

# MicroRNAs associated with small bowel neuroendocrine tumours and their metastases

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## Abstract

Novel molecular analytes are needed in small bowel neuroendocrine tumours (SBNETs) to better determine disease aggressiveness and predict treatment response. In this study, we aimed to profile the global miRNome of SBNETs, and identify microRNAs (miRNAs) involved in tumour progression for use as potential biomarkers. Two independent miRNA profiling experiments were performed ( $n=90$ ), including primary SBNETs ( $n=28$ ), adjacent normal small bowel (NSB;  $n=14$ ), matched lymph node (LN) metastases ( $n=24$ ), normal LNs ( $n=7$ ), normal liver ( $n=2$ ) and liver metastases ( $n=15$ ). We then evaluated potentially targeted genes by performing integrated computational analyses. We discovered 39 miRNAs significantly deregulated in SBNETs compared with adjacent NSB. The most upregulated (miR-204-5p, miR-7-5p and miR-375) were confirmed by qRT-PCR. Two miRNAs (miR-1 and miR-143-3p) were significantly downregulated in LN and liver metastases compared with primary tumours. Furthermore, we identified upregulated gene targets for miR-1 and miR-143-3p in an existing SBNET dataset, which could contribute to disease progression, and show that these miRNAs directly regulate *FOSB* and *NUAK2* oncogenes. Our study represents the largest global miRNA profiling of SBNETs using matched primary tumour and metastatic samples. We revealed novel miRNAs deregulated during SBNET disease progression, and important miRNA–mRNA interactions. These miRNAs have the potential to act as biomarkers for patient stratification and may also be able to guide treatment decisions. Further experiments to define molecular mechanisms and validate these miRNAs in larger tissue cohorts and in biofluids are now warranted.

## Key Words

- ▶ microRNAs
- ▶ small bowel
- ▶ neuroendocrine tumour
- ▶ biomarkers
- ▶ metastasis

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## Introduction

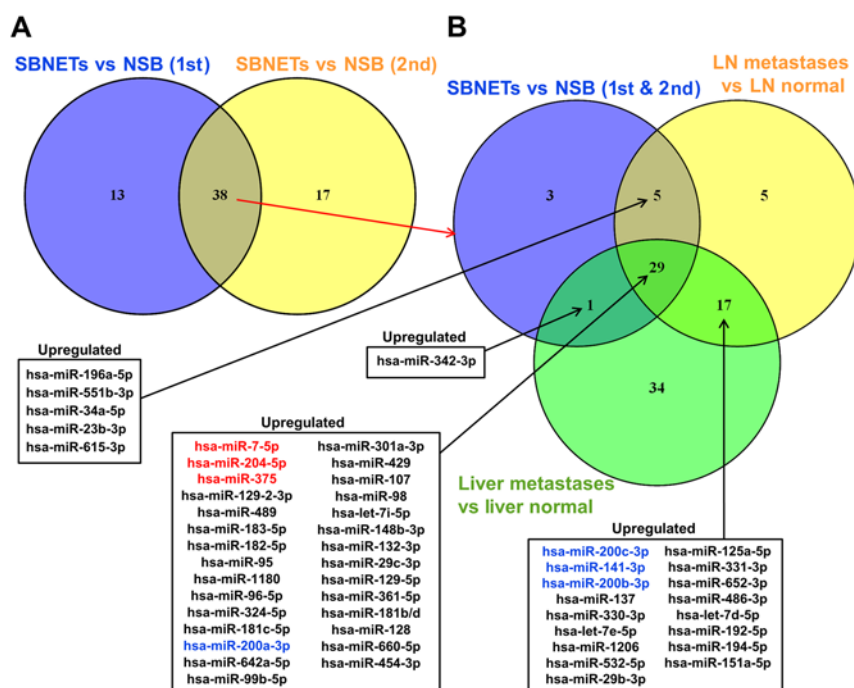
Small bowel neuroendocrine tumours (SBNETs) account for the most common neuroendocrine neoplasm of the gastroenteropancreatic (GEP) system (Lawrence *et al.* 2011). Their incidence is steadily increasing; in males, 2.7-fold overall change to 0.46 per 100,000 per year in England for the period 1971–2006 (Ellis *et al.* 2010) and from 0.38 to 1.08 per 100,000 for the period 1973–2007 based on the National Cancer Institute Surveillance, Epidemiology and End Results (SEER) cancer registry in the United States (Lawrence *et al.* 2011, Fraenkel *et al.* 2012).

Most SBNETs are low-grade lesions; nevertheless, up to 90% of patients with SBNET have lymph node metastases, and in 45–70% of cases, liver metastases are present at the initial diagnosis (Lawrence *et al.* 2011, Norlén *et al.* 2012, Miller *et al.* 2014). These intriguing characteristics contribute to a 5-year survival of less than 60% from diagnosis of liver metastases (Ahmed *et al.* 2009) compared to about 80% in patients with loco-regionally limited disease (Norlén *et al.* 2012). The lack of specific and sensitive biomarkers to stratify NETs according to subtype, determine tumour burden, assess disease progression, select patients for individualised treatment and monitor treatment efficacy is a key issue in management of NETs (Modlin *et al.* 2008, Frilling *et al.* 2014).

MicroRNAs (miRNAs) are small endogenous noncoding RNAs ~17–25 nucleotides in length that play important post-transcriptional roles in gene regulation

by targeting mRNAs, occasionally for direct cleavage, but usually for either translational repression or transcript destabilisation. miRNAs are involved in most developmental and physiological processes and their deregulation is linked to many human diseases, including cancer (Siomi & Siomi 2010, Krell *et al.* 2015). Several studies have shown that miRNAs can act as both oncogenes and tumour suppressors and expression profiling has associated specific miRNAs with a variety of cancers in the hope of developing tumour subtype-specific signatures (Calin & Croce 2006, Esquela-Kerscher & Slack 2006, Weber *et al.* 2006, Zhang *et al.* 2007). Recently, miRNAs have been identified as novel biomarkers (diagnostic and/or prognostic), as well as targets for molecular therapy in various tumours, and have the potential to be utilised in the clinical setting (Osaki *et al.* 2008, Yip *et al.* 2011, Frampton *et al.* 2014, Toiyama *et al.* 2014, Zhu *et al.* 2014, Sandhu *et al.* 2015).

In GEP NETs, data on miRNAs are limited, although their role has been well assessed in those of pancreatic origin (PNETs) (Luzi & Brandi 2011). Indeed, specific miRNAs signatures have been shown to discriminate PNETs from acinar pancreatic tumours (Roldo *et al.* 2006), cystic forms of PNETs from other pancreatic cystic lesions (Matthaei *et al.* 2012) and PNETs from pancreatic ductal adenocarcinoma (Li *et al.* 2013a). Although there have been two small miRNA profiling studies of SBNETs



**Figure 1**

Venn diagrams showing upregulated miRNAs in primary SBNETs and their metastases compared to normal tissues. (A) Upregulated miRNAs in SBNETs vs NSB. Combining the two NanoString nCounter profiling experiments (1st & 2nd) revealed 38 upregulated miRNAs in the intersection (log2 fold change (FC)  $\geq 1.5$  and adjusted  $P < 0.05$ ). (B) Upregulated miRNAs in LN and liver metastases versus primary SBNETs. A "signature" of 29 upregulated miRNAs for SBNETs and their metastases vs normal tissues was discovered (central green intersection). Furthermore, 17 upregulated miRNAs were identified in both LN and liver metastases versus normal tissues, including members of the miR-200 family (miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-141-3p). (Key: Red line indicates these common candidate miRNAs upregulated in SBNETs were used in the next blue circle; LN, lymph node; NSB, adjacent normal small bowel; SBNET, small bowel neuroendocrine tumour). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0044>.

