miR-155 Drives Telomere Fragility in Human Breast Cancer by Targeting TRF1

Roberto Dinami, Cristiana Ercolani, Eleonora Petti, Silvano Piazza, Yari Ciani, Rosanna Sestito, Andrea Sacconi, Francesca Biagioni, Carlos le Sage, Reuven Agami, Roberta Benetti, Marcella Mottolese, Claudio Schneider, Giovanni Blandino, and Stefan Schoeftner

Abstract

Telomeres consist of DNA tandem repeats that recruit the multiprotein complex shelterin to build a chromatin structure that protects chromosome ends. Although cancer formation is linked to alterations in telomere homeostasis, there is little understanding of how shelterin function is limited in cancer cells. Using a small-scale screening approach, we identified miR-155 as a key regulator in breast cancer cell expression of the shelterin component TERF1 (TRF1). miR-155 targeted a conserved sequence motif in the 3'UTR of TRF1, resulting in its translational repression. miR-155 was upregulated commonly in breast cancer specimens, as associated with reduced TRF1 protein expression, metastasis-free survival, and relapse-free survival in estrogen receptor–positive cases. Modulating miR-155 expression in cells altered TRF1 levels and TRF1 abundance at telomeres. Compromising TRF1 expression by elevating miR-155 increased telomere fragility and altered the structure of metaphase chromosomes. In contrast, reducing miR-155 levels improved telomere function and genomic stability. These results implied that miR-155 upregulation antagonizes telomere integrity in breast cancer cells, increasing genomic instability linked to poor clinical outcome in estrogen receptor–positive disease. Our work argued that miRNA-dependent regulation of shelterin function has a clinically significant impact on telomere function, suggesting the existence of “telo-miRNAs” that have an impact on cancer and aging.

Introduction

Vertebrate telomeres are composed of TTAGGG tandem repeats that recruit the shelterin complex, which protects chromosomes ends, regulates telomere length, recombination, and DNA damage checkpoints. Telomeron is composed of telomere-binding proteins, called shelterin components, which interact with POT1 via interaction with TIN2 and TPP1. TRF1, TRF2, POT1, TPP1, TIN2, and RAP1 bind to telomeres via their interaction with TRF2 (8). TRF1 and its paralog TRF2 bind to double-stranded TTAGGG repeats and interact with POT1 via interaction with TIN2 and TPP1 (2–4). POT1 interacts with single-stranded TTAGGG repeats in the telomeric 3' overhang and, together with TRF1 and TRF2, ensures the protection of chromosome ends by repressing DNA damage signaling by the ATR and ATM kinases (5–7). RAP1 binds to telomeres via its interaction with TRF2 (8). TRF1 has been shown to contribute to telomere length regulation and suppresses DNA breakage at TTAGGG repeats under replicative stress, a phenomenon described as telomere fragility (5, 6, 9, 10). Consistent with this, loss of TRF1 results in telomere replication errors and the activation of ATR signaling in S-phase (5, 6). Recently, evidence is accumulating that links altered shelterin function with human cancer. POT1 mutations have been linked to telomeric and chromosomal abnormalities in chronic lymphocytic leukemia (11). Wnt/β-catenin signaling was shown to drive TRF2 expression, improving telomere function in cancer cells (12). In addition, TRF2 protein levels were shown to be controlled by the p53-inducible E3 ubiquitin ligase Siah1, linking shelterin function with the p53 tumor suppressor pathway (13). Importantly, mice deficient for TRF1 display telomere fragility and increased cancer formation in the absence of p53. This indicates that reduced TRF1 expression can enhance cancer formation by driving telomere fragility and genomic instability (6). Together, this suggests that shelterin function is intertwined with central pathways in tumorigenesis and tumor suppression. miRNAs are 20–23 nucleotide, single-stranded RNA molecules that control gene expression by reducing the translation or stability of target mRNAs (14). Extensive studies demonstrated that miRNAs control the expression of crucial tumor suppressors or oncogenes and propagate essential features of cancer progression.

Authors’ Affiliations: 1Telomeres in Cancer and Aging Unit, 2Bioinformatics and Functional Genomics Unit, Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie (LNCIB); 3Dipartimento di Scienze della Vita, SDBM School of Molecular Biomedicine (SDBM), Università degli Studi di Trieste, Trieste; 4Telomeres in Cancer and Aging Unit, 5Translational Oncogenomics Unit, and 6Department of Pathology, Regina Elena National Cancer Institute, Rome; 7Dipartimento di Scienze Mediche e Biologiche, Università degli Studi di Udine, Udine, Italy; and 8Division of Gene Regulation, The Netherlands Cancer Institute, Amsterdam, the Netherlands

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for F. Biagioni: European Institute of Oncology (IEO) Via Adamello, 16, Milan 20139, Italy.

