Article

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Decoding of Non-Coding DNA and Non-Coding RNA: Pri-Micro RNA-Encoded Novel Peptides Regulate Migration of Cancer Cells

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Only two percent of the Human genome was shown to code for proteins and the rest of the 80–90 percent was shown to transcribe into non-coding RNAs. Micro RNAs (miRNAs) and long non coding RNAs (lncRNAs) fall into this category of non-coding RNAs. miRNAs are regulatory small RNA molecules that base pair with 3-prime-untranslated region of mRNAs and inhibit the translation or expression of mRNA. Mature miRNAs are known to be processed from large primary transcript (pri-miRNAs) in two stages. First stage involves processing of pri-miRNA into shorter pre-miRNA followed by second stage, where pre-miRNA will be processed to mature miRNAs. Earlier studies suggested that deregulation or aberrant expression of miRNAs can lead to several human diseases and cancers. Recently, it was shown that pri-miRNA codes for peptides (miPEPs) that regulate the expression of active mature miRNAs in plant cells. Since miRNAs are well conserved in humans, animals and plants, it is important to study whether pri-miRNAs code for such peptides/proteins in mammalian cells. In addition, it will be highly significant and remarkable, if one can prove the presence/absence of pri-miRNA encoded peptides in normal and cancer cells and their metastatic cells and show that they function as tumor suppressors and/or oncogenes like in the case of miRNAs. Here, we demonstrate for the first time, the presence of an ORF in pri-miRNA which codes for—peptides or small proteins that show novel biological properties in human cells. We show these pri-miRNA (miR-200a and miR-200b)-encoded peptides/proteins (miPEP-200a and miPEP-200b) to inhibit the migration of prostate cancer cells by regulating epithelial to mesenchymal transition of these cells. These miPEPs have the potential to serve as diagnostic markers for metastasis and can also be used as therapeutic agents to many cancers. We have also discussed how these novel peptides/proteins encoded by pri-miRNAs are evolved in nature and their potential role in cancer and other human diseases. These results may revolutionize our present understanding of the functional role of the so called junk DNA (non-coding DNA) and its noncoding RNAs in the biology of humans and animals.

KEYWORDS: *miRNA, miPEP, Junk DNA, lncRNA, Tumor Suppressors, Metastatic Inhibitors, Wound Healing, Vimentin, Non-Coding DNA, miRNA-200, Non-Coding RNA.*

INTRODUCTION

There are at least 2600 miRNAs have been identified and shown to regulate gene expression at the post-transcriptional level (Esquela-Kerscher, 2006; Abba et al., 2016; Christodoulatos, 2014). Among these miRNAs, miR-200a and miR-200b are of importance because they were shown to function as tumor suppressors, metastatic inhibitors, and also repressors of cancer stem cell self-renewal (Feng, 2014; Zhong, 2015; Tavazoie, 2008). These miRNAs were also shown to play a critical role in differentiation and apoptosis (Humphries, 2015). They were also shown to function as inhibitors of cancer cell migration and also shown to regulate the epithelial-to-mesenchymal transition (EMT) (Zaravinos, 2015; Humphries, 2015; Ivaska, 2011; Zhong, 2015).

Interestingly, pri-miRNA was shown to code for peptides (miPEPs) that regulate the expression of active mature miRNAs in plant cells (Lauressergues, 2015). Furthermore, recent studies have also shown the presence of conserved functional peptides MLN and DWORF encoded

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by muscle specific long noncoding RNAs (lncRNAs) (Anderson, 2015; Nelson, 2016). These represent small percentage of peptides. Since miRNAs are well conserved in humans, animals and plants, we wanted to investigate whether such pri-miRNAs code for such peptides/proteins in mammalian cells.