**Table 1** Most significantly deregulated miRNAs in SBNETs vs adjacent normal small bowel (NSB).

Deregulated miRNA (1st profiling)	Log2 fold-change	*Adjusted P value	Deregulated miRNA (2nd profiling)	Log2 fold-change	*Adjusted P value
miR-7-5p <sup>b,c,d</sup>	6.4	2.57E-114	miR-137	3.5	1.01E-05
miR-375 <sup>b,c,d</sup>	5.6	6.30E-67	miR-489 <sup>c,d</sup>	3.4	1.31E-05
miR-204-5p <sup>b,c,d</sup>	5.3	2.44E-67	miR-375 <sup>b,c,d</sup>	3.2	7.21E-05
miR-129-2-3p <sup>c,d</sup>	4.7	2.65E-28	miR-95 <sup>c,d</sup>	3.0	4.38E-05
miR-489 <sup>c,d</sup>	4.0	7.32E-30	miR-7-5p <sup>b,c,d</sup>	2.9	0.000662392
miR-183-5p <sup>c,d,e</sup>	3.9	1.43E-26	miR-301a-3p <sup>c,d</sup>	2.6	0.00021524
miR-182-5p <sup>c,d,e</sup>	3.9	1.21E-21	miR-642a-5p <sup>c,d</sup>	2.6	0.000648626
miR-95 <sup>c,d</sup>	3.8	5.83E-43	miR-204-5p <sup>b,c,d</sup>	2.6	0.002747422
miR-1180 <sup>c,d</sup>	3.6	6.29E-45	miR-129-2-3p <sup>c,d</sup>	2.5	0.003108321
miR-196a-5p <sup>c,e</sup>	3.3	1.18E-09	miR-181c-5p <sup>c,d</sup>	2.3	0.000662392
miR-96-5p <sup>c,d,e</sup>	3.2	9.31E-19	miR-183-5p <sup>c,d,e</sup>	2.3	0.009328868
miR-324-5p <sup>c,d</sup>	3.2	1.13E-24	miR-26a-5p	2.2	0.003108321
miR-181c-5p <sup>c,d</sup>	3.1	3.37E-41	miR-107 <sup>c,d</sup>	2.2	0.002747422
miR-200a-3p <sup>c,d,e</sup>	3.1	2.17E-22	miR-429 <sup>c,d</sup>	2.2	0.007482279
miR-342-3p <sup>c,d</sup>	2.9	8.62E-18	miR-98 <sup>c,d</sup>	2.1	0.002950183
miR-642a-5p <sup>c,d</sup>	2.9	3.72E-40	miR-182-5p <sup>c,d,e</sup>	2.1	0.014094932
miR-330-3p	2.8	6.36E-37	miR-34a-5p <sup>c</sup>	2.1	0.003307545
miR-551b-3p <sup>c,e</sup>	2.7	1.46E-10	miR-454-3p <sup>c,d</sup>	2.1	0.001074461
miR-135a-5p	2.7	1.63E-08	miR-200a-3p <sup>c,d,e</sup>	2.1	0.011254433
miR-486-3p	2.7	6.63E-15	miR-96-5p <sup>c,d,e</sup>	2.1	0.016459461
miR-99b-5p <sup>c,d</sup>	2.6	6.26E-18	miR-148b-3p <sup>c,d</sup>	2.1	0.003108321
miR-301a-3p <sup>c,d</sup>	2.5	9.06E-23	miR-340-5p	2.1	0.003108321
miR-429 <sup>c,d</sup>	2.5	1.18E-18	miR-551b-3p <sup>c</sup>	2.0	0.015881653
miR-331-3p	2.5	2.12E-22	miR-1206	2.0	0.016010957
miR-107 <sup>c,d</sup>	2.5	5.77E-14	miR-129-5p <sup>c,d</sup>	2.0	0.011434466
miR-98 <sup>c,d</sup>	2.4	2.88E-15	miR-582-5p	2.0	0.003108321
let-7i-5p <sup>c,d</sup>	2.3	9.27E-16	miR-660-5p <sup>c,d</sup>	2.0	0.005523635
miR-148b-3p <sup>c,d</sup>	2.3	1.74E-22	let-7f-5p	2.0	0.011434466
miR-29b-3p	2.3	1.28E-15	miR-362-3p	2.0	0.006553207
miR-532-5p	2.3	1.09E-13	miR-4284	2.0	0.003509808
miR-200b-3p	2.2	2.76E-11	miR-99b-5p <sup>c,d</sup>	2.0	0.00481433
let-7e-5p	2.2	7.68E-11	miR-29c-3p <sup>c,d</sup>	1.9	0.009328868
miR-132-3p <sup>c,d</sup>	2.2	1.71E-11	miR-30c-5p	1.9	0.005946993
miR-29c-3p <sup>c,d</sup>	2.2	1.40E-15	miR-342-3p <sup>c,d</sup>	1.9	0.012856933
miR-125a-5p	2.2	2.07E-13	miR-324-5p <sup>c,d</sup>	1.9	0.00481433
miR-129-5p <sup>c,d</sup>	2.0	8.17E-09	miR-505-3p <sup>c</sup>	1.9	0.003108321
miR-361-5p <sup>c,d</sup>	2.0	5.55E-13	miR-374b-5p	1.9	0.003899893
miR-181b/d <sup>c,d</sup>	1.9	2.83E-14	miR-128 <sup>c,d</sup>	1.9	0.003108321
let-7d-5p	1.9	5.66E-10	miR-196a-5p <sup>c,e</sup>	1.9	0.034641355
miR-34a-5p <sup>c</sup>	1.9	1.78E-08	miR-135a-5p	1.9	0.035737367
miR-128 <sup>c,d</sup>	1.8	5.95E-21	miR-30b-5p	1.8	0.019875614
miR-421	1.7	2.31E-17	let-7i-5p <sup>c,d</sup>	1.8	0.014896097
miR-652-3p	1.6	5.52E-10	miR-421	1.8	0.00065961
miR-660-5p <sup>c,d</sup>	1.6	1.32E-12	miR-132-3p <sup>c,d</sup>	1.8	0.01162531
miR-23b-3p <sup>c</sup>	1.6	1.41E-06	miR-24-3p	1.8	0.011434466
miR-615-3p <sup>c</sup>	1.6	2.77E-14	miR-27b-3p	1.7	0.020224505
let-7g-5p	1.6	3.18E-06	miR-16-5p	1.6	0.037832967
miR-15a-5p	1.5	4.07E-07	miR-1180 <sup>c,d</sup>	1.6	0.019407266
miR-505-3p	1.5	7.15E-12	miR-664-3p	1.6	0.002880957
miR-29a-3p	1.5	3.15E-08	miR-361-5p <sup>c,d</sup>	1.6	0.003108321
miR-454-3p <sup>c,d</sup>	1.5	6.38E-11	miR-23b-3p <sup>c</sup>	1.6	0.034602111
			miR-181b/d <sup>c,d</sup>	1.6	0.003108321
			let-7c	1.5	0.040776702
			miR-1468	1.5	0.020445252
			miR-615-3p <sup>c</sup>	1.5	0.009328868
miR-451a	-1.6	8.52E-05	miR-31-5p <sup>e</sup>	-1.5	0.000648626
miR-31-5p <sup>e</sup>	-1.6	7.20E-14	miR-3180	-3.3	2.71E-08

Continued

**Table 1** (Continued)

Deregulated miRNA (1st profiling)	Log2 fold-change	<sup>a</sup> Adjusted <i>P</i> value	Deregulated miRNA (2nd profiling)	Log2 fold-change	<sup>a</sup> Adjusted <i>P</i> value
miR-378g <sup>e</sup>	−1.7	5.88E-16			
miR-4516	−1.8	8.32E-05			
miR-148a-3p	−1.8	1.33E-13			
miR-378a/l <sup>e</sup>	−2.1	2.52E-22			
miR-215 <sup>e</sup>	−3.4	8.56E-22			

Key: For visualization, we included those miRNAs with log2 FC  $\pm 1.5$ . <sup>a</sup>*P* value adjusted using false discovery rate (FDR) method; <sup>b</sup>These miRNAs were validated by qRT-PCR. miRNAs highlighted in gray were deregulated in both profiling experiments; <sup>c</sup>These miRNAs are also upregulated in lymph node metastases vs normal lymph nodes; <sup>d</sup>These miRNAs are also upregulated in liver metastases vs normal liver; <sup>e</sup>These miRNAs were found to be deregulated in the study by Li and coworkers (2013b).