Corresponding Author: Stefan Schoeftner, Telomeres in Cancer and Aging Unit, Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie (LNCIB); Padriciano 99, Trieste 34148, Italy. Phone: 39-4037-58825; Fax: 390-4039-8990; E-mail: stefan.schoeftner@lncib.it

doi: 10.1158/0008-5472.CAN-13-2038
©2014 American Association for Cancer Research.
(15). Importantly, miRNA expression signatures have been established as efficient prognostic and predictive biomarkers, underlining the clinical relevance of miRNAs (15). Although telomere function is a central aspect in cancer and aging, miRNAs that modulate the expression of shelterin components are to date not known. Of interest, a recent study links miR-138 with telomerase (TERT) expression; however, the impact of miR-138 on telomere homeostasis is not known (16).

Here, we performed a small-scale screen to identify clinically relevant miRNAs that modulate telomere function in human breast cancer by targeting the expression of TRF1. We found that the oncomiR miR-155 targets a conserved sequence motif in the 3′UTR of TRF1, resulting in reduced protein expression. miR-155 is efficiently upregulated in human breast cancer specimen, correlates with reduced TERT protein levels, and is linked with poor prognosis in estrogen receptor (ER)–positive breast cancer. On the mechanistic level, we demonstrate that miR-155-dependent reduction of TERT expression results in telomere elongation, increased telomere damage, increased telomere fragility, and chromosome instability. In contrast, reducing miR-155 expression improves telomere function and genomic stability. Together, our data identify miR-155 as clinically relevant, novel telomere regulator that drives telomere fragility and genomic instability by repressing TERT expression. Our work introduces miRNAs as modulators of shelterin function, anticipating the existence of additional "telo-miRNAs" that link telomere function with fundamental processes in telomere-related diseases such as cancer and organismal aging.

Materials and Methods

Cell lines and culture

Cell lines used were obtained from ATCC and have not been cultured for longer than 6 months. MCF-7 (Michigan Cancer Foundation-7, breast adenocarcinoma), SK-BR-3 (breast adenocarcinoma derived), and HCT-116 (colorectal carcinoma) cells were cultured in RPMI-1640, 10% heat-inactivated FBS (Gibco). MDA-MB-468 (breast adenocarcinoma), HeLa (cervix adenocarcinoma), U-2 OS (osteosarcoma), and H1299 (carcinoma; non–small cell lung cancer) cells were cultured in DMEM, 10% heat-inactivated FBS. Stable MCF-7 and SK-BR-3 cells overexpressing miR-155 were selected with blasticidin (5 μg/mL). Treatments: Aphidicolin (Sigma) for 20 hours, 0.4 μmol/L; MG-132 (Millipore) for 8 hours, 12.5 μmol/L.

Luciferase reporter screening

HeLa cells were cotransfected with 3’UTR luciferase reporter plasmids (18 ng) and miRNA expression vectors (80 ng) using Lipofectamine 2000 (Life Technologies). Seventy-two hours posttransfection, Renilla/Firefly luciferase reporter activity was assayed using a Dual Luciferase Reporter Assay System (Promega) and a GloMax 96 Microplate Luminometer (Promega). A Student t test was used to calculate statistical significance.

Protein extracts and Western blotting

Whole-cell lysates were prepared using a modified RIPA buffer (20 mmol/L Tris-HCl (pH 7.5) 350 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin). Preparation of nuclear extracts: cells were resuspended in Buffer 1 [20 mmol/L HEPES-KOH (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2% NP40] and incubated for 10 minutes on ice. Nuclei were pelleted and lysed in Buffer 2 [20 mmol/L HEPES-KOH (pH 7.9), 350 mmol/L NaCl, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 10% glycerol, 1 mmol/L dithiothreitol]. All samples contained complete protease inhibitor (Roche) and were sonicated followed by centrifugation. Supernatants were recovered and used for Western blotting according to standard procedures. Used primary antibodies are listed in Supplementary Material and Methods.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde (PFA), followed by treatment with citrate buffer [0.1% (w/v), 0.05% Triton X-100] for 10 minutes at room temperature. Cells were blocked for 30 minutes in 3% BSA (1× PBS) and incubated with mouse anti-TERT [Santa Cruz (N-19) sc-6165-R], mouse anti-TRF2 [Millipore (4A794) 05-521], or rabbit anti-POT1 (Epitomics 5334-1) antibodies in 3% BSA (1× PBS), 0.1% Tween-20 for 1 hour at room temperature. Cells were washed in 0.3% BSA (1× PBS), 0.1% Tween-20 and incubated with secondary antibodies. At least 20 DAPI (Vector Laboratories) stained nuclei were used for spot IOD analysis (TFL-TELO Software). A Student t test was used to calculate statistical significance.

