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14. ABSTRACT We hypothesized that certain miRNA species are differentially expressed in the normal breast epithelium and breast cancer cells. Our concept was that these miRNAs are involved in breast cancer progression by promoting either loss or alternatively an increase (in case of miRNA downregulation) in specific target mRNA translation or stability. In order to determine if certain miRNAs are involved in breast cancer we proposed to: 1. Identify the changes in miRNA expression in primary breast tumors and/or breast cancer cell lines. 2. Establish the alterations in miRNA target(s) expression or translation in breast cancer cells. Accordingly, we investigated miRNA expression in several breast cancer cell lines, including MCF10A. We consistently observed decreased expression of mir-125b and mir-145 miRNA's and increased expression of mir-21 and mir-155. Interestingly, mir-21 was progressively upregulated during oncogenic progression of MCF10A cells. <i>In silico</i> analysis of the potential target mRNAs include oncogenes (fos, N-myc, Fli-1) and cell cycle proteins (cdc2, cyclin D1, wee1), suggesting that miRNAs might regulate expression of these genes in breast cancer cells.					
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INTRODUCTION:

We hypothesized that certain miRNA species are differentially expressed in the normal breast epithelium and breast cancer cells. Our concept was that these miRNAs are involved in breast cancer progression by promoting either loss or alternatively an increase (in case of miRNA downregulation) in specific target mRNA translation or stability.

In order to determine if certain miRNAs are involved in breast cancer we proposed to: 1. Identify the changes in miRNA expression in primary breast tumors and/or breast cancer cell lines. 2. Establish the alterations in miRNA target(s) expression or translation in breast cancer cells.

BODY:

Task1. Identify changes in miRNA expression in primary breast tumors and/or breast cancer cell lines (months 1-12).

Accordingly, we isolated RNAs from human breast cell lines MCF7, BTK20, T47D, HBL100, MDA-MB-231, MDA-MB-468 and MCF10A. Total RNA isolation was done with Trizol (Invitrogen) as described by the manufacturer. RNA labeling and hybridization was done as described previously using commercial kits (Starfire, IDTDNA Inc. or Ambion's MirVANA). We used miRNA microarrays (mirVANA v2, Ambion) to evaluate miRNA expression in seven breast cancer cell lines. As a control, we used RNA from three different batches of normal mammary epithelial cells (HMEC). To identify miRNA with difference in expression between cancer and normal cells, we used ANOVA (at $p < 0.05$).

To confirm results obtained by microarray analysis, we performed Northern blots on some of the differentially expressed miRNAs. In particular, we analyzed mir-145, mir-21, mir-125b and mir-155 expression. As a result, Northern blot confirmed variations in these four micro-RNA expression values. Among those four miRNAs, mir-145 and mir-125b showed decrease of expression and mir-21 and mir-155 showed increase of expression in breast cancer cells in comparison with normal breast epithelium cells (HMEC; fig.1).

When RNAs from 10 primary breast cancer samples were analyzed, the conclusions on mir-145, mir-21 (fig. 2), mir-125b and mir-155 expression remained the same as with breast cancer cell lines, suggesting that observed changes occur in primary tumors and not during cancer cells adaptation to growth in tissue culture.

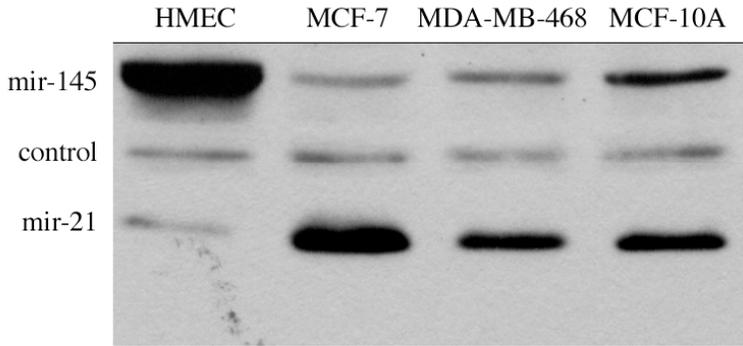


Figure 1. Northern blot analysis of mir-145 and mir-21 expression in normal human mammary epithelial cells (HMEC) and breast cancer cell lines (MCF-7, MDA-MB-468 and MCF-10A).

