

1 **Retinoic acid accelerates the specification of enteric neural progenitors from *in***  
2 ***vitro*-derived neural crest**

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21

22 **Summary**

23 The enteric nervous system (ENS) is derived primarily from the vagal neural crest, a  
24 migratory multipotent cell population emerging from the dorsal neural tube between  
25 somites 1-7. Defects in the development and function of the ENS give rise to a range  
26 of disorders, termed enteric neuropathies and include conditions such as  
27 Hirschsprung's disease. Little is known about the signalling that specifies early ENS  
28 progenitors. This has, thus far, limited progress in the generation of enteric neurons  
29 from human Pluripotent Stem Cells (hPSCs) that could provide a useful tool for  
30 disease modelling and regenerative medicine. We describe the efficient and  
31 accelerated generation of ENS progenitors from hPSCs, revealing that retinoic acid is  
32 critical for the acquisition of both vagal axial identity and early ENS progenitor  
33 specification. These ENS progenitors generate enteric neurons *in vitro* and following  
34 *in vivo* transplantation, achieving long-term colonisation of the ENS in adult mice.  
35 Thus, hPSC-derived ENS progenitors may provide the basis for cell therapy for defects  
36 in the ENS.

37

## 38 Introduction

39 The enteric nervous system (ENS) is the largest branch of the peripheral nervous  
40 system and consists of an extensive network of neurons and glia controlling critical  
41 intestinal functions such as motility, fluid exchange, gastric acid/hormone secretion  
42 and blood flow (reviewed in (Furness, 2012; Sasselli et al., 2012). In amniote embryos,  
43 the ENS is derived predominantly from the vagal neural crest (NC), a multipotent cell  
44 population that is specified at the neural plate border between the presumptive neural  
45 and non-neural ectoderm between somites 1-7. Vagal NC also contributes to  
46 structures in various other organs such as the heart, thymus and lungs (Le Douarin et  
47 al., 2004; Hutchins et al., 2018; Simkin et al., 2018; Espinosa-Medina et al., 2017).  
48 After delaminating from the dorsal neural tube, vagal NC cells migrate first in a ventro-  
49 medial direction and enter the foregut. Following entry into the gut, enteric neural  
50 progenitors colonise the entire developing gut in a rostro-caudal direction. A number  
51 of studies have provided valuable insights into the determinants of ENS progenitor  
52 migration, proliferation and differentiation. These include the RET-GDNF (Heanue and  
53 Pachnis, 2008; Durbec, Marcos-Gutierrez, et al., 1996; Durbec, Larsson-Blomberg, et  
54 al., 1996) and Endothelin3-EDNRB (Baynash et al., 1994; Hosoda et al., 1994)  
55 signalling pathways as well as the transcription factors *SOX10*, *PHOX2B* and *ASCL1*  
56 (Elworthy et al., 2005; Bondurand et al., 2006; Memic et al., 2016). However, the  
57 signals that shape an early ENS identity within vagal NC precursors remain less well-  
58 defined.

59 The axial identity of vagal NC cells is characterized by the expression of  
60 members of the HOX gene paralogous groups (PGs) 3-5 (Parker and Krumlauf, 2017;  
61 Kam and Lui, 2015; Diman et al., 2011; Fu et al., 2003) and is patterned mainly by the  
62 action of somite-derived retinoic acid (RA) signalling, which acts by “posteriorising”  
63 cranial HOX-negative NC progenitors (Stuhlmiller and García-Castro, 2012; Frith et  
64 al., 2018; Ishikawa and Ito, 2009). Studies on *Xenopus* embryos have also indicated  
65 an earlier role for RA in neural crest induction (Villanueva et al., 2002; Bang et al.,  
66 1997). Further, gain- and loss-of-function studies in embryos have pointed to a critical  
67 role for RA in the specification of downstream vagal NC derivatives (Niederreither et  
68 al., 2001; Niederreither et al., 2003; Robrini et al., 2016). This role is especially  
69 prevalent in the development of the ENS where RA signalling components have been  
70 shown to control ENS progenitor migration and proliferation (Uribe et al., 2018)  
71 (Niederreither et al., 2003).

72 Human pluripotent stem cell (hPSCs) differentiation offers an attractive  
73 approach for dissecting the molecular and signalling basis of early developmental cell  
74 fate decisions. To date, a few studies have described the *in vitro* generation of ENS  
75 progenitors and enteric neurons from PSCs providing promising indications that these  
76 cell populations can be effectively utilised for the modelling and treatment of  
77 aganglionic gut conditions such as Hirschsprung disease (HSCR) (Barber et al., 2019;  
78 Fattahi et al., 2016; Workman et al., 2016; Kawaguchi et al., 2010; Schlieve et al.,  
79 2017; Lai et al., 2017; Li et al., 2016). These protocols rely on dual TGF $\beta$ /BMP  
80 inhibition to initially induce an anterior neuroectodermal intermediate that is  
81 subsequently converted into NC through WNT and BMP signalling while a vagal axial  
82 identity is induced through RA supplementation to eventually yield ENS progenitors  
83 after 10-15 days in culture (Fattahi et al., 2016; Lau et al., 2019; Workman et al., 2016).  
84 However, there is mounting evidence, both *in vitro* and *in vivo*, that the neural crest is  
85 in fact specified directly from cells of a pre-gastrulation identity through intermediate  
86 levels of BMP and early activation of the WNT signalling pathway (Basch et al., 2006;  
87 Hackland et al., 2017; Prasad et al., 2019). This suggests a more direct route of enteric  
88 neural crest induction is possible. Furthermore, the precise timing and concentration  
89 of RA signalling that controls the positional identity of vagal neural crest cells has not  
90 been clearly defined and it is not yet clear whether RA imparts an early enteric neural  
91 identity in hPSC-derived vagal neural crest or acts solely as a positional identity  
92 specifier.

93  
94 We recently described a robust protocol for the efficient production of putative  
95 neural crest cells from hPSCs, (Hackland et al., 2017; Frith et al., 2018) that can  
96 acquire a vagal axial identity by exposure to RA (Frith et al., 2018). We have now  
97 utilised this *in vitro* differentiation system to thoroughly investigate the role of RA in  
98 both NC posteriorisation and ENS identity specification. We show that RA acts in a  
99 dose-dependent manner on pre-specified NC precursors, rather than pluripotent or  
100 neuroectodermal cells, to induce expression of *HOX* genes indicative of a vagal  
101 character. This process appears to take place in parallel with the induction of early  
102 ENS progenitor markers. Crucially, we demonstrate that this effect of RA can be  
103 exploited to direct the accelerated production of ENS progenitors (within 6 days of  
104 differentiation), which are capable of generating enteric neurons and glia *in vitro* and

105 which have the ability to colonise the ENS of adult mice following long-term  
106 transplantation. Our findings provide an efficient platform for the *in vitro* modelling of  
107 human ENS development and enteric neuropathies, as well as the development of cell  
108 therapy-based approaches for the treatment of such conditions.

109

## 110 **RESULTS**

### 111 **The timing of retinoic acid signalling affects neural crest specification *in vitro***

112 We have previously shown that RA treatment of cranial NC precursors induces a vagal  
113 axial identity as defined by expression of HOX PG members 1-5 (Frith et al 2018). To  
114 define precisely the developmental time window during which RA exerts its action as  
115 a posteriorising signal without perturbing NC specification, we exposed differentiating  
116 hPSCs to 1 $\mu$ M all-*trans* RA at different stages of our NC differentiation protocol  
117 (**Figure 1A**). Induction of the NC markers p75 and *SOX10*, was assessed by flow  
118 cytometry following antibody staining in a *SOX10*-GFP reporter hPSC line (Chambers  
119 et al., 2012). Adding RA at day 0 of differentiation (the day of plating) did not result in  
120 any *SOX10*:GFP+/p75+ cells detected at day 5, whereas addition of RA at later time  
121 points (day 3 or 4 of differentiation), was compatible with the production of  
122 considerable numbers of *SOX10*:GFP+/p75+ cells (**Figure 1B, C**). Immunostaining  
123 for *SOX10* expression in two additional independent hPSC lines (H7 and  
124 MasterShef7) confirmed the same temporal effect of RA addition on NC differentiation  
125 from hPSCs (**Figure S1**). These data suggest that RA signalling perturbs NC induction  
126 during the early stages of hPSC differentiation. Our findings also indicate that RA  
127 exerts its effects exclusively on cells committed to a NC fate rather than earlier  
128 ectodermal precursors or undifferentiated hPSCs.