Microscopy

Cells subjected to immunofluorescence, telomere DNA FISH, or immunofluorescence combined with telomere DNA FISH were analyzed using a Zeiss Axiovert 200 M microscope, equipped with a Zeiss AxioCam MRm digital camera. Images were captured using AxioVision Rel. 4.8 imaging software.

Telomere DNA FISH

Preparation of metaphases and telomere DNA FISH was performed as previously described (17). At least 20 metaphases were analyzed for metaphase chromosome aberrations (TFL-TELO software). For quantitative telomere DNA FISH analysis, at least 20 interphase nuclei were analyzed using spot IOD analysis (TFL-TELO Software). The Student t test was used to calculate statistical significance.

Immunofluorescence combined with telomere DNA FISH

For immunofluorescence, cells were fixed in 4% PFA, followed by treatment with 0.1% Triton X-100 in 1× PBS for 10 minutes at room temperature. Cells were blocked for 30 minutes in 3% BSA (1× PBS) and incubated with a mouse anti-phospho-γH2AX (S139) antibody (clone JBW301, Millipore, 06-570) in 3% BSA, 0.1% Tween-20 at room temperature for 1 hour. After incubation with secondary antibodies, cells were fixed in 1% PFA and subjected to standard telomere DNA FISH (18). Nuclei showing at least three telomere-γH2AX colocalization events were considered for the quantification (minimum 40 nuclei). The Student t test was used to calculate statistical significance.
Breast cancer specimen collection and immunohistochemistry

TRF1 expression was analyzed by immunohistochemistry (IHC) on formalin-fixed paraffin-embedded tissues using the monoclonal antibody (TRF-78, #ab10579, Abcam). Two micron–thick sections were stained with a streptavidin-enhanced immunoperoxidase technique (Supersensitive Multilink, Menarini) using a pH 6 citrate buffer antigen retrieval protocol. TRF1 was considered as positive when at least 20% of neoplastic cells showed nuclear immunoreactivity. Staining was classified as negative, score 0; low, score 1+; medium, score 2+, and high, score 3+.

Bioinformatics on clinical data from breast cancer patients

Raw data were retrieved from the gene expression omnibus (GEO) public gene expression database (GSE7390, GSE3494, GSE1456, GSE2034, GSE2603, GSE6532, GSE4922, GSE12093, GSE5327, GSE11121, and Chin dataset). To validate the correlation of TRF1 expression and the mir-155 target gene expression signature and breast cancer clinical data, a Mantel–Haenszel test was applied to the meta-dataset. Kaplan–Meier survival curve of distant metastasis-free survival and relapse-free survival (DMFS_mixed) was obtained using the GOBO tool.

Results

A candidate approach to identify miRNAs with targeting specificity for TRF1

TRF1 is a telomere repeat binding protein that acts as a negative regulator of telomere length, contributes to mitotic stability, and protects from replication-associated DNA damage (5, 6, 9, 10, 19). Using a panel of human cancer cell lines, we found evidence for divergent posttranscriptional regulation of gene expression of TRF1 and TRF2: in contrast to TRF2, TRF1 protein levels significantly diverge from mRNA levels (Fig. 1A–D). This indicates that TRF1 is subjected to efficient posttranscriptional gene regulation. To identify miRNAs that control TRF1 expression on the posttranscriptional level, we performed computational target prediction analysis to identify miRNAs with in silico target specificity for the 3’UTR of TRF1 (Supplementary Tables S1–S3). To validate targeting, luciferase reporter vectors were generated by fusing a Renilla luciferase cassette to the 3’UTR of human TRF1. HeLa cells were cotransfected with the TRF1-3’UTR luciferase reporter and 23 candidate miRNA expression vectors, obtained from a miRNA expression vector library (Fig. 1E; ref. 20). Luminometry measurements 3 days posttransfection revealed that miR-155, miR-125b1, miR-296, and miR-330 significantly reduced luciferase reporter activity (Fig. 1F). miR-125b1, miR-296, and miR-330 also reduced luciferase activity of an unrelated TRF2-3’UTR reporter construct, indicating nonspecific miRNA–luciferase reporter interaction (Supplementary Fig. S1A–S1C). In contrast, ectopic expression of miR-155 does not impact on TRF2-3’UTR or TERT-3’UTR reporter activity (Fig. 1G).

