Inducing Human Retinal Pigment Epithelium-like Cells from Somatic Tissue

Ivo Ngundu Woogeng[1], Imad Abugessaisa[2], Akihiro Tachibana[1], Yoshiki Sahara[1,3,4], Chung-Chau Hon[2], Akira Hasegawa[2], Bogumil Kaczkowski[2], Noriko Sakai[1], Mitsuhiro Nishida[1], Haiming Hu[1], Hashimita Sanyal[1], Junki Sho[1], Takeya Kasukawa[2], Minoru Takasato[1,3], Piero Carninci[2], Akiko Maeda[1], Michiko Mandai[1], Erik Arner[2], Masayo Takahashi[1], and Cody Kime[1,5]

1 RIKEN Center for Biosystems Dynamics Research, Kobe 650-0047, Japan.
2 RIKEN Center for Integrative Medical Sciences, Yokohama 230-0045, Japan.
3 Laboratory of Molecular Cell Biology and Development, Department of Animal Development and Physiology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan.
4 Department of Renal and Cardiovascular Research, New Drug Research Division, Otsuka Pharmaceutical Co. Ltd., Tokushima 771-0192, Japan.
5 Lead Contact
Corresponding Author: Correspondence should be addressed to C.K. (cody.kime@riken.jp).

16 **SUMMARY**

17 Regenerative medicine relies on basic research to find safe and useful outcomes that are only practical
18 when cost-effective. The human eyeball requires the retinal pigment epithelium (RPE) for support and
19 maintenance that interfaces the neural retina and the choroid at large. Nearly 200 million people suffer
20 from age-related macular degeneration (AMD), a blinding multifactor genetic disease among other retinal
21 pathologies related to RPE degradation. Recently, autologous pluripotent stem cell-derived RPE cells
22 were prohibitively expensive due to *time,* therefore we developed a new simplified cell reprogramming
23 system. We stably induced RPE-like cells (iRPE) from human fibroblasts by conditional overexpression of
24 broad plasticity and lineage-specific pioneering transcription factors. iRPE cells showed features of
25 modern RPE benchmarks and significant *in-vivo* integration in transplanted chimeric hosts. Herein, we
26 detail the iRPE system with comprehensive modern single-cell RNA (scRNA) sequencing profiling to
27 interpret and characterize its best cells. We anticipate that our system may enable robust retinal cell
28 induction for regenerative medicine research and affordable autologous human RPE tissue for cell
29 therapy.

30 **KEYWORDS**

31 cell biology; cell plasticity; reprogramming; regenerative medicine; retina

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INTRODUCTION

The retinal pigment epithelium (RPE) is a monolayer of cuboidal cells developed between the photoreceptors and choroid of the eye. The RPE is critical for the development, maintenance, and function of photoreceptors, and mutations in key RPE genes may cause degenerative retinal disorders such as retinitis pigmentosa (RP) with a prevalence of 1 in 4000 people as the most common inherited retinal dystrophy (Esumi et al., 2004; Verbakel et al., 2018). Supplementation of RPE cells can recover from RPE dysfunction in animal models, suggesting a potential solution for RP (Haruta et al., 2004; Maeda et al., 2013). RPE degeneration onset is also associated with preceding age-related macular degeneration (AMD), the leading cause of irreversible blindness in western countries (Esumi et al., 2004; Klein et al., 1992; Smith et al., 2001). In AMD, the RPE cells are invariably lost, therefore intervening AMD treatments to prevent blindness may require cell transplant therapy (Mandai et al., 2017).

Recent advances in regenerative medicine have motivated new strategies to develop pluripotent stem cell-derived RPE from human embryonic stem (ES) cells and autologous induced pluripotent stem cells (iPSC) (Haruta et al., 2004; Mandai et al., 2017). RPE differentiation from pluripotent stem cells was pioneered against great technological and political barriers that were overcome with demonstrably safe and functional iPSC.RPE for patient transplant (Mandai et al., 2017). Still, uncertainties about iPSC potency and genome stability remain, and such novel regenerative medicine required labor and financing that are impossible to budget in modern health systems. Therefore, simplifying the induction of autologous RPE cells with a more direct approach may be necessary.

Lately, 'direct reprogramming' systems that convert between somatic cell states (Najm et al., 2013; Pang et al., 2011; Zhang et al., 2014) have emerged, inspired by the transcription factor (TF) synergy famously uncovered by Kazu Takahashi and Shinya Yamanaka with iPSCs (Takahashi et al., 2007). Such systems posit that core TF sets should 'directly' convert between somatic cell identities (D'Alessio et al., 2015; Rackham et al., 2016). Conceptually, 'direct reprogramming' ignores epigenetic plasticity, or assumes it, with simplistic design for somatic to somatic cell state conversion. In practice, such systems usually rely on Yamanaka-factor (OCT4, SOX2, KLF4, MYC) co-induction, or transit cell progenitor and intermediate plastic states yet bound by *time* and a *surviving identity.* Unlike somatic cell identities, induced pluripotency enables increasing plasticity via pioneering TF driven epigenetically self-recursive state reinforcement, termed 'maturation', that was obviated much later (Iwafuchi-Doi and Zaret, 2014; Samavarchi-Tehrani et al., 2010; Soufi and Zaret, 2013; Soufi et al., 2012). Indeed, once minimally established, *in-vitro* pluripotency self-iterates and stabilizes its plastic pluripotent identity. To systematically induce RPE (iRPE) from fibroblasts, TFs with pioneering activity, direct roles in plasticity, and the developmental differentiation and specification of RPE may be required (Soufi et al., 2015).

To reduce the costly *time* for autologous cell production, we looked to engage epigenetic plasticity at the same time as an induced RPE state using the aforementioned criteria and found that four TFs, enhanced by CRX and small molecules, could convert human fibroblasts to bulk cultures containing RPE-like cells with characteristic function, expression, cell identity, and integration in chimeric subretinal transplants. Together, this iRPE platform and scRNA datasets might be used to develop affordable autologous biomedical-grade regenerative RPE cell therapies.

72   **RESULTS**

73   **Few Exogenes Are Necessary to Reprogram Human Somatic Cells to RPE-like Fate**
74   Inducing autologous iPSC-derived RPE (iPSC.RPE) has aided cell therapy studies (Mandai et al., 2017)
75   with a multiplier for costs based on *time*. For practicality, we set out to induce human somatic cells much
76   more quickly to RPE cells (Figure 1A). We looked to a previous 'direct reprogramming' study (Zhang et
77   al., 2014), but such factors failed in our hands (data not shown). However, we found that a conditionally
78   expressed combination of RPE cell-specific (MITF, CRX, OTX2), lineage-specific (CRX, OTX2), and
79   pluripotency/plasticity/regenerative genes (OTX2, LIN28, MYCL) could rapidly induce human foreskin
80   fibroblasts (Fib) to RPE-like cells with tacit features of RPE summarized in Figure 1. We employed
81   lentiviral transduction to introduce a molecular toolset containing a common minimized BEST1 (VMD2)
82   (Esumi et al., 2004; Masuda and Esumi, 2010; Zhang et al., 2014) synthetic reporter construct to drive
83   EGFP expression (*BEST1::*EGFP) and a constitutive polycistronic sequence with dox-inducible rTTA and
84   Puromycin resistance (Figure 1B). Fib were transduced with the toolset, selected briefly with Puromycin,
85   expanded, and then transduced with dox-inducible TetRE transgenes (Figure S1A). Importantly, the
86   *BEST1::*EGFP reporter responded to iPSC.RPE cell maturity and density, with similar expression in our
87   RPE-like cells. We sub-cultured picked colonies of such induced iRPE cells and validated RPE65 protein
88   expression among the EGFP+ cells (Figure 1C). We termed these induced RPE-like cells iRPE.
89   Individually sub-cultured iRPE colonies did not proliferate or expand much past 0.64 cm^2 (Figure S1B),
90   and thus we usually bulk passaged our full 6W well 1:2 on approximately Day 28-30 (Figure 1D). With this
91   system, we generally observed distinct morphological change and mesenchymal to epithelial transition
92   (MET) between Days 5 and 10 and specific cobblestone RPE-like morphology with variable activation of
93   *BEST1::*EGFP between Days 12 and 25 and variable stability after removal of doxycycline (Figure 1D).
94   Taken together, these observations reinforced the notion that our iRPE system may transition through
95   important MET mediated cell identity reprogramming with genome stability selectivity (Kareta et al., 2015;
96   Li et al., 2010; Marión et al., 2009; Samavarchi-Tehrani et al., 2010), and then acquire important features
97   and reporters of RPE cell identity (Maruotti et al., 2015; Masuda and Esumi, 2010; Zhang et al., 2014).