Deregulation of miRNAs, miR-200a and miR-200b were seen in several cancers including prostate cancer, breast cancer, lung cancer, renal cancer, pancreatic cancer etc. (Feng, 2014). In order to prove the hypothesis that pri-miRNA encoded peptides/proteins exist in mammalian cells, we have chosen pri-miRNA of miR-200a and miR-200b as a proof of concept because of their critical involvement in cancer progression and therapy (Feng, 2014; Zaravinos, 2015; Humphries, 2015). We have identified open reading frames that are encoded by pri-miRNA of miR-200a and miR-200b and cloned the cDNAs which encodes peptides/small proteins and tested them for their biological activities such as inhibitory effect on the migration of cancer cells and also studied their biological activities in regulating the signaling pathways that regulate cancer cell growth and migration.

MATERIAL AND METHODS

Plasmids

Expression plasmids miPEP-200a and miPEP-200b have come from Dr. Reddy laboratory (details of cloning and sequencing of these genes will be published elsewhere).

Cell Lines

Prostate cancer cell line PC-3 was obtained from the American Type Culture Collection and the cells were cultured in RPMI 1640 (Cellgro) with 10% fetal bovine serum (ATCC).

Antibodies

Horseradish peroxidase (HRP)-conjugated antibodies to mouse or to rabbit IgG were purchased from GE Healthcare. Anti-HA-Peroxidase, High Affinity (3F-10) was obtained from Roche, Anti-human vimentin polyclonal goat IgG and anti-E-cadherin polyclonal goat IgG were purchased from R&D system, anti β-catenin (β-catenin (E-5) Mouse monoclonal IgG1), anti β-Actin (β-Actin (C-4) Mouse monoclonal IgG1), and Horseradish peroxidase (HRP)-conjugated antibodies to goat were purchased from Santa Cruz Biotechnology and Sigma.

Scratch Assay

Cells were seeded in six well plates. On the following day, cells were transfected with various expression plasmids or parental expression vectors using transfection reagent (Fang, 2014). Forty-eight hours after transfection, cells were scratched with a 200 μL pipette tip and the pictures were taken immediately (at 0 h) and the wound gap was observed on the following day at 20 h and cells were photographed (Liang, 2007).

Western Blotting Analysis

Cells were seeded in six well plate or 100 mm dish. On the next day, cells were transfected with various expression plasmids or parental expression vectors using transfection reagent (Fang, 2014). Forty-eight hours after transfection, the cells were washed with cold phosphate-buffered saline (PBS) and the total cell lysates were prepared using a buffer containing 20 mM Hepes (pH 7.5), 100 mM KCl, 0.4 mM EDTA (pH 8.0), 0.2% Igepal CA630, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and PI Cocktail (Roche Complete Mini EDTA-free) one tablet in 10 ml. The cell lysates were homogenized subjected to centrifugation at 13,000 rpm in a bench top Eppendorf centrifuge (Fang, 2014). Protein concentrations of the clear cell lysates were determined using the Bradford kit (Bio-Rad). Proteins were resolved on 4–12% SDSPAGE and transferred to nitrocellulose membranes. The membranes were blocked with membrane blocking solution (Invitrogen). The membranes were incubated with primary antibodies at room temperature for 1 h. After the removal of unbound antibodies, the membranes were washed with PBS three times and then they were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and washed five times with PBST. The antibody-associated protein bands were revealed using the ECL plus system (Amersham Pharmacia Biotech) (Fang, 2014).

RESULTS

Analysis of Open Reading Frames Encoded by pri-miRNA of miR-200a and miR-200b

We have analyzed the open reading frames encoded by pri-miRNA of miR-200a and miR-200b and identified several open reading frames (Fig. 1). Based on their homology with other functional domains of known proteins, we have chosen one open reading frame encoded by each of the pri-miRNA of miR-200a and miR-200b to study their biological functions. To our surprise, we identified 187 amino acid open reading frame in the case of pri-miRNA