Clinical relevance of TRF1 targeting by miR-155 in breast cancer

MiR-155 is a reported "oncomiR" that targets multiple cancer relevant genes to promote neoplastic disease, including breast cancer (22, 23). We consequently were interested in demonstrating that miR-155-dependent regulation of TRF1 is clinically relevant in human breast cancer. Consistent with previous reports, we found a significant upregulation of miR-155 expression in not only luminal but also HER2+ triple-negative
Figure 1. A luciferase reporter screen identified miR-155 to target the 3’ UTR of TRF1. A, Western blotting for TRF1 (left) and quantification (right) in a panel of human cancer cell lines. B, quantitative real-time PCR for TRF1. C, Western blotting for TRF2 (left) and quantification (right) in a panel of human cancer cell lines. D, quantitative real-time PCR for TRF2, normalized to actin. E, strategy of luciferase reporter screen. F, luciferase reporter assay. Renilla: Firefly luciferase ratios <100 indicate miRNA-dependent targeting of the TRF1 reporter. Mouse Pax9 3’ UTR and miR206 were used as positive controls. G, luciferase reporter assays involving miR-155 and TRF1, TRF2, or TERT 3’ UTR reporter constructs. H, TRF1 Western blotting of HeLa cells transiently transfected with the miR-155 expression vector. Arrowheads, specific band for TRF1, L.E., long exposure; n, number of independent experiments. A Student t test was used to calculate statistical significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
breast cancer specimen when compared with their respective peritumoral tissue (Fig. 3A; Supplementary Table S6; refs. 24–26).

To obtain information on a possible clinical relevance of miR-155-dependent regulation of TRF1 expression, we used breast cancer gene expression datasets obtained from a total of 1,881 patients (27). Kaplan–Meier survival analysis revealed that low TRF1 mRNA expression is linked to reduced distant metastasis-free survival and relapse-free survival (P = 0.0055) of patients with ER-negative (ER−) breast cancer (Fig. 3B). Furthermore, multivariate analysis revealed that TRF1 acts as independent predictor of clinical outcome in ER− breast cancer with respect to standard clinical parameters such as lymph node status, tumor size, or age (Fig. 3C). This underlines that low TRF1 expression predicts poor clinical outcome in ER− breast cancer.

Importantly, we found that poor prognosis of patients with breast cancer characterized by low TRF1 expression was recapitulated by panel of 20 experimentally validated miR-155 target genes (Supplementary Table S5; Supplementary Fig. S3; ref. 23). This suggests that TRF1 is part of a miR-155 signature that predicts poor survival in ER− breast cancer. Of note, TRF1 does not affect distant metastasis-free survival and relapse-free survival of other breast cancer subtypes (data not shown).
Figure 3. Clinical relevance of miR-155 and TRF1 in human breast cancer. A, box plots showing miR-155 upregulation in breast cancer compared with peritumoral tissues, as determined by miRNA expression array analysis described in ref. 28. P values were calculated using the Fisher method. B, Kaplan-Meier survival curve of time considering both distant metastasis-free survival and relapse-free survival (DMFS_mixed) of patients with ER+ breast cancer classified according to the expression of TRF1 (Supplementary Material and Methods). Red line, high TRF1 mRNA expression; gray line, low expression of TRF1. C, multivariate analysis showing that low TRF1 expression behaves as independent predictor of poor clinical outcome. In the panel for each clinical variable and TRF1 expression, HRs and corresponding P values calculated by Cox regression analysis are shown. D, Immunohistochemistry for TRF1 on luminal breast cancer samples (high TRF1, top panel) used for miRNA expression studies in A. Scale bar, 30 μm. E, proportion of TRF1 staining intensities in samples analyzed in D. F, luminal cancer samples with significantly increased miR-155 expression show reduced TRF1 staining in IHC performed in D. A Pearson χ² test was used to calculate statistical significance. G, TRF1 expression is miR-155-dosage sensitive in SK-BR-3, MCF-7, and MDA-MB-468 breast cancer cell lines, as determined by Western blotting. n, number of patients.
To obtain biologic evidence that confirms in silico data, we performed IHC for TRF1 on four normal breast tissue samples and 37 ER+ luminal cancer samples previously assayed for miR-155 expression (Supplementary Table S6; Fig. 3A; ref. 28). All control and 28% of breast cancer samples were classified as TRF1 high. Seventy-two percent of cancer samples were classified medium-low or low-negative for TRF1 (33% and 39%, respectively), indicating that downregulation of TRF1 is a frequent event in luminal breast cancer (Fig. 3D and E). Importantly, 83% of specimens with reduced TRF1 protein expression demonstrate miR-155 upregulation (P = 0.0034), supporting a role for miR-155 in controlling TRF1 expression in human breast cancer (Fig. 3F). Finally, ectopic introduction of miR-155 in human luminal SK-BR-3 and MCF-7 and basal MDA-MB-468 breast cancer cells that express similar levels of endogenous miR-155 resulted in reduced TRF1 expression (Fig. 3G and Supplementary Fig. S4A). In contrast, introduction of antago-miR-155 increases TRF1 expression in all breast cancer lines tested (Fig. 3G). Our data indicate that TRF1 expression is miR-155 dosage sensitive in breast cancer cells. Reverse correlation of miR-155 and TRF1 expression and poor prognosis of patients with low expression of TRF1 or a miR-155 target gene signature underline the clinical relevance of miR-155-dependent regulation of TRF1 in ER+ breast cancer.