129

### 130 **Retinoic acid induces both vagal and enteric neural progenitor identities in a 131 dose-dependent manner**

132 RA has been shown to induce *HOX* gene expression in a dose-dependent manner *in*  
133 *vitro* (Okada et al., 2004; Simeone A, 1990) and *in vivo* (Papalopulu et al., 1991;  
134 Shimozono et al., 2013). To examine how the levels of RA signalling shape the  
135 acquisition of axial identities in hPSC-derived NC cells, we treated day 4 *HOX*-  
136 negative cranial NC precursors with varying concentrations of RA ranging from 10<sup>-9</sup>M  
137 (1nM) to 10<sup>-6</sup>M (1 $\mu$ M). This was followed by examination of the expression of various

138 HOX genes as well as NC and ENS progenitor markers (**Figure 2**). We found that  
139 expression of *HOXB1* and *HOXB2*, was induced by RA at all concentrations in a dose-  
140 dependent manner (**Figure. 2B, Figure. S2**). In contrast, HOX genes marking vagal  
141 NC (*HOXB4*, *B5* and *B7*) were only induced when higher concentrations of RA were  
142 employed (**Figure. 2B**). These data are consistent with previous findings showing that  
143 higher concentrations of RA induce more caudal identities (Okada et al., 2004;  
144 Simeone A, 1990). No expression of *HOXC9* was observed with any RA concentration,  
145 in line with other reports demonstrating that a trunk axial identity is mediated by WNT  
146 and FGF signalling (Frith et al., 2018; Frith and Tsakiridis, 2019; Abu-Bonsrah et al.,  
147 2018; Bel-Vialar et al., 2002; Lippmann et al., 2015; Mazzone et al., 2013; Metzis et  
148 al., 2018; Denham et al., 2015; Hackland et al., 2019).

149 Expression of the NC markers *SOX10*, *PAX7* and *PAX3* was unaffected by the  
150 levels of RA (**Figure 2C, Figure S2**) confirming our previous observation that NC  
151 induction is not dependent on exogenous RA signalling (**Figure 1**). The highest  
152 concentrations of RA also triggered the initiation of an ENS progenitor transcriptional  
153 profile as defined by the expression of *ASCL1* and *PHOX2B* that mark migrating ENS  
154 progenitors (Blaugrund et al., 1996; Lo et al., 1991; Elworthy et al., 2005). Collectively,  
155 these results indicate that acquisition of a vagal axial identity and ENS progenitor  
156 specification in NC progenitors are tightly coupled events that are dependent on RA  
157 signalling.

158

### 159 **RA-induced vagal NC/ENS progenitors generate putative enteric neurons *in*** 160 ***vitro***

161 To test whether day 6 RA-treated vagal NC cells treated with 1 $\mu$ M RA possess ENS  
162 progenitor potential we tested their ability to form enteric neurons *in vitro*. Day 6 RA-  
163 treated vagal NC cells were first cultured in the presence of WNT and FGF signals in  
164 non-adherent conditions to generate spheres (**Figure 3A**), as described previously  
165 (Fattahi et al., 2016). Flow cytometry and fluorescence microscopy analysis showed  
166 that these spheres retained *SOX10*:GFP expression and immunoreactivity to the NC  
167 markers p75 and CD49d (**Figure 3B, C**). Retention of an ENS progenitor identity in  
168 non-adherent culture conditions was also indicated by the sustained expression of  
169 ENS precursor markers *SOX10*, *PAX3*, *PAX7* and *ASCL1* (**Figure 3D**). Spheres were  
170 re-plated in conditions containing GDNF, ascorbic acid and NOTCH signalling  
171 inhibition, which promotes enteric neuron differentiation (Fattahi et al., 2016; Okamura

172 and Saga, 2008; Theocharatos et al., 2013). One week following plating of spheres,  
173 we observed the emergence of cells with a neuronal morphology that expressed the  
174 enteric neuronal markers TUJ1, RET, TRKC and PERIPHERIN (**Figure 3F**). Similar  
175 results were obtained with two additional independent hPSC lines (**Figure S3**).  
176 Markers of both enteric neurons and glia were detected by quantitative real time PCR  
177 (qPCR) in day 22 cultures (**Figure 3G**). Furthermore, *ChAT*, *5-HT*, *TH* and *ASCL1*  
178 expression further confirmed the presence of early enteric neurons in the cultures  
179 (**Figure 3G**). Transcripts for the early glial markers *SOX10* and *S100 $\beta$*  were also  
180 detected in day 22 cultures, but expression of *GFAP*, that is characteristic of more  
181 mature enteric glia, was not observed (**Data not shown**). Together these observations  
182 suggest that day 6 RA-induced NC cells can give rise to enteric neurons and glia *in*  
183 *vitro*.

184

#### 185 **RA-induced vagal NC/ENS progenitors colonise the adult mouse ENS *in vivo*.**

186 To assess the developmental potential of hPSC-derived vagal neural crest/ENS  
187 progenitors *in vivo*, we performed transplantations into the caecum of adult  
188 immunodeficient Rag2<sup>-/-</sup>; $\gamma$ c<sup>-/-</sup>;C5<sup>-/-</sup> mice. To track cells post transplantation, we used  
189 the human induced pluripotent stem (iPS) cell line SFCi55-ZsGr that contains a  
190 constitutive ZsGreen fluorescent reporter in the AAVS safe harbour locus (Lopez-  
191 Yrigoyen et al., 2018). ZsGreen<sup>+</sup> iPS cells were differentiated toward vagal NC/ENS  
192 progenitors as described above (**Figures 2 & 3**) and spheres were generated from  
193 sorted p75<sup>++</sup> cells (**Figure 4A, B**). The cells were then transplanted to the serosal  
194 aspect of the caecum in adult (4-8 week old) immunodeficient Rag2<sup>-/-</sup>; $\gamma$ c<sup>-/-</sup>;C5<sup>-/-</sup> mice  
195 and analysed for integration and differentiation at timed intervals. At 2 weeks post-  
196 transplantation ZsGreen<sup>+</sup> cells were observed at the serosal aspect both within the  
197 caecum and proximal colon. ZsGreen<sup>+</sup> cells expressing the neuronal marker TUJ1  
198 were also observed (**Figure 4C**; left; Arrowheads). Such ZsGreen<sup>+</sup>/TuJ1<sup>+</sup> cells were  
199 found to form filamentous and interconnecting projections along the serosal surface at  
200 this timepoint. At 4 weeks post-transplantation ZsGreen<sup>+</sup> cells were again observed  
201 on the serosal surface at the presumptive site of transplantation. Importantly,  
202 ZsGreen<sup>+</sup> cells were also found within the *tunica muscularis* at the level of the  
203 myenteric plexus. Within the *tunica muscularis* ZsGreen<sup>+</sup> cells were found to co-  
204 express the neuronal marker TUJ1 both within myenteric ganglia-like structures and



205 as intramuscular neurons (**Figure 4C**; right). Additionally, ZsGreen+ cells, which were  
206 also positive for the glial marker GFAP and located at the level of the myenteric plexus  
207 were also detected (**Figure 4C**; right) suggesting that hPSC-derived ENS progenitors  
208 have the potential to differentiate to the main enteric cell types after transplantation *in*  
209 *vivo*. Moreover, 3 months after transplantation ZsGreen+ cells could be identified  
210 across the gut wall both within individual myenteric ganglia (**Figure 4D**; left) and within  
211 the submucosa, surrounding cryptal structures (**Figure 4D**; right). At this timepoint, we  
212 observed the presence of ZsGreen+ cells which had differentiated into major enteric  
213 neuronal subtypes defined by the expression of either neuronal nitric oxide synthase  
214 (nNOS; **Figure 4D**; left) or vesicular acetylcholine transporter (vAChT; **Figure 4D**;  
215 right). Together these results suggest that hPSC-derived ENS progenitors can  
216 integrate into recipient gut tissue where they are maintained in the long-term and have  
217 the ability to differentiate to multiple neuronal subtypes and glia.

