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Original Article

Intercellular communications of microRNAs are mediated by triplex-forming complexes

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ABSTRACT

microRNAs (miRNAs) constitute a class of small (18-22 nucleotides) non-coding RNAs that bind to the 3'-untranslated region of target messenger RNAs (mRNAs) and have transcriptional or translational silencing functions in eukaryotes. Sequence-specific silencing initiated in one cell can be observed in nearby or faraway cells or even in the whole organism. In multi-cellular organisms intercellular communication is essential for survival and procreation of life. Even though exosomes are known to transport miRNAs, there is little information that addresses how these miRNAs are protected from ribonucleases that are ubiquitous in intracellular and extracellular microenvironments including those inside the intracellular organelles and in exosomal vesicles. Similar to soluble factors, miRNAs mediate cell-to-cell communications and are engaged in a diverse set of biological mechanisms and homeostatic pathways. Previously, we have hypothesized that miRNAs form triplex-forming complexes (TFCs) that become resistant to nucleases. Here, we hypothesize that miRNAs form triplex complexes in order to survive the digestive effects of endonucleases, ubiquitous in all types of extracellular and intracellular environments. In order to evaluate our hypothesis we utilized two strategies: first, we treated HeLa cells with various endonucleases (i.e. Rnase, Dnase) and second, we blocked the biosynthesis of miRNAs. We determined the presence of TFC by treating cells with a variety of single and double-stranded endo/exonucleases. By employing anti-TF-monoclonal antibodies, we demonstrate that miRNAs utilize a TFC that is resistant to various RNA and DNA nucleases. The confirmation of involvement of miRNA in TFC was further corroborated by the blocking of miRNA biosynthesis by using two polymerase II (Pol II) inhibitors: α-Amanitin, and Pol III inhibitor, Tagetin. Time-course analyses of Pol II/III inhibitors exhibited a gradual decrease in TFCs after 24, 48 and 72 hrs of treatment, with elimination of TFCs after 72 hrs. The role of TFC was further explored by transfecting HeLa cell lines with two miRNAs (miR-let-7i and miR-195) and staining the transiently transfected cells for TFC antibodies, which showed TFC transmission *via* long-micropathways. Transportation of TFCs by means of cell-cell communication (i.e. micropathways) and exosomes was also demonstrated. Our findings may have important implications in miRNA research related to the delivery of therapeutic miRNAs in malignancy, infectious diseases and other immunologic illnesses.

KEYWORDS: endonucleases, exonucleases, Tagetin, microRNA, triplex-forming complex, α-Amanitin, transient miRNA expression.

ABBREVIATIONS

bp: basepair, dsDNA: double-stranded DNA, Dnase: deoxyribonulease, EV: extracellular vesicles, HDL: high-density lipoprotein, PBS: phosphate buffered saline, miRNA: microRNA, Pol: polymerase, TFCs: triplex-forming complexes, Rnase: ribonuclease.

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1. INTRODUCTION

miRNAs are small non-coding RNA molecules of 18-22 nucleotides, found in eukaryotic species, which control gene expression by targeting mRNAs and triggering either translation repression or mRNA degradation [1, 2]. miRNAs are well conserved in eukaryotic organisms and are vital evolutionarily ancient components of genetic regulation [1-3].