(Ruebel *et al.* 2010, Li *et al.* 2013b), the role of these molecules as biomarkers in this tumour type remains largely unknown. We aimed to assess the global miRNA expression of primary SBNETs, matched LNs and liver metastases and normal tissues, to discover possible biomarkers of tumourigenesis and disease progression.

## Materials and methods

The materials and methods can be found in the [Supplementary Materials and methods](#) (see section of [supplementary data](#) given at the end of this article). This includes details about the patient samples included; RNA isolation; NanoString miRNA profiling; bioinformatic analyses; gene ontology and pathway analyses; qRT-PCR; cell culture; luciferase reporter assays and immunoblotting.

## Results

### NanoString nCounter profiling reveals a common miRNA signature for small bowel NETs and their lymph node and liver metastases compared with normal tissues

We assessed 800 known human miRNAs in 90 patient samples. The 1st profiling cohort included primary SBNETs ( $n=15$ ), adjacent normal small bowel (NSB;  $n=12$ ), matched LN metastases ( $n=9$ ), normal LNs ( $n=7$ ), normal liver ( $n=2$ ) and liver metastases ( $n=2$ ; [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). The 2nd profiling cohort included SBNET ( $n=13$ ), NSB ( $n=2$ ), LN metastases ( $n=15$ ) and liver metastases ( $n=13$ ). Combining the two NanoString nCounter experiments, we revealed 38 upregulated miRNAs (intersection in [Fig. 1A](#) and [Table 1](#)) and 1 downregulated miRNA (all log2 fold change (FC)  $\leq 1.5$  or  $\geq 1.5$ ; adjusted  $P < 0.05$ ) in SBNETs vs NSB ([Table 1](#); [Supplementary Fig. 1A](#) and [Table 2](#)).

Next, we investigated the miRNA signature of infiltrated LNs versus normal LNs, as well as liver metastases versus normal liver ([Fig. 1B](#); [Supplementary Fig. 1B](#) and [Table 2](#)). Strikingly, we found significant overlap between the upregulated miRNAs in primary SBNETs and their metastases compared with their normal tissues, and identified a 29 miRNA signature for this disease (central green intersection in [Fig. 1B](#)). We then confirmed increased expression of the top three upregulated miRNAs (miR-204-5p, miR-7-5p and miR-375) in SBNETs compared with NSB using qRT-PCR, thereby also validating our nCounter miRNA expression profile microarrays ([Fig. 2A, B and C](#)).

### NanoString nCounter profiling reveals downregulated miRNAs during metastatic spread of small bowel NETs

Next, we compared the miRNA profiles of the LN metastases to their primary SBNETs for both profiling experiments. The 1st profiling revealed upregulation of four miRNAs (miR-142-3p, miR-146a-5p, miR-150-5p and miR-548) and downregulation of four miRNAs in the infiltrated LNs (miR-1, miR-133a, miR-145-5p and miR-1233; [Supplementary Table 2](#)). The 2nd profiling discovered a further 4 miRNAs upregulated and 19 downregulated in LN metastases versus SBNETs ([Supplementary Table 2](#)). We observed that in both profiling results, four miRNAs were consistently downregulated in LN metastases (miR-1, miR-133a, miR-145-5p and miR-1233; central green intersection in [Fig. 3](#)) and also that miR-143-3p was highly downregulated in the 2nd profiling (log2 FC  $-2.2$ ; [Supplementary Table 2](#)).

Next, we examined the differential expression of miRNAs in liver metastases compared with primary SBNETs ([Supplementary Table 2](#)). This revealed five upregulated and seven downregulated miRNAs in the liver metastases (all log2 FC  $\leq 1.5$  or  $\geq 1.5$ ; adjusted  $P < 0.05$ ; [Supplementary Table 2](#)). When combining these data with the profiles

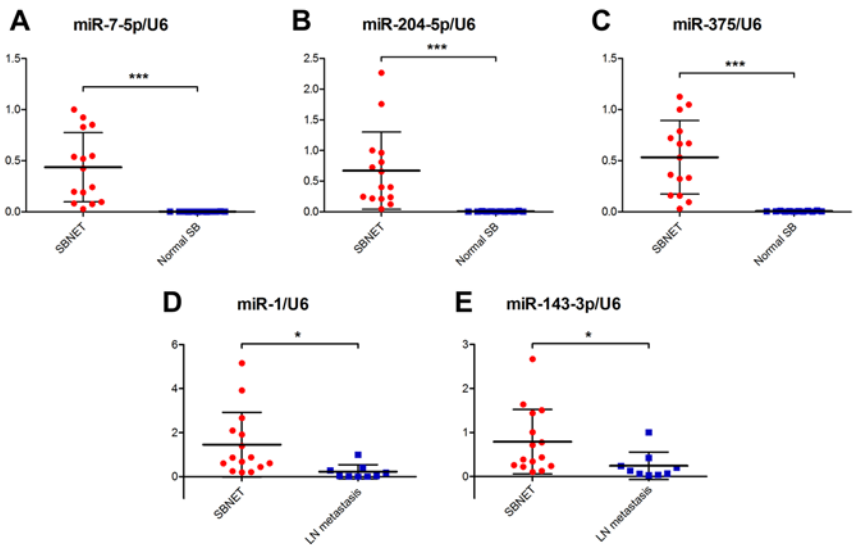
**Table 2** Most significantly deregulated miRNAs in lymph node metastases vs SBNETs.

Deregulated miRNA (1st profiling)	Log2 fold-change	<sup>a</sup> Adjusted <i>P</i> value	Deregulated miRNA (2nd profiling)	Log2 fold-change	<sup>a</sup> Adjusted <i>P</i> value
miR-142-3p	1.0	3.69E-05	miR-15b-5p	0.7	0.0143562
miR-146a-5p	0.9	0.00034256	miR-330-5p	0.7	0.04581073
miR-150-5p	0.8	0.000324898	miR-764	0.6	0.04581073
miR-548	0.5	0.006746057	miR-191-5p	0.5	0.04581073
miR-145-5p <sup>b,c</sup>	−0.7	0.018028326	miR-1825 <sup>c</sup>	−0.5	0.04792938
miR-1233 <sup>c</sup>	−0.8	0.00034256	miR-331-5p <sup>c</sup>	−0.7	0.0143562
miR-1 <sup>c</sup>	−0.8	0.000412601	miR-152	−0.9	0.0293522
miR-133a <sup>b,c</sup>	−1.0	5.75E-05	miR-574-5p	−0.9	0.0143562
			miR-28-3p <sup>c</sup>	−0.9	0.00142885
			miR-28-5p <sup>c</sup>	−1.0	0.00077043
			miR-9-5p	−1.0	0.04470614
			miR-30a-5p <sup>c</sup>	−1.1	0.00142885
			miR-10a-5p	−1.2	0.00142885
			miR-378g <sup>c</sup>	−1.5	0.00010242
			miR-378a/i <sup>c</sup>	−1.6	2.24E-05
			miR-187-3p	−1.6	2.43E-06
			miR-1233 <sup>c</sup>	−1.8	9.04E-07
			miR-139-5p <sup>c</sup>	−1.9	4.45E-07
			miR-139-3p <sup>c</sup>	−2.0	9.71E-08
			miR-145-5p <sup>b,c</sup>	−2.1	1.87E-08
			miR-143-3p <sup>c</sup>	−2.2	8.11E-09
			miR-133a <sup>b,c</sup>	−2.9	5.25E-13
			miR-1 <sup>c</sup>	−2.9	4.03E-14

Key: <sup>a</sup>*P* value adjusted using false discovery rate (FDR) method. miRNAs included were *P* < 0.05; <sup>b</sup>Comparable to our data, these miRNAs were found to be downregulated in LN/liver metastases vs primary SBNETs in the study by Ruebel and coworkers (2010); <sup>c</sup>These miRNAs are also deregulated in liver metastases vs SBNETs; <sup>d</sup>These miRNAs were validated by qRT-PCR. miRNAs highlighted in gray were deregulated in both profiling experiments.

