Developmental Biology 385 (2014) 220–229

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Vascularisation is not necessary for gut colonisation by enteric neural crest cells ☆

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ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form
4 November 2013
Accepted 8 November 2013
Available online 18 November 2013

Keywords:
Enteric nervous system
Vascular system
Neural crest cells
Blood vessels
Migration

ABSTRACT

The vasculature and nervous system share striking similarities in their networked, tree-like architecture and in the way they are super-imposed in mature organs. It has previously been suggested that the intestinal microvasculature network directs the migration of enteric neural crest cells (ENCC) along the gut to promote the formation of the enteric nervous system (ENS). To investigate the inter-relationship of migrating ENCC, ENS formation and gut vascular development we combined fate-mapping of ENCC with immunolabelling and intravascular dye injection to visualise nascent blood vessel networks. We found that the enteric and vascular networks initially had very distinct patterns of development. In the foregut, ENCC migrated through areas devoid of established vascular networks. In vessel-rich areas, such as the midgut and hindgut, the distribution of migrating ENCC did not support the idea that these cells followed a pre-established vascular network. Moreover, when gut vascular development was impaired, either genetically in *Vegfa*$^{120/120}$ or *Tie2-Cre;Nrp1*$^{fl/-}$ mice or using an *in vitro Wnt1-Cre;Rosa26*$^{Yfp/+}$ mouse model of ENS development, ENCC still colonised the entire length of the gut, including the terminal hindgut. These results demonstrate that blood vessel networks are not necessary to guide migrating ENCC during ENS development. Conversely, in *miRet*^{51} mice, which lack ENS in the hindgut, the vascular network in this region appeared to be normal suggesting that in early development both networks form independently of each other.

Introduction

During vertebrate development, organs and tissues must connect to the blood vascular system to receive fluids, nutrients and oxygen, and to the nervous system to receive and send sensory, autonomic or functional information. Consequently, both mature networks share obvious similarities at the anatomical level, and they also use similar cellular and molecular mechanisms to orchestrate their parallel developmental programs (Bates et al., 2003; Eichmann et al., 2005; Eichmann and Thomas, 2013; Tam and Watts, 2010). Recent evidence suggests that the two networks also influence each other's development through direct molecular interactions. For example, neuronal progenitors and neurons secrete the vascular growth factor VEGF to stimulate or pattern their vascular supply (Haigh et al., 2003; Mukouyama et al., 2002; Raab et al., 2004; Rosenstein et al., 2010; Ruhrberg, 2003), and vessels, comprised of endothelial cells (EC) and mural cells, release neurotrophic factors such as artemin and neurotrophin-3 to attract nerve fibres (Honma et al., 2002; Kuruvilla et al., 2004; Weinstein, 2005). Additionally, the alpha-chemokine receptor CXCR4 and its ligand SDF1, which are essential for gastrointestinal tract vascularisation (Tachibana et al., 1998), also promote the migration of cranial neural crest cells (NCC) (Rezzoug et al., 2011; Theveneau et al., 2010).

Although the nervous system of the gut contains extrinsic components, its main functional unit is the intrinsic enteric nervous system (ENS). The ENS consists of networks of interconnected ganglia embedded in the wall of the digestive tract and is the largest division of the peripheral nervous system (Furness, 2012; Gershon, 1999). The ENS is derived from NCC, which delaminate and migrate extensively to colonise the entire length of the gut (Goldstein et al., 2013; Laranjeira and Pachnis, 2009; Obermayr et al., 2013; Sasselli et al., 2012). Concomitant with the

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http://dx.doi.org/10.1016/j.ydbio.2013.11.007

migration of ENCC within the gut is the development of the gut vascular system, which has not been as extensively investigated. Studies using Tg(tie1:H2B-eYFP) quail embryos (Sato et al., 2010), in which EC express YFP, revealed the presence of scattered EC within the gut mesenchyme as early as embryonic day (E)3 (Thomason et al., 2012). These cells become organised into a honeycomb pattern by E6, and by E10 are reshaped into a branching vascular pattern that extends from the mesentery. Additionally, studies in mice have shown that cells from the serosal mesothelium undergo EMT to join the blood vessels, where they give rise to the mural cells (Wilm et al., 2005). Eventually, the digestive tract receives blood supply from three branches of the abdominal aorta: the coeliac, superior and inferior mesenteric arties that supply the foregut, midgut and hindgut respectively (Geboes et al., 2001; Tachibana et al., 1998). In the functional adult gut, recent studies have revealed close anatomical associations between the ENS and capillaries that are specific to the villi, the crypts and the myenteric plexus (Fu et al., 2013). One possibility is that this co-patterning in the mature gut arises from co-dependent interactions during early development. Indeed, recent studies in quail suggested that the pre-established hindgut microvasculature serves a critical role in promoting and directing ENCC migration along the hindgut (Nagy et al., 2009). Moreover, the intestinal vascular mesentery has been proposed to serve as an important migratory route for ENCC to colonise the terminal hindgut in mice (Nishiyama et al., 2012). Thus the aim of this study was to investigate the potential inter-relationship between the developing ENS and the gut vasculature.