98   **Orthodenticle Genes are Powerful Effectors of iRPE Reprogramming**
99   Previous reports had resolved or hypothesized (Rackham et al., 2016) iRPE system factors (Figure 2A),
100 but did not turn off reprogramming factor expression (Zhang et al., 2014), or observed a return to Fib
101 identity when doing so (D'Alessio et al., 2015). Both reports that demonstrated iRPE cell output utilized
102 Orthodenticle homeobox 2 (OTX2), a powerful gene expressed from arrangement and induction of primed
103 pluripotency through development to the eye, and then the RPE (Buecker et al., 2014; Esumi et al., 2009;
104 Hever et al., 2006; Thomson and Yu, 2012).
105 A notorious retinal development TF, Orthodenticle homeobox 3, is commonly called Cone-Rod
106 homeobox protein (CRX) (Esumi et al., 2009). In early tests, we isolated iRPE colonies for expansion and
107 performed genomic DNA PCR for our reprogramming transgenes and found that MITF was not common
108 yet the Orthodenticle homeobox TFs CRX and OTX2 were detected in all clones (Figure S1C). We found
109 that excluding CRX resulted in significant reductions in iRPE colony counts per well and smaller colony
110 diameters (Figure 1B). We therefore maintained CRX and OTX2 in our core iRPE system experiments.
111 Among all previous iRPE reports (Figure 2A), and many other 'direct reprogramming' systems, the
112 pioneering TF PAX6 (Soufi et al., 2015) was common, and we anticipated an improvement to our system.
113 We added dox-inducible PAX6 to our reprogramming set (MITF, OTX2, LIN28, MYCL, CRX) and
114 observed a broad increase in cell morphology change by Day 3 followed by a striking cell death event and
115 total ablation of iRPE colony forming cells by Day 6, leaving no visible colonies for an extended period
116 thereafter (data not shown). Alternative iRPE reprogramming factors FOXQ1 and SOX9 were also added
117 to our system. However, both factors caused premature activation of the *BEST1::*EGFP reporter,
118 rendering its RPE maturation/identity features useless (Figure 2C). Furthermore, FOXQ1+

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119   reprogramming induced small EGFP+ colonies with little to no visible proliferation by Day 9 (Figure 2C).
120   For these reasons, we did not continue to apply PAX6, FOXQ1, or SOX9 from the beginning of iRPE
121   reprogramming.

Transplanted iRPE Cells Integrate and Pigment in Albino Rat Retinas

123   In preliminary tests, *BEST1::*EGFP+ reporting iRPE cells in experiments were collected in floating
124   pigmenting balls for purification similar to our previous iPSC.RPE production method (Kuroda et al.,
125   2012). We pooled several floating pigmenting iRPE clusters to a well and sub-cultured for brief expansion
126   (Figure 2D). We prepared immune-compromised Albino rats with subretinal transplantation of iRPE cells.
127   Within 2-3 months we used Optical Coherence Tomography (OCT) scans and usually found several
128   affected areas with possible bulks of cells between the host RPE and Photoreceptors. Notably, some
129   cells in the bulked areas had structural and light characteristics that resembled the RPE layers. We also
130   saw signs of xenografted cells in the photoreceptor layer, implicating rosettes often seen in RPE
131   xenograft experiments (Figure 2E, Figure S2A).
132   We observed a weak trace of EGFP+ cells during fluorescent live fovea imaging, and fluorescent
133   imaging of all transplanted dissected retinas (Figure 2F). Cryosections of those retinas, prepared with
134   hematoxylin & eosin (H&E) staining, showed that many pigmented cells were cobblestone-morphology
135   and found interfacing with photoreceptor outer segments. These cells were sometimes fully integrated
136   into the RPE layer at various positions proximal to the injection site (Figure 2F).

Nicotinamide and Chetomin Improves iRPE Cell Reprogramming

138   Previous reports showed that Nicotinamide (NIC) and Chetomin (CTM) treatments may improve
139   pluripotent stem cell-derived RPEs, such as iPSC.RPE (Maruotti et al., 2015; Williams et al., 2012). We
140   performed bulk passage of iRPE cells to two wells and treated one well +CTM mid-reprogramming with
141   the timing shown in Figure 1D. iRPE+CTM appeared to reduce *BEST1::*EGFP maturation reporter
142   expression and cell pigmentation among significant cell debris/death while the surviving cobblestone cell
143   layer became morphologically more homogenous than control cells (Figure 3A). Next, we prepared trans-
144   well cultures with human primary RPE (hRPE), iRPE, and iRPE+CTM. We examined apical and basal
145   PEDF and VEGF concentrations by ELISA, along with TER measurements, across a 4-week period
146   (Figure S3A). Generally, iRPE and iRPE+CTM did secrete PEDF and VEGF with apical/basal secretion
147   trends like the hRPE, although weaker. TER measurements also showed a lower initial TER, and 4-week
148   increase, when compared to hRPE. Interestingly, iRPE+CTM samples were improved over iRPE alone
149   (Figure S3A).
150   To examine the effects of NIC and CTM to the iRPE system, we performed a larger bulk passage of the
151   same cells to several wells treated with NIC, CTM, or NIC&CTM (Figure S3B). Interestingly, NIC alone
152   had increased *BEST1::*EGFP expression over the control samples during treatment and afterward such
153   cells had increased cell pigmentation and bulging/blebbing as cells matured. CTM alone followed the
154   previously observed trend, decreasing *BEST1::*EGFP expression during treatment, and had mild
155   reduction in cell pigmentation as cells matured. Excitingly, the combination of NIC&CTM appeared to gain
156   the benefits of each individual treatment with no notable negative effects. We therefore termed iRPE
157   +NIC&CTM as iRPENC and generally use that treatment as standard (Figure 1D).
158   During RPE purification, individually plated pigmented clusters can reveal a subjective basic quality
159   based on outgrowth morphology, as done with iPSC.RPE (Kuroda et al., 2012). We performed parallel
160   experiments of iRPE and iRPENC originating from the same bulk passage, but post-maturation and
161   doxycycline-removal. Purified pigmented floating clusters were then plated to individual wells, and
162   outgrowths were assessed as 'good' or 'bad'. We observed that the iRPENC cultures produced
163   significantly more 'good' outgrowth cultures (Figure 3B).
164   We noted an interesting selective cell death from CTM treatments in iRPE reprogramming that was not
165   described in the previous iPSC.RPE differentiation study (Maruotti et al., 2015). Indeed, iRPE

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166   reprogramming is uniquely from fibroblasts, and involves a bulk passage such that non-iRPE cells are
167   outcompeted but may remain. We tested NIC&CTM treatments in early iRPE reprogramming and found
168   that fibroblasts and early iRPE colony formation were drastically affected. Interestingly, if treatment
169   started on or after Day 8, most colonies could survive the treatment among dying fibroblasts, indicating a
170   meaningful shift in reprogramming cell identity permissive to the small molecule treatment by that time
171   (Figure 3C). Although early NIC&CTM treatment may prove useful, for the rest of this study the timing
172   was performed as in Figure 1D and Figure 3F.