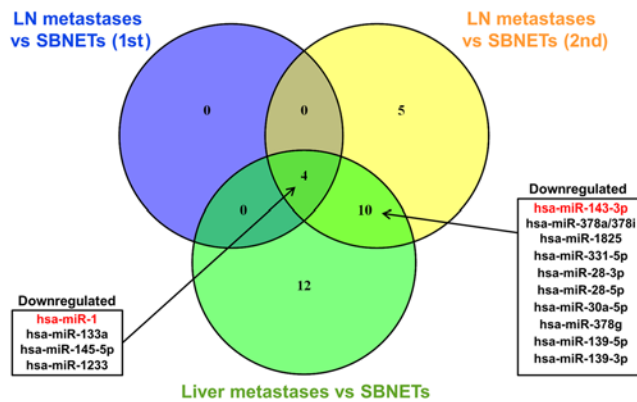
from the infiltrated LNs, we found significant overlap of 14 downregulated miRNAs in both types of metastases (green intersections in Fig. 3). Interestingly, these included reduced levels of miR-1, miR-133a, miR-143-3p, miR-145-5p and miR-1233. As miR-133a and miR-145-5p were previously found to be downregulated in metastases

from SBNETs (Ruebel *et al.* 2010, Li *et al.* 2013b), we chose to focus on miR-1 and miR-143-3p. We confirmed by qRT-PCR that they are significantly downregulated in LN metastases versus primary SBNETs (Fig. 2D and E). Unfortunately, there was insufficient RNA from the liver metastases to perform further qRT-PCR.



**Figure 2** Deregulated miRNAs in SBNETs and their lymph node metastases were validated by qRT-PCR. We confirmed upregulation of (A) miR-7-5p; (B) miR-204-5p and (C) miR-375 in SBNETs versus adjacent normal small bowel (NSB). We also confirmed downregulation of (D) miR-1 and (E) miR-143-3p in lymph node (LN) metastases versus primary SBNETs. Small nucleolar U6 was used as an endogenous control. Results are presented as mean  $\pm$  s.d. (\**P* < 0.05; \*\*\**P* < 0.0001). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0044>.



**Figure 3**

Venn diagram showing downregulated miRNAs in lymph node (LN) and liver metastases compared to their primary SBNETs. Four miRNAs (miR-1, miR-133a, miR-145-5p and miR-1233) were found to be significantly downregulated in both LN and liver metastases versus their primary SBNETs (adjusted  $P < 0.05$ ). Furthermore, miR-143-3p was found to be downregulated in LN metastases (2nd profiling) and liver metastases versus normal tissues. Interestingly, our bioinformatic analyses revealed that miR-1 and miR-143-3p share many important gene targets of disease progression. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0044>.

### microRNAs appear deregulated in liver metastases from SBNETs

Next, we further examined the miRNA expression levels in the liver metastases and normal adjacent liver, as patients with SBNETs commonly develop this type of metastasis (Supplementary Table 2). Interestingly, as mentioned earlier, we found a subset of miRNAs significantly upregulated in the primary SBNETs, as well as the LN and liver metastases, compared with the corresponding normal tissues (Supplementary Table 2; central green intersection in Fig. 1B). However, there were also 17 miRNAs upregulated in the LN and liver metastases that were not upregulated in the primary SBNETs (light green intersection in Fig. 1B). Strikingly, we also observed that many of the miRNAs deregulated in liver metastases from normal liver could be located in clusters from the same primary transcript, suggesting transcriptional regulation. Furthermore, since the probes used by the nCounter profile assay are randomly located in the platform, we regard this as further validation of our findings (Supplementary Table 2). For example, amongst the miRNAs that we found to be upregulated, miR-141-3p, miR-200a-3p, miR-200b-3p and miR-200c-3p are all miR-200 family members and cluster together in particular genomic loci (green intersections in Fig. 1B; Supplementary Table 2).

Given their importance in cancer, we next investigated changes in the miR-200 family members

in detail for the two patients for whom we had nCounter profiling of their adjacent normal liver and liver metastases (Supplementary Table 1). The miR-200 family is known to be important in epithelial-to-mesenchymal transition (EMT) and cancer progression (Craene & Berx 2013). In the patient case studies, it is clear that miR-200 family members are upregulated in LN and liver metastases, compared with the primary SBNETs and normal tissues (Supplementary Fig. 3A and B). Interestingly, for Patient 9 (T3N1M1), levels of miR-200c-3p were the most prominent in the primary tumour, and the LN and liver metastases (Supplementary Fig. 3A). Whilst for Patient 2 (T4N1M1), all miR-200 family members were elevated during metastatic dissemination, with much higher levels in the LN metastases compared with Patient 9 (Supplementary Fig. 3B). Whilst these tumours are both stage IV, this difference in miR-200 family expression may be associated with advancing T-stage, since a T<sub>3</sub> tumour has invaded the subserosa, whilst a T<sub>4</sub> tumour has gone on to invade the peritoneum and/or other organs. Nevertheless, these findings suggest that a reversal of EMT or mesenchymal-to-epithelial transition (MET) could be occurring in SBNET metastases and enforcing colonisation of distant organs. Furthermore, our case studies highlighted that in matched tissues, there appears to be a reduction in both miR-1 and miR-143-3p levels during disease progression and metastasis, compared with the originating NSB mucosae and primary SBNETs (Supplementary Fig. 4A, B, C and D).

Finally, miR-122-5p emerged as downregulated in liver metastases vs normal adjacent liver (log<sub>2</sub> FC -6.8; Supplementary Table 2). Its expression was not found to be significantly deregulated in either primary SBNET or LN metastases compared with normal tissues, but it was upregulated in liver metastases compared with SBNETs and LN metastases (log<sub>2</sub> FC 3.7 and 1.7, respectively; Supplementary Fig. 2 and Table 2).

### miR-1 and miR-143 are found to target genes crucial in the progression of small bowel NETs including *NUAK2* and *FOSB* oncogenes

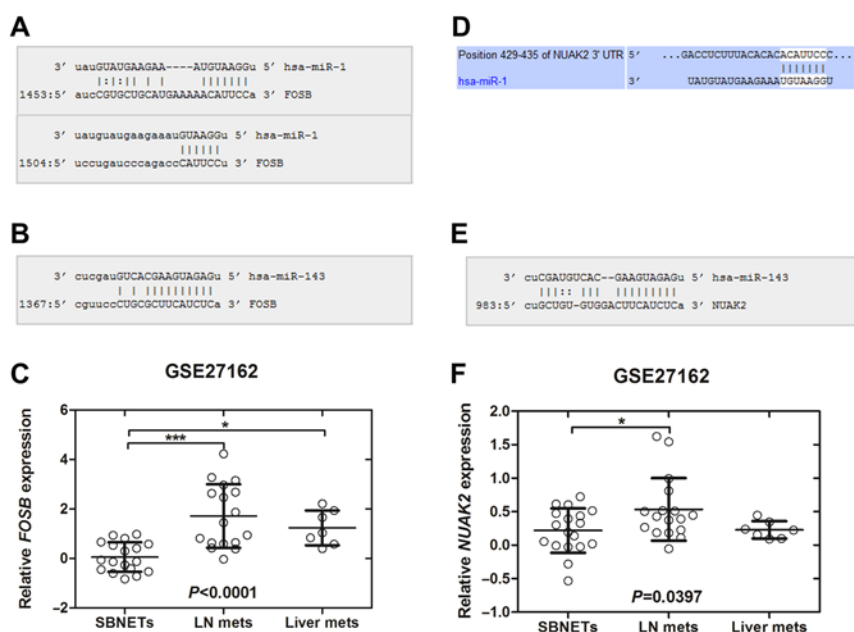
Next, we characterised the functional significance of the differentially expressed miRNAs in primary SBNETs and their LN metastases by evaluating their putative gene targets. To do this, we cross-checked the predicted targets with three publically available gene expression datasets previously assessing SBNETs vs NSB (GSE9576, GSE6272 and E-TABM-389) and the one available dataset comparing gene expression in SBNETs vs matched

LN metastases. We considered potential target genes to have expression opposite to that of the miRNA, in accordance to the antiregulation paradigm (i.e. upregulated miRNA and downregulated mRNA) (Frampton et al. 2014). We also performed enrichment analyses of gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, using DAVID (<http://david.abcc.ncifcrf.gov>) to help unravel the function of these deregulated miRNAs (Leja et al. 2009, Edfeldt et al. 2011, Kidd et al. 2014). In order to have more robust results when doing this enrichment analysis, we considered genes that appeared in  $\geq 2$  expression datasets where possible, as well as being predicted targets of the miRNA of interest.