miR-155 alters telomere structure and function

We next wished to link miR-155 to molecular pathways that modulate TRF1-related aspects of telomere function. To test whether alteration of miR-155 expression levels does not only impact on global TRF1 expression levels but also alter TRF1 abundance at telomeres, we transiently transfected SK-BR-3 luminal breast cancer cells with mimic-miR-155, antago-miR-155 siRNAs, or TRF1-specific siRNAs and performed quantitative immunofluorescence analysis using anti-TRF1 antibodies. We found that ectopically introduced miR-155 significantly reduced fluorescence signal intensity, indicative for a reduced abundance of TRF1 at telomeres (Fig. 4A). This result is also reflected by the appearance of an increased proportion of telomeres with low TRF1 loading; this is indicative for a reduced abundance of TRF1 at telomeres (Fig. 4B). This result was recapitulated when TRF1 expression was reduced by the stable overexpression of miR-155 in long-term experiments using SK-BR-3 cells (Supplementary Fig. S5A–S5E). This indicates that miR-155 promotes telomere elongation by reducing abundance of TRF1 at telomeres. Loss of TRF1 is associated with impaired telomere function, leading to the elicitation of a DNA damage response at telomeres (5, 6). In line with this, we found by Western blotting that transient transfection of luminal MCF-7 and SK-BR-3 breast cancer cell lines with mimic-miR-155 siRNAs result in elevated levels of γH2AX, indicative for the activation of a DNA damage response (Fig. 4C and D, left). DNA damage response was associated with the activation of wild-type p53 in MCF-7 cells, as indicated by increased levels of phospho-p53 (Ser15) (Fig. 4C and D, left). In SK-BR-3 cells, which express mutant p53, introduction of miR-155 was not able to significantly increase phosphorylation of p53. This is presumably due to the high basal levels of phospho-p53 (Ser15) present in this cell line (Fig. 4D). In line with results from TRF1 knockout mice, we found that miR-155-dependent reduction of TRF1 expression leads to the phosphorylation of ATM and ATR and the downstream DNA damage checkpoint kinases Chk1 and Chk2 (Fig. 4C and D, right). To test whether telomere dysfunction contributes to the increased DNA damage load in the context of ectopic miR-155, we performed γH2AX immunofluorescence combined with telomere DNA FISH. Consistent with a proposed role in controlling telomere protection, we found that ectopic introduction of miR-155 increased the abundance of γH2AX localized at telomeres in MCF-7 and SK-BR-3 cells (Fig. 4E and F). This effect was recapitulated by RNAi-mediated depletion of TRF1 and in long-term experiments using MCF-7 cells stably overexpressing miR-155 (Supplementary Fig. S6A–S6D). Importantly, introduction of antago-miR-155 reduces basal levels of telomere dysfunction in MCF-7 and SK-BR-3, indicating that chromosome end protection is miR-155 dosage sensitive (Fig. 4E and F). Together, these data indicate that miR-155 affects telomere length regulation and telomere capping by modulating the expression of TRF1.

miR-155 drives telomere fragility and chromosome instability in human breast cancer

Studies using genetic mouse models revealed that TRF1 suppresses telomere fragility under replicative stress conditions (5, 6). Fragile sites are enriched in a sequence context that challenges DNA replication. Fragility can lead to chromosome breakage and was demonstrated to drive genomic instability at sites that are subjected to amplifications or deletions, suggesting an important link between chromosome fragility and cancer formation (29). The potential of telomeric TTAGGG tandem repeats to generate guanine quadruplex (G4) DNA structures and form the so-called T-Loop structure by invasion of the 3' single-stranded telomeric DNA into the telomere duplex imposes stress to DNA replication, leading to telomere fragility (30, 31). Telomere fragility results in DNA breaks and loss of telomere DNA, leading to multiple telomere signals at the end of metaphase chromosomes (Fig. 5A). In line with a role for TRF1 in suppressing telomere fragility, we found that transient transfection of mimic-miR-155 siRNAs increased telomere fragility in SK-BR-3 cells (Fig. 5B). Targeting
**TRF1 fluorescence intensity (a.f.u.)**

- **Control**
- **miR-155**
- **Antago-miR-155**
- **siTRF1**

<table>
<thead>
<tr>
<th>Group</th>
<th>TRF1 a.f.u. intensity categories (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 8.36</td>
</tr>
<tr>
<td>miR-155</td>
<td>69.1 ± 28.37</td>
</tr>
<tr>
<td>Antago-miR-155</td>
<td>10.8 ± 5.18</td>
</tr>
<tr>
<td>siTRF1</td>
<td>2.0 ± 0.84</td>
</tr>
</tbody>
</table>