218  
219

## 220 **Discussion**

221 We have described an efficient differentiation system that can be employed in multiple  
222 hPSC lines, based on the use of retinoic acid to drive the concomitant induction of  
223 both a vagal and an ENS progenitor identity in cranial neural crest progenitor cells.  
224 The generation of early ENS progenitors after just 6 days, is quicker than other  
225 previously published protocols that describe the production of similar cell populations  
226 from hPSCs (Fattahi et al., 2016; Workman et al., 2016; Barber et al., 2019). The  
227 common feature of these protocols is the induction of an anterior neuroectodermal  
228 intermediate by dual-SMAD inhibition to produce NC that then becomes  
229 “posteriorised” into a vagal axial identity by RA to give rise to ENS progenitors after  
230 10-15 days. A likely reason for the accelerated production of similar progenitors in the  
231 protocol we describe is that we employ a strategy that generates an NC precursor  
232 population from hPSCs through WNT and intermediate levels of BMP signalling, which  
233 then acquires a vagal/ENS identity through the action of RA. This is in line with our  
234 previous findings (Frith et al., 2018), as well as with other studies (Basch et al., 2006;  
235 Leung et al., 2016; Prasad et al., 2019) suggesting that NC specification occurs  
236 independently of a neurectodermal precursor intermediate. Our protocol does not  
237 include any serum replacement and is fully defined to reduce variability within  
238 components of the differentiation media. We also use ‘Top-Down Inhibition’ to control

239 BMP signalling to reduce further variability and improve robustness across  
240 independent hPSC lines (Hackland et al., 2017).

241 RA also appears to drive specification of early ENS progenitors as indicated by  
242 expression of *SOX10*, *ASCL1* and *p75* (**Figure 2**), consistent with previous studies  
243 that reveal a critical role for RA signalling in promoting ENS progenitor migration,  
244 proliferation and differentiation (Niederreither et al., 2003; Uribe et al., 2018; Simkin et  
245 al., 2013). Further, ENS progenitor identity is acquired alongside a vagal axial identity  
246 during neural crest specification, in a manner that is dependent on the dose of RA.  
247 This finding suggests that the gene regulatory networks that control axial identity and  
248 cell fate are inter-linked and inter-dependent. RA signalling may control these  
249 processes through the induction of vagal level HOX family members such as *HOXB3*  
250 and *HOXB5* as well as their TALE family co-factors, such as the *MEIS* family of genes,  
251 which have been reported to regulate downstream ENS development (Chan et al.,  
252 2005; Kam et al., 2014; Kam and Lui, 2015; Uribe et al., 2018; Uribe and Bronner,  
253 2015) by acting upstream of *Ret* (Zhu et al., 2014) and preventing apoptosis (Kam et  
254 al., 2014).

255 Critically, our differentiation strategy rapidly yields (by day 6 of differentiation)  
256 a promising well-defined cell population that can efficiently generate enteric neurons  
257 and glia in vitro (**Figure 3**), and so may provide a potential cellular donor for the  
258 treatment of enteric neuropathies. In contrast to previous *in vivo* studies utilising  
259 hPSC-derived ENS progenitors, we chose to transplant our ENS progenitors into the  
260 gut of immunodeficient *Rag2<sup>-/-</sup>;γc<sup>-/-</sup>;C5<sup>-/-</sup>* mice. This approach eliminates the  
261 requirement for chemical immunosuppression and allows long-term study of donor cell  
262 survival and integration within a “normal” host ENS microenvironment. Crucially, we  
263 found that the hPSC-derived neurons were present within endogenous ENS ganglia  
264 of adult mice up to 3 months post-transplantation, expressing the same characteristic  
265 markers (nNOS and vAChT positive neurons) as the mouse neurons in their host  
266 environment (**Figure 4**). Further, the transplanted human cells populated both the  
267 myenteric and submucosal plexuses of the gut, demonstrating that they are able to  
268 migrate extensively within the gut wall and form neuronal networks even though the  
269 host ENS remained intact (**Figure 4**). Recent studies have demonstrated the potential  
270 of cellular transplantation for the treatment of enteric neuropathies. Importantly, both  
271 postnatally-derived human and murine endogenous enteric neural stem cells (ENSC)  
272 have been used for *in vivo* applications, in mice, demonstrating functional integration



273 (J.E. Cooper et al., 2017; J.E. Cooper et al., 2016; Stamp et al., 2017), and functional  
274 rescue of an enteric neuropathy (McCann et al., 2017). Similarly, transplanted hPSC-  
275 derived ENS progenitors generated through dual-SMAD inhibition have been shown  
276 to integrate and migrate extensively within a mouse model of Hirschsprung disease  
277 leading to increased survival (Fattahi et al., 2016). Our work here extends and  
278 complements these studies providing further evidence in support of the use of hPSCs  
279 as a promising platform for the development of cell therapies to treat ENS dysfunction.

280

## 281 **Methods and Materials**

### 282 **hPSC culture**

283 The human pluripotent stem cell lines H7 (WA07), H9 (WA09) (Thomson, 1998),  
284 H9:SOX10 (Chambers et al., 2012), Mastershef7 (Gouti et al., 2014), and SFCi55-  
285 ZsGr (Lopez-Yrigoyen et al., 2018) were grown in mTESR (Stem Cell Technologies #  
286 85850) on 1:100 dilution of Geltrex (ThermoFisher A1413202) in DMEM/F12 (Sigma  
287 D6421). Cells were passaged at 80-90% confluency using ReLeSR (Stem Cell  
288 Technologies Catalog # 05873). Cells were incubated at 37°C in 5%CO<sub>2</sub>.

289

### 290 **Directed Differentiation**

291 For vagal neural crest differentiation, we use a previously described protocol (Frith et  
292 al., 2018). hPSCs at approximately 80% confluency were detached using Accutase  
293 (Sigma-Aldrich A6964) for 10 minutes at 37°C to generate single cells. Cells were  
294 counted manually and plated at 50,000 cells/cm<sup>2</sup> on Geltrex coated plates  
295 (ThermoFisher A1413202). Neural Crest differentiation media is comprised of  
296 DMEM/F12 (Sigma-Aldrich), supplemented with 1x N2 (ThermoFisher 17502048),  
297 NEAA (ThermoFisher 11140050), Glutamax (ThermoFisher 35050061), 1μM  
298 CHIR99021 (Tocris 4423), 2μM SB431542 (Tocris 1614/1), 1μM DMH-1 (Tocris  
299 4126/10), 20ng/ml BMP4 (ThermoFisher PHC9533). All-Trans Retinoic Acid (Sigma  
300 R2625) was diluted in DMSO. 10μM Y-27632 dihydrochloride (Tocris 1254/1) was  
301 added at day 0 until day 2 to assist attachment. For all vagal neural crest induction all-  
302 trans Retinoic acid was added at a final concentration of 1μM on day 4 unless specified  
303 in the results. Media was changed every other day until day 5/6.