First, we considered those miRNAs highly expressed in SBNETs (i.e. miR-7, miR-204 and miR-375). Unfortunately, no significantly enriched GO terms or pathways were identified for the targets of these upregulated miRNAs, than would be obtained for randomly picked miRNAs (Supplementary Table 3) (Bleazard et al. 2015). Next, we considered miR-1 and miR-143, as they were downregulated in LN and liver metastases compared with SBNETs in the nCounter profiling, although not differentially expressed compared with normal tissues (Supplementary Table 2). This suggests that specific transcriptional networks in the primary tumour cells have changed during the metastasis of these cells. To further test this hypothesis, we analysed the genes upregulated, upon downregulation

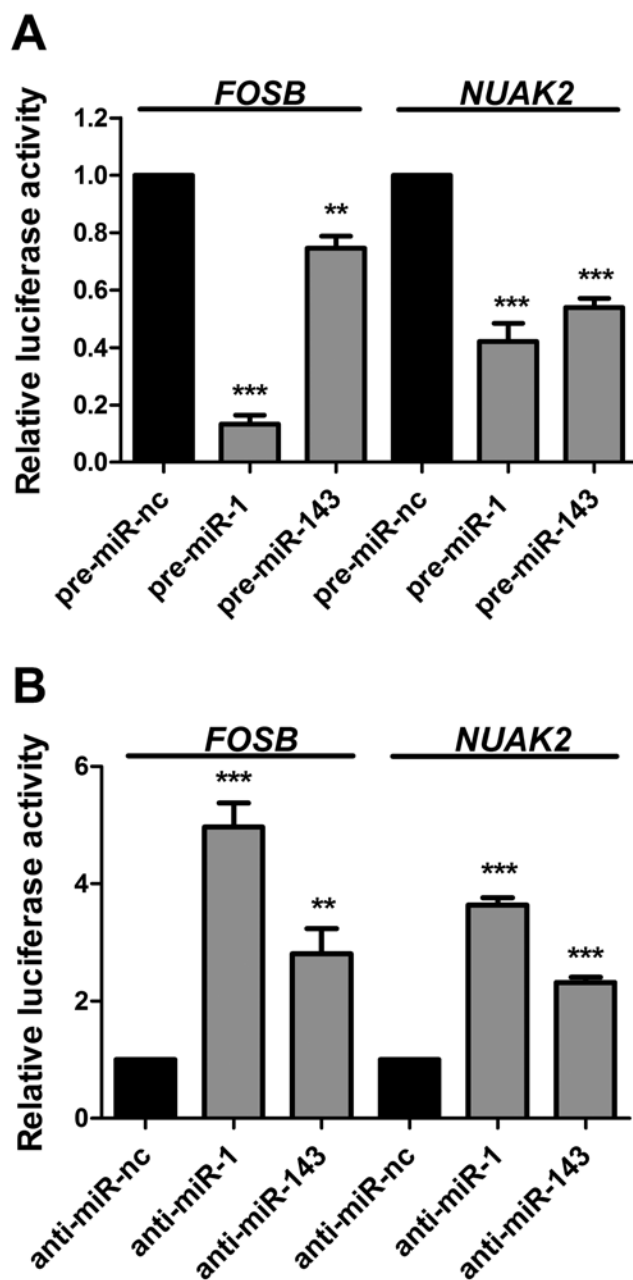
of miR-1 and miR-143 to assess their potential biological functions (Supplementary Table 4).

For miR-1, we found significant enrichment of GO terms relevant to tumour progression and a metastatic phenotype, such as GO:0042981 'regulation of apoptosis' (Benjamini-Hochberg  $P=0.011$ ), GO:0043067 'regulation of programmed cell death' (Benjamini-Hochberg  $P=0.008$ ) and GO:0010941 'regulation of cell death' (Benjamini-Hochberg  $P=0.006$ ). Strikingly, the exact same GO terms were enriched for miR-143, GO:0042981 'regulation of apoptosis' (Benjamini-Hochberg  $P=0.049$ ), GO:0043067 'regulation of programmed cell death' (Benjamini-Hochberg  $P=0.033$ ) and GO:0010941 'regulation of cell death' (Benjamini-Hochberg  $P=0.025$ ). Target genes encompassed by these GO terms for both miRNAs included those normally upregulated in oncogenesis, such as *NUAK2* (Namiki et al. 2011), *EGFR* (copy number gain seen in 4% SBNETs (Banck et al. 2013)), *KRAS*, *NRAS*, *IGF1* (Svejda et al. 2011, Reidy-Lagunes et al. 2012), *MAPK1* (ERK1) (Svejda et al. 2011), *BCL2*, *ARHGEF7* and *BMP7* (Supplementary Table 4). Target genes specific only for miR-1 included *HGF* (Svejda et al. 2013) and *VEGFA*. These data suggest that downregulation of these two miRNAs could be key in the development of SBNET metastases through reduced repression of these genes and increased cancer cell survival. Accordingly, we validated a few of these target genes using the dataset GSE27162



**Figure 4**

Reduction in miR-1 and miR-143 levels allows release of important oncogenes during SBNET progression. Using the miRanda-mirSVR target prediction algorithm, we identified (A) miR-1 and (B) miR-143 both target *FOSB*. Furthermore, assessing a publicly available dataset of gene profiling (GSE27162), we found that (C) *FOSB* expression is increased in lymph node (LN;  $n=17$ ) and liver metastases ( $n=7$ ) compared to primary SBNETs ( $n=18$ ). Similarly, using miRanda-mirSVR and TargetScan prediction algorithms, we found that (D) miR-1 and (E) miR-143 both regulate *NUAK2*. (F) *NUAK2* expression is also increased in LN metastases compared to primary SBNETs. These data suggest that the reduction in miR-1 and miR-143 in metastases from SBNETs may allow reduced repression of important oncogenes *FOSB* and *NUAK2*, and therefore contribute to disease progression.  $P$  values were calculated using one-way analysis of variance (ANOVA) to compare gene levels between groups followed by Tukey's multiple comparison tests. Scatterplots are shown for each group and the horizontal lines represent the mean gene expression level and s.d. (\* $P < 0.05$ , \*\*\* $P < 0.001$ ). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0044>.

**Figure 5**

*FOSB* and *NUAK2* oncogenes are directly targeted by miR-1 and miR-143. HEK293T cells were cotransfected with (A) precursor microRNA negative control mimic (pre-miR-nc) or precursors to miR-1 or miR-143 (100 nM), or (B) anti-microRNA negative control (anti-miR-nc) or anti-miR-1 or anti-miR-143 (100 nM), together with *FOSB* or *NUAK2* 3'UTR reporter constructs (100 ng/well). Luciferase activity was measured 24-h post-transfection. Horizontal lines represent the mean luciferase activity and s.e.m. from independent experiments, each measured in triplicate (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

and found that *KRAS* and *BCL2* were upregulated in LN metastases vs primary SBNETs (Supplementary Fig. 5A and B), while *HGF* and *VEGFA* were upregulated in

both LN and liver metastases vs primary SBNETs (Supplementary Fig. 6A and B).