173   **Coordinated scRNA and *In-Vivo* Experiments**
174   Given the transplanted iRPE data (Figure 2D,E,F), and the implications of NIC&CTM treatments (Figure
175   3, Figure S3), we designed an experiment to prepare iRPE from the same bulk passage with or without
176   NIC&CTM treatment (iRPE/iRPENC). For each sample, the matured *BEST1::*EGFP positive cells were
177   purified from one 6W by flow cytometry into 2 subsequent 24W wells for brief expansion and maturation
178   with variable RPE-like stability (Figure 3D, Figure 3F). iRPENC treated cells maintained higher
179   *BEST1::*EGFP expression thereafter, and with more lasting RPE-like 'bleb' that are common in high-
180   quality iPSC.RPE cultures when cell junctions are tight and apical-basal flow bulges the RPE from the
181   plate (Figure 3A, D, E; Video S3A, Video S3B). Unfortunately, several iRPENC 'blebs' had puckered and
182   released from the plate to supernatant and were lost during media changes. For each, iRPE and
183   iRPENC, one 24W well was sourced for subretinal transplantation into 4 immune-compromised albino rat
184   retinas, and the other well was sourced for ~3500 cells in scRNA sequencing that also included standard
185   Fib and iPSC.RPE culture cells prepared at the same time (Figure 3F).

186   **scRNA-sequence Profiling of iRPE System Including Starting and Target Cell States**
187   The parallel cultures of Fib, iRPE, iRPENC, and iPSC.RPE were prepared for scRNA sequencing (Figure
188   3F). To prepare the reads for downstream analysis, we performed a workflow with several QC and read
189   detection steps that allow for comprehensive uses in Seurat, Monocle, Velocyto, and SCENIC tools
190   (Figure 4A) (Abugessaisa et al., 2020; Aibar et al., 2017; Butler et al., 2018; La Manno et al., 2018;
191   Petukhov et al., 2018; Stuart et al., 2019; Trapnell et al., 2014).

192   **SkewC Improves Upon scRNA-sequenced Sample Distinction**
193   To QC the resulting scRNA-seq data and decide the dataset for downstream analysis and interpretation,
194   we employed workflows that combined commonplace manual filtering of poor quality cells and SkewC
195   (Abugessaisa et al., 2020), a scRNA-seq quality assessment method. In short, SkewC uses gene body
196   coverage of each single-cell to segregate skewed quality cells from typical cells based on the skewness
197   of the gene body coverage.
198   From Cell Ranger filtered output, we performed two workflows, with manual filtering and SkewC filtering
199   in parallel (Workflow 1; Figure S4A) or in series (Workflow 2; Figure 4B, Figure S4A). Samples were
200   processed manually in Seurat (Butler et al., 2018; Stuart et al., 2019) with nFeatures, nCounts, and
201   mitochondrial read frequency (percent.mt) for basic filtering parameters that output "Pass" or "Fail";
202   SkewC was implemented on the same cells with output "Typical" or "Skewed". While manual and SkewC
203   filtering both detected most low-quality cells, SkewC also frequently detected cells that clustered within
204   the anticipated good cell clusters (Figure S4A, Figure S4B).
205   Interestingly, the Workflow 2 "Skewed" cells appeared to have normal counts and features and
206   clustered accordingly. However, when we compared the feature average expression level for RPE marker
207   genes, variable features, and all detected features, "Skewed" cells had a higher average expression than
208   "Typical" cells, or no detection at all (Figure S4C). We believe SkewC possibly detected the single-cell
209   libraries that had over-represented highly expressed reads and under-represented lowly expressed reads
210   and were therefore possibly less dynamic. While both workflows improved filtering for clustering,
211   Workflow 2 increased "Skewed" cell detection sensitivity within clusters (Figure S4A) and was used as the
212   filter for the rest of the study (Figure 4B).

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iRPENC is Notably Improved and has 'High-Quality' Cells Approaching Subjective RPE Identity

213 Uniform Manifold Approximation and Projection (UMAP) (McInnes et al., 2018) analysis in Seurat
214 clustered Fib, iPSC, and iRPE/iRPENC samples separately, but a distinct population closer to iPSC.RPE
215 was predominately from iRPENC cells, suggesting that NIC&CTM treatments may have meaningfully
216 improved iRPENC cells with lasting effect (Figure 4C). We therefore compared iRPE to iRPENC, and
217 while many cells did cluster similarly in UMAP, the iRPENC mostly occupied the distinct population with
218 *BEST1::*EGFP counts and enriched with important RPE features (*CRX, TYR, MERTK, LHX2*) (Figure 4D).
220 We then focused on iRPENC alone and performed Seurat clustering revealing clusters 1 and 4 to retain
221 the highest expression of *BEST1::*EGFP and RPE features (Figure S4D); we labeled the iRPENC cells as
222 HQ1 (cluster 1) and HQ2 (cluster 4), or 'other'. We passed the UMAP coordinates to Monocle and
223 employed Pseudotime analysis originating from the highest RPE feature rich region (Figure 4E). We
224 found that, across Pseudotime, HQ1 and HQ2 cells tended to be high in RPE genes and low In
225 Fibroblastic genes (Tomaru et al., 2014), while 'other' cells showed the opposite trend (Figure 4E). We
226 thereafter combined HQ1 and HQ2, termed iRPENC.HQ, and the remaining cells as iRPENC.other. We
227 were surprised that the distinction of iRPENC.HQ and iRPENC.other from the sample neatly showed that
228 iRPENC.HQ had 'percent.mt' ratios matching iPSC.RPE cells while iRPENC.other had not (Figure S4E).
229 Expectedly, iRPENC.HQ broadly retained important RPE gene expression (Figure 4F), while
230 iRPENC.other did not (Figure S4F). Apparently, the iRPENC culture met a breaking point and diverged
231 into a stable 'HQ' RPE-like population and a variably destabilized 'other' population with some donor cell
232 (Fib) TFs.

iRPENC and High-Quality Subset May Approach Objective RPE Identity

234 We sought to understand the identity of iRPENC cells objectively, and thus we employed Random Forest
235 (RF) machine learning and RNA Velocity to do so (Breiman, 2001; La Manno et al., 2018).
236 With our Fib, iPSC.RPE, iRPENC.HQ, and iRPENC.other samples, we imported the public 5K Human
237 PBMC dataset to increase the size and diversity of the RF and labeled 10 PBMC Seurat clusters (PBMC-
238 CL0 to 9) (Figure 5A). The inclusion of more diverse cell types caused the iRPENC cells to cluster closer
239 to the iPSC.RPE area of the UMAP (Figure 5A), while the Fib cells became more distant. We *trained* the
240 RF on the PBMC, Fib, and iPSC.RPE samples, and then *tested* the iRPENC.HQ and iRPENC.other
241 samples against that RF. RF Response is an absolute determination of singular identity and labeled most
242 iRPENC cells with iPSC.RPE identity (Figure 5A, right). RF Probability of iRPENC was perhaps more
243 informative, with high iPSC.RPE probability in iRPENC.HQ where Fib probability was often zero, and
244 mediocre iPSC.RPE probability in iRPENC.other where Fib probability was more frequent (Figure 5A,
245 lower).
246 Since we could parse exon and intron counts from scRNA samples with DropEst (Petukhov et al.,
247 2018), we prepared these matrices for RNA Velocity analysis with Velocyto (La Manno et al., 2018) on a
248 Seurat UMAP with samples integrated on our reference iPSC.RPE (Figure 5B). Such integration placed
249 the iRPENC cells much closer to the reference than without (Figure 5B). RNA Velocity showed that most
250 clusters generally had arrows pointing inward, implicating a stable state. Surprisingly, a smaller group of
251 iRPENC.HQ had significant RNA Velocity in the direction of iPSC.RPE, implicating state change toward
252 iPSC.RPE state (Figure 5B). We therefore specifically labeled those 'RNA Velocity Implicated' cells on the
253 UMAP, and found that they neatly overlapped with 51 of the highest RF probability RPE-like cells of
254 iRPENC (iPSC.RPE.prob >75%, Fib.prob <10%) (Figure 5B). Taken together with recurring cell clustering
255 trends, we concluded that unique machine learning and bioinformatics tools could find agreement among
256 highly dynamic and specific criteria among unique cell systems.
257 We further observed from the unspliced (u) and spliced (s) Veloctyo plots of important RPE genes, that
258 some RPE RNAs (*CDH1, FRZB, LHX2, TJP1*) were expressed typically in iRPENC.HQ as in iPSC.RPE,
259 while other RPE genes (*RLBP1, TRPM1, TYR*) showed splice variance that related important epigenetic
260 regulation was involved, and that the 'RNA Velocity Implicated' cells were perhaps the most like

