW cm⁻²) for 15 min and washed 3 times. Fluorescence was re-measured for strepAF647–biotin–PL–Jag1 + UV. Protein G coated well plates (plastic) were used for analytical characterization of streptavidin modification of biotin–PL–Jag1 and photoinduced cleavage to maximize the use of the hydrogels.

Synthesis of Streptavidin Caged Jag1-HA and DLL4-HA Gels and Temporal and Spatial Control of Notch Activation: Streptavidin caged Jag1 HA (strep–biotin–PL–Jag1–HA or simply Jag1 OFF) gels were synthesized similarly to Jag1-HA hydrogels with the addition of streptavidin in PBS (100 µg mL⁻¹, 70 µL) to biotin–PL–Jag1–HA gels for 2 h at 37 °C. The Jag1 OFF HA gels were washed 3-times with PBS and either directly used for cell seeding or uncaged by UV-irradiation at 365 nm for 15 min (1.9 mW cm⁻²) to produce “Strep–biotin–PL–Jag1–HA + UV” (or simply Jag1 ON) gels. The same process was used for DLL4 with the following minor changes: 200 equiv. photolinker was used instead of 250 equiv. during conjugation and 60 µg mL⁻¹ of DLL4-Fc was added to the ProGmal-HA gels instead of 100 µg mL⁻¹. To demonstrate temporal control, two time points were investigated. For 1-day cultures, Jag1 OFF HA gels were exposed to UV light for 15 min to photoactivate Jag1 (Jag1 ON) and then seeded with 1×10⁴ number of N2-CHO cells in a 384 well plate overnight (Culture time: 1 day). For 4-day cultures, N2-CHO cells were seeded and cultured on Jag1 OFF HA gels for 3 d, then exposed to UV light to photoactivate Jag1 (i.e., Jag1 ON), and then cultured for an additional day prior to imaging with confocal microscope. For the control group, cells were cultured on photocaged, Jag1 OFF gels for 1 and 4 d. The Notch2-induced citrine fluorescence intensity was measured using imageJ, normalized to total number of cells, and then normalized to Jag1 OFF control at respective time points. Similar protocols were used to demonstrate the temporal activation of Notch signaling with strep-biotin-PL-DLL4 gels. To demonstrate spatial control, N2-CHO cells were cultured on photocaged Jag1 OFF HA gels for 1 d in a custom-made acrylic chamber of 4 mm x 4 mm x 1 mm dimensions, and then half of the gels (2 mm x 4 mm x 1 mm) were exposed to UV light (8.7 mW cm⁻²) for 5 min through a photomask using a mask aligner (Model 30, OAI; Milpitas, CA, USA) (workflow shown in Figure S15a in the Supporting Information). Following exposure, the cells were cultured for an additional day and imaged with the confocal microscope. For the photocontrolled differentiation of hepatoblasts, cells were cultured on photoactivated (Jag1 ON) and photocaged (Jag1 OFF) gels for 5 days and then analyzed for Sox9, CK19, CK7, and CFTR gene expression and Sox9 protein expression as described previously.

Statistical Analysis: All statistical analyses were performed using GraphPad Prism version 7. Student's *t*-test was used to determine statistical significance for all the data except for characterization of Jag1 immobilization on HA (one-way ANOVA and Dunnett's post hoc test) and characterization of N2-CHO cells fluorescence (Two-way ANOVA and Sidak's post hoc). *P* values are represented as **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

M.R. and A.F. contributed equally to this work. The authors are grateful to the Canada First Research Excellence Fund to Medicine by Design at

the University of Toronto (MSS, CEB, SO, and GK) for funding and to members of the Shoichet lab for thoughtful discussions and review of this manuscript. The authors acknowledge financial support from the Swedish Foundation for Strategic Research (RMA15-0010). The authors thank Dr. Michael B. Elowitz (California Institute of Technology) for providing the fluorescent Notch2 reporter cell line.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

hepatoblast differentiation, hyaluronan, hydrogel, liver, notch signaling, photocaging

Received: July 20, 2020
Revised: September 11, 2020
Published online:

- [1] M. Ogawa, S. Ogawa, C. E. Bear, S. Ahmadi, S. Chin, B. Li, M. Grompe, G. Keller, B. M. Kamath, A. Chanekar, *Nat. Biotechnol.* **2015**, *33*, 853.