Furthermore, when assessing individual putative gene targets for these miRNAs that were differentially expressed in the dataset GSE27162, we noticed an important interaction that could be related to SBNET progression. Our bioinformatic analyses revealed that both miR-1 and miR-143 are predicted to target *NUAK2* and *FOSB*, and these genes are significantly upregulated in SBNET LN metastases, more so than in hepatic disease, compared with primary SBNETs (Fig. 4A, B, C, D, E and F). Next, we investigated the direct binding of miR-1 and miR-143 to the seed sequences in the 3'-untranslated regions (UTRs) of *NUAK2* and *FOSB*. We used 3'-UTR constructs for each gene in a 3'-UTR luciferase reporter assay (SwitchGear Genomics, Menlo Park, CA, USA) to prove that these genes are miRNA targets. Indeed, cotransfection of HEK293T cells with precursors for miR-1 or miR-143 resulted in significant reduction in luciferase activity compared with the negative control precursors for both *FOSB* and *NUAK2* oncogenes (Fig. 5A). Conversely, transfection with anti-miRs for miR-1 and miR-143 significantly increased luciferase activity compared with negative controls for both *FOSB* and *NUAK2* (Fig. 5B), demonstrating that both miRNAs target these genes. Next, we tried to confirm these results using KRJ-I cells (neoplastic enterochromaffin cells) derived from a localised human ileal carcinoid (Pfragner et al. 1996). However, over-expression or silencing of both miR-1 and miR-143 in KRJ-I cells (Supplementary Fig. 7) did not show any changes in *FOSB* and *NUAK2* transcript or protein expression (Supplementary Fig. 8), indicating that these miRNA-mRNA interactions probably occur in selected subtypes or metastatic SBNET cells. Whilst KRJ-I cells have been used as a model for the *in vitro* investigation of SBNETs, it may be that other cells, especially those derived from metastatic disease, would have been better models for examining these miRNA-mRNA interactions. Unfortunately, we did not have access to other SBNET cells, such as P-STS (derived from primary tumour), L-STS (from LN metastasis), H-STS (from liver metastasis) or CNDT2 (from liver metastasis) to validate these miRNA-mRNA interactions (Van Buren et al. 2007, Pfragner et al. 2009).

## Discussion

Current staging and grading systems used to classify SBNETs have attempted to stratify tumours, in order to predict survival outcomes and the risk of developing metastatic disease, but can often be ineffective.



Consequently, novel molecular biomarkers are required to aid diagnosis, prognosis and the development of targeted therapies. The current study represents the most extensive assessment of miRNA profiling in SBNETs and their metastases. We also gained some insight into changes in miRNA expression in patients with matched liver metastases. We identified miRNA–mRNA interactions, which could have a key role in disease progression in SBNET. Important hypotheses have been generated from these data that will provide the basis for further investigations.

### Global miRNA profiling reveals a signature specific for small bowel NETs

Our strategy consisted of identifying differentially expressed miRNAs in SBNETs vs adjacent NSB tissues, and then assessing changes in miRNA expression between SBNETs and their LN and liver metastases. We selected 90 patient samples (primary tumours, metastases and normal tissues) for miRNA profiling. From the global miRNA profiling, we identified an miRNA signature for SBNETs compared with NSB tissues, consisting of 38 upregulated miRNAs and 1 downregulated miRNA (Supplementary Table 2; intersection in Fig. 1A; Supplementary Fig. 1A). Next, we confirmed the upregulation of miR-204-5p, miR-7-5p and miR-375 by qRT-PCR to validate our SBNET miRNA signature and profiling methods (Fig. 2A, B and C). Interestingly, previous profiling by Li and coworkers (Li et al. 2013b) found significant altered expression in miR-31, miR-96, miR-129-5p, miR-182, miR-196a, miR-200a and miR-215 in SBNETs and our findings were consistent with this (Table 1). We also found that miR-133a and miR-145-5p, as well as others, were significantly downregulated in LN metastases compared with SBNETs (Fig. 3 and Table 2), in keeping with findings of a previous smaller profiling study assessing only 95 miRNAs (Ruebel et al. 2010). This suggests that these miRNAs could be reliable for detecting SBNETs in other patient cohorts also, especially since we identified these in both profiling experiments (Fig. 3 and Table 2). Furthermore, we also found novel miRNAs deregulated in SBNETs (Fig. 1; Supplementary Fig. 1) and, importantly, also in their associated LN and liver metastases (Fig. 3; Supplementary Fig. 2) that have not been previously considered in this tumour type.

Interestingly miR-7 was upregulated in our SBNETs (Fig. 1B and Table 1; Supplementary Table 2), in contrast to other cancers where it has been identified as downregulated, functioning as a tumour suppressor. Indeed, reduced miR-7 levels are seen in highly invasive

breast cancer stem cells (Zhang et al. 2014), metastatic gastric cancer (Zhao et al. 2013), colorectal cancer (Xu et al. 2014) and pancreatic cancer (Ma et al. 2014). Recently, a circular RNA, ciRS-7, has been identified to act as an endogenous miR-7 inhibitor/sponge (Hansen et al. 2013). Thus, ciRS-7 expression results in reduced miR-7 activity and consequently increased levels of miR-7 target transcripts, since it harbours more than 70 binding sites for miR-7 (Hansen et al. 2013). However, miR-7 has also been shown to have oncogenic properties and, therefore, is tissue-specific (Chou et al. 2010). In lung cancer, EGFR is able to induce miR-7 expression through a Ras/ERK/Myc pathway (Chou et al. 2010). Over-expression of miR-7 *in vitro* promotes lung cancer cell growth and increases the mortality of nude mice with orthotopically implanted lung tumours (Chou et al. 2010). Thus, the role and mechanism of miR-7 in SBNETs merits further investigation, and it would also be important to evaluate ciRS-7 expression. Similarly, whilst we found upregulation of miR-204 in SBNETs, it has been seen as downregulated in gastric cancer (Sacconi et al. 2012, Zhang et al. 2013), colorectal cancer (Yin et al. 2014) and clear cell renal cell carcinoma (Mikhaylova et al. 2012). Thus, its increased expression and role in SBNETs remains unknown. We found elevated miR-375 levels in SBNETs, which is also more often downregulated in cancers. However, high expression of miR-375 has been seen in ER $\alpha$ -positive breast cell lines and it is a key driver of their proliferation (de Souza Rocha Simonini et al. 2010).

There were fewer miRNAs significantly downregulated in SBNETs compared with adjacent NSB (Table 1; Supplementary Fig. 1A and Table 2). Of these, miR-215 was downregulated in the 1st profiling of SBNETs vs NSB (Table 1). miR-215 is also reduced in metastatic renal cell carcinomas and over-expression is able to decrease cell migration and invasion *in vitro* (White et al. 2011). Interestingly, miR-215 functions as a tumour suppressor with its activation inducing cell cycle arrest in a p53-dependent manner (Georges et al. 2008). We also found that miR-31-5p (miR-31) was significantly downregulated in SBNETs compared with NSB mucosae in both profiling experiments (Supplementary Fig. 1B and Table 2). The study by Li and coworkers also identified miR-215 and miR-31 as downregulated in SBNETs compared with normal enterochromaffin (EC) cells, with further downregulation in LN and liver metastases compared with the primary tumours (Li et al. 2013b). Indeed, miR-31 has been shown to be a tumour suppressor with antimetastatic properties in breast and liver cancers (Viré et al. 2014, Kim et al. 2015). We, however, did not see any

reduction in the levels of miR-31 and miR-215 in SBNET metastases (Supplementary Table 2).

### miR-1 and miR-143 are downregulated during metastatic spread to regional lymph nodes and the liver

Next, we examined miRNAs deregulated in SBNET metastases. We identified that miR-1 and miR-143-3p (miR-143) are significantly downregulated in LN and liver metastases compared with the primary SBNETs (Figs 2D, E and 3). However, these miRNAs were not differentially expressed compared with normal LNs (Supplementary Table 2). This suggests that loss of expression may be associated with the development of metastases, especially since the majority of the SBNETs profiled were LN positive (N1) and developed liver deposits. Further assessment of miR-1 and miR-143 in SBNETs that did not develop metastases is now warranted to see if primary tumours with lower levels of these miRNAs are more likely to metastasise.