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261 iPSC.RPE (Figure 4B).
262   Integrating on iPSC.RPE as reference brought iRPENC and its iRPENC.HQ closer to iPSC.RPE in
263 UMAP (Figure 5B), with RPE/RPE-like cells overlapping in principal component space (Figure S5A).
264 However, we sought to include external RPE data to improve our analyses. We imported a count matrix
265 from a recently published ES cell-derived RPE study (Lidgerwood et al., 2019) as 'ES.RPE.Young' and
266 integrated the data with iPSC.RPE reference. We found that ES.RPE.Young intermixed with iPSC.RPE
267 and our iRPE system trend of reprogramming from Fib toward RPE became more apparent (Figure 5C).
268 While positions across UMAP_2 appeared dynamic, positions across UMAP_1 appeared to better reflect
269 the spectrum across Fib and RPE identities. The trend was not obvious by simpler principal component
270 space where the ES.RPE.Young, iPSC.RPE, and iRPENC.HQ shared the same general area while Fib
271 and iRPENC.other were usually further away (Figure S5B). A heatmap of gene expression based on
272 specific molecular signatures of primary RPE cells and clinical iPSC.RPE cells (Kamao et al., 2014; Liao
273 et al., 2010) showed that ES.RPE.Young, iPSC.RPE, and iRPENC.HQ had a shared pattern, while Fib
274 and iRPENC.other also had a different shared pattern (Figure 5C, lower), supporting the trends seen in
275 the Pseudotime gene expression plots (Figure 4E). To confirm that observation, we then checked Seurat
276 expression for (*CRX, TYR, RLBP1, RPE65, BEST1, RAX*), and found relatively comparable expression
277 between the ES.RPE.Young, iPSC.RPE, and iRPENC.HQ cells (Figure 5D).

278 **Gene Regulatory Network Analysis Reveals Distinct Cell Type Signatures**
279 To better understand the reprogramming status of iRPENC cells, we employed SCENIC (Aibar et al.,
280 2017) to determine upstream 'regulons' from putative downstream expression at the single-cell level yet
281 often analyzed in specified clusters (Figure S6A). We compared Fib, iPSC.RPE, iRPENC.HQ, and
282 iRPENC.other at equal cell numbers per sample and performed tSNE in Seurat (Figure S6B). Global AUC
283 per sample indicated iPSC.RPE AUC patterns resembled iRPENC.HQ (Figure S6C) despite a notable
284 difference at the few lowest row-clusters where differences were obvious.
285   Many TFs operate in OFF/ON states relative to dosage and cofactor availability. In SCENIC analysis,
286 manually thresholding AUC histograms for binarization (OFF/ON) of the regulons provides an easier to
287 interpret binarized plot (Figure 6A, left) than without (Figure S6D). Still, in all cases, the iPSC.RPE
288 clustered with iRPENC, and more closely to iRPENC.HQ (Figure 6A, S6D). A distinct large set of Fib
289 regulons was effectively OFF in the iPSC.RPE and iRPENC.HQ samples (Figure 6A, Figure S6A). Among
290 the top 281 regulons, the pattern of iPSC.RPE and iRPENC.HQ regulon activity was similar in the higher
291 and lower activity regulons (Figure 6A, left). To succinctly understand the most relevant data, and the cell
292 reprogramming, we reduced the plot to the Top 55 Regulons affecting >95% of cells in the analysis
293 (Figure 6A, mid) and saw that most Fib regulons were OFF in iRPENC.HQ where most iPSC.RPE
294 regulons were ON. Not surprisingly, the iRPENC samples also had a specific subset of regulons whose
295 expression likely restricts iRPENCs from closer clustering to RPEs. Of interest, the MITF and CRX
296 regulons were strong in iRPENC.HQ, as in iPSC.RPE, indicating that the removal of conditional
297 reprogramming left these endogenous regulatory networks intact (Figure 6A). However, the SOX9 and
298 PAX6_extended regulons were very poorly represented in iRPENC.HQ restating the potential role for
299 these factors in our iRPE system despite the antagonistic effects when expressed from Day 0.
300 Expectedly, checking average gene expression for the TFs of the same regulons correlated the
301 relationship between most TF gene expression and putative downstream regulatory activity (Figure 6A,
302 right).
303   On these samples, we performed a Seurat UMAP plot (Figure S6E), and tSNE plots based on AUC
304 (Figure 6B, left) and Binarized Regulons (Figure 6B, right). Expectedly, UMAP provided the distinct
305 clustering that informed sample differences (Figure 6E), and as reported in SCENIC (Aibar et al., 2017)
306 the AUC based and Binarization based tSNE plots improved cell clustering more than when tSNE is
307 based on gene expression (Figure 6B, Figure S6B,E). Curiously, in these plots, and others in this report,
308 a few iPSC.RPE plot neatly among the iRPENC.HQ cluster.

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309   To further understand our cells, we selected the Binarization based tSNE plot to coordinate AUC and
310   Binary Regulon activity of MITF, CRX, and HOXC6 regulons. Expectedly, iRPENC.HQ and iPSC.RPE
311   showed similar RPE regulons (MITF, CRX) while iRPENC.other and Fib showed the HOXC6 fibroblastic
312   regulon (Figure 6C). Binarization thresholding can be variable, for example we set the CRX threshold to
313   the highest of three normal distributions of AUC activity where the iRPENC.HQ and iPSC.RPE were
314   comparable and mixed, yet the middle distribution represented iRPENC.other cells, and the lowest was
315   Fib cells (Figure S6F). We also looked at candidate iRPE factors PAX6_extended and SOX9 (Figure 6C,
316   lower, Figure 2A), which interestingly showed that a few iRPENC cells strongly represented the
317   PAX6_extended regulon, and a cluster of iRPENC.HQ had near full SOX9 regulon activity. We then
318   plotted the individual AUC plots for HOXC6, CRX, MITF, PAX6_extended, and SOX9 regulons across the
319   UMAP and tSNE coordinates to clarify local enrichment (Figure S6G). Interestingly, plotting the 'RNA
320   Velocity Implicated' iRPENC.HQ cells on the Binarization based tSNE coordinates showed that the
321   separated subset of cells toward iPSC.RPE were predominately those with RNA Velocity in that same
322   identity direction (Figure S6H, Figure 5B). Taken together, gene regulatory network inference for cell
323   identities from SCENIC and cell state change inference from RNA Velocity/splicing (Velocyto), could unify
324   to implicate an ideal subset of cells for focused analysis.

325   **scRNA Sampled iRPENC Survived and Integrated to Host Retina *In-Vivo***
326   Parallel cultures of iRPENC used for scRNA were also transplanted to immune compromised albino rat
327   retinas (Figure 3F, Figure 6D). Transplanted iRPENC had apparent pigmentation noted in standard fovea
328   imaging just 6 weeks after transplant, and with obvious pigmented tissue in the retina about 5 months
329   after dissection for cryosection and immunohistochemistry (IHC) (Figure 6D). H&E stains of transplanted
330   retinas showed pigmented cobblestone cells in many areas, particularly interfacing with some host
331   photoreceptor outer segments (Figure 6D). Transplanted iRPENC cells appeared to sit atop host albino
332   RPEs, with notably similar morphology and H&E stain characteristics. In some cases, RPE-like cells with
333   weak pigment speckles were noted atop the retina ganglion cell layer, which can happen when transplant
334   areas leak cells to the vitreous. Curiously, some pigment, or pigmented cells, appeared at the outer
335   nuclear layer *membrana limitans externa*, which piqued concern for material transfer or mis-localization
336   between the pigmented iRPENC and host cells (Figure 6D).
337   To validate the RPE positioning and polarity, we then prepared IHC of intervening cryosections proximal
338   to the H&E stained sections in Figure 6D. We found that the apical RPE marker protein BSG (Deora et
339   al., 2004) had labeled the apical face of the RPE cell layer (Figure 6E). Expectedly, many BSG+ cells co-
340   localized apical to STEM121, a common human-specific cytosolic marker (Tu et al., 2019) (Figure 6E). To
341   validate our apical BSG protein detection in host RPE cells and IHC methods, we stained untreated
342   control retina cryosections with BSG and the RPE marker RPE65 (Figure 6D).