304

305 Spheres were generated as previously described (Fattahi et al., 2016). Day 6 cells  
306 were treated with accutase to form a single cell suspension and replated in a media  
307 containing a 1:1 mix of DMEM/F12 (Sigma) with Neurobasal (ThermoFisher  
308 21103049) supplemented with 1x N2, 1x B27, 1x NEAA, 1x Glutamax, 3 $\mu$ M  
309 CHIR99021, 10ng/ml FGF2 (R&D systems 233-FB/CF). Sphere media supplemented  
310 10 $\mu$ M of Y-27632 dihydrochloride (Tocris) to ensure sphere formation and left until day  
311 10. One well of a 6 well plate was plated into one well of an Ultra-Low Attachment 6  
312 well plate (Corning 3471) or 6 well plates with a coating of 1% w/v agarose.

313

314 For enteric neuronal differentiation, day 10 spheres were plated onto Geltrex coated  
315 plates in BrainPhys (Stem Cell Technologies 05790) supplemented with 1x N2, 1x B27  
316 (ThermoFisher 17504044), 100 $\mu$ M Ascorbic Acid (Sigma A8960), 10ng/ml GDNF  
317 (Peprotech 450-10) and 10 $\mu$ M DAPT (Sigma D5942). Media was changed every other  
318 day and once a week supplemented with Vitronectin (ThermoFisher A14700)

319

#### 320 **RNA extraction, cDNA synthesis & qPCR**

321 RNA was extracted using a Total RNA purification plus kit (Norgen BioTek #48300)  
322 per manufacturer's instructions. RNA concentration was measured using a nanodrop  
323 (ThermoFisher). RNA was stored at -80°C. cDNA was synthesised using the High-  
324 Capacity cDNA Reverse Transcription kit (ThermoFisher 4368813) and stored at -  
325 20°C.

326 qPCR was performed on QuantStudio 12K Flex thermocycler (Applied Biosystems).  
327 CT values were calculated against GAPDH for each sample. Relative quantities  
328 calculated using the  $-2^{\Delta\Delta CT}$  method.

329

#### 330 **Immunofluorescence**

331 Cells were fixed with 4% PFA for 10 minutes at room temperature and washed 3 times  
332 with 1x PBS (no Mg<sup>2+</sup>/ Ca<sup>2+</sup>). Cells were permeabilised and blocked with 1x PBS (no  
333 Mg<sup>2+</sup>/ Ca<sup>2+</sup>) supplemented with 10% FCS, 0.1% BSA and 0.3% Triton-X 100 for 1 hour  
334 at room temperature. Primary antibodies were diluted in permeabilisation buffer and  
335 incubated at 4°C overnight. Secondary antibodies were diluted in permeabilization  
336 buffer and stained in the dark at 4°C for one hour. Nuclei were counterstained with

337 Hoechst 33342 (ThermoFisher H3570). Images were taken on an InCell Analyser  
338 2500 (GE Healthcare).

339

### 340 **Image Analysis.**

341 Images were quantified using custom made pipelines on CellProfiler 2.2 (Carpenter et  
342 al., 2006). Individual nuclei were identified by Hoechst staining and the fluorescence  
343 intensity following staining was measured and related to nuclei. Positive staining was  
344 scored based on having greater fluorescence intensity values than a threshold value  
345 from a secondary only staining control for each biological repeat.

346

### 347 **Flow Cytometry**

348 Flow cytometry was carried out as described in (Frith et al., 2018). In short, a single  
349 cell suspension was generated using Accutase as described above. Cells were  
350 pelleted and resuspended in FACs buffer (DMEM/10% v/v FCS) at  $1 \times 10^6$  cells/ml.  
351 Gating for positive cells was based on a negative control consisting of cells not  
352 carrying a reporter or cells stained with P3X, an antibody from the parent myeloma  
353 (KÖHLER and MILSTEIN, 1975; Hackland et al., 2017).

354

### 355 **Animals**

356 Animals were maintained, and experiments were performed, in accordance with the  
357 UK Animals (Scientific Procedures) Act 1986 under license from the Home Office  
358 (P0336FFB0) and approved by the University College London Biological Services  
359 Ethical Review Process. Animal husbandry at UCL Biological Services was in  
360 accordance with the UK Home Office Certificate of Designation.

361 Rag2<sup>-/-</sup>;γc<sup>-/-</sup>;C5<sup>-/-</sup> mice, which lack innate immunity and are deficient in all lymphocytes  
362 (R.N. Cooper et al., 2003; Silva-Barbosa et al., 2005), were used as recipients for all  
363 hPSC-derived vagal neural crest/ENS progenitor transplantations.

364

### 365 **In vivo Cell Transplantation**

366 ZsGreen+ spheres were transplanted to the caecum of 4-8 week-old immunodeficient  
367 Rag2<sup>-/-</sup>;γc<sup>-/-</sup>;C5<sup>-/-</sup> mice, via laparotomy under isoflurane anesthetic. Briefly, the  
368 caecum was exposed and ZsGreen+ spheres, containing 1 million cells each, were  
369 subsequently transplanted to the serosal aspect of the caecum by mouth pipette, using  
370 a pulled glass micropipette. Each transplanted tissue typically received 3 ZsGreen+

371 spheres which were manipulated on the surface of the caecum with the bevel of a 30G  
372 needle to ensure correct positioning. Transplanted Rag2<sup>-/-</sup>;γc<sup>-/-</sup>;C5<sup>-/-</sup> mice were  
373 typically maintained for up to 3 months post-transplantation before sacrifice and  
374 removal of the caecum and proximal colon for analysis.

375

### 376 **Wholemount Gut Immunohistochemistry**

377 Wholemount immunohistochemistry was performed on transplanted caecal and  
378 proximal colon segments after cervical dislocation and excision. Tissues were fixed in  
379 ice cold 4% PFA for 45 min at 22°C. After fixation, tissues were washed for 24h in 1x  
380 PBS at 4°C. Cells were permeabilised and blocked with 1x PBS supplemented with  
381 1% Triton X-100 and 10% sheep serum. Primary antibodies were diluted in  
382 permeabilisation buffer and incubated at 4°C for 48h. Secondary antibodies were  
383 diluted in permeabilization buffer and stained in the dark for one hour at 22°C. Nuclei  
384 were counterstained with DAPI (Sigma). Before mounting, tissues were washed  
385 thoroughly in 1x PBS for 2h at 22 °C. Tissues were examined using a LSM710 Meta  
386 confocal microscope (Zeiss). Confocal micrographs of whole mounts were digital  
387 composites of the Z-series of scans (0.5-1µm optical sections, 10–50µm thick).

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**Antibodies**

	<b>Antibody</b>	<b>Species</b>	<b>Source</b>	<b>Dilution</b>
<b>In Vitro</b>	SOX10	Rabbit	Cell Signalling Technology (D5V9L) #89356	1:500
	RET	Rabbit	Abcam ab134100	1:1000
	TUJ1	Mouse	Abcam ab78078	1:1000
	TRKC	Rabbit	Cell Signalling Technology (C44H5) #3376	1:1000
	PERIPHERIN	Rabbit	Millipore AB1530	1:100
	P3X	Mouse	In House myeloma P3X63Ag8 (Kohler & Milstein 1975)	1:10
	p75	Mouse	In House Hybridoma Clone ME20.4 (Ross et al., 1984)	1:20
	CD49d	Mouse	BioLegend 304302 Clone 9F10	1:100
<b>Transplants</b>	TUJ1	Mouse	BioLegend MMS-435P	1:500
	GFAP	Rabbit	Millipore AB5804	1:500
	nNOS	Rabbit	Invitrogen 61-7000	1:400
	vAChT	Goat	ThermoFisher Scientific OSH00003W	1:200
	DAPI		Sigma D8417	1:1000