Indeed, looking at our two case studies, we realised that from NSB tissue to SBNET, to LN infiltration and finally liver metastasis, there appears to be a gradual reduction in both miR-1 and miR-143 expression (Supplementary Fig. 4A, B, C and D). Whilst the levels of these miRNAs do not appear to be altered from normal LN and liver, their biological relevance in the infiltrating cancerous cells originating from matched primary tumours is still of importance.

To further explore the possible functional consequences of the downregulation of these miRNAs, we performed gene- and pathway-enrichment analyses on putative targets and publically available gene expression datasets. Strikingly, we found that both miRNAs affect genes involved in the regulation of apoptosis and this could help to explain their role in SBNET progression (Supplementary Table 4). Our bioinformatic analyses also revealed that both miR-1 and miR-143 are predicted to target *FOSB*, and this gene is significantly upregulated in LN and liver metastases versus primary SBNETs (GSE27162; Fig. 4C). Indeed, we show that miR-1 and miR-143 both directly bind to the 3'UTR of *FOSB* (Fig. 5). Interestingly, *FOSB* transcription is induced by metastasis-associated protein 1 (MTA1), and consequently represses E-cadherin expression in TGF- $\beta$ 1-stimulated breast cancer cells (Pakala et al. 2011). Thus, the miR-1/*FOSB* and miR-143/*FOSB* axes may be able to regulate EMT and, therefore, metastasis in SBNETs also. Furthermore, it has been shown in KRJ-I (neoplastic EC) cells that

TGF- $\beta$ 1 stimulation results in increased cell proliferation (Kidd et al. 2007). However, this is not due to classical SMAD signalling, but rather upregulation of *c-MYC*, concomitant activation of *c-MYC* transcriptional targets and inhibition of *p21<sup>WAF1/CIP</sup>* (Kidd et al. 2007). In addition, TGF- $\beta$ 1 stimulation increases the expression of *MTA1* transcript and decreases E-cadherin expression in KRJ-I cells (Kidd et al. 2007). *MTA1* has also been shown to be over-expressed in malignant primary SBNETs and their metastases (Kidd et al. 2006, 2007). Therefore, reduced post-transcriptional repression of *FOSB*, by downregulation of miR-1 and miR-143, could potentiate invasion and metastasis caused by the TGF $\beta$ 1-pathway via *MTA1* in SBNET cells.

miR-1 and miR-143 have both been noted as downregulated in numerous tumour types. miR-1 is reduced in primary prostate cancer compared with normal tissue, and levels are further decreased in metastatic disease (Liu et al. 2015). Indeed, in aggressive prostate cancer mouse models, loss of miR-1 enhances mesenchymal commitment, invasiveness and tumourigenesis (Liu et al. 2013). Accordingly, re-expression of miR-1 in bladder cancer (Yoshino et al. 2011), hepatocellular carcinoma (Datta et al. 2008), lung cancer (Nasser et al. 2008) and rhabdomyosarcoma (Yan et al. 2009) inhibits tumour cell growth and metastatic traits. Deep sequencing has also found miR-1 levels to be reduced in colorectal cancers (CRCs) and a case of colorectal NET (Hamfjord et al. 2012). miR-1 downregulation in human CRCs has been correlated with over-expression of the *MET* gene, especially in advanced stages of progression (Migliore et al. 2012). The *MET* oncogene encodes a tyrosine kinase receptor that binds hepatocyte growth factor (HGF) and drives the malignant progression of several tumour types (Reid et al. 2012). Experiments in CRC cells have confirmed the tumour-suppressive ability of miR-1, as enforced expression impairs cell scattering, migration, wound-healing and proliferation in response to HGF (Migliore et al. 2012). Thus, activation of *MET*, due to a decrease in miR-1 is likely to be associated to cancer progression and to the acquisition of an invasive phenotype and metastatic dissemination (Migliore et al. 2012, Reid et al. 2012). Interestingly, our bioinformatic analyses revealed *HGF* to also be targeted by miR-1 and we found *HGF* levels significantly increased in LN and liver metastases compared with primary SBNETs (Supplementary Fig. 6A). However, we could not see an increase in *MET* expression between primary SBNETs and metastases (GSE27162; data not shown).

Nevertheless, *MET* proto-oncogene over-expression has been correlated with metastatic ability in well-differentiated PNETs (Hansel *et al.* 2004), and further investigation in a larger cohort of SBNETs is warranted. We also noticed that miR-1 targets *VEGFA*, and found *VEGFA* levels are indeed significantly higher in LN and liver metastases compared with primary SBNETs (Supplementary Fig. 6B). This is very relevant to SBNET biology, as these tumours are highly vascular, and currently there are several clinical trials investigating VEGF signalling as a prime therapeutic target. Importantly, recent whole-exome sequencing has shown that *VEGFA* is not mutated in SBNETs (Banck *et al.* 2013); thus, loss of post-transcriptional regulation by miR-1 in SBNET metastases could explain higher levels of transcript in these lesions.

Reduced miR-1 expression is also thought to play an oncogenic role via release of specific target genes such as *LASP1*, *IGF1*, *IGF1R* or *BCL2* (antiapoptotic gene) in CRC (Migliore *et al.* 2012). *IGF1R* is highly expressed in a large percentage of primary SBNETs (46%), LN metastases (50%) and liver metastases (68%) (Gilbert *et al.* 2010). Thus, the miR-1/*IGF1R* interaction should be further validated. Furthermore, we identified *BCL2* as upregulated in LN metastases compared with primary SBNETs, and it is not only targeted by miR-1, but also by miR-143 (Supplementary Fig. 5B). These novel interactions, miR-1/*BCL2* and miR-143/*BCL2*, deserve further investigation. Finally, we also revealed that miR-1 and miR-143 target *NUAK2*, which has been shown to be oncogenic in melanoma (Namiki *et al.* 2015) and gastric cancer (Kim *et al.* 2013), and levels of this transcript were also upregulated in LN metastases compared with SBNETs (Fig. 4F). Importantly, we also show that miR-1 and miR-143 both directly target the 3'UTR of *NUAK2* (Fig. 5).

miR-143 has been shown to have an antimetastatic effect and is downregulated in several cancers (Takagi *et al.* 2009, Kent *et al.* 2010, Peng *et al.* 2011). miR-143 and miR-145 are often cotranscribed and are usually investigated together as tumour suppressors (Kent *et al.* 2014). In prostate cancer, over-expression of miR-143 and -145 reduces migration and invasion *in vitro* and tumour development and bone invasion *in vivo* (Peng *et al.* 2011). Furthermore, lower expression of miR-143 and miR-145 in primary prostate cancers was significantly associated with tumour progression and the development of bone metastases (Peng *et al.* 2011). Interestingly, deregulation of miR-143 and miR-145 has not been seen in LN metastasis compared with primary prostate tumours (Spahn *et al.* 2010). This suggests that in prostate cancer, the functional loss of miR-143 or miR-145 may be cell-type-specific and

results in bone metastasis, instead of LN metastasis (Peng *et al.* 2011). Reduced miR-143 expression also plays a crucial role in the invasion and metastasis of pancreatic cancer (Hu *et al.* 2012). In a metastatic mouse model of pancreatic cancer, miR-143 expression significantly reduced the formation of liver metastases (Hu *et al.* 2012). Furthermore, xenograft pancreatic tumour growth was reduced by miR-143 through downregulating *ARHGEF1*, *ARHGEF2* and *KRAS*, and reducing MMP-2 and MMP-9 protein levels, whilst increasing E-cadherin protein levels (Hu *et al.* 2012). Importantly, putative interactions exist between miR-1/MMP-8 and miR-1/*KRAS*, and miR-143/*KRAS* and miR-143/MMP-19 (Supplementary Figs 5A and 9A, B, C, D). Indeed, our bioinformatic analyses revealed that *KRAS*, *MMP-8* and *MMP-19* are upregulated in LN metastases vs SBNETs (GSE27162; Supplementary Figs 5A and 9A, B, C, D). These miRNA-mRNA interactions deserve validation *in vitro* and *in vivo*. Our analyses showed that there is an increase in the *KRAS* transcript during SBNET progression (GSE27162; Supplementary Fig. 5A); however, recent deep sequencing has shown that *KRAS* is not actively mutated in SBNETs (Banck *et al.* 2013). This suggests that there may be post-transcriptional regulation occurring by loss of miR-1 and miR-143, thereby allowing an increase in *KRAS*.

