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The enteric neural crest progressively loses capacity to form enteric nervous system

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ABSTRACT

Cells of the vagal neural crest (NC) form most of the enteric nervous system (ENS) by a colonising wave in the embryonic gut, with high cell proliferation and differentiation. Enteric neuropathies have an ENS deficit and cell replacement has been suggested as therapy. This would be performed post-natally, which raises the question of whether the ENS cell population retains its initial ENS-forming potential with age. We tested this on the avian model in organ culture *in vitro* (3 days) using recipient aneural chick midgut/hindgut combined with ENS-donor quail midgut or hindgut of ages QE5 to QE10. ENS cells from young donor tissues (≤ QE6) avidly colonised the aneural recipient, but this capacity dropped rapidly 2–3 days after the transit of the ENS cell wavefront. This loss in capability was autonomous to the ENS population since a similar decline was observed in ENS cells isolated by HNK1 FACS. Using QE5, 6, 8 and 10 midgut donors and extending the time of assay to 8 days in chorio-allantoic membrane grafts did not produce 'catch up' colonisation. NC-derived cells were counted in dissociated quail embryo gut and in transverse sections of chick embryo gut using NC, neuron and glial marker antibodies. This showed that the decline in ENS-forming ability correlated with a decrease in proportion of ENS cells lacking both neuronal and glial differentiation markers, but there were still large numbers of such cells even at stages with low colonisation ability. Moreover, ENS cells in small numbers from young donors were far superior in colonisation ability to larger numbers of apparently undifferentiated cells from older donors. This suggests that the decline of ENS-forming ability has both quantitative and qualitative aspects. In this case, ENS cells for cell therapies should aim to replicate the embryonic ENS stage rather than using post-natal ENS stem/progenitor cells.

1. Introduction

The enteric nervous system (ENS) in vertebrates in the wall of the gastrointestinal tract is the largest and most complex division of the autonomic nervous system, and controls gut peristalsis, water and electrolyte balance and intestinal blood flow (Furness et al., 2014). The genetic and cell biological bases of ENS formation is highly conserved between vertebrates species including humans, as are defects in the development of the ENS (Brooks et al., 2005).

The ENS is derived from neural crest (NC) cells and in particular mostly from the vagal level neural tube overlapping caudal hindbrain and rostral trunk levels (Kuo and Erickson, 2011; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). There is an additional contribution by sacral NC cells (Kapur, 2000; Le Douarin and Teillet, 1973). Recently in the mouse; late-originating ENS neurons derived from trunk NC-derived Schwann cell precursors have been identified (Uesaka et al., 2015). The overlap of this ENS-source with the sacral source is not clear, and its importance in other vertebrates is at present unknown.

The vagal NC-derived cells occupy the foregut first and are then termed enteric NC (ENC) cells as they acquire gut-colonising competence (Simkin et al., 2013). The ENC cells then occupy the midgut and hindgut as a rostro-caudal wave (Allan and Newgreen, 1980; Fairman et al., 1995). In mice, this sequence is similar, but with the distinction that ENC-derived cells from the midgut 'short cut' across the mesentery directly into the hindgut then colonise the hindgut in both rostral and caudal directions (Nishiyama et al., 2012).

The starting vagal NC cell population that colonises the foregut by E2.5 in quail embryos is around 1500 ENC cells, and by E7, soon after the gut is entirely colonised, the NC-derived population is over 500,000 (Zhang et al., 2018), by which time about half of the cells display neuron differentiation markers (Rollo et al., 2015). The first indication

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of the importance of cell number for ENS development arose from experiments in which the premigratory vagal NC cell population in chick embryos was partially ablated: this early quantitative reduction resulted in the distal intestine failing to be colonised (Yntema and Hammond, 1954), an outcome resembling the human enteric neuropathology Hirschsprung disease (see below). Subsequent investigations have pointed to the importance of ENC cell numbers in driving the rostro-caudal wave to complete colonisation of the growing gut (Barlow et al., 2008; Newgreen et al., 1996) and mathematical and experimental models have extended this (Landman et al., 2007; Simpson et al., 2006, 2007; Zhang et al., 2018). In avian, murine and fish models, colonisation is driven by proliferation especially of ENC cells at the wavefront by a process termed frontal expansion (Harrison et al., 2014; Nishiyama et al., 2012; Simpson et al., 2007), while in the ENS behind the wavefront proliferation keeps pace with the intestine's growth (Young et al., 2005).

The increase of the ENS as a cell population is achieved with enormous diversity of proliferation at the single cell level, with a relatively few ENC progenitor cells contributing disproportionately to the final ENS (Cheeseman et al., 2014). We argued that all or many ENC cells are capable of this massive clonal expansion, but the few actually realise this capability (Newgreen et al., 2017). Followed to its conclusion, this argument would hold that only a relatively few ENS progenitor cells would be necessary to form the ENS, and this has experimental backing in avian and murine models (Barlow et al., 2012; Rothman et al., 1993; Sidebotham et al., 2002; Zhang et al., 2018).

A potentially fatal developmental defect of the ENS in humans is Hirschsprung disease or enteric aganglionosis (Puri, 2000). In this disease, enteric ganglia are absent from the most caudal segment of the intestine which leads to megacolon rostral to the aganglionic segment. Hirschsprung disease is detected in early post-natal life and treated by surgical resection of the aganglionic bowel segment but distressing functional problems often persist (Sander and Powell, 2004). Consequently, therapeutic options with stem or progenitor cells that might form a functional ENS in the aneural segment are being pursued (Burns et al., 2016; Hotta et al., 2009). Since this disease is detected in the neonate, this cell therapy would have to be accomplished in patients at this stage. Recently, embryonic and post-natal mouse ENS cells introduced into the post-natal colon of normal and Hirschsprung-model mice have been shown to assemble at the appropriate plexus site where they form ENS cells (Hotta et al., 2013; Stamp et al., 2017). For human Hirschsprung disease patients (Stamp and Young, 2017), the most immunologically favourable source of potentially corrective cells would be the same patient. Recently, patient ENS cells have been shown to colonise and differentiate in human autologous aganglionic colon smooth muscle when combined in organ culture (Cheng et al., 2017; Rollo et al., 2016).