- [2] N. Dianat, H. Dubois-Pot-Schneider, C. Steichen, C. Desterke, P. Leclerc, A. Raveux, L. Combettes, A. Weber, A. Corlu, A. Dubart-Kupperschmitt, *Hepatology* **2014**, *60*, 700.
- [3] F. Sampaziotis, M. Cardoso de Brito, P. Madrigal, A. Bertero, K. Saeb-Parsy, F. A. C. Soares, E. Schrupf, E. Melum, T. H. Karlens, J. A. Bradley, W. T. H. Gelson, S. Davies, A. Baker, A. Kaser, G. J. Alexander, N. R. F. Hannan, L. Vallier, *Nat. Biotechnol.* **2015**, *33*, 845.
- [4] S. Matsui, M. Ochiai, K. Yasuda, S.-i. Mae, M. Kotaka, T. Toyoda, T. Yamamoto, K. Osafune, *Stem Cell Res.* **2019**, *35*, 101400.
- [5] S. Llamas, E. García-Pérez, Á. Meana, F. Larcher, M. del Río, *Tissue Eng., Part* **2015**, *21*, 345.
- [6] E. H. Nguyen, W. T. Daly, N. N. T. Le, M. Farnoodian, D. G. Belair, M. P. Schwartz, C. S. Lebakken, G. E. Ananiev, M. A. Saghiri, T. B. Knudsen, N. Sheibani, W. L. Murphy, *Nat. Biomed. Eng.* **2017**, *1*, 0096.
- [7] F. Geisler, M. Strazzabosco, *Hepatology* **2015**, *61*, 382.
- [8] K. Si-Tayeb, F. P. Lemaigre, S. A. Duncan, *Dev. Cell* **2010**, *18*, 175.
- [9] Y. Zong, B. Z. Stanger, *Int. J. Biochem. Cell Biol.* **2011**, *43*, 257.
- [10] Y. Zong, A. Panikkar, J. Xu, A. Antoniou, P. Raynaud, F. Lemaigre, B. Z. Stanger, *Development* **2009**, *136*, 1727.
- [11] A. A. Louis, P. Van Eyken, B. A. Haber, C. Hicks, G. Weinmaster, R. Taub, E. B. Rand, *Hepatology* **1999**, *30*, 1269.
- [12] M. Huch, C. Dorrell, S. F. Boj, J. H. van Es, V. S. W. Li, M. van de Wetering, T. Sato, K. Hamer, N. Sasaki, M. J. Finegold, A. Haft, R. G. Vries, M. Grompe, H. Clevers, *Nature* **2013**, *494*, 247.
- [13] M. Takeda, S. Ogino, R. Umemoto, M. Sakakura, M. Kajiwara, K. N. Sugahara, H. Hayasaka, M. Miyasaka, H. Terasawa, I. Shimada, *J. Biol. Chem.* **2006**, *281*, 40089.
- [14] J. E. Rowley, G. E. Rubenstein, S. L. Manuel, N. L. Johnson, J. Surgnier, P. P. Kapitsinou, F. E. Duncan, M. T. Pritchard, *J. Histochem. Cytochem.* **2019**, *68*, 75.
- [15] C. M. Nimmo, S. C. Owen, M. S. Shoichet, *Biomacromolecules* **2011**, *12*, 824.
- [16] B. G. Ballios, M. J. Cooke, L. Donaldson, B. L. Coles, C. M. Morshead, D. van der Kooy, M. S. Shoichet, *Stem Cell Rep.* **2015**, *4*, 1031.
