Developmental Biology 416 (2016) 255–265

Contents lists available at ScienceDirect

Developmental Biology

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

Genomes and Developmental Control

# Regulators of gene expression in Enteric Neural Crest Cells are putative Hirschsprung disease genes

Duco Schriemer [a], Yunia Sribudiani [b,c], Arne IJpma [d], Dipa Natarajan [e], Katherine C. MacKenzie [b], Marco Metzger [e,f], Ellen Binder [e], Alan J. Burns [b,e], Nikhil Thapar [e,g], Robert M.W. Hofstra [b,e], Bart J.L. Eggen [a,*]

[a] Department of Neuroscience, section Medical Physiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[b] Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands
[c] Department Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia
[d] Department of Bioinformatics, Erasmus University Medical Center, Rotterdam, The Netherlands
[e] Stem Cells and Regenerative Medicine, Birth Defects Research Centre, UCL Great Ormond Street Institute of Child Health, London, United Kingdom
[f] Fraunhofer IGB, Wuerzburg branch and Chair Tissue Engineering and Regenerative Medicine, University Hospital Wuerzburg, Wuerzburg, Germany
[g] Division of Neurogastroenterology and Motility, Department of Gastroenterology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom

ARTICLE INFO

*Article history:*
Received 30 September 2015
Received in revised form
17 May 2016
Accepted 2 June 2016
Available online 4 June 2016

*Keywords:*
Hirschsprung disease
Enteric nervous system
Transcriptome
Upstream Regulators

ABSTRACT

The enteric nervous system (ENS) is required for peristalsis of the gut and is derived from Enteric Neural Crest Cells (ENCCs). During ENS development, the RET receptor tyrosine kinase plays a critical role in the proliferation and survival of ENCCs, their migration along the developing gut, and differentiation into enteric neurons. Mutations in *RET* and its ligand *GDNF* cause Hirschsprung disease (HSCR), a complex genetic disorder in which ENCCs fail to colonize variable lengths of the distal bowel. To identify key regulators of ENCCs and the pathways underlying RET signaling, gene expression profiles of untreated and GDNF-treated ENCCs from E14.5 mouse embryos were generated. ENCCs express genes that are involved in both early and late neuronal development, whereas GDNF treatment induced neuronal maturation. Predicted regulators of gene expression in ENCCs include the known HSCR genes *Ret* and *Sox10*, as well as *Bdnf*, *App* and *Mapk10*. The regulatory overlap and functional interactions between these genes were used to construct a regulatory network that is underlying ENS development and connects to known HSCR genes. In addition, the adenosine receptor A2a (*Adora2a*) and neuropeptide Y receptor Y2 (*Npy2r*) were identified as possible regulators of terminal neuronal differentiation in GDNF-treated ENCCs. The human orthologue of *Npy2r* maps to the HSCR susceptibility locus 4q31.3-q32.3, suggesting a role for NPY2R both in ENS development and in HSCR.



## 1. Introduction

The enteric nervous system (ENS) of the gastrointestinal tract is composed of neurons and glial cells that are organized in interconnected ganglia in the myenteric and submucosal plexuses. The ENS controls the peristaltic movements of the bowel, fluid exchange between the gut and its lumen, and local blood flow (Furness, 2006; Gershon, 2005). In vertebrates, the ENS is entirely derived from neural crest cells (Le Douarin and Kalcheim, 1999). Vagal (hindbrain) neural crest cells invade the foregut at around E9 in mice (Durbec et al., 1996b), at which point they are referred to as Enteric Neural Crest Cells (ENCCs). The intestinal wall is colonized by ENCCs in a rostral to caudal direction between E9 and E15 in mice and sacral neural crest cells contribute to colonization of the hindgut late during ENS development (Burns and Douarin, 1998; Druckenbrod and Epstein, 2005). A subset of Schwann cell precursors invades the gut postnatally and contributes up to 20% of the neurons in the colon (Uesaka et al., 2015).