Despite these encouraging outcomes in small scale assays, construction of an ENS in a human neonate is a large-scale undertaking. Cells of the embryonic ENS have high proliferation ability (Simpson et al., 2007) so that surprisingly small starting numbers of ENC cells are effective at forming the ENS (Barlow et al., 2012; Rothman et al., 1993; Sidebotham et al., 2002; Zhang et al., 2018). However, the qualitatively appropriate ENS cells obtainable from human patients (see Rollo et al., 2016) are from post-natal stages when most ENS cells are differentiated neurons and glial cells. This raises the question of whether the ENS cell population retains its initial colonisation, proliferation and differentiation potential with age.

Previously, ENS-containing avian gut of various embryonic ages was juxtaposed to uncolonised hindgut in chorio-allantoic membrane (CAM) organ cultures. The results suggested that colonisation capacity of the ENC cells from the donor gut tissue was restricted in time (Meijers et al., 1992). Comparison *in vitro* of rat ENC stem cells from embryonic and adult gut also suggested the adult-derived cells were reduced in renewal and proliferation capacities as well as being impoverished for some lines of differentiation (Kruger et al., 2002).

We explore the question of retention or loss of ENS-forming ability in further detail here with the avian model system. The results suggest that ENC-derived cells retain high ability to colonise aneural gut tissue for several days after the normal colonisation phase, but this ability then rapidly declines. The age-related decline broadly matches the progressive reduction in proportion of donor ENS cells lacking neuronal and glial markers. However the ENS colonisation deficit with older donor age was more extreme than that produced by simply reducing the number of early ENC cells (Zhang et al., 2018). We conclude that the ability of avian ENS-derived cells to form ENS declines rapidly due to quantitative reduction in undifferentiated ENC-like cells and also in qualitative changes in these cells.

2. Materials and methods

2.1. Ethics

Procedures were approved by the Royal Children's Hospital Animal Ethics Committee (A596 and A650) and the Institutional Animal Care and Use Committee of Semmelweis University.

2.2. Embryos

Fertilised White Leghorn and White Leghorn/Black Australorp cross chicken (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs were obtained from commercial sources. Eggs were stored at 14 °C and incubated at 38 °C in a 60% humidity incubator. Chick (Ch) and quail (Q) embryos were staged according to the number of embryonic days (E) and Hamburger and Hamilton stage (HH) (Ainsworth et al., 2010; Hamburger and Hamilton, 1951) and gut stages were referenced to the chick embryo gut staging table (Southwell, 2006).

2.3. Intestinal tissues

2.3.1. Intestinal tissues for ENC cell donors

Neural gut segments, that is segments already colonised by vagal-derived ENC cells, were obtained from quail midgut and hindgut at ages QE4 to QE10 (Suppl. Fig. 1). These segments were cut to a standard length of about 250 μm, and were used for ENC cell donors in organ culture experiments (see below). To test the colonisation ability of small numbers of ENC cells, very small fragments of QE3.5 (about 180 μm diam.) were obtained from the distal foregut/rostral midgut. Previous work showed these contain about 40 ENC cells (Zhang et al., 2018). We will term these 'tiny donors'.

2.3.2. Intestinal tissues for NC cell recipients

Aneural post-umbilical midgut, caeca and hindgut to act as recipients for ENC cell immigration in organ culture was dissected from E4.5 (HH25) chick embryos. At this stage, this gut segment is not yet invaded by vagally originating NC cells (Allan and Newgreen, 1980).

2.3.3. Cell dissociation and FACS for ENC cell donors

Intestinal tissues (midgut from bile duct to umbilicus) from QE5 to QE9 embryos were pooled (N = 30 to N = 10) and dissociated by digestion for 35 min at 37 °C in F12 media (Gibco Cell Culture, Invitrogen, USA) supplemented with 2 U/ml Dispase II (Roche, USA) and 0.05% *w/v* CLSAFA Collaganase (Worthington, USA), then EDTA (to 1 mM) was added for 10 min. The digested tissue was mechanically triturated and the cell suspension was washed in F12 media supplemented with 5% BSA. Intestinal cells were labelled in suspension with mouse anti-HNK-1 antibody (30 min on ice, 1/20 of supernatant), pelleted, washed and resuspended twice followed by secondary labelling with goat anti-mouse Ig Alexafluor 488 antibody (30 min on ice, 1/400). Antibody details are in Suppl. Table 1. Cells were filtered through a 30 μm strainer (BD-Falcon, USA) and dead cells were stained

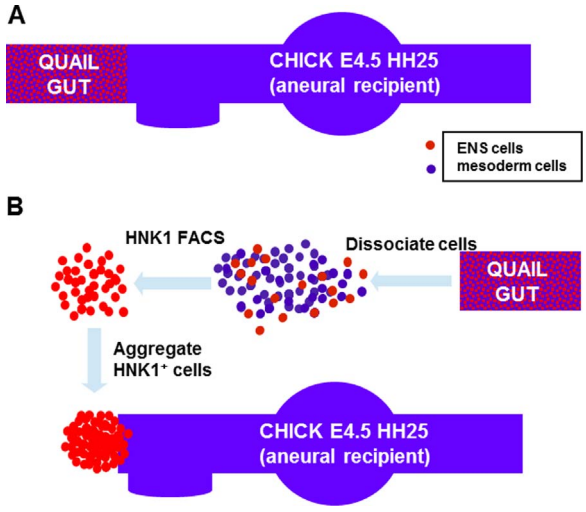

Fig. 1. Scheme for organ culture assays to test the ability of ENS cells to colonise aneural midgut/hindgut. ENS cell donors are either (**A**) segments of colonised quail gut, or (**B**) ENS cells isolated by HNK1-FACS.

with propidium iodide (Sigma-Aldrich, USA; 10 μg/ml). Alexa 488-positive cells that were negative for propidium iodide were sorted using a MoFlo cell sorter (MoFlo, USA) as detailed in Rollo et al. (2015). These cells were aggregated by brief centrifugation then cultured overnight before use in co-cultures (Fig. 1).