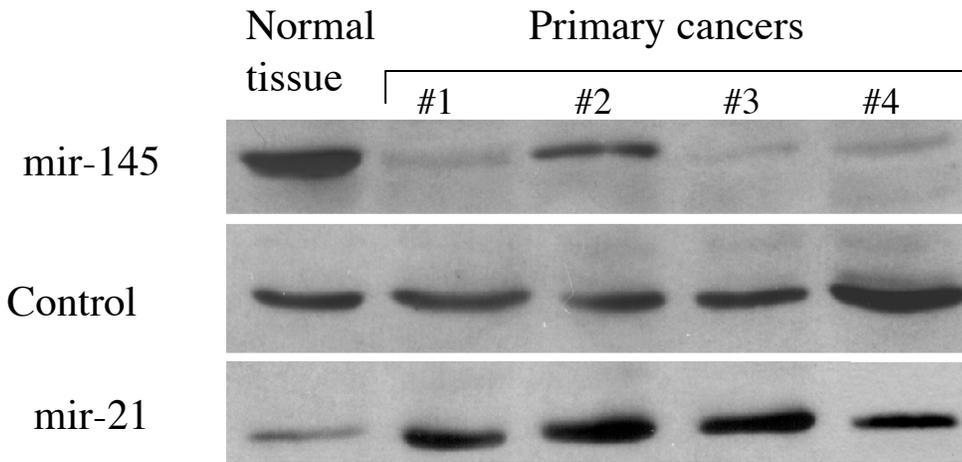
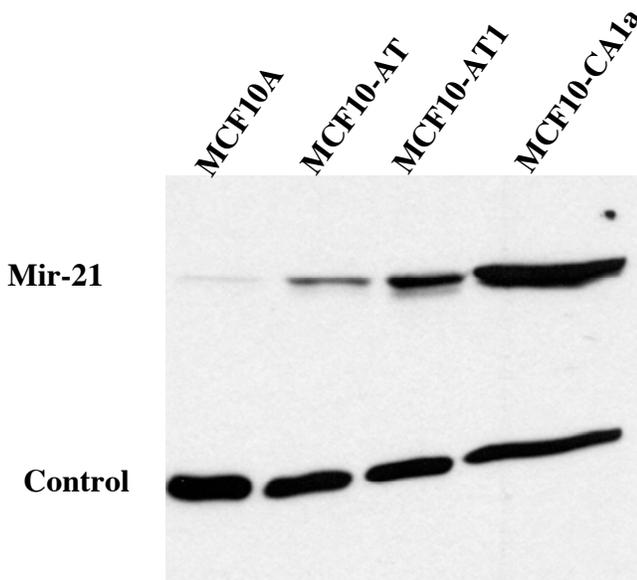


Figure 2. Northern blot analysis of mir-145 and mir-21 expression in normal breast cells and primary breast cancers. Cancer #2-stage I, cancer #1,3,4-stage III.



In additional experiments (Task 1-3), we determined miRNA expression profile in breast cancer cell line MCF10A and its progressively carcinogenic derivatives (MCF-10-AT, MCF10-AT1 and MCF10-CA1a; Fig.3).

Figure 3. Northern blot analysis of mir-21 expression in MCF10 series. MCF10-A-non-transformed variant, MCF10-AT-transformed variant; MCF10-AT1 and MCF10-CA1a-aggressive carcinogenic variants.

As the result, we observed that, as MCF10A cells become more oncogenic, expression of mir-145 stayed low or decreased even more (not shown) while expression of mir-21 progressively increased, becoming even more pronounced in most aggressive variants of MCF10 cell line (Fig. 3).

Task2. Establish alterations in miRNA target(s) expression or translation in breast cancer cells (months 6-12).

We analyzed the predicted targets of the most significantly down- (mir-145 and mir-125b) and up-regulated miRNA (mir-21 and mir-155) to ascertain the potential effects of these miRNAs alterations in expression using three commonly used algorithms: Targetscan, miRanda and PicTar (Lewis et al., 2003; Enright et al., 2004; Krek et al., 2005). We proceeded to identify targets that were identified by at least two out of three programs.