miRNAs are well-established epigenetic control mechanisms and are evolutionarily conserved [1]. miRNAs are found in almost all metazoan genomes and even in some viruses [1, 4]. Bioinformatics data suggest that miRNAs can regulate almost all mRNAs and a single miRNA can interfere in multiple mRNA silencing [1-3]. All miRNAs are originally transcribed in the cell nucleus as long primary miRNA (pri-miRNA) transcripts containing a 5' cap and 3' poly A tail, by polymerase II (Pol II), (or in some cases (Pol III)). The pri-miRNA is further cleaved in the nucleus by DROSHA/ DGCR8 (a class 2 endoribonuclease III or RNase III) enzyme, into characteristic hairpin (hp) miRNA precursor (pre-miRNA) transcripts [1-3, 5]. These pre-miRNA transcripts can be shuttled outside of the nucleus into the cytoplasm with the help of exportin-5 [1, 5, 6]. Final production of the mature miRNA species requires further cytoplasmic processing by another RNase III enzyme, Dicer, which catalyzes further cleavage of these pre-miRNAs to produce an ~18-22 bp mature product with a two-base overhang on the 3' end [1, 6]. These miRNAs are capable of being incorporated into the RNA-induced silencing complex (RISC). Dicer interacts with several partner proteins: trans-activation response RNA-binding protein or TRBP [7-8] in human cells and Dicer-2 (dsRNA binding protein or R2D2) in Drosophila [1, 5, 6]. These Dicer/protein complex-associated "mature" miRNA strand sequences specifically bind to complementary or near-complementary mRNAs, promoting their degradation and suppressing translation [7]. The RISC, in turn, is able to use the 'seed sequence' located at the 3'-untranslated region (3'UTR), between 2-8 basepair (bp) of the miRNA, to recognize complementary target messenger RNA (mRNA) transcripts for degradation or translational suppression [1, 7, 8]. miRNAs play numerous pivotal roles in cellular functions and gene regulation and it is particularly apparent that miRNAs play an essential role in the regulation of the immune response [1, 3, 9]. It is well documented that *in vivo* miRNAs are transported to both surrounding and distant cells by a variety of mechanisms [7-11]. Additionally, extracellular miRNA can be encapsulated into high-density lipoprotein (HDL) particles and extracellular vesicles (EVs), microvesicles or exosomes (Figure 1). Recent analysis of a post-transcriptional gene silencing mediated by miRNAs suggests that cells of a multicellular organism also communicate with other cells by transport of miRNAs [8-12]. Such communication is involved in antiviral immunity as well as in gene regulation, differentiation, and proliferation [8]. In the present study we attempted to answer one of the most crucial questions: how do miRNAs survive degradation by endonucleases and more particularly Rnases? In this study we present one of the survival mechanisms and show that miRNAs are preferably transported over variable distances in triplex-forming complexes or TFCs. TFCs are relatively resistant to endonucleases and this stealth form allows the miRNAs to reach its targets at long distances. Previously, we have shown that miRNAs can bind to the major groove of homopurine-homopyrimidine stretches of double-stranded RNA or DNA in a sequence-specific manner through Hoogsteen hydrogen bonding to form TFCs [13]. This is a novel form of gene transport mechanism that has likely evolved to protect silencing and gene regulation mechanisms from destruction by endogenous nucleases, which are highly ubiquitous in intracellular and extracellular microenvironments of eukaryotic cells. Our data suggest that TFCs of miRNA may represent a major evolutionary mechanism of safe miRNA transport through extracellular vesicles (exosomes) or *via* ribbon-like micropathways between cells.

2. MATERIALS AND METHODS

2.1. Cell culture

All the cell lines were obtained from the NIH-HIV reagent Program (the NIH AIDS Reagent Program, Division of AIDS, NIAID). The epithelial HeLa cell line 1022 was cultured in RPMI-1640 media (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum and 5 ml L-Glutamine-Penicillin-Streptomycin (Sigma, St. Louis, MO).

2.2. Small RNA inhibition and immuno-histochemistry for triplex-forming miRNA

HeLa-cell suspensions were seeded into Nunc 8-well chambered slides in 1 mL of complete RPMI, containing 10% fetal calf serum (CFS) and 1% penicillin/streptomycin/L-glutamine (Sigma Chemicals, St. Louis, MO) media in a humidified, CO_2 chamber at 37 °C until 70-80% confluency was reached. The adherent cells were washed with serum-free 1x phosphate buffered saline (PBS), three times to remove serum. These cells were fixed as described below [11, 13]. These cells were incubated with various nucleases, i.e. dsDnase, ssRnase, and ssDNA (S1) endonuclease in buffers specified by the manufacturers for 24, 48 and 72 hrs at optimal temperatures and at optimal concentrations according to the manufacturer's protocols. The cells were also treated with different combinations of the nucleases according to the optimal protocols. Enzymatic digestions were performed for 24, 48 and 72 hrs at 37 °C in a humidified CO_2 chamber. The digestion process was terminated by washing the wells with 3x PBS once and in 1x PBS twice and the cells were evaluated for the presence of TFCs by utilizing triplex-specific monoclonal antibodies (Abs) as described previously [11, 13-14]. All the experiments were run in duplicates. Each experiment was repeated at least four times.