- [17] N. Mitrousis, R. Y. Tam, A. E. G. Baker, D. van der Kooy, M. S. Shoichet, *Adv. Funct. Mater.* **2016**, *26*, 1975.

- [18] J. A. Burdick, G. D. Prestwich, *Adv. Mater.* **2011**, *23*, H41.
- [19] G. Khandekar, J. Llewellyn, A. Kriegermeier, O. Waisbourd-Zinman, N. Johnson, Y. Du, R. Giwa, X. Liu, T. Kisseleva, P. A. Russo, N. D. Theise, R. G. Wells, *J. Hepatol.* **2020**, *72*, 135.
- [20] M. I. Yovchev, P. N. Grozdanov, B. Joseph, S. Gupta, M. D. Dabeva, *Hepatology* **2007**, *45*, 139.
- [21] D. Dhar, L. Antonucci, H. Nakagawa, J. Y. Kim, E. Glitzner, S. Caruso, S. Shalapour, L. Yang, M. A. Valasek, S. Lee, K. Minnich, E. Seki, J. Tuckermann, M. Sibilia, J. Zucman-Rossi, M. Karin, *Cancer Cell* **2018**, *33*, 1061.
- [22] Y. He, G. D. Wu, T. Sadahiro, S. I. Noh, H. Wang, D. Talavera, H. Wang, J. M. Vierling, A. S. Klein, *Am. J. Physiol.* **2008**, *295*, G305.
- [23] T. E. Riehl, S. Santhanam, L. Foster, M. Ciorba, W. F. Stenson, *Am. J. Physiol.* **2015**, *309*, G874.
- [24] X.-M. Chen, S. P. O'Hara, J. B. Nelson, P. L. Splinter, A. J. Small, P. S. Tietz, A. H. Limper, N. F. LaRusso, *J. Immunol.* **2005**, *175*, 7447.
- [25] S. Khunmanee, Y. Jeong, H. Park, *J. Tissue Eng.* **2017**, *8*, 204173141772646.
- [26] V. Delplace, P. E. B. Nickerson, A. Ortin-Martinez, A. E. G. Baker, V. A. Wallace, M. S. Shoichet, *Adv. Funct. Mater.* **2020**, *30*, 1903978.
- [27] A. E. G. Baker, L. C. Bahlmann, R. Y. Tam, J. C. Liu, A. N. Ganesh, N. Mitrousis, R. Marcellus, M. Spears, J. M. S. Bartlett, D. W. Cescon, G. D. Bader, M. S. Shoichet, *Adv. Mater.* **2019**, *31*, 1901166.
- [28] L. J. Smith, S. M. Taimoory, R. Y. Tam, A. E. G. Baker, N. Binth Mohammad, J. F. Trant, M. S. Shoichet, *Biomacromolecules* **2018**, *19*, 926.
- [29] R. Y. Tam, J. Yockell-Lelièvre, L. J. Smith, L. M. Julian, A. E. G. Baker, C. Choey, M. S. Hasim, J. Dimitroulakos, W. L. Stanford, M. S. Shoichet, *Adv. Mater.* **2019**, *31*, 1806214.
- [30] L. Meloty-Kapella, B. Shergill, J. Kuon, E. Botvinick, G. Weinmaster, *Dev. Cell* **2012**, *22*, 1299.
- [31] W. R. Gordon, B. Zimmerman, L. He, L. J. Miles, J. Huang, K. Tiyanont, D. G. McArthur, J. C. Aster, N. Perrimon, J. J. Loparo, S. C. Blacklow, *Dev. Cell* **2015**, *33*, 729.
- [32] P. D. Langridge, G. Struhl, *Cell* **2017**, *171*, 1383.
- [33] H. Safaee, M. A. Bakooshli, S. Davoudi, R. Y. Cheng, A. J. Martowirogo, E. W. Li, C. A. Simmons, P. M. Gilbert, *Cell Mol. Bioeng.* **2017**, *10*, 501.