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**Primers**

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>	<b>Roche UPL Probe</b>
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaattcc	60
HOXB1	ccagctaggggctgtc	atgctgaggaggatg	39
HOXB2	aatccgccacgtctcctt	gctgctgttggtgtaagc	70
HOXB4	ctggatgcgcaaagttcac	agcggttgtagtgaattcctt	62
HOXB5	aagcttcacatcagccatga	cggttgaagtggaactcctt	1
HOXB7	ctaccctggatgcaag	caggtagcgattgtagtgaattct	1
HOXC9	gcagcaagcacaagagga	cgctggacttggtgtaggg	85
SOX10	ggctccccatgtcagat	ctgtctcgggggtggtg	21
PAX3	aggaggccgacttgaga	cttcatctgattgggtgct	13
PAX7	gaaaaccagcatgttcag	gctgctaatcgaactcactaa	66
ASCL1	cgacttcaccaactggttctg	atgcaggttgtgcatca	38
PHOX2A	cactacccgacatttacag	gctcctgttgcggaactt	17
PHOX2B	ctaccccgacatctacactcg	ctcctgcttgcgaaactg	17
SST	acccagactccgtcagttt	acagcagctctgccaagaag	38
CHAT	cagccctgatgccttcat	cagtcttcgatggagcctgt	78
TH	acgccaaggacaagctca	agcgtgtacgggtcgaact	42
5-HT	tgatgtcacttgcatagctg	caggtaaattccagactgcacaa	3
S100 $\beta$	ggaaggggtgagacaagga	ggtggaaaacgtcgatgag	78
GFR $\alpha$ 1	caccattgccctgaaagaat	cgcttttaggggttcaggctc	36

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## 409 **Acknowledgements**

410 This work was supported in part by grants from the Medical Research Council  
411 Confidence in Concept awarded to IB & PWA (MC\_PC\_14115), BBSRC  
412 (BB/P000444/1) awarded to AT, H2020-EU.1.2.2 (Grant agreement ID: 824070)  
413 awarded to AT & PWA. CM is supported by Guts UK (Derek Butler Fellowship). NT is  
414 supported by Great Ormond Street Hospital Children's Charity (GOSHCC - V1258).  
415 This work was partially funded through a GOSHCC grant (W1018C) awarded to NT  
416 (Principal Investigator) and AJB (Co-Investigator).

417 The authors would like to acknowledge the NIHR Great Ormond Street Hospital  
418 Biomedical Research Centre which supports all research at Great Ormond Street  
419 Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child  
420 Health. The views expressed are those of the author(s) and not necessarily those of  
421 the NHS, the NIHR or the Department of Health. The authors acknowledge the support  
422 of Prince Abdullah Ben Khalid Celiac Research Chair, College of Medicine, Vice-  
423 Deanship of the Research Chairs, King Saud University, Riyadh, Saudi Arabia.

424

## 425 **Author Contributions**

426 TF, PWA, JOSH, CM, AJB, NT conceived the project. TF, CM designed, performed  
427 and analysed experiments with help from AT & AG.

428 IB, PWA, AT, NT, AJB and CM provided financial support. TF, AT, CM and PWA  
429 wrote the manuscript.

430 **Figure Legends**

431 **Figure 1: Retinoic Acid affects neural crest specification in a time-dependent**  
432 **manner**

433 **(A)** Schematic showing the neural crest differentiation protocol and timepoints  
434 corresponding to addition of all-trans retinoic acid (RA).

435 **(B)** FACs plots after 5 days of differentiation showing representative SOX10:GFP and  
436 p75 expression after RA addition at indicated timepoints during neural crest  
437 differentiation. Gates were set based on the negative control from a GFP negative line  
438 stained with P3X as a staining control.

439 **(C)** Quantifications of the percentage of cells that display SOX10 expression in three  
440 different lines following FACs or immunofluorescence analysis. Graphs show  
441 percentages of SOX10+ cells normalised to a control condition where no RA was  
442 added. Bars state the mean  $\pm$  standard deviation of 4 biological repeats for  
443 SOX10:GFP hPSCs and 3 biological repeats for H7 & MasterShef7. \* P<0.05 \*\*  
444 P<0.01 One-way-Anova.

445

446 **Figure 2: Retinoic induces a vagal axial and an early ENS progenitor identity in**  
447 **a dose dependent fashion.**

448 **(A)** Schematic showing the differentiation protocol with RA addition at day 4.

449 **(B)** qPCR plots showing the induction of *HOX* genes in day 6 neural crest cells after  
450 exposure to different concentrations of retinoic acid. Data presented are relative  
451 quantities compared to *HOX* negative cells that were not treated with retinoic acid.  
452 Bars represent mean + standard deviation from 3 biological repeats of SOX10:GFP  
453 hPSCs.

454 **(C)** qPCR plot showing the expression of the neural crest markers *SOX10*, *PAX3* and  
455 the enteric neural precursor marker *ASCL1* in day 6 cells following 2 days exposure  
456 to different concentrations of retinoic acid. Bars represent the mean + standard  
457 deviation of 3 biological repeats of SOX10:GFP hPSCs.

458 **(D)** qPCR plots showing the expression of indicated neural crest and enteric neural  
459 precursor markers in day 6 cells that have been subjected to two days exposure of  
460 1 $\mu$ M retinoic acid.

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464 **Figure 3: Day 6 putative enteric neural precursors can generate putative enteric**  
465 **neurons *in vitro*.**

466 **(A)** Schematic to depict the culture of day 6 cells in non-adherent conditions.

467 **(B)** FACs plots showing the retention of SOX10:GFP and p75/CD49d co-expression  
468 from day 6 to day 10 after culture in non-adherent conditions with WNT/FGF.

469 **(C)** Representative photomicrograph of neural crest spheres at day 8 showing  
470 SOX10:GFP positive cells forming spheres.

471 **(D)** qPCR graph showing that the expression of the neural crest markers *SOX10*,  
472 *PAX7*, *PAX3* and the enteric neural precursor marker *ASCL1* is retained between day  
473 6 and day 10 following culture in non-adherent conditions. Bars represent  
474 mean±standard deviation of 3 biological repeats.

475 **(E)** Schematic depicting the conditions to generate enteric neurons from day 10  
476 spheres following plating.

477 **(F)** Immunofluorescence analysis shows the presence of cells that are positive for  
478 TUJ1, RET, TRKC and PERIPHERIN at day 17 of differentiation. Scale bars are 50µm.

479 **(G)** qPCR analysis shows the induction of markers for early enteric neurons and early  
480 glial progenitors at day 22 of differentiation. Expression is shown as relative quantity  
481 compared to hPSCs. Bars represent mean + standard deviation of 3 biological  
482 repeats.

483 All data shown have been obtained using SOX10:GFP hPSCs.

484

485 **Figure 4: hPSC derived enteric neuronal precursors integrate into the mouse**  
486 **ENS after transplantation.**

487 **(A)** Schematic depicting the experimental procedures for transplantation of hPSC-  
488 derived ENS progenitors

489 **(B)** Sorting strategy to isolate ZsGreen+/p75++ labelled putative ENS progenitors  
490 following *in vitro* differentiation. P3X antibody is control for antibody staining.

491 **(C)** Wholemout images of gut tissue corresponding to the indicated regions obtained  
492 from immunodeficient mice at 2 weeks and 4 weeks post-transplantation and showing  
493 the presence of ZsGreen+ cells that are positive for the neuronal marker TUJ1  
494 (Arrows) amongst endogenous TUJ1+ neurons (Arrowheads), and glial marker GFAP  
495 after immunostaining. Pr. Colon, proximal colon.

496 **(D)** Images showing the differentiation of hPSC-derived ENS progenitors into different  
497 subtypes of enteric neurons including nNOS positive and vAChAT positive neurons in  
498 the caecum of Rag2<sup>-/-</sup>;γc<sup>-/-</sup>;C5<sup>-/-</sup> mice at 3 months post-transplantation. Arrows are  
499 transplanted ZsGreen<sup>+</sup> cells; Arrowheads are endogenous enteric neurons.

500 **(E)** Table showing the numbers of mice in which ZsGreen<sup>+</sup> cells were identified over  
501 the total number of transplanted mice analysed at indicated timepoints post-  
502 transplantation.

503

#### 504 **Supplementary Figure 1: RA timing conserved across other hPSC lines.**

505 **(A-B)** Immunofluorescence images showing SOX10 expression at day 5 after RA was  
506 added at different times during neural crest differentiation of H7 **(A)** and MasterShef7  
507 **(B)** hPSCs.