343   Taken together, these observations strengthen the notion that the optimized iRPE reprogramming system
344   conditions may reprogram human somatic cells into stabilized cells with subjective and objective metrics
345   for RPE identity that could mature, integrate, and interface in transplanted host retinas.

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347 **DISCUSSION**

348 **Bulking Up**
349 In basic research we can explore nature willy-nilly, yet medicine is bound by economies of scale and
350 practical finance. The adage '*time is money'* holds true in medicine, inspiring research for shortcuts
351 toward autologous or compatible cell therapies. In that context, individual colonies of iRPE were irrelevant
352 and only bulk cultures proved efficient with robust expansion toward the excess, then purity, that is often
353 necessary in biomedical products. In bulk, many stabilized iRPE cells could be found after ~2 months'
354 time which suggests significant savings in contrast with iPSC.RPE generation that requires > 6 months.
355 To our surprise, Nicotinamide and Chetomin provided an unforeseen role in iRPE cell selection, although
356 a complete purification of high-quality iRPEs remains unaddressed. For that reason, we foresee
357 necessary co-development of the iRPE reprogramming system with plausible cell purification
358 technologies (Miki et al., 2015; Ota et al., 2018; Plaza Reyes et al., 2020). Relatedly, earlier iRPE may
359 prove better given the fact that FACS purified *BEST1::*EGFP+ iRPENC cells later divided into 'HQ' and
360 'other' in time, and that several studies find that overly-mature RPEs are poor candidates for cell therapy.

361 **Cell Reprogramming**
362 This iRPE system may advance timely somatic cell conversion toward RPE given the hallmark of stability
363 after the removal of conditional reprogramming; a state not shown in other iRPE systems (D'Alessio et al.,
364 2015; Rackham et al., 2016; Zhang et al., 2014). We recognize the importance of previous reports
365 variably employing MITF, OTX2, and CRX in wet cultures to induce RPE features (D'Alessio et al., 2015;
366 Zhang et al., 2014). Still, prior iRPE reports ostensibly showed 'direct reprogramming' while relying on the
367 pluripotency factor OTX2 (Buecker et al., 2014; Thomson and Yu, 2012) and optionally relying on the
368 Yamanaka factor KLF4 (Zhang et al., 2014). Given the state of the cell reprogramming field, 'direct
369 reprogramming' may be a misnomer since most reprogramming system cell state changes, over
370 significant time, is diverse. We wonder how a slow/non-dividing somatic state could generate another
371 slow/non-diving somatic state, effectively, without the proliferative precursor programs that predicate
372 important *survival* and *generative* identity. Conversely, this study sought to exploit that hypothesis,
373 focusing on plasticity, precursor, lineage, and end-state. Given that iPSC-like reprogramming MET was
374 evident in the first week of iRPE reprogramming, and cell death/survival was obvious, we anticipate that
375 tumor suppressors like p53 or Rb may perturb iRPE reprogramming, or selectively affect surviving cell
376 states and genomic stability (Kareta et al., 2015; Marión et al., 2009; Samavarchi-Tehrani et al., 2010).
377 Among the TFs in this iRPE system, Microphthalmia-Associated Transcription Factor (MITF) is a
378 regulator of RPE differentiation (Adijanto et al., 2012; Hansson et al., 2015) that critically cooperates with
379 Orthodenticle homeobox 2 (OTX2) for RPE development (Bharti et al., 2006, 2012; Ramón Martínez-
380 Morales et al., 2004). Both MITF and OTX2 are pioneering TFs (Soufi et al., 2015), and OTX2 is
381 developmentally retained from primed pluripotency as an organizer/specifier/reprogrammer (Buecker et
382 al., 2014; Shahbazi et al., 2017; Thomson and Yu, 2012), through the neural plate, optic vesicle, and RPE
383 specification (Hever et al., 2006). LIN28 and MYCL, the 'hUL' cassette for iPSC reprogramming
384 enhancement (Okita et al., 2011), are also involved in natural retinal regenerating reprogramming (Luz-
385 Madrigal et al., 2014). LIN28 binds and neutralizes *Let-7*, a promiscuous and broadly expressed miRNA
386 somatic cell identity 'lock' against plasticity, reprogramming, and state change. MYCL is a form of MYC,
387 which cooperates with reprogramming TFs by holding open freshly pioneered nucleosome-bound
388 genomic DNA (Soufi et al., 2015). The combination (MITF, OTX2, LIN28, MYCL) was improved by CRX,
389 a powerful Orthodenticle homeobox TF involved in various aspects of retinal differentiation and early RPE
390 fate (Esumi et al., 2009; Furukawa et al., 1997).
391 CRX was important to this iRPE system and was identified by SCENIC as a distinguishing regulon for
392 iPSC.RPE and iRPENC.HQ cells. However, CRX is lowly expressed or turned off *in-vivo* as RPE
393 matures, highlighting a potential role in regenerative medicine. Comparable MITF and CRX regulons in

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394 iPSC.RPE and iRPENC.HQ, among absence OTX2 regulon relevance, provides and strong backdrop for
395 interpreting necessary transient, or lasting, reprogramming TFs; indeed, most iRPENCs did not express
396 detectable endogenous OTX2. Weak iRPENC.HQ signatures for PAX6_extended and SOX9 regulons
397 reiterate their value in other iRPE systems; these TFs could not be used from Day 0 in our iRPE system
398 context, and thus an exploration of differential timing, or other TF candidates, may significantly improve
399 iRPENCs to match the iPSC.RPE model. Importantly, the current iRPENC.HQs stability may exist due to
400 the loss Fib-specific regulon activity. With stability, perhaps most of the reprogramming was achieved,
401 leaving a fraction of meaningful donor cell gene regulatory networks to address, and only if they may
402 affect the safety or function of the target autologous RPE cell product.

403 **Cell Identity**
404 iRPENC displayed numerous characteristics of RPE *in-vitro*, and *in-vivo* when transplanted to immune
405 compromised albino rat retinas where cells survived, expressed a maturation reporter, pigmented, and
406 apparently integrated into host RPE layers sometimes interfacing with host photoreceptor outer
407 segments. The scRNA analysis of iRPEs revealed a distinct sub-population of 'high-quality' cells that
408 were better stabilized toward RPE cell identity and whose generation was drastically improved by the
409 addition of Nicotinamide and Chetomin during cell reprogramming. Both machine learning Random Forest
410 and RNA Velocity approaches objectively strengthened that subjective segregation and further identified
411 subsets of iRPEs with high RPE expression and genetic regulation. Taken together, the bioinformatics
412 tools employed here provided a window for analysis that helped us characterize and understand the iRPE
413 system and important cell identities, while providing extensive data that we have yet to fully explore.
414   Of note, the inclusion of other cell samples, and cell numbers, strongly affected bioinformatics analysis.
415 In Random Forest, more distant blood cell types caused iRPENC to cluster closer to iPSC.RPE in UMAP
416 and strengthened the RF Response and Probability clarity between Fib and iPSC.RPE identities. In
417 Veloctyo, the absence of other cells brought iPSC.RPE and iRPENCs yet closer. Conversely, when the
418 ES cell-derived RPE model was included, distance increased between the iRPENC.HQ and iPSC.RPE
419 UMAP space despite overlapping principal component space coverage. Even adjustments to cell
420 numbers often had notable effects on the positioning and clustering of cells. For these reasons, careful
421 per-sample consideration may be critical for scRNA analysis.

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422 **EXPERIMENTAL PROCEDURES**

423 **Animal Use**
424 Rat handling and transplant experiments were carried out with humane methods in compliance with
425 animal ethical standards approved by RIKEN Kobe Safety Center.

426 **Human Fibroblast Culture**
427 BJ Human Foreskin Fibroblasts (ATCC), were cultured in fibroblast culture media (FCM) that consisted of
428 90% DMEM-high glucose, 10% Tetracycline free Fetal Bovine Serum (FBS), and 1% Penicillin
429 Streptomycin (P/S) with no filtering. For most cultures, 10cm cell culture plates were coated with 0.1%
430 gelatin in CMF-DPBS for 1 hour. Cryopreserved fibroblasts were thawed, diluted with FCM, and
431 centrifuged at 200 x g for 4 min, and then the pellet was resuspended for cell counting and then diluted
432 appropriately in FCM to yield ~650,000 cells/10mL, triturated to mix evenly. The gelatin/PBS coating was
433 aspirated from the 10cm plate and 10mL of diluted cells in FCM were plated. Cells were incubated at
434 37°C until ~ 85-90% confluent and then passaged or used for reprogramming experiments.