2.4. Organ Culture

2.4.1. Organ co-cultures in vitro and in chorio-allantoic membrane (CAM) grafts

Chick E4.5 recipient aneural post-umbilical midgut through hindgut tissues were mounted on a rectangle of Millipore filter paper (type HA black, 0.45 μm pore; Millipore Corp., MA, USA) with a V-shaped cut-out. The intestinal tissue was anchored at each end, but most of the intestine was suspended catenary-wise across the cut-out to prevent cell attachment and spreading and loss of tubular organ morphology (Hearn et al., 1999). The quail standard-length midgut and hindgut NC donor tissue (Fig. 1A) or the FACS-derived HNK1$^+$ aggregates (Fig. 1B) were placed in contact with the rostral end of the aneural chick intestine. These were maintained for 3 days in a 38 °C cell culture incubator in Ham's F12 buffered with 10 mM HEPES with 10% foetal bovine serum, 5% QE4 embryo extract and pen/strep as described (Simpson et al., 2007).

Additionally, the CAM of chick hosts of E7–8 were prepared as in Zhang et al. (2010) and the same combination grafts were placed on the CAM, the eggs were sealed with tape and returned to the egg incubator. Both *in vitro* and CAM organ culture methods allow ENC cell migration and differentiation of both ENC and gut cells. The difference between these two methods is that the *in vitro* duration was for only 3 days and the gut shows minimal growth (Hearn et al., 1999; Simpson et al., 2007), while most CAM grafts were for 8 days with gut growth (Zhang et al., 2010). For the tests with the tiny donor fragments, CAM grafts were for 8 days and 10 days. Grafts that were small or malformed or that lacked quail cells were discarded.

2.4.2. Fluorescence labelling and imaging of organ cultures

Gut organ culture wholemounts were fixed in 4% paraformaldehyde in PBS (1 h) then blocked and permeabilized (1% horse serum and 0.1% Triton X100 in PBS; > 1 h). The specimens were incubated overnight (*in vitro* organ cultures) or for 2 days (CAM grafts) at 4 °C sequentially in primary and secondary antibodies in blocking solution. Primary and secondary antibodies and treatments are given in Suppl. Table 1. Between treatments, the specimens were washed in blocking medium over a day. Labelled specimens were mounted in glycerol with 200 mM DABCO (Sigma-Aldrich) antifade reagent and sandwiched between two coverslips. Controls were performed by replacing first antibodies with rabbit IgG or normal mouse serum (Jackson 011-000-002 and 015-000-001 respectively) and by omitting first antibodies.

Wholemounts were analysed using an Olympus IX 70 microscope (Olympus Optical Co., Tokyo, Japan), with Texas Red, FITC and AMCA band pass filters and with a Leica TCS SP2 (Wetzlar, Germany) and Zeiss LSM 780 (Oberkochen, Germany) confocal microscopes. Image capture and processing used Leica and Zeiss proprietary software and Image-ProPlus. Myenteric area and percentage occupancy of this area by ENS cells in CAM graft wholemounts (donor QE6 N = 10, QE8 N = 10, QE10 N = 16) was calculated using Fiji software, based on the assumption that the gut in cross-section was circular.

2.5. Detection of cell differentiation

2.5.1. Cell dissociation and immunolabelling for differentiation markers

Midguts (from bile duct to umbilicus) at QE5 (N = 14), QE6 (N = 16), QE8 (N = 8) and QE10 (N = 4) were collected and imaged for length measurements (using Fiji software), then each age was pooled and dissociated as for FACS. Total cells were counted using a haemocytometer and then fixed in 4% paraformaldehyde (30 min), blocked and permeabilized as above, and multi-labelled overnight. For antibodies, refer to Suppl. Table 1. After 2 cycles of centrifuging and washing in blocking solution, cells were labelled for 2 h with isotype and species-specific second antibodies and, to identify SoxE, with streptavidin:AMCA (see Suppl. Table 1). Cells were then washed and plated onto glass coverslips and imaged with Olympus IX 70 microscope (Olympus Optical Co., Tokyo, Japan), with Texas Red, FITC and AMCA band-pass filters; cell counts (Fiji) were made from images. HNK1 antibody was assumed to identify all NC-derived cells, HuCD antibody identified neurons and B-FABP antibody identified glial cells. Subsequently the same samples were labelled with DAPI and the proportion of ENS cells (HNK1$^+$) to total cells (DAPI$^+$)was counted; this also checks for conformity with previous counts obtained by different methods (Rollo et al., 2015; Zhang et al., 2018).

2.5.2. Section immunolabelling for glial differentiation markers

Chick embryo guts were fixed as above and infiltrated with 15% sucrose/PBS overnight at 4 °C. The medium was changed to 7.5% gelatin (Sigma G-2500) containing 15% sucrose at 37 °C for 2 h, and the tissues frozen at −60 °C in isopentane (Sigma). Frozen sections (10 μm) were collected on polyL-lysine-coated slides (Sigma) for immunolabelling for 45 min. Antibodies are listed in Suppl. Table 1.

2.6. Statistics

Data were expressed as mean ± standard error of mean (SEM). All statistical tests were performed using GraphPad Prism version 6. For differences among multiple groups, statistical comparisons were performed using one-way analyses of variance (one-way ANOVA) followed with Tukey's multiple comparisons test. A P value of < 0.05 was considered significant.