Glial cell line Derived Neurotrophic Factor (GDNF) acts as a chemoattractant to migrating vagal ENCCs and directs the migration of ENCCs along the gut (Young et al., 2001). ENCCs express the REarranged during Transfection (*RET*) receptor tyrosine kinase, and RET with its co-receptor GFRα1 is the receptor for GDNF (Durbec et al., 1996a). Mice lacking *Ret*, *Gfrα1* or *Gdnf* fail to colonize the bowel beyond the stomach, leading to intestinal aganglionosis (Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1994). Besides its role in migration, RET signaling is important for proliferation of ENCCs

* Corresponding author.
*E-mail address:* b.j.l.eggen@umcg.nl (B.J.L. Eggen).

http://dx.doi.org/10.1016/j.ydbio.2016.06.004
0012-1606/

at E12 and for their survival at E14-E16 in mice (Chalazonitis et al., 1998; Gianino et al., 2003; Taraviras et al., 1999; Uesaka et al., 2008). In order to develop into a mature ENS, post-migratory ENCCs differentiate into various neuronal subtypes and glial cells. GDNF promotes neuronal, but not glial differentiation of ENCCs in vitro, and acts as a chemoattractant to outgrowing neurites (Hearn et al., 1998; Natarajan et al., 2002; Taraviras et al., 1999; Young et al., 2001). The mature ENS contains sensory neurons, interneurons and excitatory and inhibitory motor neurons that use a wide range of neurotransmitters, similar to those found in the CNS (Hao and Young, 2009).

Mutations in *RET* and *GDNF* in humans contribute to Hirschsprung disease (HSCR), a congenital disorder that is characterized by intestinal aganglionosis in a variable segment of the distal gut. HSCR patients present with tonic contraction of the muscle layers in the affected segment, which consequently leads to dilatation of the proximal bowel. The prevalence of HSCR is 1:5000 live births and it is considered an inherited disease. Based on the families reported, the mode of inheritance can differ. Most non-syndromic familial HSCR cases show a dominant pattern of inheritance, mostly with reduced penetrance, whereas many syndromic HSCR families show a recessive pattern of inheritance. The sporadic cases are considered polygenic or oligogenic. Of the approximately 15 HSCR genes identified so far, the *RET* gene is by far the most important gene. Coding mutations in *RET* are found in ~50% of familial and 15–35% of sporadic HSCR patients and represent the great majority of genetic mutations found in HSCR patients (Attié et al., 1995; Hofstra et al., 2000). Mutations in all other HSCR-associated genes, including *GDNF*, are rare (Alves et al., 2013; Amiel et al., 2008; Brooks et al., 2005a).

The colonization of the intestine by ENCCs, followed by their neuronal and glial differentiation, is a complex process controlled by various signaling pathways. These include Hedgehog, NOTCH, WNT, Retinoic acid and TGF-β/BMP signaling (Goldstein et al., 2005; Ikeya et al., 1997; Okamura and Saga, 2008; Ramalho-Santos et al., 2000; Sato and Heuckeroth, 2008). Several other pathways that are important for ENS development have been identified in gene expression profiling studies. Iwashita et al. (2003) reported that the HSCR genes *Ret*, *Gfrα1*, *Ednrb* and *Sox10* are highly expressed by ENCCs in mice, thereby explaining how mutations in these genes contribute to aganglionosis. A subsequent expression study by Heanue and Pachnis (2006) showed that at E15.5 in mice, ENCCs express markers of early and late neuronal development and glial differentiation, representing different stages of ENS development. These authors identified *Sox2*, *Cart*, *Sema*, *Fgf* and *Jnk* as novel signaling pathways in ENCCs (Heanue and Pachnis, 2006). In addition, Vohra et al. (2006) found that E14 mouse ENCCs highly express genes with synaptic functions and showed that these genes are important for ENCC migration and neurite outgrowth. Some insight in the pathways downstream of RET signaling has come from expression profiling of GDNF-treated ENCCs by Ngan et al. (2008), who analyzed the changes in gene expression in ENCCs after 8 and 16 h of GDNF treatment. These authors showed that the Prokineticin pathway is activated by RET signaling, but found that few other genes were differentially expressed by the short-term GDNF treatment of ENCCs (Ngan et al., 2008).