**Telomere a.f.u intensity categories (%)**

- **Control**
- **miR-155**
- **Antago-miR-155**
- **siTRF1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Telomere a.f.u. intensity categories (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 8.36</td>
</tr>
<tr>
<td>miR-155</td>
<td>69.1 ± 28.37</td>
</tr>
<tr>
<td>Antago-miR-155</td>
<td>10.8 ± 5.18</td>
</tr>
<tr>
<td>siTRF1</td>
<td>2.0 ± 0.84</td>
</tr>
</tbody>
</table>

**Telomere fluorescence units (a.f.u.)**

- **Control**
- **miR-155**
- **Antago-miR-155**
- **siTRF1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Telomere fluorescence units (a.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 8.36</td>
</tr>
<tr>
<td>miR-155</td>
<td>69.1 ± 28.37</td>
</tr>
<tr>
<td>Antago-miR-155</td>
<td>10.8 ± 5.18</td>
</tr>
<tr>
<td>siTRF1</td>
<td>2.0 ± 0.84</td>
</tr>
</tbody>
</table>

**γH2AX Merge**

- **Control**
- **miR-155**
- **Antago-miR-155**
- **siTRF1**

<table>
<thead>
<tr>
<th>Group</th>
<th>γH2AX Merge (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 8.36</td>
</tr>
<tr>
<td>miR-155</td>
<td>69.1 ± 28.37</td>
</tr>
<tr>
<td>Antago-miR-155</td>
<td>10.8 ± 5.18</td>
</tr>
<tr>
<td>siTRF1</td>
<td>2.0 ± 0.84</td>
</tr>
</tbody>
</table>

**MCF-7**

- **Control**
- **miR-155**
- **Antago-miR-155**
- **siTRF1**

<table>
<thead>
<tr>
<th>Group</th>
<th>TIF frequency (&gt;3 TIFs; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 8.36</td>
</tr>
<tr>
<td>miR-155</td>
<td>69.1 ± 28.37</td>
</tr>
<tr>
<td>Antago-miR-155</td>
<td>10.8 ± 5.18</td>
</tr>
<tr>
<td>siTRF1</td>
<td>2.0 ± 0.84</td>
</tr>
</tbody>
</table>

**SK-BR-3**

- **Control**
- **miR-155**
- **Antago-miR-155**
- **siTRF1**

<table>
<thead>
<tr>
<th>Group</th>
<th>TIF frequency (&gt;3 TIFs; %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 8.36</td>
</tr>
<tr>
<td>miR-155</td>
<td>69.1 ± 28.37</td>
</tr>
<tr>
<td>Antago-miR-155</td>
<td>10.8 ± 5.18</td>
</tr>
<tr>
<td>siTRF1</td>
<td>2.0 ± 0.84</td>
</tr>
</tbody>
</table>