3. Results

3.1. Does colonisation capacity of gut segment ENS donor tissue alter with its embryonic age?

To test the effect of increasing donor age on colonisation ability, co-cultures of ChE4.5 aneural post-umbilical midgut, caeca and hindgut with progressively older quail midgut ENC donor gut segments were prepared and maintained *in vitro* for 3 days. Neural pre-umblilical midgut segments from quail ENC donors at the initiation of colonisation (QE4; just after colonisation of the pre-umbilical midgut) had

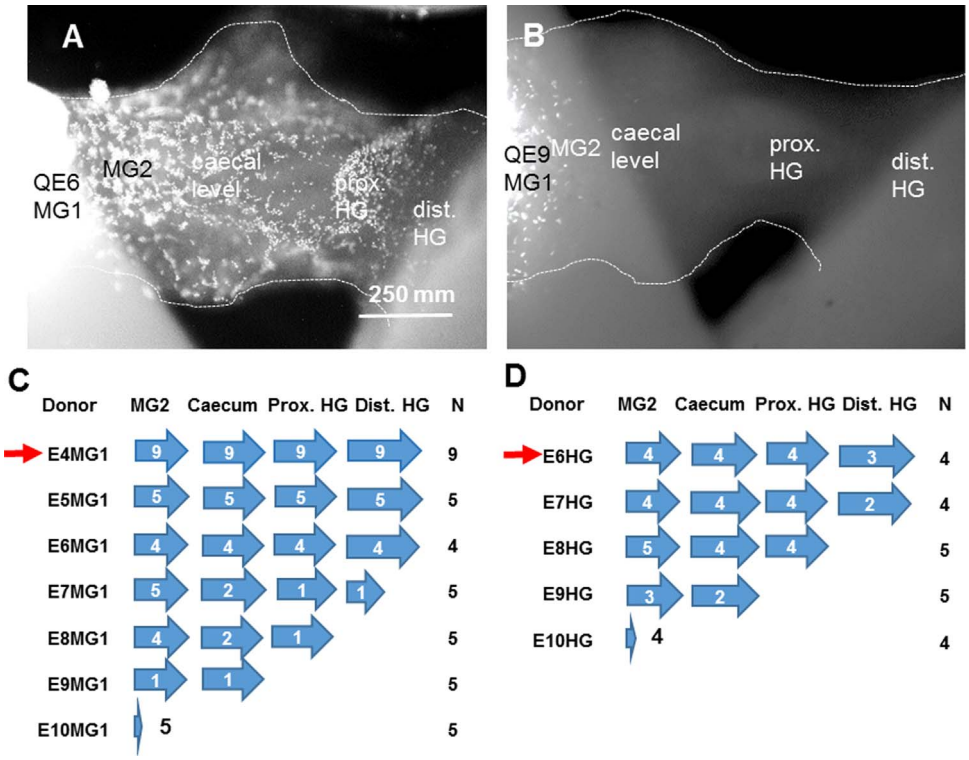

Fig. 2. Colonisation capacity of ENS cells from donor gut declines rapidly with donor age. A. QE6 quail embryo midgut donors strongly colonises recipient aneural chicken gut in organ culture *in vitro*. **B.** QE9 quail embryo midgut donors have restricted colonisation of recipient gut. **C, D.** Scheme of colonisation from midgut (MG) and hindgut (HG) donors of ages E4 to E10. Both MG and HG show strong colonisation ability at the wavefront zone (red arrow) and both lose colonisation capacity with age, but for the HG this loss is more rapid.

great ability to supply aneural chick mid and hindgut (ChE4.5) with chains of migrating ENC cells which were QCPN$^+$ and either SoxE$^+$ or HuCD$^+$ after 3 days co-culture *in vitro*. The same midgut region but slightly older (QE5 and 6; about 1 and 2 days after colonisation) possessed similar ability (Fig. 2A). A similar region from QE7 donors (ie. about 2.5 days after colonisation) had lower capacity; in only one of 5 grafts did ENC cells reach the hindgut in the 3 day period. Midgut segments from a QE8 and 9 donors (Fig. 2B) showed progressively less colonisation capacity in terms of length of gut occupied. Using QE10 ENC donors, only a few scattered quail cells were observed proximally in the recipient gut. These results are shown schematically in Fig. 2C and an overview of all procedures is supplied in Suppl. Table 2.

We conclude that colonisation capacity of midgut segment ENS donor tissue declines with donor age, and/or with distance behind the ENC cell wavefront.

3.2. Is the age-related decline in colonisation capacity related to the region of the gut?

The ENC cell donor hindgut segment at or near the wavefront was obtained from QE6 embryos; these strongly colonised aneural midgut plus hindgut, similar to that of midgut wavefront ENC cells. This capacity declined with older hindgut ENC cell donors until QE10, as with the older midgut donors. However, the hindgut ENC donors at intermediate chronological ages of E7–9 achieved caecal-level colonisation, and so were superior to E7–9 midgut ENC donors. These results, shown schematically in Fig. 2D, suggest that the ability to rapidly colonise recipient aneural midgut and hindgut is present in the donor ENS cell population of both midgut and hindgut levels at the time of first colonisation (*i.e.* at the wavefront position, chronologically later at hindgut level than midgut) and that this ability persists in each region for a time limited to several days after the wavefront has passed. Later, this colonisation capacity declines (*i.e.* behind or rostral to the wavefront), and this decline seems more rapid from donors of later-colonised levels, that is, when the ENS donor is of hindgut origin, compared to donors of midgut origin.

3.3. Is the age-related decline in colonisation capacity of ENC cells due to changes in the ENS cells or in their local tissue?

The above ENS donors included gut mesoderm so it is possible that age-related effects of this tissue retard donor ENC cell egress. Therefore midgut ENS cells were isolated by FACS for HNK1 and aggregated. In *in vitro* assays these showed colonisation capacities similar to those of the cells delivered within entire midgut segments: cells of QE5 origin showed high colonising ability but this progressively declined with aggregates from older donors (Fig. 3).

We conclude that the age-dependent decline in colonisation ability is inherent in the ENS cell population.

3.4. Does age-related decline in colonisation capacity reflect a 'slow start' that can be corrected by increasing the assay duration?