### Reduction in miR-122 may play a role in small bowel NET progression and act as a biomarker for liver metastases

miR-122-5p appeared strongly downregulated in liver metastases versus adjacent normal liver (Supplementary Fig. 1B and Table 2), although still significantly upregulated in liver metastases vs SBNETs (Supplementary Fig. 2 and Table 2). Indeed, miR-122 is known to be highly expressed in the liver (~70% of all miRNA content in liver) and is tissue-specific (Jopling 2012). Furthermore, miR-122 levels are frequently reduced in hepatocellular carcinoma (HCC) compared with normal liver, and lower miR-122 expression is associated with worse prognosis (Jopling 2012). miRNA-122 expression has been found to be regulated by DNA methylation and correlates with apoptosis in HCC cells (Xing *et al.* 2013). Thus, in HCC, miR-122 acts as a tumour suppressor, and its loss correlates with gain of metastatic properties and suppression of the hepatic phenotype (Coulouarn *et al.* 2009). Interestingly, in CRC liver metastases, miR-122 levels are higher compared with primary CRCs and/or normal colonic mucosae (Iino *et al.* 2013, Ellermeier *et al.* 2014). Possible reasons for this include either detecting residual liver tissue within the CRC liver metastases,



as primary CRCs have low miR-122 expression (Ellermeier *et al.* 2014), or that miR-122 is upregulated in CRC cells during the process of liver metastasis, rather than during carcinogenesis (Iino *et al.* 2013). Thus, following the proposed 'seed and soil' theory, during the formation of CRC liver metastasis, cancer cells try to adapt to their new environment by expressing miR-122, whilst HCC cells prepare to metastasise out of the liver by reducing miR-122 levels (Iino *et al.* 2013). Clearly, the role of miR-122 in cancer progression depends on the primary site. We speculate, therefore, that miR-122 may be a biomarker for SBNET liver metastases; however, further samples need to be assessed.

### miR-200 family members are deregulated in small bowel NET progression accompanying a reversal of epithelial-to-mesenchymal transition

Several studies have shown that the TGF $\beta$  and EMT pathways contribute to tumour growth and metastasis in SBNETs. Indeed, E-cadherin expression is often reduced in SBNETs compared with NSB mucosae and has been correlated with malignant behaviour (Kawahara *et al.* 2002, Li *et al.* 2002). E-cadherin levels are also lower in larger (>2 cm) SBNETs and those with transmural invasion (Li *et al.* 2002). Furthermore, recent whole-exome sequencing has revealed that *SMAD2* and *SMAD4* genes are frequently deleted (Banck *et al.* 2013). However, we found significant upregulation of miR-200a-3p in SBNETs vs NSB (Fig. 1B; Supplementary Table 2), and this can be used as a surrogate for E-cadherin levels (Craene & Bex 2013). Unexpectedly, we also noticed elevated levels of miR-200 family members in SBNET metastases compared with normal tissues (Fig. 1B), and consistent with this, when assessing previous gene profiling, we found stable or reverse expression of EMT markers (*ZEB1/2*, *CDH1*) in liver metastases compared with primary SBNETs (Supplementary Fig. 10A, B and C). Indeed, whilst SBNETs commonly metastasise to the liver, with the development of multiple lesions, these behave in a relatively indolent manner from an oncologic perspective (Reddy & Clary 2010). Thus, a 'seed and soil' phenomenon may explain these findings that miR-200s appear to be elevated in SBNET metastases (Fig. 1B), allowing possible repression of *ZEB1/2* and consequent stabilisation of E-cadherin (*CDH1*) expression, thereby allowing colonisation of large parts of the liver. Interestingly, over-expression of miR-200s has also been shown to be pro-metastatic and promote metastatic colonisation in breast cancer by influencing not only E-cadherin-dependent epithelial traits,

but also the Sec23a-mediated tumour cell secretome (Korpai *et al.* 2011). Thus, the exact role of miR-200s in SBNET liver metastases remains uncertain.

### Limitations

We were unable to dissect out the differences in miRNA expression between different grades of SBNETs. However, this is not surprising since 90% of patients have G1 tumours and only 10% have G2 lesions. Furthermore, we do not resect patients with G3 tumours, as they are extremely rare and usually go for chemotherapy and not surgical treatment (Clift *et al.* 2016). Therefore, it would be appropriate, but extremely difficult, for future studies to include a larger number of primary SBNETs in each grade. Similarly, it would be interesting, but almost impossible, to assess miRNA profiles in those with N0 vs N1, as nearly all patients have G1 tumours and 90% are N1 (Clift *et al.* 2016). However, it would be possible and important to assess those primary SBNETs with and without liver metastases, as well as circulating miRNAs in such patients.

We had difficulty confirming *NUAK2* and *FOSB* as direct targets of miR-1 and miR-143 in KRJ-I cells. Indeed, miRNA-mRNA interactions occur in a context-dependent, cell-type-specific manner (Kedde *et al.* 2007, Erhard *et al.* 2014), and we would have liked to investigate these in an additional SBNET cell line, but were unable to do so. However, we did validate these miRNA-mRNA interactions in HEK293T cells. Furthermore, these miRNAs are clearly downregulated in metastases vs primary SBNETs in patients in two independent miRNA profiling experiments (Fig. 3), whilst *NUAK2* and *FOSB* are more expressed in LN metastases vs primary SBNETs (Fig. 4C and F), suggesting that they are being regulated *in vivo*.

### Future work

Further investigations will include validation of these miRNAs in larger cohorts of patient samples and correlation with clinicopathologic factors. We also plan to study miRNAs in blood samples and compare the results with those obtained from tumour tissues. This will enable us to examine miRNAs involved in disease progression and identify clinically useful biomarkers. The availability of serum from patients also raises the possibility of applying a liquid biopsy approach to SBNETs, potentially enabling the noninvasive early detection of micrometastases or treatment response monitoring using circulating-free miRNAs (Miller *et al.* 2015). Furthermore, work *in vitro* and *in vivo* using cell lines derived from SBNETs, and other models, will help



to validate further key target genes of these miRNAs and phenotyping studies will elucidate the functions of these miRNAs in SBNETs (Pfragner *et al.* 1996).

## Conclusions

We have identified novel miRNAs that may potentially differentiate between primary SBNETs and NSB, including the upregulation of miR-204-5p, miR-7-5p and miR-375. Our data also suggest that miRNAs could be used to further classify SBNETs according to their biological behaviour. Indeed, we show that miR-1 and miR-143-3p are downregulated in SBNET and their metastases, and that their target gene pathways are crucial for tumour development and disease progression. Additional experiments will help to define the mechanistic functions of these miRNAs and their potential use as biomarkers and/or therapeutic targets.

### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0044>.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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### Author contribution statement

A F, L C and A E F were involved in study concept and design. H C M, L C, R F, E A S, D K, O F, G H, A M and S O were involved in acquisition of data. A E F, H C M, L C, E A S, R F, A M, S O, G S and R P were involved in analysis and interpretation of data. A E F, H C M, A F and L C were involved in drafting of the manuscript. A E F, H C M, A F, J S, L C, D K, O F, G H, G S, R P and B K were involved in Revision of manuscript. A F, L C, J S, R F, E A S and B K were involved in supervision of work.

### Ethics approval

This study is part of our project R12025: Genetic signature, metabolic phenotyping and integrative biology of neuroendocrine tumours. Ethics approval REC number: 07/MRE09/54.

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