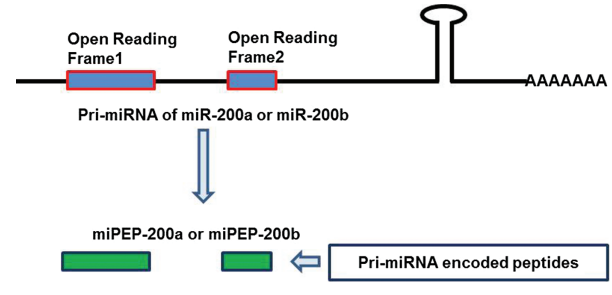

Figure 1. Biogenesis of miPEP-200a and miPEP-200b from pri-miRNA of miR-200a and miR-200b.

of miR-200a. This 187 aa open reading frame showed significant homology to breast tumor suppressor AZU-1 and transforming acidic coiled-coil-containing protein 2 (Chen, 2000; Lappin, 2002) suggesting a potential role to function as a tumor suppressor gene.

We have also chosen 54 aa open reading frame in the case of pri-miRNA of miR-200b. This 54 aa open reading frame showed significant homology to Flavin-containing monooxygenase FMO, Phospholipase D zeta 2 and Endoribonuclease YbeY. Interestingly, Flavin-containing monooxygenase FMO was shown to promote longevity and health span (Leiser, 2015).

Molecular Cloning and Expression of Novel Genes Encoding miPEP-200a and miPEP-200b

We have cloned these open reading frames of pri-miRNA of miR-200a and miR-200b into pcDNA3-HA vector so that they are expressed as HA-tagged peptides or proteins. We have named these open reading frames of pri-miRNA of miR-200a and miR-200b as miPEP-200a and miPEP-200b respectively. We have transfected expression plasmids pcDNA3-HA-miPEP-200a and pcDNA3-HA-miPEP-200b into prostate cancer cells and tested the expression of HA-fusion peptides/proteins of miPEP-200a and miPEP-200b. Our results demonstrate significant expression of miPEP-200a and miPEP-200b in PC3 cells (Fig. 2).

miPEP-200a and miPEP-200b Inhibit the Migration of Cancer Cells by Regulating Vimentin-Mediated Pathway

We have also tested the effect of miPEP-200a and miPEP-200b on the migration of prostate cancer cells using wound healing/scratch assay (Fig. 3). These results demonstrate that miPEP-200a and miPEP-200b inhibit significantly the migration of prostate cancer cells (Figs. 3(A and B)).

In order to understand the role of miPEP-200a and miPEP-200b in regulating the migration of prostate cancer cells, we have also studied the effect of miPEP-200a and miPEP-200b on the expression of key proteins involved in molecular signaling pathways that regulate epithelial to mesenchymal transition (Williams, 2013). Interestingly, our results demonstrate that miPEP-200a and miPEP-200b inhibit significantly the expression of vimentin (Fig. 4).

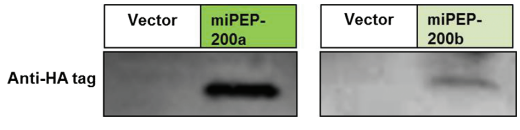

Figure 2. Expression of miPEP-200a and miPEP-200b in prostate cancer cells. PC3 cells were transfected with vector or expression plasmid pcDNA3-HA-miPEP-200a and pcDNA3-HA-miPEP-200b. Total extracts were subjected to HA-affinity matrix followed by Western blot analysis using anti-HA peroxidase.

We also observed slight induction of E-cadherin in the case of miPEP200a but no significant effect on β-catenin expression (Fig. 4).