The *in vitro* organ cultures were of only 3 days duration with a

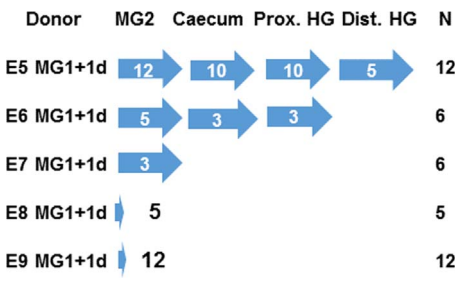

Fig. 3. Colonisation capacity of isolated ENS cells declines rapidly with donor age. ENS cells were obtained from midgut by FACS using HNK1 antibody, aggregated for a day and placed in contact with recipient aneural gut.

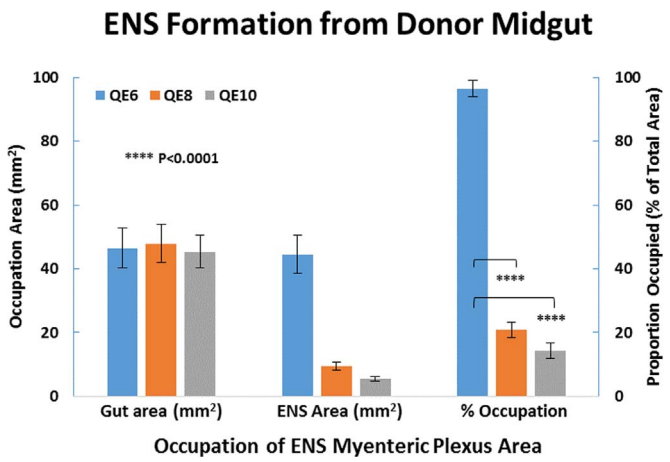

Fig. 4. ENS colonisation area decreases with ENS donor age. Histograms of average total myenteric area of CAM grafted intestines potentially available for colonisation by ENS cells from quail midgut donors of E6 (N = 10), E8 (N = 10) and E10 (N = 16) days age (left), average area actually colonised (middle) and percentage of total that was colonised (right). Error bars: SEM.

non-growing gut, so we grew similar cultures on the CAM for 8 days to test whether, with more time, there might be 'catch-up' ENS colonisation. These grafts also allow cell differentiation and ENS ganglion morphogenesis to be examined.

Pre-umbilical midgut from QE5 (N = 7) and QE6 (N = 10) donors completely colonised recipient aneural chick gut with ENS except for a short cloacal region (Suppl. Fig. 2A), as previously described (Zhang et al., 2018). In these grafts over 96% of the potential myenteric plexus area was colonised (Fig. 4). Quail (QCPN$^+$) cells formed myenteric and submucosal plexuses with SoxE$^-$/HuCD$^+$ neurons and SoxE$^+$/HuCD$^-$ support cells in a ratio of about 1:1 (Fig. 5A).

QE8 donor pre-umbilical midgut (N = 10) supplied QCPN$^+$ cells which after 8 days formed a network in a layer corresponding to the myenteric plexus in recipient CAM-grafted gut, but this was restricted to the proximal part of the recipient midgut adjacent to the donor tissue (Suppl. Fig. 2B). Donor cells never occurred in the distal part of the recipient post-umbilical midgut, in the caeca or in the hindgut. About 20% of the potential plexus area contained ENS cells (Fig. 4) but SoxE$^+$ cells in this area were relatively sparse and deficient in HuCD$^+$ neurons, which formed smaller, looser groups (Fig. 5B), compared to those produced by QE5 and QE6 donors. QE10 pre-umbilical midgut (N = 16) donor cells were even more restricted in distribution, typically to the proximal quarter of the recipient midgut (Suppl. Fig. 2C). On average, about 8% of the potential plexus area contained ENS cells (Fig. 4). Moreover these cells, which were mostly QCPN$^+$/SoxE$^+$, were even fewer and sparser than those from QE8 donors and did not form typical ganglionated ENS networks (Fig. 5C).

We conclude that midgut ENS cells of QE6 or younger have great ENS colonisation, ganglion morphogenesis and differentiation ability, but that ENS cells of QE8 or older provenance have limited ENS formation ability, even when extra 'catch-up' time is allowed.

3.5. Does age-related decline in colonisation capacity reflect a reduction in NC-derived cells lacking differentiation markers?

We dissociated pooled quail pre-umbilical midgut at QE5 (N = 14), QE6 (N = 16), QE8 (N = 8) and QE10 (N = 4) and labelled with antibodies to HNK1 (for all NC-derived cells), HuCD (for neurons) and B-FABP (for glia). With developmental age, the length of the midgut and the total number of gut cells increased dramatically. The number of ENS cells (HNK1$^+$) increased from QE5 to QE10 and the proportion of ENS cells rose from QE5 to QE8 then declined slightly by QE10 (Suppl. Fig. 3A, B).

The number and proportion of ENS cells that were neurons (HuCD$^+$) increased over the period QE5 to QE10 (Fig. 6A). These trends closely match those from previous *in situ* counts (Rollo et al., 2015). The remainder of the HNK1$^+$ cells were SoxE$^+$, which identifies both progenitor ENC cells and ENS glial cells. As in mice (Young et al., 2003) an early marker for avian ENS glial cells is B-FABP (Nagy et al., 2012). Therefore B-FABP antibody was used to detect how many of the HNK1$^+$ ENS cells displayed the glial marker B-FABP, or were neither HuCD$^+$ nor B-FABP$^+$. B-FABP$^+$ cells increased in number and proportion of ENS cells (Fig. 6A). The proportion of HNK1$^+$ cells that *did not* express HuCD or B-FABP fell from almost 40% at QE5 to 12% at QE10 although the number of such cells increased in the pre-umbilical midgut (Fig. 6B).