508 **(C)** Quantification of the number of GFP positive cells from SOX10:GFP hPSCs (4  
509 biological repeats) and SOX10 positive cells in H7 and MasterShef7 after addition of  
510 RA at different time points from 3 biological repeats. Bars are mean ± standard  
511 deviation. \*P<0.05, N.S= not significant One-Way Anova compared to day 5 cells not  
512 treated with RA.

513

#### 514 **Supplementary Figure 2: HOX gene induction and early enteric neural marker 515 induction is dependent on the concentration of RA**

516 qPCR analysis showing HOX gene and early ENS progenitor marker induction after 6  
517 days of differentiation following RA exposure of H7 **(A)** and MasterShef7 **(B)** hPSCs  
518

#### 519 **Supplementary Figure 3: Generation of enteric neurons in 2 further independent 520 hPSC lines.**

521 **(A)** Schematic of enteric neuron differentiation protocol.

522 **(B)** Immunofluorescence showing the cells that are positive for TUJ1 and TRKC at day  
523 17 of differentiation. Scale bars are 50μm.

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Figure 2

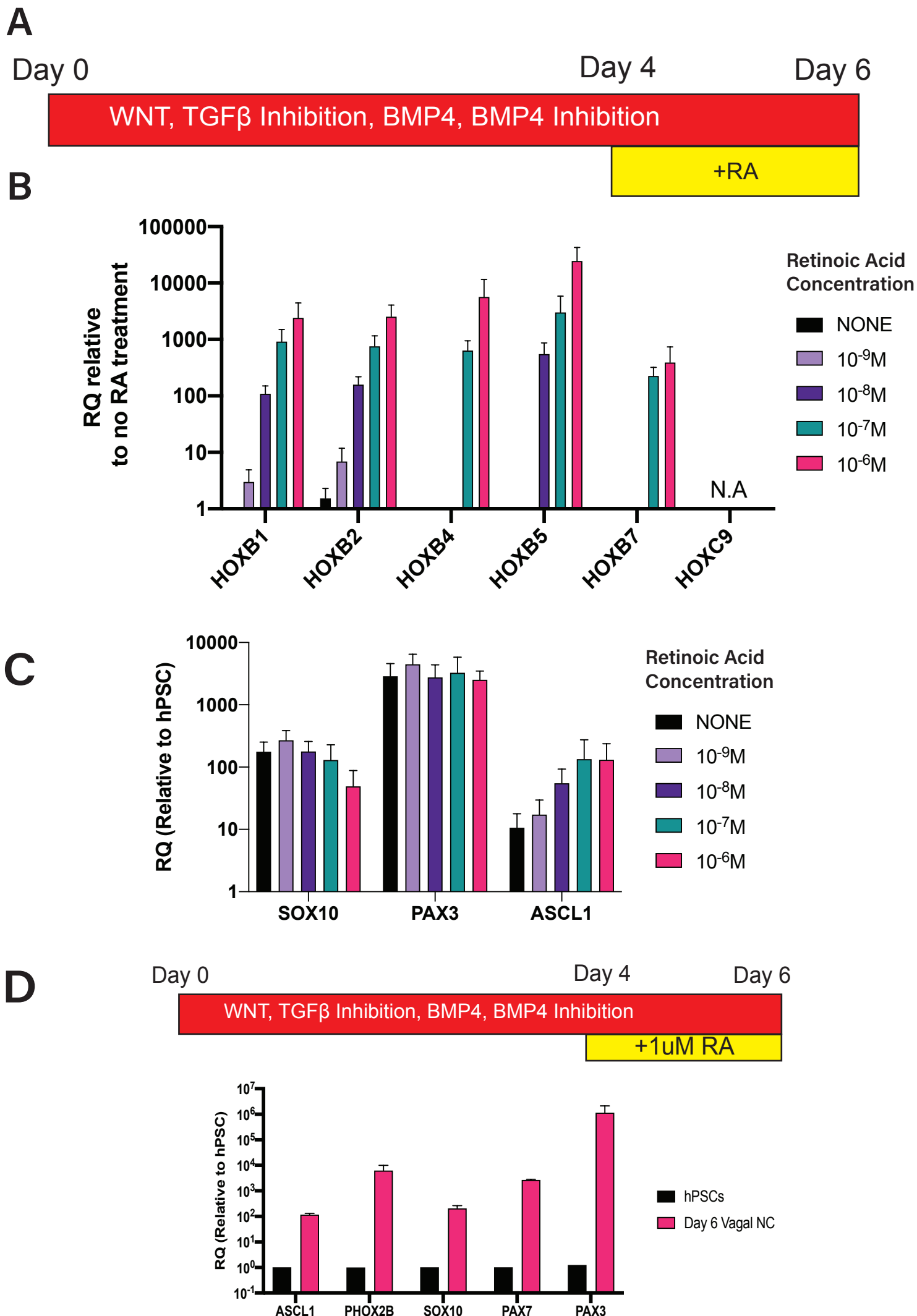
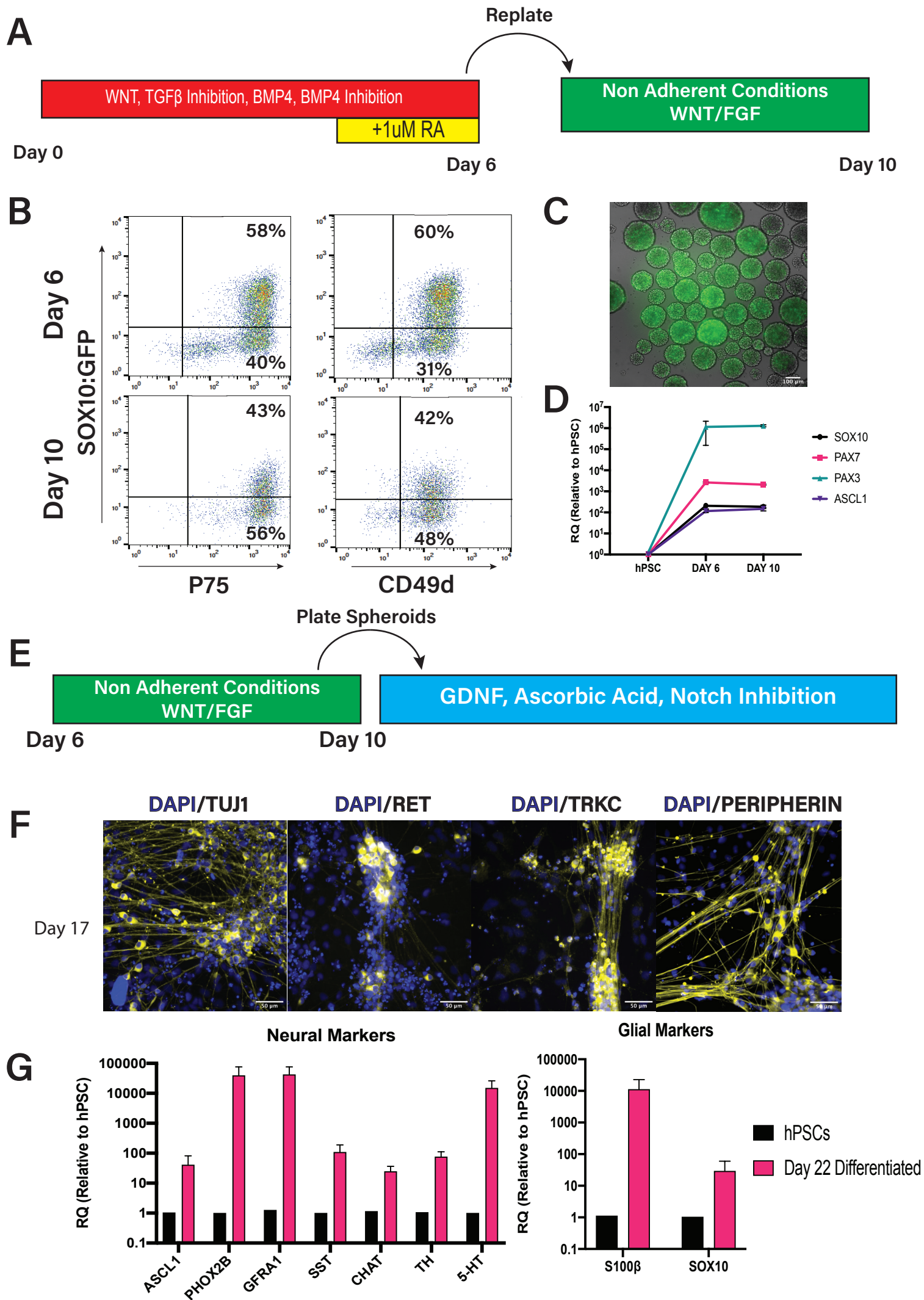
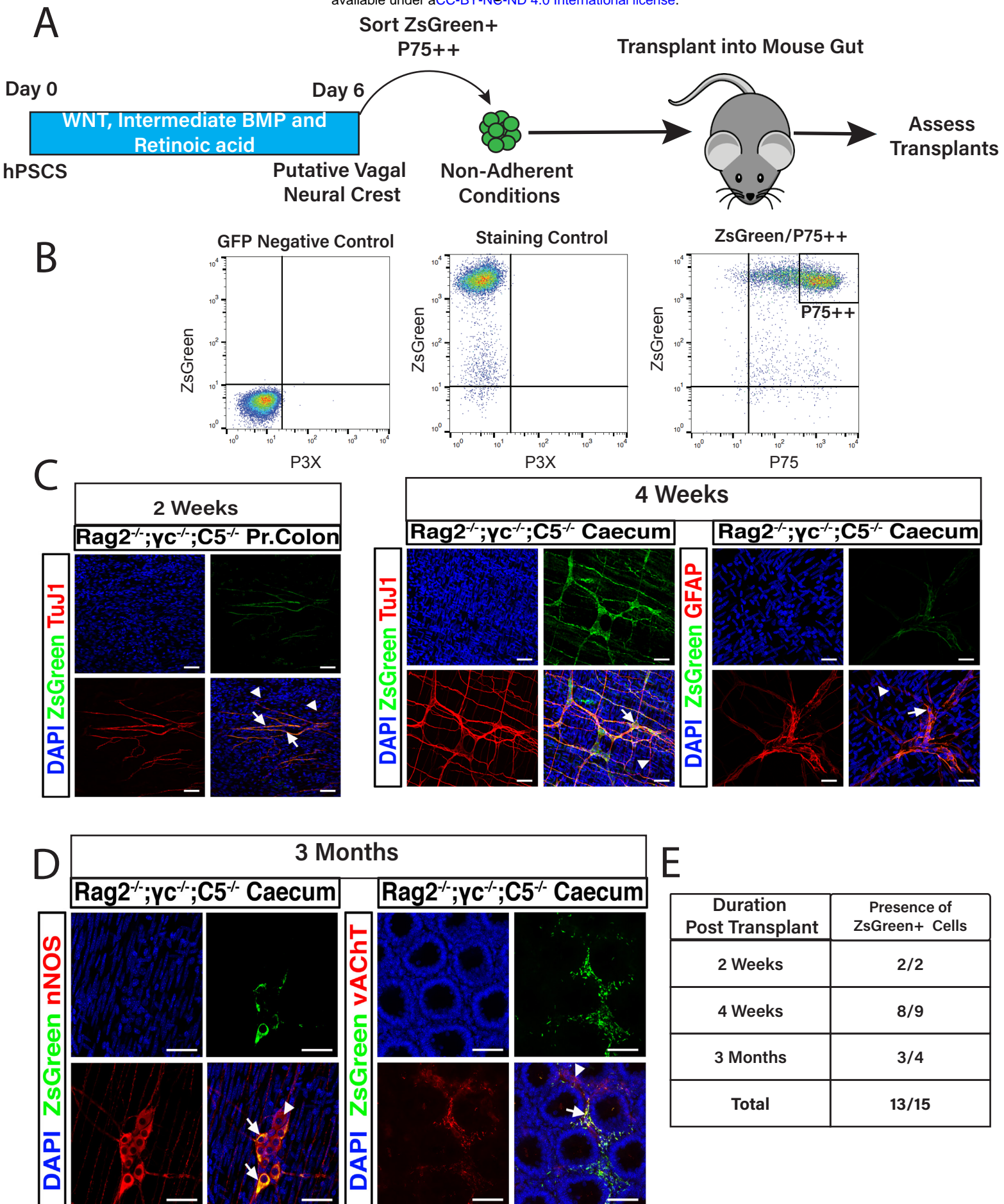