DISCUSSION

It appears from our results that novel peptides/proteins namely miPEP-200a and miPEP-200b function as cancer cell migration inhibitors by regulating vimentin-mediated pathway. Previously, it was shown that miR-200a and miR-200b also inhibit vimentin-mediated pathway (Ivaska, 2011) and regulate epithelial to mesenchymal transition (Ivaska, 2011). These results suggest that these miPEPs may function by activating miRNAs (miR-200a and miR-200b) like in the case of plant cells (Lauressergues, 2015). In addition to activating miRNAs, it is also possible miPEPs may also act independently (independent of miRNAs) by regulating the key proteins (like vimentin, E-cadherin) involved in signaling pathway responsible for the regulation of migration of cancer cells (Ivaska, 2011). In support of this view, previously, it was shown that Silibinin functions as a strong anti-metastatic agent by inhibiting the expression of vimentin in prostate cancer cells (We, 2009). Irrespective of the mechanism, our results suggest that these pri-miRNA encoded peptides miPEP-200a and miPEP-200b have the potential to be used as anti-metastatic agents and therapeutic agents against many cancers including prostate cancer, breast cancer, lung cancer, renal cancer etc. (Feng, 2014).

Since pri-miRNA of miR-200a and miR-200b are expressed at high levels in normal cells compared to cancer cells or metastatic cells, it is tempting to speculate that these novel peptides/small proteins (miPEP-200a and miPEP-200b) may also serve as diagnostic markers for metastasis of many cancers like their corresponding miRNAs. In addition, since it was observed that low level expression of pri-miRNA of miR-200a and miR-200b were correlated with poor survival, it is possible that the above pri-miRNA encoded peptides/proteins can be used as prognostic markers for cancer patients. Since miPEP-200a and miPEP-200b show homology to tumor suppressors and also to proteins that promote longevity and health span, it is possible that these peptides/small proteins can be used not only as tumor/metastatic suppressors but also to increase longevity and health of mammals and people.

Since our results demonstrate that novel peptides/proteins encoded by mammalian pri-miRNAs (miPEP-200a and miPEP-200b) possess novel biological functions suggesting that it is the time to reconsider the junk DNA (98% of the genome) as a treasured DNA that codes for several thousands of novel and valuable biologically-active peptides/proteins in mammalian cells. It is tempting to speculate that the peptide-RNA world seen in primordial soup that were responsible for creating single cells (Wolfenden, 2015) were retained during the evolution to regulate the growth and development of

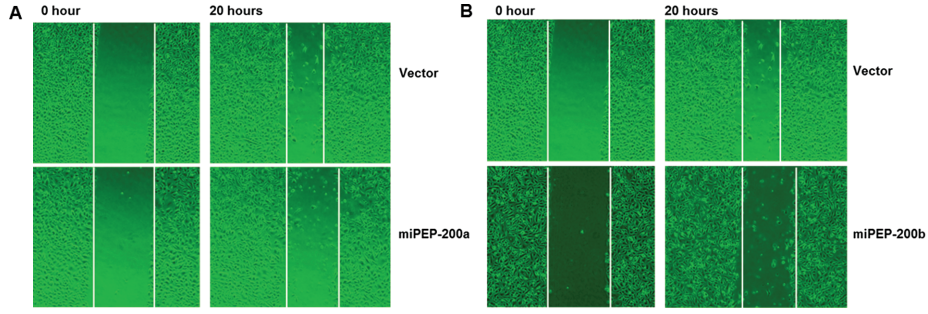

Figure 3. **miPEP-200a and miPEP-200b inhibit the migration of prostate cancer cells. PC3 cells were transfected with vector or expression plasmid (A) pcDNA3-HA-miPEP-200a and (B) pcDNA3-HA-miPEP-200b and the migration was measured in scratch wound assay. Representative pictures of the size of the scratch wound at 0 and 20 hours after wound were shown.**

all human, animal and plant kingdoms and other living creatures. Thus, these results will probably revolutionize our understanding of biology per se.

We propose that noncoding DNA (so called junk DNA representing 98% of the genome) can use its transcriptionally active non-coding RNAs (such as miRNAs and lncRNAs) and also its translatable peptide/proteins as double edged sword to control cell growth and development of not only humans, animals and plants but also all the living organisms on this planet. Thus, these results will probably transform our understanding of biology per se. In addition, it is also the time to rethink about the new role of messenger RNA (mRNA) of known genes as regulators of biological function of cells like in the case of miRNAs and lncRNAs. In future, if one proves that mRNA may act like miRNA or lncRNA, this will totally change our present understanding of the biological function of coding and non-coding DNAs and RNAs.