In addition, sections of chicken embryo gut were co-labelled for Sox10 and B-FABP (Fig. 7A, B). At ChE8, overall about 20% of the Sox10$^+$ cells (400 cells counted) in the hindgut did not express the glial marker B-FABP (Suppl. Table 3), in good agreement with the counts from dissociated cells. The sections also revealed spatial differences that were undetectable in dissociated cells. There was a marked difference between the myenteric plexus (over 20% of Sox10$^+$ cells were B-FABP$^-$) and the sub-mucosal plexus (about 7% of Sox10$^+$ cells were B-FABP$^-$), and 5 of the 12 Sox10+ cells outside the plexuses were B-FABP$^-$. In addition, in the myenteric plexus in the dorsal (mesenteric) quadrant close to 40% of Sox10$^+$ cells lacked B-FABP (Fig. 7C) whereas in the ventral quadrant, only about 8% of Sox10+ cells in the myenteric plexus cells lacked B-FABP (Suppl. Table 3).

We conclude that the proportion of ENS cells that lack both neuronal and glial differentiation markers decreases with time, but at least until QE10 at least 10% of all ENS cells lack detectable

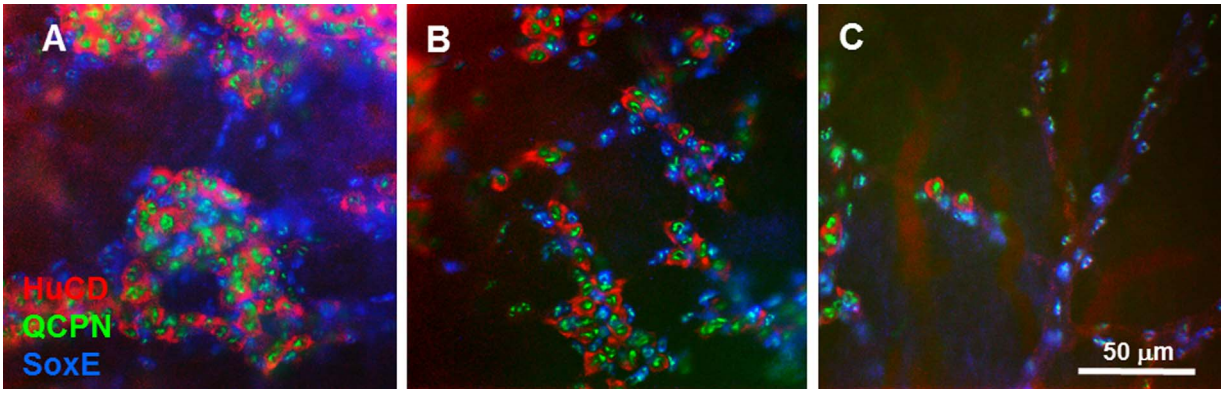

Fig. 5. Donor quail ENS cells show donor age-dependent decrease in ENS formation in chick embryo midgut in 8 day CAM grafts. A. Quail ENC cells (QCPN: green) derived from a QE6 midgut donor provide a dense ENS plexus in the myenteric layer with ganglia of neurons (HuCD: red) and support cells (SoxE: blue). **B.** Quail ENC cells derived from a QE8 midgut donor gives a sparsely ganglionated ENS in the myenteric plexus. **C.** Quail ENC cells derived from a QE10 midgut donor form strands with few ganglia, and with few neurons in and near the myenteric plexus layer.

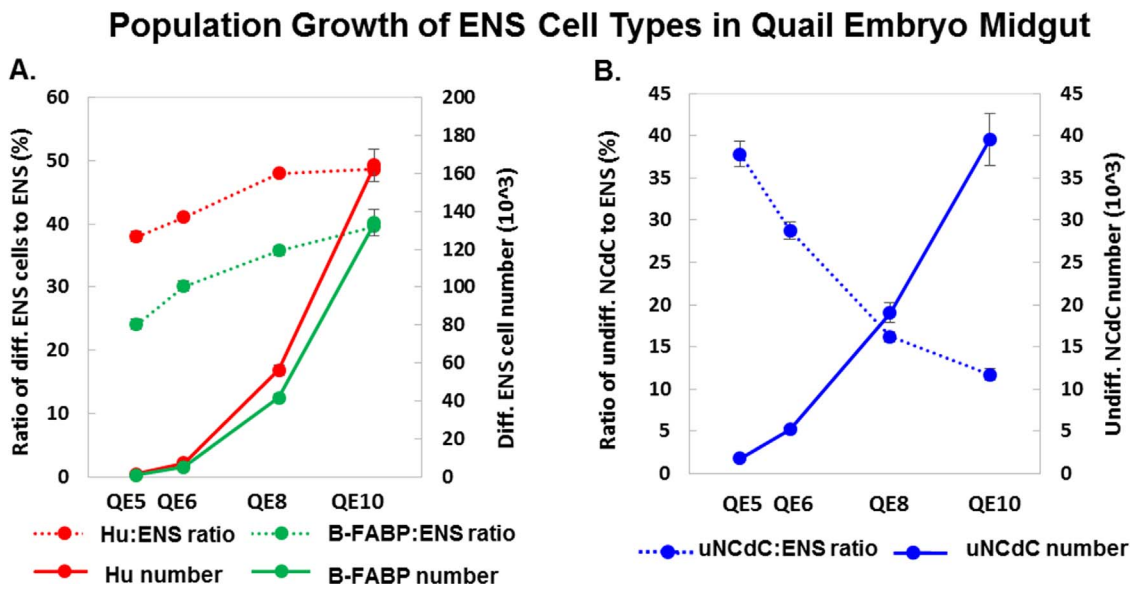

Fig. 6. Progressive differentiation of ENS cells is shown in counts from dissociated and immunolabelled quail embryo pre-umbilical midguts of ages QE5 to QE10. A. The number of differentiated ENS cells (neurons: HuCD$^+$ and glia: B-FABP$^+$) rises with age as does their proportion relative to total ENS cells (HNK1$^+$). B. The number of apparently undifferentiated NC-derived cells (uNCdC: HuCD$^-$/ B-FABP$^-$/HNK1$^+$) rises with age but their proportion declines. Error bars: SEM.

differentiation markers. The lower proportion of Sox10$^+$ ENS cells showing the glial differentiation marker in the myenteric plexus of the hindgut compared to the sub-mucosal plexus parallels the more rapid development in general of the latter plexus in the avian hindgut (Conner et al., 2003).