**P-values**

- **Control vs. miR-155**
P = 0.0001
- **miR-155 vs. Antago-miR-155**
P = 0.0001
- **Antago-miR-155 vs. siTRF1**
P = 0.0001
- **Control vs. miR-155**
P = 0.0001
- **miR-155 vs. Antago-miR-155**
P = 0.0001
- **Antago-miR-155 vs. siTRF1**
P = 0.0001
- **Control vs. miR-155**
P = 0.0001
- **miR-155 vs. Antago-miR-155**
P = 0.0001
- **Antago-miR-155 vs. siTRF1**
P = 0.0001
- **Control vs. miR-155**
P = 0.0001
- **miR-155 vs. Antago-miR-155**
P = 0.0001
- **Antago-miR-155 vs. siTRF1**
P = 0.0001
- **Control vs. miR-155**
P = 0.0001
- **miR-155 vs. Antago-miR-155**
P = 0.0001
- **Antago-miR-155 vs. siTRF1**
P = 0.0001
endogenous miR-155 by transfecting antago-miR-155 resulted in a significant suppression of telomere fragility. Increased telomere fragility was recapitulated by classic RNAi-mediated depletion of TRF1 and by stable overexpression of a miR-155 mini gene in SK-BR-3 cells (Supplementary Fig. S7A and S7B). Together, this indicates that telomere fragility is miR-155 dosage sensitive. We next wished to exclude that alternative miR-155 targets drive TRF1-independent telomere fragility. Cotransfection of SK-BR-3 cells with a mix of mimic-miR-155 and TRF1-specific siRNAs did not augment telomere fragility induced by mimic-miR-155 siRNAs, suggesting that miR-155 and TRF1 siRNA act on the same pathway controlling telomere fragility (Fig. 5C). Furthermore, we were able to rescue increased telomere fragility induced by ectopic miR-155 in SK-BR-3 cells when miR-155 was cotransfected with an expression vector encoding Flag-tagged TRF1 lacking the respective 3’UTR (Fig. 5D and E). Finally, miR-155 target prediction analysis ( TargetsCan) did not reveal target sites in the 3’ UTR of reported suppressors of telomere fragility ( Supplementary Table S4B). Together, this demonstrates that miR-155 drives telomere fragility by specifically targeting TRF1. Next, we aimed to demonstrate that antagonizing miR-155-dependent regulation of TRF1 expression could mediate resistance to telomere fragility induced by Aphidicolin, a selective inhibitor of DNA Polymerase α (5, 6, 32). As expected, telomere fragility was reduced by antago-miR-155 and increased by Aphidicolin treatment (Fig. 5F; Supplementary Fig. S7C). Importantly, we found that introduction of antago-miR-155 was able to protect from Aphidicolin-induced telomere fragility, thus reducing telomere fragility to levels observed in untreated, control miRNA-transfected cells (Fig. 5F). We conclude that miR-155 efficiently modulates telomere fragility by controlling the expression of TRF1. We next were interested whether miR-155-driven reduction of TRF1 leads to increased genomic instability by quantifying telomere sister chromatid fusions, a reported consequence of TRF1 loss-of-function (6). We found that ectopic miR-155 increases the frequency of telomeric sister chromatid fusions in metaphase spreads that were protease treated before telomere-specific DNA FISH (Fig. 5G; ref. 17). Augmented telomere sister chromatid fusion was rescued when miR-155 was cotransfected with Flag-tagged TRF1. This result indicates that miR-155 drives telomere sister chromatid fusions by targeting TRF1 and excludes miR-155 off-target effects (Fig. 5D and G). Aphidicolin-treated SK-BR-3 cells display significantly increased frequency of telomere sister chromatid fusions (Supplementary Fig. S7D). Remarkably, transient transfection of antago-miR-155 reduced telomere sister chromatid fusions ($P = 0.01$) and also protected from telomere sister chromatid fusions driven by Aphidicolin (Fig. 5H). This indicates that telomere-related genomic instability is miR-155 dosage sensitive. Altogether, we show that miR-155-dependent regulation of TRF1 is an efficient mechanism to control telomere fragility and genomic stability in human breast cancer cells. The consistent upregulation of miR-155 in breast cancer suggests that impaired telomere function is a central aspect of the oncogenic function of miR-155 that promotes genomic instability in human breast cancer and contributes to reduced distant metastasis-free survival and relapse-free survival in ER+ breast cancer.

Discussion

Telomere dysfunction is a major type of chromosome damage in cancer and interfering with regulators of telomere function drives central features of cancer such as loss of heterozygosity, chromosomal rearrangements, aneuploidy, and the repression of DNA damage response checkpoints (33, 34). The importance of telomere regulation in human cancer is underlined by the upregulation of telomerase activity in 90% of human cancers and frequent alteration of the expression levels of shelterin components (35–43). Increased cancer formation upon loss of TRF1 in the context of compromised tumor suppressor suppression suggests that alteration of TRF1 expression drives genomic instability, a hallmark feature of human cancer (6, 11). Using a small-scale miRNA screening approach, we show that miR-155 efficiently regulates TRF1 expression by targeting a partially conserved sequence motif in the TRF1 3’UTR, miR-155 is a classic oncomiRNA that is processed from a noncoding transcript encoded by the BIC locus and has been linked to lymphomagenesis (44, 45). However, more recent studies indicate a general role for miR-155 in human cancer, as demonstrated by a robust upregulation in breast, colon, and lung cancer (46). Ectopic miR-155 was shown to drive the proliferation of breast cancer cell lines in vitro but also, when xenografted into nude mice, to underline the role of miR-155 as oncomiRNA in breast cancer (47, 48). In addition to its role in promoting cell proliferation, a large panel of targets has been identified that link miR-155 to cancer relevant pathways, including apoptosis, inflammation, or DNA mismatch repair (22, 23, 49). miRNA expression signatures have been established as efficient prognostic and predictive biomarkers, underlining the clinical relevance of miRNAs (15).