HeLa-cells were cultured in the presence of two miRNA inhibitors α-Amanitin, and Tagetin™, that preferentially inhibit Pol II and Pol III, respectively [15-19]. Actinomycin D, which is a DNA intercalating agent and inhibits all RNA transcriptions, was utilized as a positive control [13]. Cells were treated with Tagetin™ (Sigma Chemicals) at 100 pmol/10 ul, α-Amanitin at 4 ug/ml and Actinomycin D at 1 ug/ml. A combination of Tagetin and α-Amanitin was also used and cells were incubated for 24, 48, 72 hrs before fixation [15-19].

After treatment, cells were fixed with Streck tissue fixative (STF: Streck labs, Omaha, NE 68128), a non-ionic fixative [13]. After overnight fixation, the slides were washed twice in 1x PBS for immunofluorescent staining. Non-specific binding was blocked in a blocking solution (containing 2% bovine serum albumin, BSA) for 30 minutes [11, 13]. For detection of TFCs a combination of two murine monoclonal antibodies Jel 318 and 466 (a gift from Dr. Jeremy S. Lee, University of Saskatchewan, Canada: 14) at an equal volume of 20 ug/ml were mixed with the blocking buffer (containing 2% bovine serum albumin in 1x PBS). These monoclonal antibodies can recognize any combination of TFCs including triplexes between dsRNAs-ssDNA, TF-RNAs and dsDNA and ssRNA [14]. Cells were incubated in primary antibody for one hour at 37 °C in a humidified chamber. After incubation, cells were washed in 1x PBS, 3 times. A fluorescent conjugated secondary antibody for anti-mouse antibody diluted in blocking buffer was used for staining triplexes. The cells were incubated in secondary antibody for one hour at 37 °C. After one hour, cells were washed in 1x PBS, 3 times, to remove unbound antibodies. Slides were then mounted in 50% PBS/Glycerol solution and covered with glass cover slips.

The purpose of utilizing miRNA transcription inhibitors was to confirm if the observed triplex formation in the HeLa cells and in micropathways was due to TFCs, on the assumption that if Pol II/Pol III inhibitors decreased TFCs in a significant manner this would provide further confirmation of our hypothesis. Four groups were employed: 1) Tagetin™ treatment, 2) α-Amanitin treatment, 3) combined Tagetin and α-Amanitin, used to block the global RNA Pol II/Pol III transcriptional system and, 4) control (untreated). Actinomycin D, which inhibits all types of RNA transcription, was used as the positive control.

2.3. Evaluation of TFCs in transiently transfected cell lines

HeLa-CD4+ cells were transfected with two miRNA expression vectors each expressing hsa-miR-let-7i and hsa-miRhas-195 (hsa-let-7i: GAGGTAGTA ATTAGATCTG, and hsa-miR-195: GCAGCAG CAGAACAATTTGC), respectively. These two human miRNAs are abundantly expressed in HeLa cell lines [[13] and unpublished data]. We utilized pSuper.GFP.Neo vector to drive the expression of these miRNAs. The vector was purchased from OligoEngine (cat# VEC-PBS-0006). The two small hairpin miRNAs (shmiRNAs), i.e. has-let-7i and has-miR-195, were cloned between the unique BgIII and HindIII sites by restriction enzyme digestion and ligated by the standard method [13]. Positive clones were confirmed by sequencing on