3.6. Does reduction in number of colonisation-competent ENC cells lead to a decline in colonisation extent?

We therefore asked if reducing the number (as opposed to the proportion) of apparently undifferentiated ENC cells would mimic the effect of older donors, that is, would the spatial extent of occupation be reduced. We therefore used tiny donor fragments from young embryos (QE3.5). Previously we showed these contained about 40 SoxE$^+$ ENC cells (Zhang et al., 2018). Unlike the result with older (QE8, QE10) ENS donors (Suppl. Fig. 2B, C), the ENS in these grafts was not restricted to the rostral midgut. In these tiny donor grafts (N = 12) the ENS usually (10/12) extended throughout the midgut including both caeca and into the distal hindgut after 8 days on the CAM. In one graft the ENS only extended through about half the midgut and in another the hindgut and one caecum were not colonised. Relative to larger midgut standard donors (QE6 and younger), the ENS in the grafted gut was sparser especially in the hindgut where there were frequently fewer HuCD$^+$ neurons relative to SoxE$^+$ cells (Suppl. Fig. 4A, B); this resembled the normal chain pattern of younger stages of ENS formation. To test whether this could be rectified by increasing the graft duration, we performed similar CAM grafts with time extended to 10 days (N = 12). Ten of the grafts were fully colonised and in 2 the midgut was colonised but the hindgut was not colonised. In these two extra days the ENS attained a mature ganglionated appearance even distally (Suppl Fig. 4C, D).

We conclude that the restriction of colonisation of an aneural gut field by older ENS cell donors is not simply due to too few ENC cells lacking differentiation markers.

4. Discussion

4.1. ENS cells undergo a developmental reduction in ability to colonise aneural gut

The colonisation of avian aneural intestine by ENS-derived cells is chiefly by undifferentiated highly proliferative ENC cells at the wavefront (Simpson et al., 2007). Results by Meijers et al. (1992) and in the present more detailed experiments show that the ability to rapidly colonise an aneural intestinal field by ENC cells decreased with donor age. Here, this decline commenced about 2–3 days followed the transit of the ENC cell wavefront and was minimal after another 2–3 days. The timing of this decline in colonisation efficiency in

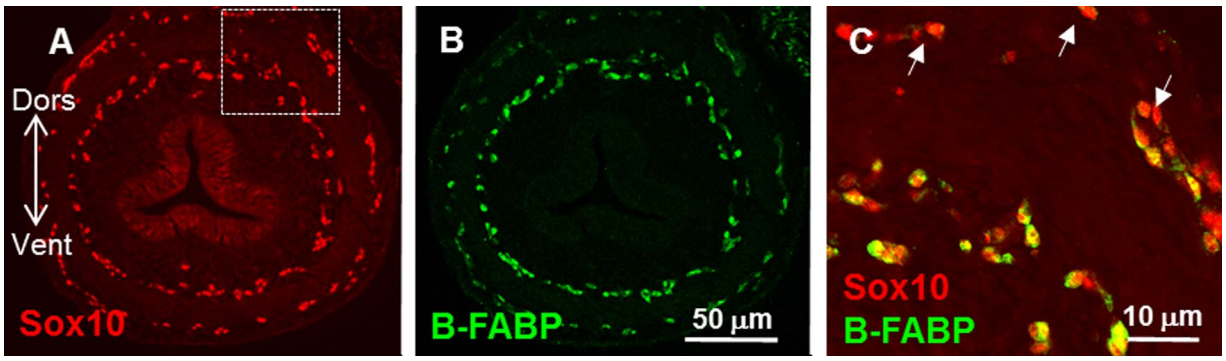

Fig. 7. Some Sox10$^+$ ENS cells lack expression of the glial marker B-FABP. Section from ChE8 hindgut. **A.** Sox10$^+$ cells in the myenteric (outer) plexus and submucosal (inner) plexus. **B.** B-FABP$^+$ cells in the same section. **C.** Boxed area in dorsal quadrant in A showing some Sox10$^+$ cells have undetectable B-FABP labelling (arrows), especially in the dorsal part of the myenteric plexus.

experiments paralleled the stage *in vivo* where the increase in ENS cell numbers falls behind that of the gut mesoderm cells (Suppl. Fig. 3B).

This population-level decline occurred more rapidly in the hindgut than in the midgut, suggesting that as avian ENC cells approached completion of colonisation they - as a population – have already had a decline in reserve capacity for further colonisation, as previously suggested (Allan and Newgreen, 1980). A similar age-dependent decline in central nervous system regeneration capacity has been recorded in the spinal cord of avian embryos (Ferretti and Whalley, 2008).

4.2. Reduction of ENS-colonisation ability reflects changes in the ENC-derived cell population

The decline in colonisation ability might be due to age-changes in the NC population itself, but since ENS cells are surrounded by gut mesoderm, this decline might be also mediated indirectly by the surrounding older gut mesodermal tissues acting, for example, to prevent the movement of ENC cells out of the donor tissue. This is consistent with reports of older mouse gut mesoderm becoming less supportive of ENC cell migration (Druckenbrod and Epstein, 2009; Hotta et al., 2010). In contrast, in the avian system young ENC cells were tested for ability to colonise progressively older and more differentiated recipient gut mesoderm. The results showed that mature mesoderm still permitted colonisation by young ENC cells (Meijers et al., 1987). Likewise, grafts of ENS cells to post-natal mouse colon showed extensive spreading of the ENS cells (Hotta et al., 2013). These suggest that older mesoderm can support colonisation, at least to some degree, by ENC cells.

To explore this directly, we isolated avian ENS cells from gut mesoderm at progressively older ages by HNK1-FACS and tested their ability to colonise aneural gut mesoderm in 3 day organ cultures. We conclude that the midgut ENS donors have an age-dependent reduction of colonisation ability, and that this change occurs in the ENS cell population itself.