We expected that targets of down-regulated miRNAs might include potential oncogenes and/or other positive growth regulators. Indeed, among putative targets of mir-125b we found oncogenes (Ets1, Akt3 and Yes), growth factor receptor FGFR2 and several members of the mitogen-activated signal transduction pathway (MAPK10, MAPK11, MAPK14). Among predicted targets of mir-145, we found oncogenes FOS, MYCN, YES and FLI1. Additional targets include cell-cycle proteins, such as cyclin D2.

For the up-regulated in breast cancer miRNAs (mir-21 and mir-155) we expected that some negative growth regulators (such as tumor suppressors and negative growth regulators) might be affected. Indeed, we found that known negative growth regulator, TGFB, was a potential target of mir-21. Tumor suppressors APC and SOCS1, and negative cell cycle regulator Wee1 were predicted to be the targets of mir-155. Hypoxia-inducible transcription factor HIF1A was also among mir-155 targets.

Of particular note was the observation that mir-145 was progressively reduced from normal breast to cancers with higher tumor stage (see Fig. 2). Reciprocally, mir-21 was progressively up-regulated in the same samples, suggesting that, perhaps, changes in these two miRNAs regulation contributes to the onset or severity of breast cancer phenotypes.

More recent re-analysis of our data suggested that mir-21 also targets PDCD4, a known human tumor suppressor. In order to investigate (task 2-3) whether mir-21 specifically targets PDCD4, we obtained 2'-O-methoxyethyl phosphorothioate antisense oligoribonucleotides directed against mir-21 (anti-mir-21; Ambion, Inc.) and transfected these oligonucleotides into MCF10-AT cells, expressing moderate to high levels of mir-21. As the result, we observed that inhibition of mir-21 by anti-mir-21 oligonucleotides caused accumulation of putative mir-21 target, PDCD4, suggesting that inhibition of tumor suppressor PDCD4 during progression of MCF10 cell lines toward higher levels of oncogenic transformation is at least in part caused by mir-21 (Fig. 4).

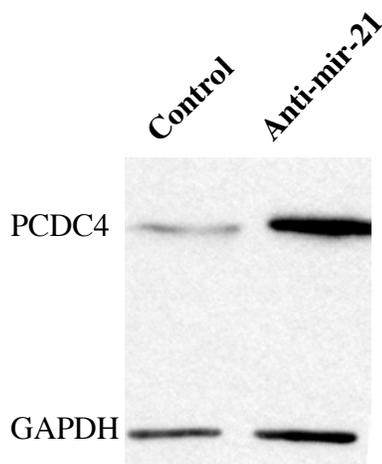


Figure 4. Western blot analysis of PDCD4 or GAPDH (loading control) expression in MCF10-AT cells following transfection with anti-mir-21.

KEY RESEARCH ACCOMPLISHMENTS:

1. MicroRNAs mir-145 and mir-125b were identified as being downregulated in breast cancer cells.
2. MicroRNAs mir-21 and mir-155 were identified as being upregulated in breast cancer cells.
3. Oncogenes, growth control and cell cycle proteins were identified as potential targets of miRNAs affected in breast cancer.
4. Observed expression of mir-21 result in specific decrease in certain tumor suppressors, such as PDCD4.

REPORTABLE OUTCOMES:

1. ABSTRACT presented at the 70th Cold Spring Harbor Symposium on "Molecular approaches to controlling cancer", 2005.
2. NIH RO1-A1 grant "Seladin-1 in RAS induced senescence and tumorigenesis" applied for in March of 2006.
3. Abstract submitted to Era of Hope Meeting, 2008

CONCLUSIONS:

1. MicroRNAs mir-145 and mir-125b are downregulated in breast cancer cells.
2. MicroRNAs mir-21 and mir-155 are upregulated in breast cancer cells.
3. Among mir-145 and mir-125b targets are oncogenes and positively acting growth regulators.

Individuals receiving pay from this Award:

Konstantin Galaktionov, PhD

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SELADIN-1 MUTATION ASSOCIATED WITH MULTIPLE HUMAN TUMORS

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Expression of multiple oncogenes and inactivation of tumor suppressors is required to transform primary mammalian cells into cancer cells. Activated Ha-*RasV12* (Ras) is usually associated with cancer, but it also produces paradoxical premature senescence in primary cells by inducing reactive oxygen species followed by accumulation of tumor suppressors p53 and p16^{INK4a}. We identified, using a direct genetic screen, *Seladin-1* as a key mediator of Ras-induced senescence (Wu *et al.* 2004, *Nature* 432, 640). Following oncogenic and oxidative stress, Seladin-1 binds p53 amino terminus and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. This suggested that *Seladin-1* is a potential human tumor suppressor.