These gene expression studies have focused on individual genes that are highly expressed by ENCCs and showed that some of these genes are critical for the development of the ENS. However, for many highly expressed genes it is still unknown if and how they contribute to ENS development. In this study, we used an unbiased, systematic pathway analysis to identify the biological processes that are important for ENS development. Moreover, we treated ENCC cultures with GDNF for 14 days to identify the stable, long-term gene expression changes induced by RET signaling. We show that the expression of differentially expressed genes is regulated by known ENS-related genes, and by several genes not previously implicated in ENS development. These regulatory genes present novel candidates genes for HSCR.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 mice carrying Wnt1-Cre$^{+/-}$ were mated with C57BL/6 females that were homozygous for the Rosa26-LoxP-Stop-LoxP-YFP reporter locus. The stage of embryonic development was set to E0.5 on the day the vaginal plug was seen. Embryos carrying both Wnt1-Cre and Rosa26-LoxP-Stop-LoxP-YFP were identified based on YFP expression in the gastrointestinal tract and craniofacial tissues.

### 2.2. ENCC isolation, culture conditions and RET activation

Full-length guts (foregut to hindgut) were dissected from E14.5 embryos. Single cell suspensions were made using 0.05 mg/ml Collagenase XI/Dispase II (Sigma Aldrich, St. Louis, MO, USA), followed by incubation for 3–5 min at 37 °C and trituration. YFP-positive ENCCs were FACS sorted and directly seeded in Fibronectin-coated plates containing DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 20 ng/ml FGF (Peprotech EC, London, UK), 20 ng/ml EGF (Peprotech EC) and 1% Penicillin-Streptomycin (Invitrogen). YFP-positive ENCCs from one gut were equally divided over two wells; one of which was supplemented with 50 ng/ml GDNF (Peprotech EC). ENCCs were incubated at 37 °C for 14 days in a humidified atmosphere with 5% $CO_2$. Neurosphere-like bodies appeared after 3–5 days and were kept in culture for 14 days without passaging.

### 2.3. Immunostaining

Wells with neurosphere-like bodies were fixed with 4% PFA for 15 min at room temperature (RT) and washed with PBS + 1%Triton X 100 (PBT) for 2 × 5 min. They were incubated in blocking solution (PBT + 1%BSA + 0.15% glycine) for 1 h at RT followed by primary antibodies diluted in blocking solution overnight (O/N) at 4 °C: rabbit anti-human p75 (1:500, Promega, UK), mouse-anti βIII-Tubulin (1:500, Covance UK). After several washes in PBT, secondary antibodies were applied: goat anti-rabbit Alexa Fluor 568 and goat anti-mouse Alexa Fluor 568 (1:500, Invitrogen) O/N. DAPI was added to secondary antibodies (1:1000). Antibody was washed off (3 × 15 min with PBT). Slides were mounted using Vectashield (Vector labs, UK) and visualized using confocal microscopy imaging on Zeiss LSM 710 (Zeiss, Cambridge, UK).

### 2.4. RNA isolation and gene expression quantification

RNA was isolated from untreated and GDNF-treated ENCC cultures, as well as from uncultured E14.5 embryonic guts using the RNeasy Mini Kit (Qiagen, Crawel, UK). RNA yield, purity and integrity were determined using the Agilent 2100 BioAnalyzer with 6000 Nano Chips (Agilent Technologies, Amsterdam, The Netherlands). DNA microarray analyses and RNA quality controls were performed by ServiceXS (Leiden, The Netherlands) using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). For the ENCC vs gut experiment, 3 ENCC samples and 8 mouse gut samples entered the analysis. For the ENCC + GDNF vs ENCC experiment, 7 pairs of untreated and GNDF-treated ENCC cultures were analyzed.