435 **iRPE Reprogramming**
436 iRPE Media consisted of 85% DMEM/F12+GlutaMax (1X), 15% KnockOut Serum Replacement, 1% MEM
437 Non-Essential Amino Acids, 1% N-2 Supplement (all filtered via a 0.22µm polyethersulfone - PES) and
438 then prepared as frozen aliquots. 1% Penicillin Streptomycin (P/S) is added fresh, and in the first 10 days
439 of reprogramming Basic Fibroblast Growth Factor (bFGF) is added to a final concentration of at 10ng/mL
440 with 2-mercaptoethanol (2ME) at 1:1000 dilution. Doxycycline is added at 1µg/mL (See Figure 1) for the
441 Phase 1 media. Phase 2 media consisted of all the above-mentioned reagents except for bFGF and 2ME.
442   Generally, a 10cm plate was coated with 0.1% gelatin and plated with 650,000 fibroblasts containing
443 conditional doxycycling-inducble reprogramming sets we generally designated as 'programs' (e.g.
444 Program 2, Set 2 = P2.2). Fibroblasts ready to reprogram were incubated 37°C until ~ 85-90% confluent.
445 Fibroblasts were reprogrammed in 6W plates. Briefly, target wells of 6W plates were coated with 1.5 mL
446 of 1:150 iMatrix511/CMF-DPBS substrate for 1 hour at room temperature. Fibroblasts were passaged to
447 yield ~125,000 cells/1.5 mL per well, and then incubated at 37°C 16-24 hours before reprogramming
448 medium (Phase 1 iRPE medium) was added. Fibroblasts were checked prior to reprogramming to ensure
449 that the cells plated as single evenly dispersed cells. The addition of the reprogramming medium marks
450 the beginning of the reprogramming or conversion process and is noted as Day 0. The Phase 1 medium
451 is added fresh at 2mL every day together with any molecule or supplement for 9 more days – Day 0 to
452 Day 9, which makes 10 continuous days of feeding the cells with the Phase 1 media. On the Day 10, the
453 Phase 1 medium was replaced with Phase 2 medium (no bFGF and no 2ME) at 2mL every-other-day.
454   Phase 2 media was used until ~Day 28-32 when the cells were passed in bulk, combining all colonies,
455 to an iMatrix511 coated 6W plate. Cell passage was as described with fibroblasts, but with Phase 2
456 medium containing 10% FBS to neutralize trypsin. In some cases, defined trypsin inhibitor was used.
457 Reprogramming cells were plating between 300,000 to 500,000 cells/well of a 6W plate in Phase 2
458 medium and incubated at 37°C until for ~48 hours before refreshing the Phase 2 medium. On ~Day 36
459 Phase 2 medium was prepared with fresh Nicotinamide [5-10mM] and Chetomin [40-80nM] (NC) and fed
460 every other day for 10 days. Cells that did not receive NC, were fed the same medias excluding those
461 molecules. After the last feed with NC, the reprogramming cells were fed Phase 2 medium once more,
462 before culture in Maturation Medium ~Day 50. *Note: If necessary, colonies were counted on Day 9 and*
463 *the diameter of the colonies were measured on Day 13.*

464 **iRPE/iPSC.RPE Maturation Media Culture (SFRM-B27)**
465 Maturation Media contained 70% DMEM-low glucose, 30% Nutrient Mixture F-12 Ham, 1% GlutaMax
466 (100X), 2% B-27 Supplement (all filtered via a 0.22µm polyethersulfone - PES) and stored in frozen
467 aliquots. Fresh 1% P/S, 0.5-1µM SB431542, and 10ng/mL bFGF is added before use.

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468   On ~Day 50 of iRPE reprogramming, the medium is changed to the Maturation Media (SFRM-B27) with
469   1µg/mL Doxycycline. The Doxycycline concentration is gradually reduced from 1µg/mL for the first and
470   second feeds, and then 0.5µg/mL for the next two feeds. From the fifth feed with Maturation Media,
471   doxycycline was not added. iRPE or iPSC.RPE were fed Maturation Media every-other-day, as
472   necessary. *Note: iPSC.RPE could be fed Maturation Media with or without doxycycline without*
473   *consequence since they did not have doxycycline-inducible reprogramming factors.*

Single-cell RNA Sampling
475   Cells were dissociated and passed through cell screen cuvettes to isolate mostly healthy single-cells that
476   were prepared with 10x Chromium Single Cell 5' Library & Gel Bead Kit (PN-1000014) skipping steps 4/5
477   per standard protocol for non-VDJ samples. Sample libraries were finalized and sequenced on one Hiseq
478   X lane (150bp PE; Macrogen) for each. Standard Cell Ranger protocol detected sample chemistry and
479   produced 'possorted' BAM files from which the subsequent Primary Analysis workflow in Figure 4A was
480   performed.

Albino Rat Xenografts of iRPE/iRPENC
482   Briefly, cells for transplant were prepared similar to cell passage, but kept on ice, and at 50,000 cells per
483   µL. Recipient rats were prepared with isofluorane and accommodated, and then 1-2µL of cells added to
484   the subretinal space between the neural retina and RPE/Choroid. Rat health was checked frequently, and
485   transplanted animals were generally maintained for 4-5 months prior to sacrifice.

Sample Fixation and Cryosection
487   Rats were sacrifice humanely, and the eyes extracted and fixed with 4% paraformaldehyde in PBS
488   solution, overnight. The eyes were then desiccated in 30% sucrose solution for ~24-48 hours. Upon later
489   dissection, based on prior fundus imaging, the exact pigmented or transplanted parts of the eye were
490   retained, and the rest discarded. Samples were placed in a 10mm X 10mm X 5mm size Tissue cryomold
491   and filled with optimal cutting temperature (O.C.T.) compound, and then stored at -80°C.
492   For cryosection, the frozen cryomold containing the eye tissue was sectioned with a Thermo Scientific
493   Micron HH 560 cryomictrotome. Each section had a fine size of 12micron, placed on glass slides, and
494   then stored at -80°C until further use.

H&E Staining
496   Generally, for each eye sample, every fifth cryosection slide was air dried for 1 hour, and then subjected
497   to common H&E stains procedures.
498   Briefly, residual O.C.T. compound was dissolved by immersing in CMF-PBS for 1 hour. The slides were
499   then placed in Hematoxylin stain for 2.5mins, followed by two MilliQ water washes of 2.5 minutes. The
500   slides were then placed in Eosin stain for 1 minute, followed by MilliQ water washes for 1-2 seconds. The
501   slides are then moved to 80% ethanol, then 90% ethanol washes for 30 seconds each. Then, slides pass
502   through two 100% ethanol washes for 3 minutes each. Finally, the slides pass through two washes in
503   xylene for 3 minutes each. After the xylene, the slides are air dried and then mounted with Malinol and
504   nail polish prior to imaging.

Immunohistochemistry and Confocal Microscopy
506   Immunohistochemistry in Figure 6E was performed on previously noted cryosections. Sample slides were
507   removed from the freezer and then air dried at room temperature for ~1 hour. Slides were submerged in
508   100°C 1X citrate buffer for 20 mins for heat induced epitope retrieval. Generally, blocking buffer (BB) was
509   4% horse serum in CMF-DPBS. Samples were permeabilized for 30 minutes with 0.2% Triton X-100 in
510   BB, and then blocked for ~1 hour in BB. Primary antibody solutions were prepared in BB and then added
511   to slides and incubated overnight at 4□°C.
512   All samples were washed with BB 3 times, followed by secondary antibody solution for ~1.5 hours at

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513 room temperature. The solution is replaced with DNA stain (Hoechst 33342 1:2000) for ~10 minutes. The
514 slides are washed two more times with CMF-DPBS, and then dried and mounted FluorSave and a
515 coverslip prior to imaging with Zeiss LSM 700 Confocal Microscope.