In summary, if our logical reasoning that pri-miRNA encoded peptides/proteins are present in all mammalian cells (Fig. 5) and they may regulate the growth and development of normal and cancer cells is true, pri-miRNA encoded peptide signatures will be tested in future as novel clinical biomarkers to further subtyping of cancers and their potential for predicting metastasis. In addition, pri-miRNA encoded peptides that function as tumor suppressors or metastatic suppressors can be used as therapeutic agents to target cancer cell growth and their metastatic potential. Therefore, identification of normal and cancer specific pri-miRNA encoded peptides will greatly accelerate early detection of all cancers, diseases and also their treatment. In addition, identification of disease specific pri-miRNA encoded peptides/proteins will also accelerate early detection of other human diseases such as Alzheimer's, Parkinson etc. and also their treatment. These pri-miRNA encoded peptides/proteins can be synthesized and also can be altered so that they function as better therapeutic agents. Thus, these results (proving the existence of pri-miRNA encoded biologically active peptides/proteins in mammalian cells) will revolutionize the biology field per se as they may be involved in growth and development of all humans, animals, plants and other living creatures. Based on our results, we predict that there will be a great number of publications describing novel pri-miRNA encoded peptides/small proteins in eukaryotic and prokaryotic cells will be forth

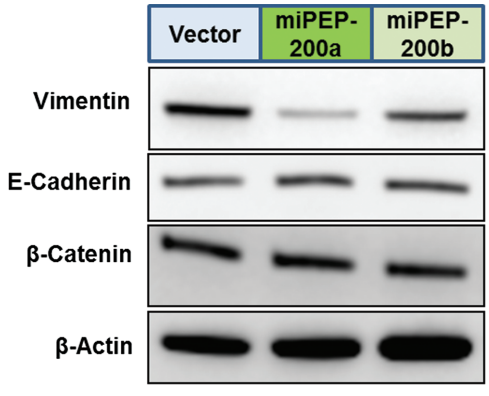

Figure 4. **miPEP-200a and miPEP-200b inhibit the expression of Vimentin in prostate cancer cells. PC3 cells were transfected with vector or expression plasmid pcDNA3-HA-miPEP-200a (A) and pcDNA3-HA-miPEP-200b (B). Total cell extracts were prepared at 48 hours after transfection and were subjected to SDS-PAGE followed by western blot analysis using specific antibodies.**

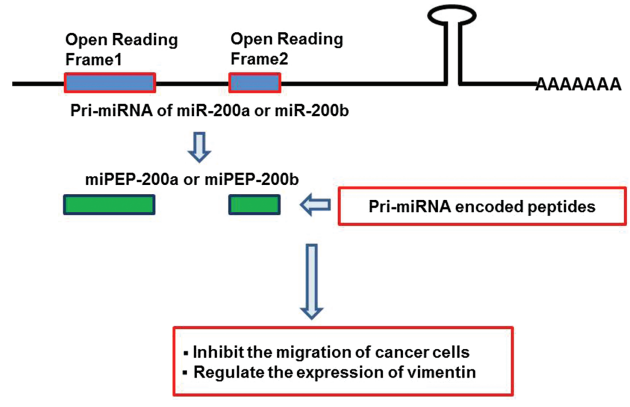

Figure 5. **pri-miRNA encoded peptides miPEP-200a and miPEP-200b and their function.**

coming in future that may change the landscape of biology per se.

List of Abbreviations

miRNA:	microRNA
pri-miRNAs:	Primary microRNA
miPEP:	MicroRNA encoded peptides
ORF:	Open Reading Frame.

Conflict of Interest

We acknowledge that we have no conflict of interest or any financial interest that could influence our work.

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