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Malignant transformation of colonic epithelial cells by a colon-derived long noncoding RNA ☆

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ABSTRACT

Recent progress has been made in the identification of protein-coding genes and miRNAs that are expressed in and alter the behavior of colonic epithelia. However, the role of long non-coding RNAs (lncRNAs) in colonic homeostasis is just beginning to be explored. By gene expression profiling of post-mitotic, differentiated tops and proliferative, progenitor-compartment bottoms of microdissected adult mouse colonic crypts, we identified several lncRNAs more highly expressed in crypt bottoms. One identified lncRNA, designated non-coding *Nras* functional RNA (*ncNRFR*), resides within the *Nras* locus but appears to be independent of the *Nras* coding transcript. Stable overexpression of *ncNRFR* in non-transformed, conditionally immortalized mouse colonocytes results in malignant transformation, as determined by growth in soft agar and formation of highly invasive tumors in nude mice. Moreover, *ncNRFR* appears to inhibit the function of the tumor suppressor *let-7*. These results suggest precise regulation of *ncNRFR* is necessary for proper cell growth in the colonic crypt, and its misregulation results in neoplastic transformation.

1. Introduction

The intestinal epithelium is one of the most rapidly proliferating, self-renewing epithelia in the body [1]. Stem cells reside at the bottom of cellular invaginations called crypts of Lieberkühn. Stem cells continuously divide, giving rise to daughter cells that differentiate as they move up the crypt column and are ultimately shed into the lumen of the intestine [1].

Characterization of the intestinal progenitor cell compartment (PCC) has largely focused on identification of protein-coding genes that define the PCC and several of these have been shown by lineage labeling to mark stem cells [2–6]. The importance of non-coding RNAs in the PCC has been largely restricted to miRNA function [7,8], although long non-coding RNAs (lncRNAs) are now recognized as regulators of stem cell function in general [9]. The transcriptome consists of many lncRNAs that map mostly to intronic and extragenic regions of the genome [10] with estimates of total lncRNAs varying from 7% to greater than 50% of total transcripts [11–13]. The miRNAs are among the best characterized regulatory ncRNAs that bind through seed sequences, 2–8 base pairs in length at the 5′ end of the miRNA [14], which directly hybridize to target transcripts. Some miRNAs, like *mir-17–92*, may function as oncogenes, while others, like *let-7*, have tumor-suppressor functions [15,16]. LncRNAs have been found to participate in epigenetic regulation through chromatin remodeling and transcriptional

Abbreviations: lncRNAs, long non-coding RNAs; *ncNRFR*, non-coding Nras functional RNA; PCC, progenitor cell compartment; *ISH*, in situ hybridization; UTRs, untranslated regions; YAMC, Young adult mouse colon; PCR, polymerase chain reaction; dpc, days postcoitus; aRNA, amplified RNA; BrdU, bromodeoxyuridine; siRNA, small interfering RNAs; GSEA, gene set enrichment analysis.

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regulation, with direct consequences for cancer progression [17,18]. In addition, non-coding pseudogenes can act as miRNA binding decoys that inhibit miRNA function, thus driving oncogenesis [19]. Herein, we have identified a colonic lncRNA that is localized to the PCC. This RNA is from the *Nras* locus of the mouse genome and we have designated it non-coding *Nras* Functional RNA (*ncNRFR*). Overexpression of ncNRFR in immortalized mouse colonocytes results in transformation *in vitro* and *in vivo*.

2. Materials and methods

2.1. Computer analysis

Programs used were the RNA-fold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and the UCSC browser. BLASTX confirmed there are no homologous coding sequences in *ncNRFR* cDNA.

2.2. siRNA knockdown of ncNRFR

Oligonucleotides coding for *ncNRFR* siRNAs (see Supplementary Table 1) were cloned into pSilencer 4.1 hygro (ABI/Ambion, Austin, TX) and control non-targeting siRNAs in this vector were used as controls. Nucleofection (Lonza/Amaxa Gaithersburg, MD) was used to transfect with solution L with program X-005 (Lonza). Transfection was near 90–100% as measured by fluorescence with a pmaxGFP expression construct (Lonza).

2.3. RNA identification

Colonic crypt epithelium was isolated [20] and crypts were bisected with an opthalmic microscalpel. Approximately 7500 cells from 50 crypt fragments were pooled, yielding 0.5 μg of RNA that was linearly amplified (aRNA) yielding 30 μg [21]. These aRNAs were used to probe a microarray that identified a few candidate genes that had differential expression in the crypt epithelium. Three micrograms of aRNA was fluorescently labeled with Cy3 or Cy5 dyes and hybridized to a 5000-gene cDNA Research Genetics microarrray.