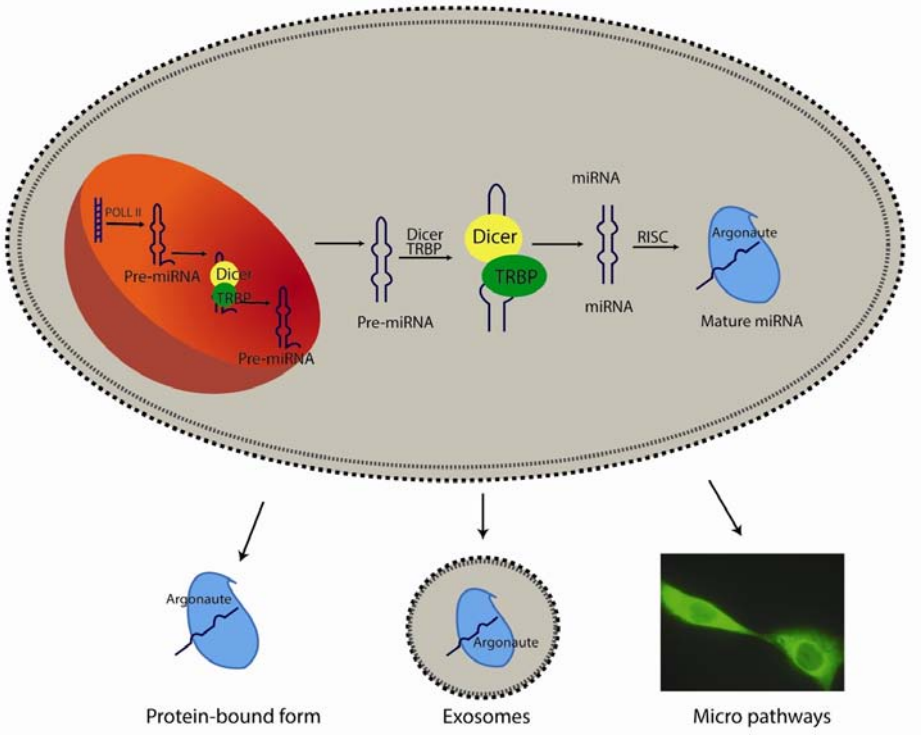
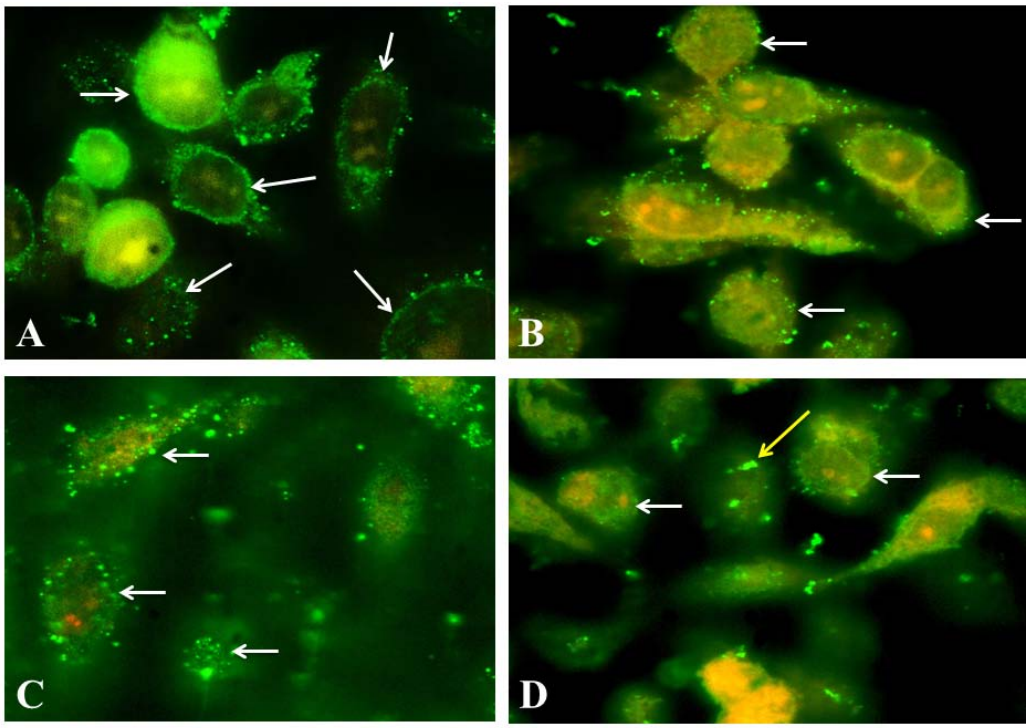


Figure 1



Dnase treatment

Figure 2

an ABI PRISM 3130 sequencer as well as by BgIII-HindIII restriction enzyme digestion. The HeLa-CD4+ cells were transfected by electroportation utilizing 10 ng of the cloned plasmids (Invitrogen, NeonTM Transfection System, Cat# MPK50000). [13]. Cells were maintained in complete media for a week, without antibiotic. Immediately after one week the cells were grown in 8-well glass slides for 48 hrs and fixed in STF. After overnight fixation, the slides were washed and were stained for TFCs as described above [13].