In order to test this hypothesis, we performed the sequence analysis of coding exons of Seladin-1 in 97 human sporadic cancers of various types. As the result, we observed that approximately 21% of all tested patients (20 out of 97) had the mutation at the single common site, suggesting that *Seladin-1* function is frequently affected in patients with multiple major tumor types. No known SNPs exist at this location and those were not detected at this site in 64 randomly chosen unaffected donors. The cancer mutation data and the significance of this mutation for Seladin-1 function will be presented.

The remarkably high frequency of the *Seladin-1* single-site mutation in patients developing multiple human tumors suggests a potentially unique requirement for, or a mechanism of, *Seladin-1* mutation in human cancers in comparison with the previously described tumor suppressors.

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We hypothesized that certain miRNA species are differentially expressed in the normal breast epithelium and breast cancer cells. Accordingly, we isolated RNAs from human breast cell lines MCF7, BTK20, T47D, HBL100, MDA-MB-231, MDA-MB-468 and MCF10A. Total RNA isolation was done with Trizol (Invitrogen) as described by the manufacturer. RNA labeling and hybridization was done as described previously using commercial kits (Starfire, IDTDNA Inc. or Ambion's MirVANA). We used miRNA microarrays (mirVANA v2, Ambion) to evaluate miRNA expression in seven breast cancer cell lines. As a control, we used RNA from three different batches of normal mammary epithelial cells (HMEC). To identify miRNA with difference in expression between cancer and normal cells, we used ANOVA (at $p < 0.05$). To confirm results obtained by microarray analysis, we performed Northern blots on some of the differentially expressed miRNAs. In particular, we analyzed mir-145, mir-21, mir-125b and mir-155 expression. As a result, Northern blot confirmed variations in these four micro-RNA expression values. Among those four miRNAs, mir-145 and mir-125b showed decrease of expression and mir-21 and mir-155 showed increase of expression in breast cancer cells in comparison with normal breast epithelium cells (HMEC). When RNAs from ten primary breast cancer samples were analyzed, the conclusions on mir-145, mir-21, mir-125b and mir-155 expression remained the same as with breast cancer cell lines, suggesting that observed changes occur in primary tumors and not during breast cancer cells adaptation to growth in tissue culture. We analyzed the predicted targets of the most significantly down- (mir-145 and mir-125b) and up-regulated miRNA (mir-21 and mir-155) to ascertain the potential effects of these miRNAs alterations in expression using three commonly used algorithms: Targetscan, miRanda and PicTar (Lewis et al., 2003; Enright et al., 2004; Krek et al., 2005). We proceeded to identify targets that were identified by at least two out of three programs. We expected that targets of down-regulated miRNAs might include potential oncogenes and/or other positive growth regulators. Indeed, among putative targets of mir-125b we found oncogenes (Ets1, Akt3 and Yes), growth factor receptor FGFR2 and several members of the mitogen-activated signal transduction pathway (MAPK10, MAPK11, MAPK14). Among predicted targets of mir-145, we found oncogenes FOS, MYCN, YES and FLI1. Additional targets include cell-cycle proteins, such as cyclin D2. For the upregulated in breast cancer miRNAs (mir-21 and mir-155) we expected that some negative growth regulators (such as tumor suppressors and negative growth regulators) might be affected. Indeed, we found that known negative growth regulator, TGF- β , was a potential target of mir-21. Tumor suppressors APC and SOCS1, and negative cell cycle regulator Wee1 were predicted to be the targets of mir-155. Hypoxia-inducible transcription factor HIF1A was also among mir-155 targets. Of particular note was the observation that mir-145 was progressively reduced from normal breast to cancers with higher tumor stage. Reciprocally, mir-21 was progressively upregulated in the same samples, suggesting that, perhaps, changes in these two miRNAs regulation contributes to the onset or severity of breast cancer phenotypes.

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