- [34] A. Ranga, S. Gobaa, Y. Okawa, K. Mosiewicz, A. Negro, M. P. Lutolf, *Nat. Commun.* **2014**, *5*, 4324.
- [35] U. Blache, Q. Vallmajo-Martin, E. R. Horton, J. Guerrero, V. Djonov, A. Scherberich, J. T. Erler, I. Martin, J. G. Snedeker, V. Milleret, M. Ehrbar, *EMBO Rep.* **2018**, *19*, 45964.
- [36] K. B. Kaylan, I. C. Berg, M. J. Biehl, A. Brougham-Cook, I. Jain, S. M. Jamil, L. H. Sargeant, N. J. Cornell, L. T. Raetzman, G. H. Underhill, *eLife* **2018**, *7*, 38536.
- [37] W. Choe, T. A. Durgannavar, S. J. Chung, *Material* **2016**, *9*, 994.
- [38] L. A. Tiemeijer, J. P. Frimat, O. M. J. A. Stassen, C. V. C. Bouten, C. M. Sahlgren, *Sci. Rep.* **2018**, *8*, 6392.
- [39] A. P. Feranchak, *Hepatology* **2018**, *67*, 833.
- [40] Y. Zong, A. Panikkar, J. Xu, A. Antoniou, P. Raynaud, F. Lemaigre, B. Z. Stanger, *Development* **2009**, *136*, 1727.
- [41] J. J. Hofmann, A. C. Zovein, H. Koh, F. Radtke, G. Weinmaster, M. L. Iruela-Arispe, *Development* **2010**, *137*, 4061.
- [42] N. Tanimizu, A. Miyajima, *J. Cell Sci.* **2004**, *117*, 3165.
- [43] N. Nandagopal, L. A. Santat, M. B. Elowitz, *eLife* **2019**, *8*, 37880.
- [44] C. M. Farinha, P. Matos, M. D. Amaral, *FEBS J.* **2013**, *280*, 4396.
- [45] S. H. Cheng, R. J. Gregory, J. Marshall, S. Paul, D. W. Souza, G. A. White, C. R. O'Riordan, A. E. Smith, *Cell* **1990**, *63*, 827.
- [46] M. I. Dishowitz, F. Zhu, H. G. Sundararaghavan, J. L. Ifkovits, J. A. Burdick, K. D. Hankenson, *J. Biomed. Mater. Res., Part A* **2014**, *102*, 1558.
- [47] Y. M. Yang, M. Nouredin, C. Liu, K. Ohashi, S. Y. Kim, D. Ramnath, E. E. Powell, M. J. Sweet, Y. S. Roh, I. F. Hsin, N. Deng, Z. Liu, J. Liang, E. Mena, D. Shouhed, R. F. Schwabe, D. Jiang, S. C. Lu, P. W. Noble, E. Seki, *Sci. Transl. Med.* **2019**, *11*, eaat9284.
- [48] F. Geisler, F. Nagl, P. K. Mazur, M. Lee, U. Zimmer-Strobl, L. J. Strobl, F. Radtke, R. M. Schmid, J. T. Sivek, *Hepatology* **2008**, *48*, 607.
- [49] Y. Kodama, M. Hijikata, R. Kageyama, K. Shimotohno, T. Chiba, *Gastroenterology* **2004**, *127*, 1775.
- [50] K. Shin, S. Straight, B. Margolis, *J. Cell Biol.* **2005**, *168*, 705.
- [51] J. Ikenouchi, K. Umeda, S. Tsukita, M. Furuse, S. Tsukita, *J. Cell Biol.* **2007**, *176*, 779.
- [52] M. A. Odenwald, W. Choi, A. Buckley, N. Shashikanth, N. E. Joseph, Y. Wang, M. H. Warren, M. M. Buschmann, R. Pavlyuk, J. Hildebrand, B. Margolis, A. S. Fanning, J. R. Turner, *J. Cell Sci.* **2017**, *130*, 243.