3. RESULTS

3.1. Endonuclease digestion and immunohistochemistry of triplex-forming nucleic acid complexes

In our previous studies we have established, by triplex-binding monoclonal antibodies and fluorescent melting-curve analyses, that miRNAs form stable TFCs in untransfected HeLa cells [11]. In the following section we evaluate the relative resistance of TFC to Dnase, Rnase and S1 endonuclease. We aimed to determine if TFCs are selectively resistant to only one of the endonucleases or if the resistance is a global phenomenon. In the following section we examine the relative resistance of TFC to various endonucleases by treating them with each of the endonucleases for 24, 48 and 72 hrs.

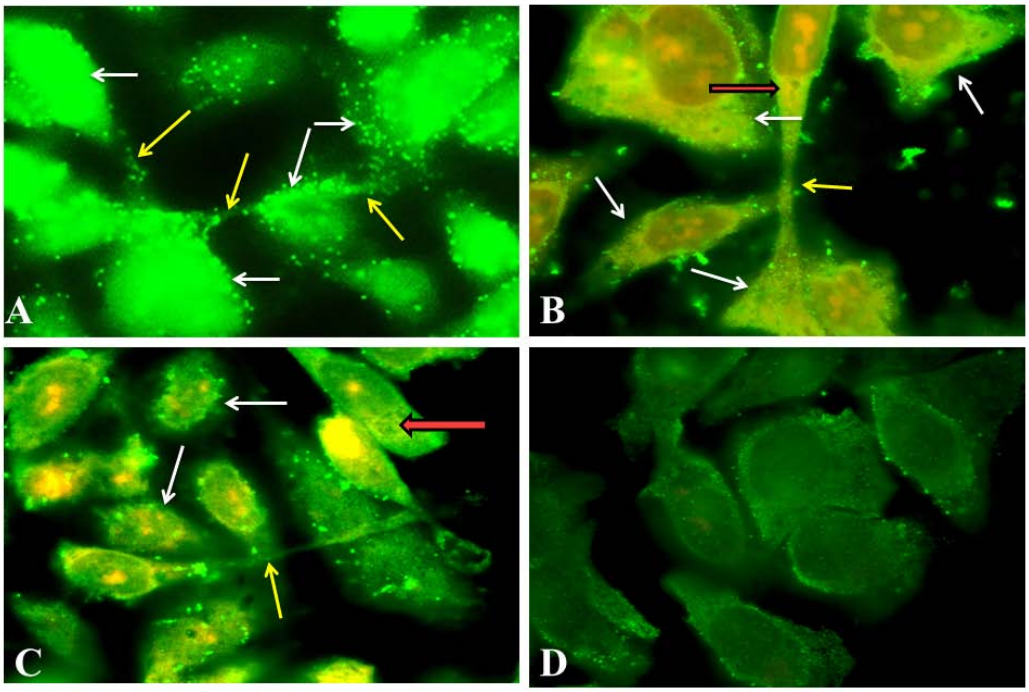
After enzymatic treatment, immunohistochemistry was performed to detect TFCs. In untreated cells triplex dots were significantly distinct and well defined. In comparison to untreated cells, treated cells depicted fewer triplex dots and granular structures. Digestion of ssRNA/dsDNA made triplexes more discernible but the number of triplexes declined after 24 to 48 hrs of enzymatic digestion. Thereafter, triplexes began to disappear as the result of enzymatic digestion by specific endonucleases. A time-course analysis of 24, 48 and 72 hrs of incubation was undertaken to ensure that enzymatic degradation of the dsDNA/ssRNA structures occurred. After digestion, indirect immunohistochemistry was utilized to detect triplexes.