516 *Antibodies Used*

Reagent	Maker / Catalog #	Dilution
Mouse anti-RPE65	Merk Millipore MAB5428	1:250
Goat anti-BSG (CD147/EMMPRIN)	R&D Systems AF972	1:20-40
Mouse anti-STEM121	Takara Bio Y40410	1:250
Donkey anti-Mouse Alexa Fluor 647	Thermo Fisher A-31571	1:1000
Donkey anti-Goat Alexa Fluor 546	Thermo Fisher A-11056	1:1000
Hoechst 33342	Thermo Fisher H3570	1:2000

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AUTHOR CONTRIBUTIONS

Conceptualization, C.K.; Methodology, C.K. and I.N.W; Cryosections, J.S., M.N., H.H. and H.S.; Formal Analysis, C.K. and I.N.W.; Investigation, C.K., I.N.W., I.A., B.K, HH.C., and M.M.; Resources, C.K., A.T., J.S., I.A., B.K., P.C., E.A., Mi.T., Y.S., M.M. and Ma.T.; Writing – Original Draft, C.K.; Writing – Revision & Editing, C.K., I.N.W., A.M., P.C., I.A., and E.A.; Visualization, C.K., H.S., M.N., and I.N.W.; Project Supervision, C.K., and Ma.T.; Retina Aspect Supervision: M.M., A.M., and Ma.T.; Bioinformatics Aspect Supervision: E.A., I.A., T.K., B.K., and P.C.; Project Administration, C.K. and Ma.T.; Funding Acquisition, C.K., M.M., and Ma.T.

CONFLICTS OF INTEREST

C.K., M.T., and I.N.W have filed for patent on related technology.

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693 **FIGURE LEGENDS**

694 **Figure 1: iRPE System Overview**
695 **A)** A schematic representing the iRPE system objectives and estimated time frames when comparing
696 autologous iRPE to autologous iPSC.RPE.
697 **B)** The *BEST1::EGFP* synthetic promoter reporter construct and constitutive (CMV) driven conditional
698 dox-inducible system (rTetR/rtTA) with Puromycin Resistance (PuroR). Construct is integrated and
699 expressed in iPSC.RPE (left) and iRPE (right). *Scale bars = 200µm.*
700 **C)** A sub-cultured iRPE colony expressing *BEST1::EGFP* stained for DNA (light-blue; Hoechst 33342)
701 and RPE65 (red). *Scale bar = 100µm.*
702 **D)** A schematic (upper) and pictorial (lower) representation of iRPE reprogramming with basal media
703 compositions and supplementations, timing of molecules and conditional reprogramming (doxycycline).
704 *Scale bars = 100µm.*

705 **Figure 2: iRPE Systems, TF Testing, and Preliminary Subretinal Transplantation**
706 **A)** A Venn-diagram of the validated TFs from our iRPE system and each previous iRPE study (D'Alessio
707 et al., 2015; Rackham et al., 2016; Zhang et al., 2014).
708 **B)** iRPE reprogramming +/- CRX, with colonies counted per 6W on Day 9 (left) and average colony
709 diameters measured on Day 13 (right).
710 **C)** Day 9 iRPE system +FOXQ1 colony expressing *BEST1::EGFP*. *Scale bars = 100µm.*
711 **D)** Pre (upper) and Post (lower) iRPE cluster purification culture images to show morphology,
712 pigmentation, and *BEST1::EGFP* expression. *Upper scale bars = 500µm, Lower scale bars = 200µm.*
713 **E)** Optical Coherence Tomography scans of untreated albino rat retina (left) and iRPE transplanted albino
714 rat retina (right). Host and transplanted RPE/RPE-like layers are indicated (white arrows) along with
715 potential retinal transplant rosette formations (yellow arrow).
716 **F)** Live fovea fluorescent imaging for *BEST1::EGFP* (upper left), with the same explanted dissected retina
717 fluorescent imaging for *BEST1::EGFP* (left), and that retina's cryosections with H&E staining to reveal
718 pigmented human iRPE cells (black arrows) in RPE layers and subretinal space, interfacing with host
719 photoreceptor outer segments. *Scale bars = 100µm.*

720 **Figure 3: iRPE Reprogramming Cells Treated with Nicotinamide and Chetomin**
721 **A)** A split iRPE culture +/- CTM treatment, during treatment, with *BEST1::EGFP* expression (left) and
722 brightfield imaging (right). *Scale bars = 200µm.*
723 **B)** Subjective qualification (bad/good) of 24 individually plated iRPE cluster outgrowths from 2 iRPE and 2
724 iRPENC cultures.
725 **C)** Effects of NIC&CTM treatments on early iRPE reprogramming cultures shown with brightfield
726 microscopy (left) and iRPE colony counts on Day 9 (right). *Scale bars = 200µm.*
727 **D)** A split iRPE culture that had previous +/- NIC&CTM treatments, prior to retinal transplant, expressing
728 *BEST1::EGFP* among variable quality cells visible by brightfield (right). *Scale bars = 500µm.*
729 **E)** A representative bulging RPE-like bleb of iRPE cells imaged at two z positions (upper/lower) with
730 obviated pigmentation and cell nuclei (upper). *Scale bars = 200µm.*
731 **F)** A schematic overview of how the iRPE and iRPENC cells were prepared for scRNA analysis and
732 albino rat subretinal transplant.

733 **Figure 4: iRPE System scRNA Data Preparation and Analysis**
734 **A)** An overview of the Primary and Secondary Analysis of scRNA samples in this study.
735 **B)** Workflow 2 (serial) SkewC analysis total gene body mapped read traces, per cell after Cell Ranger
736 (upper), after manual filtering in Seurat (mid) and then separated to Skewed cell traces (lower left) and
737 Typical cell traces (lower right).
738 **C)** Seurat UMAP plot of Fib, iRPE, iRPENC, and iPSC.RPE samples.

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739   **D)** Seurat UMAP plot of iRPE and iRPENC samples with inset coordinate plot of *BEST1::*EGFP counts
740   (upper), and RPE gene expression feature plots (lower).
741   **E)** Seurat UMAP coordinates of iRPENC plotted in monocle for HQ1, HQ2, and other, with pseudotime
742   originating at the point of highest RPE gene and *BEST1:*EGFP expression (Figure S4D). Expression of
743   RPE genes (left) and Fibroblastic genes (right) are plotted across pseudotime and labeled with HQ1,
744   HQ2, and other.
745   **F)** Seurat UMAP based RPE gene expression feature plots of iRPENC.HQ (HQ1 + HQ2).

746   **Figure 5: Machine Learning and Bioinformatics Interpretation of RPE Identity in iRPENC.HQ**
747   **A)** Seurat UMAP plots of RF analysis samples labeled by Original Identity (upper left), RF Response
748   (upper right), and by Fib/iPSC.RPE probability (lower).
749   **B)** Seurat UMAP plots with cell velocity (upper), identity (mid), and RF / RNA Velocity Implicated RPE-like
750   iRPENC.HQ cells indicated in the cell velocity map (orange arrow) and highlighted in red (mid). RPE gene
751   expression detection by spliced/exon (s) and unspliced/intron (u) reads.
752   **C)** Seurat UMAP plot of samples with reference based integration on iPSC.RPE cells (upper), including
753   RPE gene heatmap (lower).
754   **D)** The Seurat UMAP plot of Figure 5C with RPE gene feature plot for CRX, TYR, RLBP1, RPE65,
755   BEST1, and RAX.