- [53] J. Roignot, X. Peng, K. Mostov, *Cold Spring Harbor Perspect. Biol.* **2013**, *5*, a013789.
- [54] R. K. Rao, G. Samak, *Tissue Barriers* **2013**, *1*, 25718.
- [55] D. Vyas, P. M. Baptista, M. Brovold, E. Moran, B. Gaston, C. Booth, M. Samuel, A. Atala, S. Soker, *Hepatology* **2018**, *67*, 750.
- [56] S. Tang, Z. Wan, Y. Gao, J. S. Zheng, J. Wang, Y. Y. Si, X. Chen, H. Qi, L. Liu, W. Liu, *Chem. Sci.* **2016**, *7*, 1891.
- [57] S. Takamori, S. Yamaguchi, N. Ohashi, T. Nagamune, *Chem. Commun.* **2013**, *49*, 3013.
- [58] R. J. Mart, R. K. Allemann, *Chem. Commun.* **2016**, *52*, 12262.
- [59] S. Petersen, J. M. Alonso, A. Specht, P. Duodu, M. Goeldner, A. del Campo, *Angew. Chem., Int. Ed.* **2008**, *47*, 3192.
- [60] O. Shaya, U. Binshtok, M. Hersch, D. Rivkin, S. Weinreb, L. Amir-Zilberstein, B. Khamaisi, O. Oppenheim, R. A. Desai, R. J. Goodyear, G. P. Richardson, C. S. Chen, D. Sprinzak, *Dev. Cell* **2017**, *40*, 505.
- [61] C. A. DeForest, D. A. Tirrell, *Nat. Mater.* **2015**, *14*, 523.
- [62] H. Ma, A. S. Caldwell, M. A. Azagarsamy, A. G. Rodriguez, K. S. Anseth, *Biomaterials* **2020**, *255*, 120205.
- [63] R. G. Wylie, S. Ahsan, Y. Aizawa, K. L. Maxwell, C. M. Morshead, M. S. Shoichet, *Nat. Mater.* **2011**, *10*, 799.
- [64] J. C. Grim, T. E. Brown, B. A. Aguado, D. A. Chapnick, A. L. Viert, X. Liu, K. S. Anseth, *ACS Cent. Sci.* **2018**, *4*, 909.
- [65] J. A. Shadish, G. M. Benuska, C. A. DeForest, *Nat. Mater.* **2019**, *18*, 1005.
- [66] R. Mogaki, K. Okuro, R. Ueki, S. Sando, T. Aida, *J. Am. Chem. Soc.* **2019**, *141*, 8035.
- [67] E. R. Ruskowitz, C. A. DeForest, *ACS Biomater. Sci. Eng.* **2019**, *5*, 2111.
- [68] C. M. Morell, M. Strazzabosco, *J. Hepatol.* **2014**, *60*, 885.
- [69] A. Holtzinger, P. R. Streeter, F. Sarangi, S. Hillborn, M. Niapour, S. Ogawa, G. Keller, *Development* **2015**, *142*, 4253.
- [70] O. Laselva, T. A. Stone, C. E. Bear, C. M. Deber, *Biomolecules* **2020**, *10*, 334.
- [71] O. Laselva, G. Marzaro, C. Vaccarin, I. Lampronti, A. Tamanini, G. Lippi, R. Gambari, G. Cabrini, C. E. Bear, A. Chilin, M. C. Dechecchi, *Front. Pharmacol.* **2018**, *9*, 719.
- [72] O. Laselva, P. D. Eckford, C. Bartlett, H. Ouyang, T. N. Gunawardena, T. Gonska, T. J. Moraes, C. E. Bear, *J. Cystic Fibrosis* **2020**, *19*, 717.
- [73] Y. S. Wu, J. Jiang, S. Ahmadi, A. Lew, O. Laselva, S. Xia, C. Bartlett, W. Ip, L. Wellhauser, H. Ouyang, T. Gonska, T. J. Moraes, C. E. Bear, *Mol. Pharmacol.* **2019**, *96*, 515.