3.1.1. Resistance to Dnase treatment

Figure 2A shows that the HeLa cell line immunostained for TFCs with triplex-specific monoclonal antibodies, exhibits clear evidence of TFCs. The TFCs can be identified as green microdots in the cytoplasmic areas of all the cells (white arrows). They are more obvious in the perinuclear and cytoplasmic areas. The density of the TFCs appears to vary from cell to cell. For example, a large accumulation TFCs can be observed in the three cells located at 9, 10 and 11 O' clock where an over-abundance of TFCs appears as semi-circular green areas (arrow). Also evident are the variations in the nuclear, perinuclear and cytoplasmic staining, since the cell-growth is not synchronized and cells

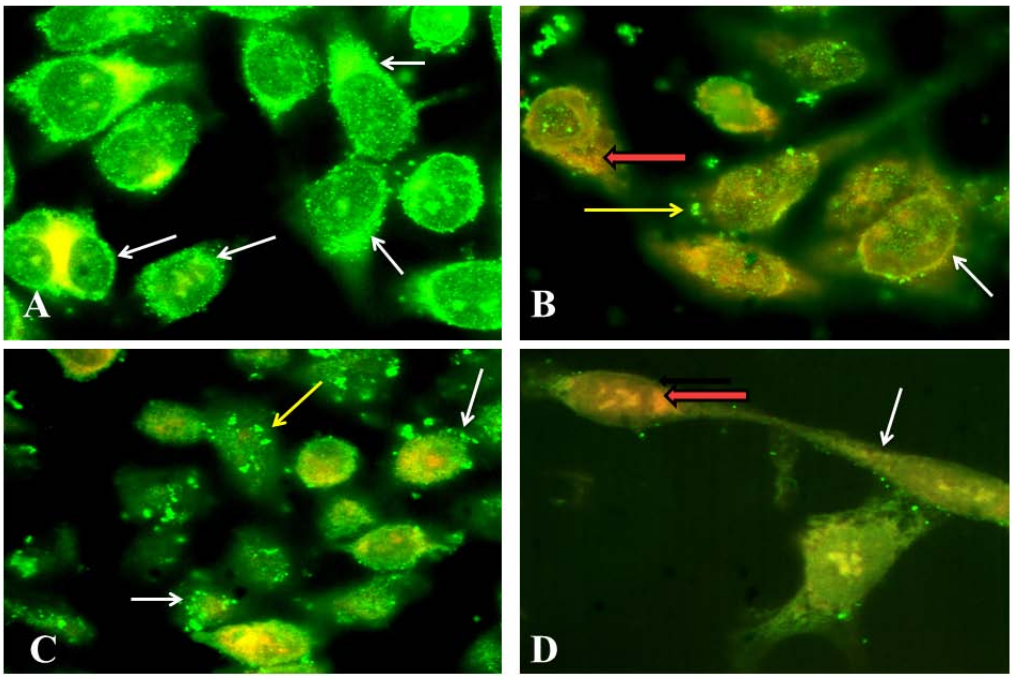
Legend to Figure 1. The biogenesis of miRNAs starts in the cell nucleus with the generation of primary miRNA (pri-miRNA) transcripts. pri-miRNAs are cleaved by the microprocessor complex Drosha/DGCR8 into shorter miRNA precursors (pre-miRNA). They are later transported to the cytoplasm and further cut by the endonuclease Dicer into ~22 nt miRNA duplexes. Finally, one of the miRNA strands is incorporated into a protein of the Argonaute family (AGO1, AGO2, AGO3, or AGO4). The mature miRNA strand eventually serves as the guide for RISC-mediated mRNA targeting resulting in either mRNA cleavage or translational interference. Extracellular miRNA can be either solely AGO protein-associated or additionally encapsulated into apoptotic bodies, microvesicles, and HDL particles.

