Knockdown of long noncoding RNAs in breast cancer

[1]Jennii Luu, [2]Jesper Maag, [1]Yanny Handoko, [3]Richard Redvers, [3,4]Robin L. Anderson, [5]Maren M. Gross, [2]Marcel E. Dinger, and [1,3]Kaylene J. Simpson

[1]Victorian Centre for Functional Genomics, Peter MacCallum Cancer Centre; [2]Genome Informatics, The Kinghorn Cancer Centre, The Garvan Institute of Medical Research; [3]Metastasis Research Laboratory, Peter MacCallum Cancer Centre, [4]Sir Peter MacCallum Department of Oncology, University of Melbourne; [5]Dharmacon, now part of GE Healthcare, 2650 Crescent Drive, Suite #100, Lafayette, CO 80026, USA

Abstract

Traditionally genetics has held a protein centric view with RNA seen as an intermediate step between DNA and protein. Recently, the emerging evidence of pervasive transcription throughout the genome has challenged this view[1,2]. Long noncoding RNAs (lncRNA) are selectively expressed during different cell cycles[3] as well as transcribed differently in specific cell types[4], which emphasizes their importance in regulating cell specification. lncRNAs can work on every stage of transcription from chromatin remodeling, controlling transcription to post-transcriptional processing through various mechanisms such as directly binding to transcription activation sites, working as decoys for transcript suppressors/activators or as guiding/scaffold molecules for chromatin remodeling complexes[5].

Increasing numbers of studies have associated disease with lncRNAs. However, such studies have typically only focused on exploring the function of individual lncRNAs. In preliminary studies, we investigated the functional consequences of lncRNA knockdown in the breast cell lines MCF 10A and MDA-MB-231 using cell viability and morphology as readouts. Using high throughput siRNA screening protocols established in the Victorian Centre for Functional Genomics, we have knocked down all targets in the Dharmacon™ Lincode™ siRNA Library collection (currently 2,231) in both cell lines and quantitated changes using high content imaging. Here we report the functional consequences of lncRNA knockdown in breast cell lines and correlate with patient tumor data.

Introduction

Long noncoding RNA (≥ 200 nt) can be found throughout the genome. They can be transcribed from either strand of a protein coding locus, can have multiple exons, may or may not be poly-adenylated and their expression varies greatly across cell types, tissue types, and developmental stages **(Figure 1)**. Additionally, long noncoding RNAs can be localized to the nucleus or cytoplasm.

Long noncoding RNAs have been identified as differentially expressed in breast cancer cell lines **(Figure 2)** suggesting a role for them in tumor progression. To further characterize the role of long noncoding RNAs in breast cancer we performed a high throughput screen of siRNAs targeting long noncoding RNAs using the Dharmacon Lincode siRNA Library. This library consists of 2,231 siRNAs; **Figure 3** shows the genomic location of each long noncoding RNA targeted by the Lincode library. The siRNAs target each chromosome and are distributed between plus and minus strands.

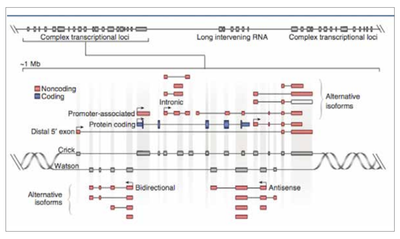


Figure 1. Diagram of long noncoding RNA transcription from Mercer and Mattick 2013[6].

lncRNAs show differential expression between tumor and normal samples

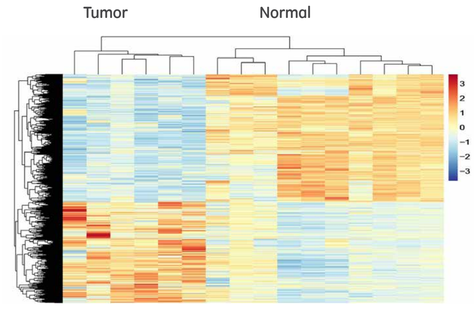


Figure 2. Heatmap of differentially expressed lncRNAs between the matched normal and tumor tissues from TCGA. The samples cluster into two distinct groups based on the lncRNA expression profiling showing that lncRNAs are dysregulated, and therefore might have a function, in breast cancer. Of the 650 significantly differentially expressed lncRNAs 245 were present in the Lincode library. These were subsequently knocked down.

Genomic location of Lincode library siRNAs

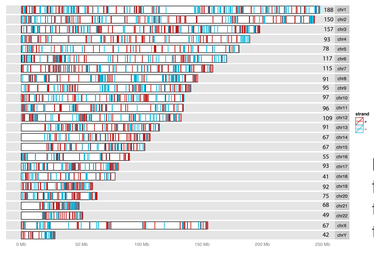


Figure 3. Distribution of siRNAs from the Lincode library targeting long noncoding RNAs throughout the genome.