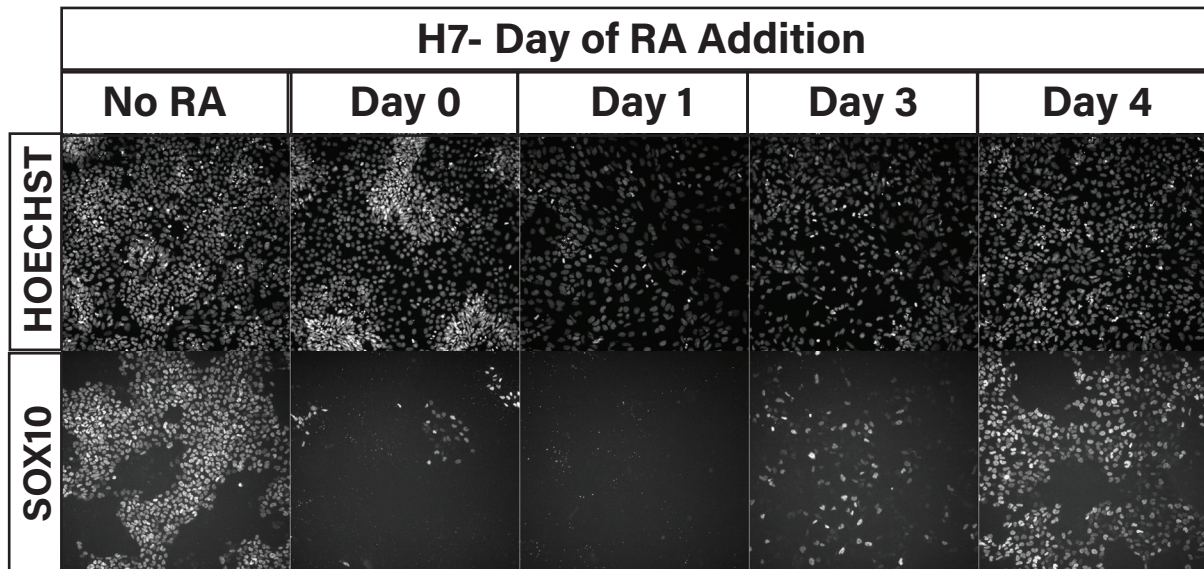
Figure 3



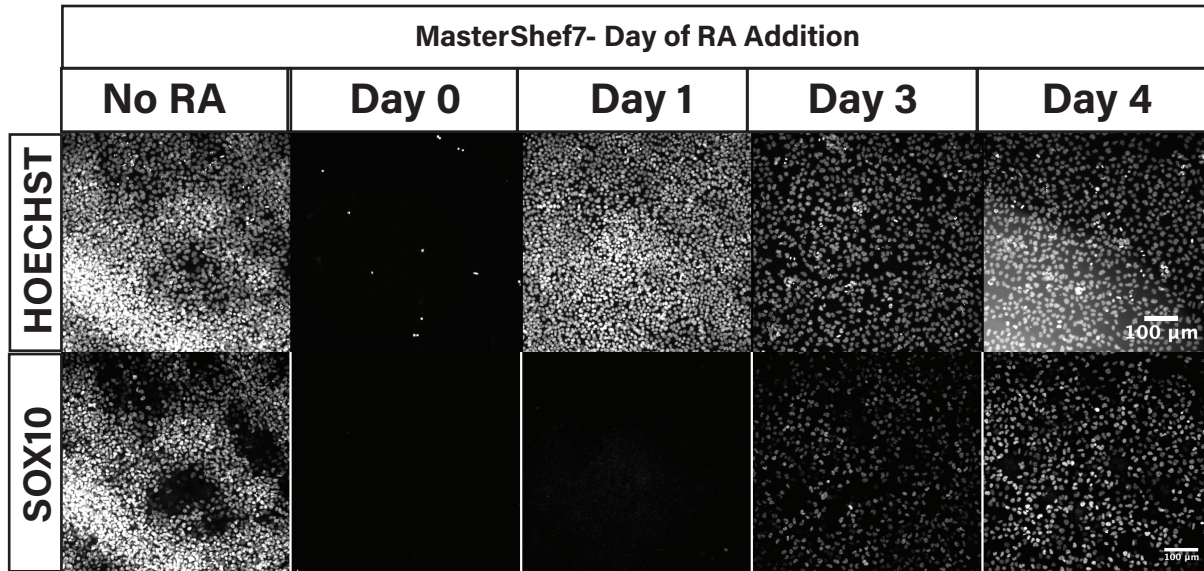




A



B



C

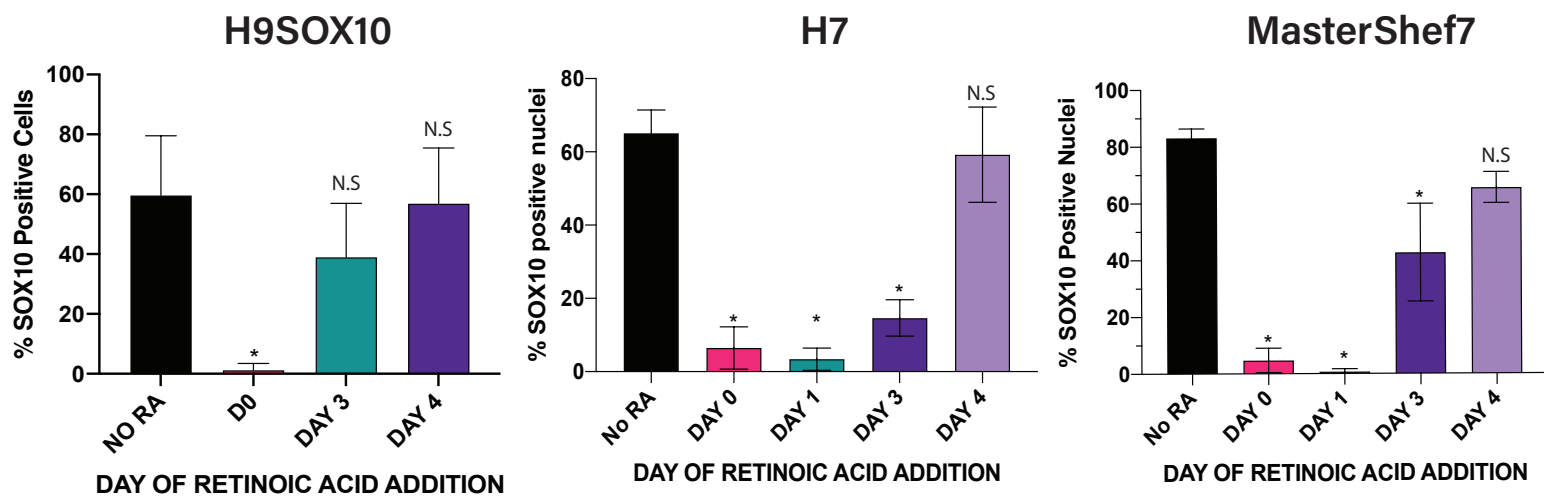
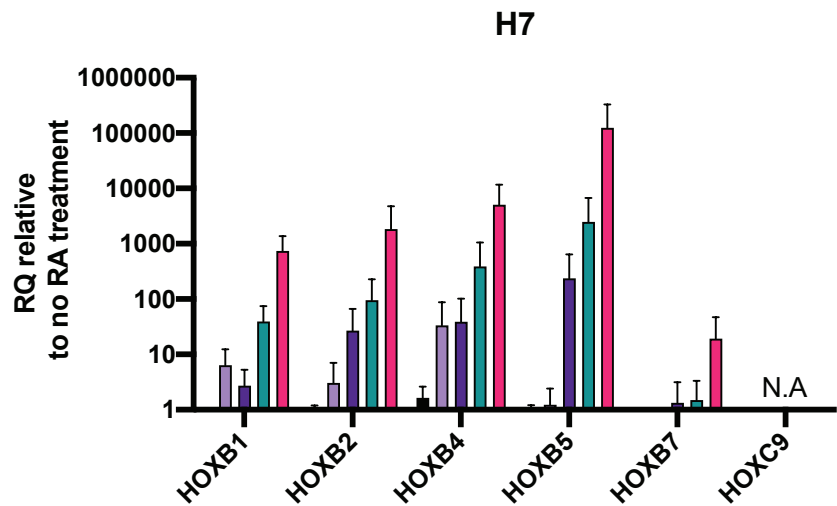
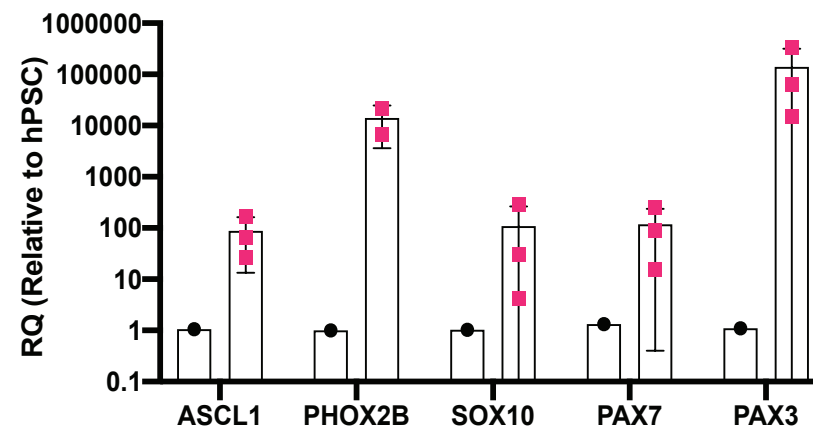


Figure S2

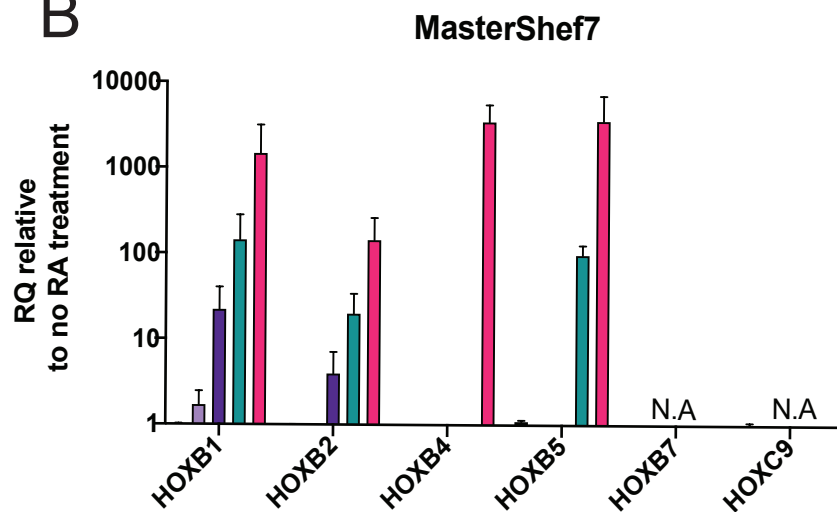
A



**H7**



B



**MASTERSHEF7**