2.4. Northern blot analysis

Total RNA was purified with TRIzol reagent (Invitrogen) and RNAeasy (Qiagen, Valencia, CA). *Nras* cDNA probe (Thermo Fischer Open Biosystems clone ID: 6475312) was purified from an EcoRI 518 bp digested fragment, and *ncNRFR* probe fragment (bases 679–990, see Supplementary Table 1) was made by PCR amplification from a non-repeat region of the cDNA. These were used for Northern blots as described [22], using 10 μg of total RNA per lane.

2.5. Nude mouse cell injections

Three to six million cells were injected subcutaneously into the right flank of athymic nude mice. This study was carried out in strict accordance with animal care and use guidelines and approval of the Vanderbilt IACUC. Mice were monitored throughout the experiment for signs of distress and tumor growth greater than 2 cm.

2.6. Isolated whole crypt RNA in situ hybridization (ISH)

RNA whole mount *ISH* was performed as described [23]. Fluorescent *ISH* was performed using the Tyramide Signal Amplification (TSA) kit with manufacturer's instructions (Perkin Elmer, Waltham, MA).

3. Results and discussion

3.1. ncNRFR is expressed in the colonic PCC

To identify genes expressed in the mouse colonic PCC, we microdissected crypt tops (differentiated cells) and bottoms (PCC), and analyzed RNA expression from the two populations by microarray profiling. These results were validated for 10 randomly selected genes by *ISH* on isolated whole colonic crypts (Fig. 1, Supplementary Fig. 1 and gene list Supplementary Table 2). In general, *ISH* showed expression in discrete crypt regions rather than in a gradient along the crypt axis. Many of the genes expressed in the PCC corresponded to apparent lncRNAs.

We identified a lncRNA 3′ to the coding portion of the *Nras* locus (Fig. 2A). This would represent a 1357 bp transcript, *ncNRFR*, which is transcribed in the same direction as *Nras*. It overlaps untranslated regions (UTRs) of some *Nras* splice variants and the intronic regions of others, but it does not overlap with other *Nras* transcripts (Fig. 2A). Two mouse Riken clones were found that overlap the 5′ and 3′ ends, respectively, of *ncNRFR*, suggesting transcriptional initiation occurs within this region. Based on annotated cDNA transcript information, there are numerous other ncRNAs in the *ncNRFR* locus that are independent from the *Nras* transcriptional locus, none of which map to known miRNAs. A BLASTX analysis showed no homologous coding sequences are present in *ncNRFR*; however, this analysis does not exclude the presence of short protein sequences that might code for unrecognized proteins in this transcript.

To confirm that *ncNRFR* was preferentially expressed in the PCC, we performed RNA *ISH* that showed *ncNRFR* is expressed most

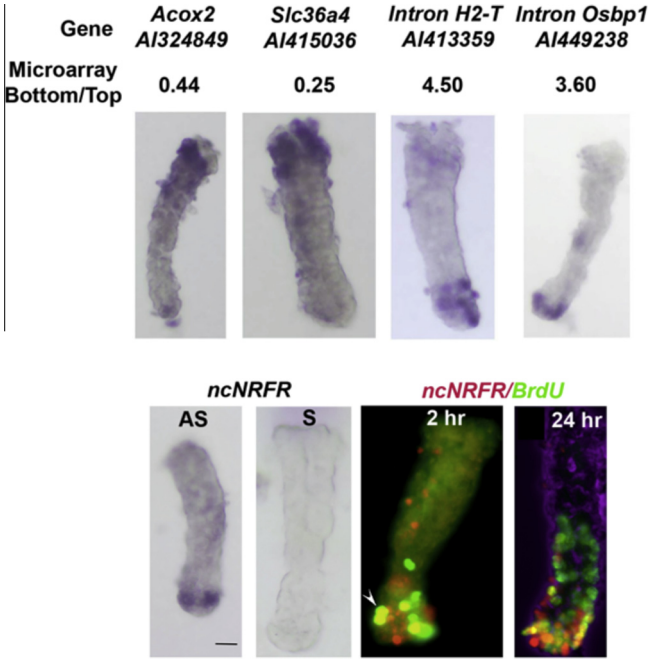

Fig. 1. *In situ* hybridization (*ISH*) for genes expressed in the tops and bottoms of crypts. Top panels, examples of confirmatory RNA *ISH* with bottom to top microarray signal ratios and clone accession numbers shown. RNA *ISH* is shown for *Acox2*, *Slc36a4*, intronic lncRNA in *H2-T*, intronic lncRNA in *Osbp1*. Bottom panels from left to right, RNA *ISH* for *ncNRFR* and its negative sense control. Following a pulse of BrdU, colonic crypts were isolated and processed 2 and 24 h later and whole-mount staining of crypts for BrdU (green) and fluorescent *ISH* for *ncNRFR* (red) was performed. Shown are two representative crypts. The arrowhead points to a double stained cell. The far right panel has a DIC view overlaid in the blue channel to give an impression of the 3-D relationship of cells. The bottom to top microarray ratio for *ncNRFR* is 2.7. The scale bar is 50 uM.

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