Proof of principal: differential expression is reproduced in *in vitro* assay

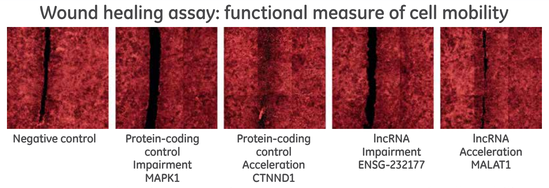


Figure 4. Wound healing assay is a surrogate measure of cell mobility. Representative examples of protein-coding controls and lncRNAs that positively or negatively alter cell motility and progression of wound healing after 12 hours of knockdown.

Screening methods

Cell lines: MCF 10A (nontumor) & MDA-MB-231 (tumor)

Library: Dharmacon™ Lincode™ siRNA Library (2,231 genes, 4 siRNAs pooled, RefSeq 54)

Transfection: 40 nM Dharmacon™ SMARTpool™ siRNA, DharmaFECT™ Transfection Reagents, 2 technical replicates

Assays: High content imaging cell count (viability) & length vs. width ratio (cell morphology) at 72 hours

- Viability: low in tumor, high in nontumor
- Cell morphology: differences from control

Primary screen results: viability

Hit identification: knockdown of long noncoding RNAs resulting in decreased viability in both MCF 10A and MDA-MB-231 cells

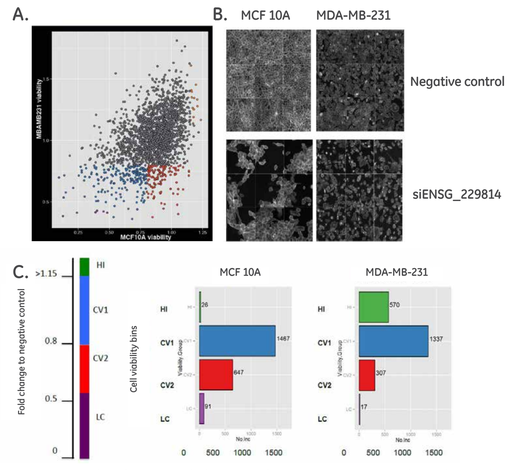


Figure 5. Screen results from viability analyses. A. Scatter plot of viability of MCF 10A and MDA-MB-231 cells 72 hours post-transfection showing differences in viability between nontumor and tumor cells populations. **B.** Phase contrast images of MCF 10A and MDA-MB-231 cells transfected with either a non-targeting siRNA or Lincode siRNA targeting ENSG_229814 long noncoding RNA. **C.** Viability cutoffs to identify "hits" from high content analysis; 0-0.5 fold change Low Cell Count (LC), 0.5-0.8 fold change Cell Viability 2 (CV2), 0.8-1.15 fold change Cell Viability 1 (CV1) and > 1.15 fold change High Cell Count (HI).

Primary screen results: cell morphology

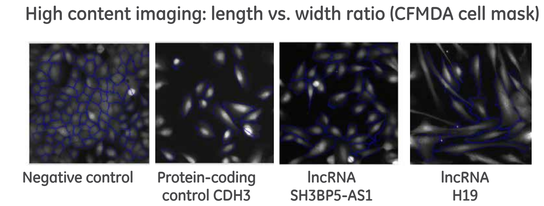


Figure 6. High content imaging of length verses width is a measure of changes in cell morphology. Representative examples from the long noncoding siRNA screen show changes in cell morphology after 72 hours of knockdown.

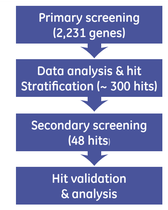
Hit identification workflow

Primary screening with two high content assays investigating cell viability and morphology in tumor and nontumor cell culture models

Resulting data gives hundreds of potential hits showing changes in cell viability and morphology

Prioritized 48 hits for secondary screening (4 individual siRNAs per gene)

More hit validation in additional assays (wound healing)



Conclusion

Here we show data from an initial high throughput knockdown study that reveals a few hundred previously uncharacterized transcripts that affect the viability, invasiveness and cell morphology of cells. Around 30% of the lncRNA knocked down with siRNAs displayed decreased viability compared to the negative control.

Currently, secondary validation screens are in progress including deconvolution of the prioritized primary hits. At the same time, the functional genomics group is developing a new algorithm to more precisely calculate morphology changes to find additional hits. Additionally, the wound healing assay will be performed on prioritized hits. Finally, Dharmacon has reversioning the Lincode Library to RefSeq 65 which now targets almost 3,500 lncRNAs which can additionally be tested for roles in breast cancer.

References

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