We used chick^{GFP}–chick intraspecies grafting to permanently label and fate-map vagal ENCC with GFP, combined with intravascular injection of the lipophilic dye DiI to stain the nascent blood vessel network. Because DiI highlighted only patent blood vessels, we also labelled quail embryos with QH1 and HNK1 antibodies to mark endothelial and neural crest-derived networks, respectively. The analysis of wholemount preparations by high magnification confocal imaging showed little correlation between migrating NCC and blood vessel development. Consistent with these observations we showed, using mouse models defective in vascular development, and an *in vitro* assay, that ENCC can colonise the entire length of the gut independently of the vascular network.

Materials and methods

Chick^{GFP}–chick intraspecies tissue grafting and blood vessel labelling with DiI injection

Fertile chicken eggs, obtained from commercial sources, and transgenic GFP chicken eggs, obtained from The Roslin Institute, The University of Edinburgh (McGrew et al., 2004), were incubated at 37 °C and staged according to the embryonic day of development (E), and by using the developmental tables of Hamburger and Hamilton (1951). For chick^{GFP}–chick grafting, the neural tube and associated neural crest, adjacent to somites 2–6 inclusive, was microsurgically removed from normal chick embryos at embryonic day E1.5 and replaced with equivalent stage-matched tissue obtained from chick^{GFP} embryos (Fig. 1), as previously described (Burns and Le Douarin, 1998; Freem et al., 2012) Following grafting, eggs were returned to the incubator, and embryos allowed to develop up to a further 10 days such that GFP+NCC colonised the gut and lungs. For DiI injection in chick embryos at stages E4.5–E11.5, CellTracker™ CM-DiI (Molecular Probes®) was injected into the peripheral vascular network using a fine glass needle connected to a mouth pipette (Fig. 1). The dye was injected slowly and allowed to diffuse within the blood flow for two to three minutes to ensure staining of the entire lumenised blood vessel network to its finest capillaries. The labelled embryos were harvested and fixed in 4% paraformaldehyde (PFA) shortly after DiI injection and mounted under a bridged coverslip using Vectashield mounting medium (Vector Laboratories).

Mouse strains

To obtain mouse embryos of defined gestational ages, mice were mated in the evening, and the morning of vaginal plug formation was counted as 0.5 days post coitum (dpc). Mice carrying the *Vegfa^{120}* mutation (MGI:1931047) (Carmeliet et al., 1999) have been described (Ruhrberg et al., 2002). Conditional null mutants for *Nrp1* (*Nrp1$^{fl/fl}$*) (MGI:3512103) (Gu et al., 2003) were mated to mice expressing Cre recombinase under the control of the endothelial-specific *Tie2* promoter (MGI:2450311) (Kisanuki et al., 2001). Mice carrying a conditional *Yfp* allele in the Rosa26 locus (MGI:2449038) (Srinivas et al., 2001) were mated to mice expressing Cre recombinase under the control of the Wnt1 promoter/enhancer (MGI:2386570) (Danielian et al., 1998). Mice carrying the *miRet^{51}* mutation (MGI:2387446) have been described (de Graaff et al., 2001). Genotyping protocols can be supplied on request. Mouse husbandry was performed in accordance with UK Home Office and Institutional guidelines.

Culture of foetal mouse intestine

Foetal mouse intestines were cultured *in vitro* as previously described (Natarajan et al., 1999). Briefly, transgenic *Wnt1-Cre; R26R$^{yfp/+}$* intestines with the mesentery attached were dissected from E11.5 embryos and placed in culture with OptiMEM (Invitrogen, UK) supplemented with 1 mM L-Glutamine (Invitrogen, UK) and 1 mM Penicillin/Streptomycin antibiotic mixture (Invitrogen, UK). Gastrointestinal tracts were cultured up to 4 days then fixed in 4% PFA for 1 h and processed for immunofluorescence labelling as described below.

Immunofluorescence

For wholemount immunofluorescence labelling of quail (obtained from commercial sources) and mouse tissues, dissected gastrointestinal tracts were fixed for 1–2 h in 4% PFA in PBS then rinsed 3 times in PBS at room temperature and processed as previously described (Freem et al., 2010). Briefly, antibody blocking solution (10% sheep serum, 1% Triton-X-100 in PBS) was applied for 1 h at room temperature then samples were rinsed extensively in PBS and incubated in endomucin (Santa Cruz) and TuJ1 (Covance) primary antibodies diluted in antibody blocking solution overnight at 4 °C. Samples were then washed three times in PBS for 20 min and incubated with fluorescently tagged secondary antibodies (anti-rat Alexa568 and anti-mouse Alexa488, respectively) for 4 h at room temperature. Samples were washed for 1 h, and stained for 10 min with DAPI, before being mounted under a coverslip using Vectashield mounting medium (Vector Laboratories). Images were acquired with a Zeiss Axioskop fluorescent microscope or a Zeiss LSM 710 confocal microscope. For counting ENS cells in mouse tissues, sequential z-stack images were analysed, and TuJ1/DAPI+ cells counted using the Image-J Fiji cell counter plugin. Once imaged using confocal microscopy, the same samples were un-mounted, then cryoprotected in 15% sucrose in PBS, and put in gelatin blocks as previously described (Barlow et al., 2008). Frozen sections were cut at 12 μm using a Leica CM1900 cryostat at −22 °C. In the case of the foetal mouse intestine explants, the sections we re-immunostained using PECAM/CD31 (BD Pharmingen™). Imaging was carried out on a Zeiss Axioskop fluorescent microscope as above.