Figure 4. miR-155 drives alterations in telomere function in breast cancer cell lines. A, quantitative immunofluorescence for TRF1 on SK-BR-3 cells transfected with the indicated mimic-miRNAs or siRNAs; top, representative images. Bottom left, TRF1 fluorescence intensity analyzed for each telomere. Bottom right, individual telomere signal intensities were categorized in three subgroups. B, telomere length measurements by quantitative telomere DNA FISH after repeated transfection of SK-BR-3 cells with the indicated siRNAs; top, representative images. Bottom left, telomere fluorescence intensity analyzed for each telomere. Bottom right, individual telomere signal intensities were categorized in three subgroups. P values and SDs are indicated. N, number of independent experiments; n, total signals analyzed; at least 20 nuclei were analyzed. a.f.u., arbitrary fluorescence units. C and D, ectopic introduction of miR-155 induces DNA damage signaling in SK-BR-3 and MCF-7 cells. Representative images are shown; actin/tubulin A/C was used as loading control. Arrowheads, specific bands. E and F, ectopic introduction of miR-155 in MCF-7 (E) and SK-BR-3 (F) cells induces DNA damage as demonstrated by colocalization of γH2AX with telomeres in immunofluorescence FISH experiments. N, number of independent experiments; n, number of analyzed nuclei; A–F, a Student t test was used to calculate statistical significance; P values are shown.
Dinami et al.

Here, we found that elevated miR-155 levels reduce TRF1 abundance at telomeres and competing endogenous miR-155 expression increases TRF1 at telomeres. This indicates that alterations in miR-155 expression do not only affect the nuclear pool of TRF1, but directly impact on TRF1 abundance at telomeres. miR-155-dependent modulation of the stoichiometry of shelterin complex components resulted in impaired telomere function related to reduced TRF1 expression. We show that miR-155 is a positive regulator of telomere length in telomerase-positive SK-BR-3 a luminal breast adenocarcinoma-derived cell line. This is in line with previous results from TRF1 gain- and loss-of-function experiments (10, 50). Of note, miR138 has been proposed to regulate the expression of human telomerase; however, the relevance of this interaction for telomere homeostasis remains unaddressed (16). In line with reports demonstrating a central role of TRF1 in controlling the replication of telomeres, we show that elevated miR-155 levels promote telomere fragility coupled with the recruitment of the DNA damage marker γH2AX at telomeres (5, 6, 32). Importantly, miR-155-dependent telomere fragility resulted in increased genomic instability exemplified by an increased telomere...
sister chromatid fusions in SK-BR-3 cells that are characterized by impaired tumor suppression due to the expression of a mutant form of p53. In line with this, experimental reduction of endogenous miR-155 levels improves genomic stability at telomeres. These findings are in line with increased cancer occurrence in TRF1 knockout mice lacking p53 (6). Our results show that driving telomere fragility and genomic instability is a central aspect in the repertoire of cancer-promoting functions of miR-155. This result is of special relevance in the light of the recent finding that more than 50% of recurrent amplifications/deletions in human diffuse large B-cell lymphoma map to fragile sites (29). We show that increased miR-155 expression correlates with reduced TRF1 protein levels in specimen from luminal breast cancer that is typically ER$. Importantly, low TRF1 expression is an independent predictor of clinical outcome that is associated with reduced distant metastasis-free survival and relapse-free survival in patients with ER$ breast cancer. This pattern is recapitulated by a panel of validated miR-155 targets, underlining the role of miR-155 as “oncomiR.” However, this also indicates that TRF1 expression is an integral component of a miR-155-dependent signature that predicts poor clinical outcome in ER$ breast cancer.

In conclusion, our work identifies the “oncomiR” miR-155 as the first miRNA that controls the expression of a shelterin component to alter telomere function. Our finding that promotion of telomere-related genomic instability by miR-155 is linked with poor clinical outcome in an ER$ breast cancer underlines the relevance of mechanisms that control telomere function during cancer formation and/or progression.

Our work also anticipates the existence of multiple miRNAs that affect telomere function and homeostasis. Identification and functional characterization of “tery-miRNAs” is expected to provide inroads into the understanding and potential therapeutic treatment of telomere-related maladies such as cancer and aging.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Dinami, R. Benetti, C. Schneider, G. Blandino, S. Schoeftner
Development of methodology: R. Sestito, F. Biagioni, M. Mottolse
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Escolani, M. Mottolse
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): E. Petti, S. Piazza, Y. Ciani, A. Sacconi
Writing, review, and/or revision of the manuscript: R. Dinami, E. Petti, S. Piazza, Y. Ciani, R. Benetti, C. Schneider, S. Schoeftner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. le Sage, R. Agami
Study supervision: S. Schoeftner

Grant Support
This work was supported by the Italian Association for Cancer Research (AIRC) grant cod. 10299, a Fondazione Veronesi grant (S. Schoeftner), and a Young Investigator Grant, Ministry of Health GR-2007-683407 (R. Benetti).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 24, 2013; revised April 22, 2014; accepted May 12, 2014; published OnlineFirst May 29, 2014.


miR-155 Drives Telomere Fragility in Human Breast Cancer by Targeting TRF1

Roberto Dinami, Cristiana Ercolani, Eleonora Petti, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-2038

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/07/21/0008-5472.CAN-13-2038.DC1

Cited articles
This article cites 49 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/15/4145.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/74/15/4145.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/74/15/4145.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.