4.3. Reduction of ENS-colonisation ability parallels a decrease in the proportion of ENS cells lacking differentiation markers

HuCD antibody labels enteric neurons but SoxE (and Sox10) antibody labels not only undifferentiated ENC cells but also differentiated enteric glial cells. In the chick ENS, differentiation of glial cells can be detected by antibodies to B-FABP (Nagy et al., 2012) and GFAP (Conner et al., 2003).

We investigated the differentiation status in greater temporal detail using dissociated quail gut tissues and sections of chicken gut. Our counts of marker expression for NC cells neurons and glia confirmed that the decline of colonisation ability coincided with reduction of proportion of apparently undifferentiated ENS cells (*i.e.* in dissociated cells: $HNK1^+/HuCD^-$ cells that were $B\text{-}FABP^-$ and in sections: $Sox10^+$ cells that were $B\text{-}FABP^-$).

4.4. Does reduction of ENC cell numbers contribute to loss of colonisation capacity?

With age the midgut length and total cell number increase (Suppl. Fig. 3A), and the number of ENS cells in the midgut also increases (Suppl. Fig. 3B) although the proportion of ENS cells lacking differentiation marker expression decreases. We can therefore calculate that there is an *increase* in the number of apparently undifferentiated ENS cells in standard donor midgut segments (about 250 μm long) with age, from about 250 cells in QE5 donors to triple this number in QE8 and QE10 donors (Suppl. Fig. 3C). Superficially this is not consistent with the loss of colonisation capacity reflecting only a numerical decline of apparently undifferentiated NC-derived cells.

Furthermore, if the age-dependent reduction of colonisation capacity is simply due to a low number of fully colonisation-competent undifferentiated ENC cells (ie. equivalent to young ENC cells) in older ENS at the start, then two predictions can be made. Firstly the reduced colonisation when using older donors should be rescued by allowing more time for the residual ENC cells to build up numbers by proliferation and hence drive migration more distally, and secondly the pattern of age-related restriction of colonisation should be reproduced by using ENC cells from young donors, but in low numbers. To test the first prediction, the co-culture period was extended from 3 days *in vitro* to 8 days on the CAM, which also allowed differentiation to be observed. In this case the QE8 donors and more drastically the QE10 donors produced a network in a position expected of the ENS but this was still strongly restricted spatially. In addition, the ENS cells were sparser, ganglionation was impaired and neuronal differentiation was low, similar to immature ENS.

To test the second prediction, we reduced the number of ENC cells from young donors to about 40 cells (see also Zhang et al. (2018). In this case the colonisation extent of the ENS in 8 day CAM grafts was in most cases not spatially truncated as it was with older donors; the resultant ENS extended throughout the midgut, caeca and into the hindgut. However, the ENS cell density, ganglionic morphogenesis and degree of differentiation, especially in the hindgut, was less than that achieved with larger starting numbers of young ENS cells (see also Zhang et al., 2018). However this shortfall was rectified simply by allowing 2 days more time for differentiation. Thus neither of these predictions was borne out.

5. Conclusions

We conclude that there is an age-related decrease in the vagal NC-derived ENS cell population's ability to form extensive new ENS, and this decrease is autonomous to this cell population. This parallels a reduction in the proportion of the ENS population of cells without glial or neuronal differentiation markers. However, this reduction also has age-related qualitative traits since ENS cells lacking differentiation markers from younger gut donors outperform larger numbers of apparently similar cells of older ENS origin.

5.1. Implications for progenitor cell therapy

ENS neuropathies are common causes of intestinal dysfunction and a potential cell therapeutic option is to generate and administer enteric neural progenitors to replace missing ENS cells in total (eg. in Hirschsprung disease), or to replace a missing or dysfunctional subtype (eg. in achalasia and gastroparesis) (Burns et al., 2016).

Cell obtained from patients would avoid problems of immune-rejection (Stamp, 2017), and cells derived directly from the patients' ENS would possess appropriate lineage-specific programming. Since enteric neuropathies are diagnosed post-natally, these cells would have to be obtained post-natally. Neural lineage cells obtained directly from post-natal mouse and human gut can form new ENS cells in post-natal animal gut *in vivo* and in post-natal human gut tissue in organ culture (Cheng et al., 2017; Cooper et al., 2016; Hetz et al., 2014; Hotta et al., 2013; Rollo et al., 2016). These are assumed to derive from post-natal ENS stem/progenitor cells. However, the studies described here with avian tissues suggest that ENS cells undergo a developmental decline in ability to form ENS even in permissive young aneural gut tissue. Likewise, clonal comparison of potential ENS stem/progenitor cells from rat gut suggests these cells undergo a decline from foetal to post-natal stages in proliferative potential, in self-renewal and in multi-lineage differentiation capacity (Kruger et al., 2002).

Given that post-natal ENS stem/progenitor cells have limitations in ENS-forming efficiency, we conclude that patient-derived cells reverted to an embryonic ENS cell-like stage may provide a more favourable proliferative and differentiative capacity for ENS cells for therapeutic applications. These would be obtained eg. *via* patient iPSCs induced to form enteric NC cells (Fattahi et al., 2016).

However, three different NC sources contribute to the ENS (see Introduction) and their properties are not identical (Burns et al., 2000; Hearn and Newgreen, 2000; Newgreen et al., 1980; Wang et al., 2011). Vagal cells make the largest contribution in terms of fate (Burns and Le Douarin, 1998) and have greatest ENS-forming capacity (Delalande et al., 2008; Newgreen et al., 1980; Zhang et al., 2010) and are clearly indispensable. The size and nature of the contributions of the sacral and Schwann cell precursor sources in the human ENS are not known, and nor is it known whether NC-derived cells of the three types are all required for normal or at least acceptable gut function. Facsimiles of these three related but distinct NC-derived cell lineages requires different inductive protocols. It is not known at present whether all three types would need to be induced separately *ex vivo* and combined to reconstitute a functional ENS, or whether the gut microenvironment would resolve this problem by diversifying one basal, presumably vagal, enteric NC population.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.11.017.

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