756   **Figure 6: iRPE System Genetic Regulatory Analysis and Validation *In-Vivo***
757   **A)** SCENIC Binarized regulons total (281, left), top 55 (mid) with heatmap(pheatmap) clustering and
758   regulon activity (red scale), with row-matched TF expression (log-transformed TF gene expression
759   averages per sample) (right).
760   **B)** SCENIC AUC Based (left) and Binarization Based (right) tSNE clusters of samples.
761   **C)** AUC (left) and Binarized (right) activity of MITF, CRX, HOXC6, PAX6_extended, and SOX9 regulons.
762   **D)** Live fovea imaging of a iRPENC transplanted albino rat retina (left), with apparent pigmentation during
763   dissection (mid). Cryosection H&E stains (right, low mag (upper) high mag (lower x2)) show pigmented
764   RPE-like cells (black arrows) in expected areas sometimes interfacing photoreceptor outer segments.
765   *Note: A non-pigmented RPE layer is indicated (yellow arrows), and out-of-place pigmentation is also*
766   *noted (red arrows). Upper Scale bar = 200μm, Lower Scale bars = 50μm.*
767   **E)** A proximal cryosection (upper) to Figure 6D H&E stains was used for IHC against BSG (red) and
768   STEM121 (light blue), with DNA counterstain (light grey). An untreated control retina (lower) was used for
769   comparable IHC against BSG (red) and RPE65 (yellow), with DNA counterstain (light grey). *Scale bars =*
770   *100μm.*

771

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Related to Figure 1 & Figure 2
A) A representative dox-inducible system viral construct with tetracycline (doxycycline) response elements upstream of an example TF/exogene, MITF.
B) A picked and sub-cultured pigmented iRPE colony after 100 days of reprogramming (left), was passaged for expansion (mid) and developed into large pigmented cells resembling aged RPE based on size, polynuclei, and morphology (right). *Scale bars = 500µm.*
C) A table of the initial reprogramming factor virus set in the parental culture (e.g., MITF, OTX2, LIN28, MYCL, CRX) and the Genomic DNA PCR detection (YES/NO) for those virii in individually sub-cultured/cloned iRPE colonies.

Figure S2: Related to Figure 2
A) An iRPE transplanted albino rat retina cryosection with H&E staining to reveal pigmented human iRPE cells (black arrows) in 'rosettes' in the neural retinal space.

Figure S3: Related to Figure 3
A) PEDF (left) and VEGF (mid) secretion ELISA detection from transwell Apical (upper) and Basal (lower) cell supernatants of hRPE, iRPE, and iRPE+CTM treated cells, sampled after Week 1 and Week 4. TER for the same cultures was taken weekly and plotted (right).
B) A bulk iRPE passage was prepared and treated with NIC, CTM, and NIC&CTM, or control. *BEST1::*EGFP expression during treatment is represented (left) and pigmented morphological imaging after treatment, in maturation, is represented (right). *Left scale bars = 100µm, Right scale bars = 500µm.*

Figure S4: Related to Figure 4
A) Seurat UMAP plots of Manual Filtering and SkewC labeled or filtered cells in Workflow 1 (parallel; upper), and Workflow 2 (series; lower).
B) SkewC cell clustering by gene body coverage with 'Typical cells highlighted in red.
C) Box plots of the log-transformed Feature Average expression levels for all samples in Figure S4A, based on feature sets of RPE Marker Genes, Seurat Variable Features, and all Features. Skewed cell 'no detection' is indicated with red arrow.
D) Seurat UMAP plot of iRPENC cell clusters (upper left), *BEST1::*EGFP expression counts (upper right), and monocle gene expression for CRX, TYR, MERTK, and LHX2 plotted on the Seurat UMAP coordinates.
E) Seurat violin plots for calculated 'percent.mt' for Fib, iPSC.RPE, iRPENC.HQ, and iRPENC.other samples.
F) Seurat UMAP based RPE gene expression feature plots of iRPENC.other.

Figure S5: Related to Figure 5
A) Seurat Principal Component (PC1 vs PC2) plots of cells analyzed in Figure 5B.
B) Seurat Principal Component (PC1 vs PC2) plots of cells analyzed in Figure 5C.

Figure S6: Related to Figure 5 & Figure 6
A) SCENIC boxplots for the number of cells per regulon, and the number of regulons per cell.
B) Seurat tSNE plot for the samples used in SCENIC analysis.
C) SCENIC total AUC regulon activity.
D) Heatmap (pheatmap) with clustering of all AUC regulon activity per sample type.
E) Seurat UMAP plot for the samples used in SCENIC analysis.
F) Example 'Binarization' AUC threshold setting for CRX regulon, indicating the OFF and ON determination.
G) Gene set AUC for HOXC6, CRX, MITF, PAX6_extended, and SOX9, plotted across Seurat UMAP

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817 coordinates (upper) and SCENIC Binarized tSNE (lower) coordinates.

818 **H)** Seurat tSNE plot using SCENIC Binarized tSNE coordinates to highlight RNA Velocity Implicated

819 iRPENC.HQ cells.

820 **Video S3: Related to Figure 3**

821 **A)** A representative video of a slow change of focal (Z) position of iRPE pigmented 'bleb' cells and

822 attached culture.

823 **B)** A representative video, matching Video S3A, imaged for *BEST1::*EGFP.

824 /end/

A — Induced RPE (iRPE) ~40–60 Days; Mature RPE; iPSC.RPE ~150–200 Days; RPE Transplant

B — BEST1-0.6KB-PROMOTER; EGFP; WPRE; CMV enhancer; CMV promoter; rTetR; rtTA-Advanced; IRES; PuroR; iPSC.RPE; iRPE; *BEST1*::EGFP

C — Sub-cultured iRPE Colony; DNA; *BEST1*::EGFP; RPE65

D — iRPE Media: DMEM/F12; 15%KSR; NEAA; N2 — Daily :: Bi-Daily; Maturation Media: SFRM-B27; 2.5-10ng/mL bFGF; 0.5-1uM SB :: Bi-Daily

Pass; iRPE Media (10 Days); iRPE Media (44 Days); Maturation Media; 1000 ng/mL DOXYCYCLINE 500 ng/mL; 10ng/mL bFGF; NICOTINAMIDE & CHETOMIN

Day 0; Day 5; Day 10; Day 15; Day 20; Day 25; Day 30; Day 35; Day 40; Day 45; Day 50; Day 58; *BEST1*::EGFP

A Our System

MITF
OTX2
LIN28
MYCL
CRX

D'Alessio et al.

GLIS3
FOXD1

OTX2
MITF

SIX3

RAX
MYC
CRX

PAX6

MYBL2
SMAD6
JUNB
E2F5
SOX9
FOXQ1

Zhang et al.

Rackham et al.

B

Colony Counts Per Well

Averaged Colony Diameters (μm)

(MITF, OTX2, LIN28, MYCL) n=3
(MITF, OTX2, LIN28, MYCL, CRX) n=13

(MITF, OTX2, LIN28, MYCL) n=27
(MITF, OTX2, LIN28, MYCL, CRX) n=42

C +FOXQ1

BEST1::EGFP

D

BEST1::EGFP

BEST1::EGFP

E Control Retina

iRPE Transplanted Retina

F Fovea Imaging

Dissected Retina

BEST1::EGFP

BEST1::EGFP

A Control
BEST1::EGFP
+CTM
BEST1::EGFP

B RPE-like Plated Cluster Quality

Outgrowth QC Count

iRPE.1　iRPE.2　iRPENC.1　iRPENC.2

■ bad　■ good

E

C

Colony Counts
Per Well on Day 9

■ (NIC&CTM Treated Days 6&8) n=3
■ (NIC&CTM Treated Day 8) n=3
■ (Control /No Treatment) n=3

D Control
BEST1::EGFP
+NIC&CTM
BEST1::EGFP

F

Pass (2 Wells)

NIC&CTM Treatment
No Treatment

FACS *BEST1*::EGFP+ Cells
2 wells for each

1 well = Transplant
to 4 Retinas for each

1 well = scRNA Sample
Hiseq X Lane for each

iRPE Media　　iRPE Media　　**Maturation Media**
(Reprogramming Complete)

1000 ng　　1000 ng　　DOXYCYCLINE (Reprogramming Factor Expression)　　500 ng

bFGF + 2ME

A Primary Analysis | Secondary Analysis

B iRPENC(n=3500)

C scRNA Samples — Fib, iPSC.RPE, iRPE, iRPENC

D scRNA Samples — iRPE, iRPENC

CRX, TYR, MERTK, LHX2

E Quality.ID — HQ1, HQ2, other — pseudotime

RPE Genes: CRX, LHX2, MERTK, MITF, RAX, RPE65, TRPM3, TYR, TYRP1

Fibroblastic Genes: FOXD1, HOXC4, HOXC6, HOXC8, LHX9, OSR1, RUNX1T1, TBX3, TWIST2

F Isolated iRPENC.HQ(1+2)

CRX, TYR, MERTK, LHX2, RLBP1, TJP1