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The long intergenic non-coding RNA CCR492 functions as a let-7 competitive endogenous RNA to regulate c-Myc expression

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ABSTRACT

In mammals the cell-cycle progression through the G1 phase is a tightly regulated process mediated by the transcriptional activation of early genes in response to mitogenic stimuli, whose dysregulation often leads to tumorigenesis. We here report the discovery by RNA-seq of cell-cycle regulated (CCR) long intergenic non-coding RNAs (lincRNAs), potentially involved in the control of the cell-cycle progression. We identified 10 novel lincRNAs expressed in response to serum treatment in mouse embryonic fibroblasts (MEFs) and in BALB/c fibroblasts, comparably to early genes. By loss-of-function experiments we found that lincRNA CCR492 is required for G1/S progression, localizes in the cell cytoplasm and contains 4 let-7 microRNA recognition elements (MREs). Mechanistically, CCR492 functions as a competing endogenous RNA (ceRNA) to antagonize the function of let-7 microRNAs, leading to the de-repression of c-Myc. Moreover, we show that ectopic expression of CCR492 along with a constitutively active H-Ras promotes cell transformation. Thus, we identified a new lincRNA expressed as an early gene in mammalian cells to regulate the cell-cycle progression by upregulating c-Myc expression.

1. Introduction

The portion of the genome deputed to the transcription of protein-coding genes in mammals is limited to <2%, while the majority of the transcripts is represented by non-coding RNA (ncRNAs). To date, the function and regulation of many short non-coding RNAs such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), microRNAs (miRNAs) and small nuclear RNAs (snRNAs) have been well studied. Much less is known about the other group of ncRNAs so far defined as long non-coding RNAs (lncRNAs). LncRNAs have been described as transcripts longer than 200 nucleotides with no evidence of coding for functional proteins. Long intergenic non-coding RNAs (lincRNAs) are lncRNAs that lie within a genomic region devoid of annotated genes. Large-scale sequencing of cDNA libraries estimated that several thousand lncRNAs are transcribed in mammalian cells in a tightly regulated cell and developmental specific way, although only a few of them have been studied [1]. They can exert a variety of different functions, such as epigenetic regulation, transcriptional, post-transcriptional and post-translational processing [2,3].

The cell cycle is the sequence of events that occurs in cells stimulated to grow. A complex network of signalling pathways regulates the entry into the cell cycle and it is crucial for the cell homeostasis. Normal cells, like fibroblasts, require growth factors to proliferate, which are provided in vitro by the addition of fetal bovine serum (FBS). In serum-deprivation conditions, the cells enter in a quiescent state, termed G0, while the addition of serum triggers a proliferative response. This system has been widely used as a model for studying growth control and cell-cycle progression and the identification of the early genes that are induced by mitogens to progress into the in G1 phase and whose genetic and epigenetic alterations led to cell transformation.

miRNAs are short ncRNAs (~22 nucleotides long) that serve as guides for targeting the RNA to imperfectly complementary microRNA recognition elements (MREs) within target mRNAs inducing both translational repression, and mRNA decapping/deadenylation [4]. Several studies have shown that miRNAs can control the expression levels of genes involved in the cell-cycle regulatory machinery [5,6]. The let-7 miRNA family was initially discovered in *Caenorhabditis elegans* [7], and has been shown to be highly evolutionarily conserved [8]. Let-7 is undetectable in embryonic stem cells, but it is expressed at high levels

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in adult tissues and its expression is deregulated or lost in many cancers [9,10].

In order to characterize the control circuitry underlying cell proliferation, we set up a screening approach to identify novel cell-cycle regulated (CCR) lincRNAs. We found a number of new lincRNAs specifically upregulated by mitogens as early genes. Moreover, by loss-of-function experiments we identified one lincRNA that promotes G1/S progression by upregulating c-Myc expression and whose deregulation contributes to cell transformation.

2. Materials and methods

2.1. Cell culture

Primary mouse embryonic fibroblasts (MEFs) were derived from 13.5d pregnant female mice and BALB/c fibroblasts (ATCC, Manassas, VA, USA) were cultured as previously described [11]. Briefly, the cells were maintained in high-glucose DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1 mM sodium pyruvate (Invitrogen), 50 U/ml of penicillin/ml, and 50 μg/ml of streptomycin/ml. Cell synchronization was performed as previously described [12] by starving MEFs in 0.2% serum for 48 h and BALB/c fibroblasts in 0% serum for 48 h then released into cell cycle by adding 20% serum for the times indicated (30 min, 1 h and 2 h). 60 nM pool of let-7 miRNA Inhibitors (Ambion) and 60 nM pool of let-7 miRNA Mimics (Ambion) were respectively used for let-7 inhibition and overexpression experiments. For 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) treatment, 40 μM DRB (diluted in DMSO) were added directly to the cell culture medium and incubated for the indicated times.

2.2. RNA extraction and quantitative real-time PCR (RT-qPCR)

RNA extraction and RT-qPCR were performed as previously described [13]. Briefly, RNA was extracted using TRIzol reagent (Invitrogen), following manufacturer instructions. RNA integrity measurements were performed using Fragment Analyzer™ (Advanced Analytical). All samples had RNA Quality Number (RQN) >9.8. RT-qPCR was performed using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen) following the manufacturer's instructions. Primers sequences are shown in Suppl. Table 2. Expression levels of mature let-7 were quantified by RT-qPCR using TaqMan MicroRNA Assays Kit (Applied Biosystems, Carlsbad, CA). In addition, the expression levels of miR-16 were evaluated as a negative control of RNA precipitation. The reverse transcription reaction was carried out with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions.

2.3. RNA-seq library preparation

For RNA-seq library preparations, 2 μg of total RNA were used as input for the TruSeq RNA Library Prep Kit v2 (Illumina), and libraries were prepared following manufacturer instructions.

2.4. Sequencing and bioinformatics analysis

Libraries were normalized, pooled, and sequenced on the Illumina HiScanSQ Platform. Reads were pre-processed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), to remove poor-quality reads, and clip adapter sequences. After pre-processing, the obtained high quality paired-end reads were mapped to mouse genome (*Mus musculus*, assembly mm9) using the TopHat v2.0.0 spliced-mapper (http://tophat.cbcb.umd.edu/), a gapped aligner able to discover new splice-junctions ab initio. This resulted in approximately 1.4 billion mappings. Reads mapped from TopHat were then assembled into a parsimonious set of transcripts using Cufflinks v2.0.2. Cufflinks uses TopHat output to build the minimal set of transcripts that can explain the majority of mapped reads. To increase Cufflinks accuracy, we provided it with an annotation of both annotated and predicted transcripts from Ensembl release 68 (July 2012). At this stage, Cufflinks produced approximately 3×10^5 transcripts, many of which are single exon transcripts deriving from transcriptional noise, residuals of pre-mRNAs, and exons that Cufflinks was unable to connect due to the lack of spliced-read information. To clean our annotation from background noise, we designed a filtering pipeline, which allowed us to simultaneously remove low-fidelity transcripts, and to select putative previously unannotated transcripts lacking coding potential. (1) All single exon transcripts, and multi-exon transcripts shorter than 200 bases were excluded; (2) Using Cufflinks read coverage estimation we excluded those transcripts with a maximal coverage below 3 reads per base; (3) We removed all transcripts that have at least an exon overlapping a transcript from RefSeq, UCSC, Ensembl, and Vega annotation sets, and any transcript overlapping known rRNAs, tRNAs, miRNAs and snoRNAs; (4) To estimate the coding potential of novel transcripts, we used two different Support Vector Machines (SVM) trained on different sets of lncRNAs, namely iSeeRNA and Coding Potential Calculator {Kong:2007hx}. Transcripts identified by at least one of these two SVMs as coding, were excluded. The final annotation yielded approximately 750 high-fidelity long non-coding transcripts. To determine which transcripts may have a role in cell proliferation, we then performed differential expression analysis across time-course samples, using Cuffdiff, and selected only those showing significant upregulation upon serum induction with respect to serum-starved cells (point 0 of our time-course). miRNA target prediction on lincRNA CCR492 was performed using three algorithms: MREdictor [14], RNAhybrid {Kruger:2006ek}, and Pita [15]. The RNA-seq of the present manuscript are deposited in http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mhgdymwgxloxvij&acc=GSE77324

2.5. Proliferation assay and flow cytometry analysis

For cell-growth assay, 5×10^4 cells were plated in 35 mm wells and counted at the indicated time point using Scepter™ Automated Cell Counter (Millipore). A growth curve was plotted to examine the effects of the shRNAs on cell proliferation. 72 h post-transfection, the cells were harvested, fixed in 70% ethanol, and stained for flow cytometry analysis. For the One-dimensional cell-cycle analysis, the cells were stained with propidium iodide (PI) solution (0.1% TRITON X-100, 200 mg/ml RNase, 20 mg/ml PI in PBS buffer) for 30 min at room temperature. Two-dimensional cell-cycle analysis was performed using Click-iT EdU-Cell Cycle 633-red assay from Invitrogen according to the manufacturer's protocol with 1 h of EdU pulse. Acquisition was performed using Becton Dickinson FACS Canto and analysis was done with FACS FlowJo Software.

2.6. Northern blot and rapid amplification of cDNA ends (RACE)

Northern blot analysis was performed with NorthernMax kit (Ambion) following manufacturer instructions. In brief, 4 μg of polyA+ RNAs was purified from total RNA of serum-induced fibroblasts and run on formaldehyde 1% agarose gel, transferred to BrightStar-Plus Membrane (Ambion) followed by cross-linking through UV irradiation. The membrane was subjected to hybridization with 0.1 nM of Antisense probe overnight at 68 °C.

The probe complementary to CCR492 was transcribed in vitro with T7-FlashScribe Transcription kit (CellScript) and labeled with biotin-16-UTP (Roche) for a length of 351 nt (561–912), using the following primers: AS-PROBE-FW: 5′-GAGATAATACGACTCACTATAGGGAGAAGC ATCATGTGTCTGTGTACAAG-3′ and AS-PROBE-REV: 5′-TATCTTTATCCCTG AACTTTCTTTC-3′. After washing, membranes were exposed for 2 h. 5′ and 3′ RACE were performed using SMARTer RACE cDNA Amplification kit (Clontech) and followed by DNA sequencing. The Gene-Specific Primers used for PCR of RACE analysis were as follows: CCR492-REV: 5′-TTCCAGCTCCTAAGGTCACTG-3′ (5′RACE) and CCR492-FW: 5′-CTACTCTT ATAAAGGACCCGACT-3′ (3′RACE).