Legend to Figure 2. Resistance to Dnase treatment. HeLa cells were immunostained for TFCs with TF-specific monoclonal antibodies. As shown in Figure 2A, control cells exhibited numerous nuclear, perinuclear and cytoplasmic TFCs (white arrows). In addition, in the large perinuclear areas, where TFCs are over-abundant, one can observe green circular zones (white arrow). Also evident are the variations in the nuclear, perinuclear and cytoplasmic staining since the cell-growth is not synchronized and cells in different phases of cell cycle can express TFCs at varying degrees. As shown in Figure 2B-C, after treatment with dsDNA-nuclease for 24 and 48 hours, there was a gradual but remarkable decrease in the number of very tiny TFCs seen in Figure 2A, whereas, larger-sized TFCs are now clearly visible as TFC grains in the cytoplasmic and perinuclear areas (white arrows). At 72 hrs of dsDnase digestion, the majority of TFCs have disappeared and occasional larger, irregular-shaped TFCs are prominently visible (yellow arrow). The numbers of small and medium-size TFCs have decreased significantly (white arrows).



Rnase treatment

Figure 3



Dnase + Rnase treatment

Figure 4

in different phases of the cell cycle can express TFCs at varying degrees. As shown in Figures 2B-C, Dnase treatment resulted in significantly decreased number of TFCs as compared to the control. In Figure 2B one can observe concentrated areas of TFC (white arrows) in the cytoplasm, whereas, in Figure 2C the number of TFCs appears to be much lower as compared to that in Figure 2B. However, we observed dense TFCs in the cytoplasm of some cells (white arrows). After treatment with dsDNA-nuclease for 72 hrs, we observed a remarkable decrease in the number of TFCs (white arrows). However, we observed a few highly dense TFCs that appeared to be resistant to the Dnase treatment (yellow arrow). The clear presence of perinuclear TFCs was evident both 24 and 48 hrs post treatment. Significantly, even after 72 hrs of digestion, there were still visible "TFC-grains", suggesting that some TFCs are relatively resistant to Dnase even after extended digestion, which also suggests that TFCs are not entirely composed of triplex-DNA strains [14].

3.1.2. Rnase treatment

As shown in Figure 3A, numerous TFC grains can be observed in the HeLa cell line untreated with any nuclease. In Figure 3B-D, the cells treated for 24 hrs with Rnase expressed a decrease in TFCs compared to untreated cells which exhibited normal morphology (Figure 3B). Significant increase in cell-to-cell micropathways (yellow arrows) and decreased number of TFCs were observed at 24 and 48-hr post exposure to Rnase. There was no apparent evidence of change in cell morphology (Figure 3C). There appears to be no significant difference between post-24 and -48 hrs treatment with Rnase. We also noticed that large TFCs were increasingly visible and appeared to be more resistant to Rnase treatment (Figure 3B and Figure 3C). TFC-loaded micropathways, connecting cell to cell were visible in control and in 24-48 hrs Rnase-treated cells (Figure 3A-C). After 72 hrs of enzymatic treatment with Rnase, cells showed significant reduction in the numbers of TFCs and the TFC diameters decreased precipitately even though numerous perinuclear and cytoplasmic TFCs were still visible (Figure 3D).

3.1.3. Combined Rnase and Dnase treatment

As shown in Figure 4A, numerous TFCs are visible throughout the cytoplasmic and perinuclear areas. However cells treated with a combination of Rnase and Dnase for 24 hrs showed a significant reduction ($P > 0.001$) in number of TFCs when compared to the control (Figure 4B). Cell structure was intact and cellular micropathways for cell-to-cell communication were observable but were faint (Figure 4B). Both, cells treated for 24 and 48 hrs

Legend to Figure 3. Rnase treatment. As shown in Figure 3A, numerous TFC grains can be observed in the untreated HeLa cells (control). The nuclear, perinuclear and cytoplasmic TFC grains are visible (white arrows). TFCs in micropathways are also clearly visible (yellow arrows). In Figure 3B-C, the cells treated for 24 and 48 hrs with Rnase showed a significant decrease in tiny TFCs, but one can clearly visualize a large number of large-size TFCs that became visible after digestion with Rnase (Figure 3B-C, white arrows). TFCs in micropathways are clearly visible (yellow arrows). After 24-48 hrs of digestion P-bodies are also visible (red arrows). There was no apparent evidence for change in cell morphology. There appears to be no significant difference between post-24 and 48 hrs treatment with Rnase. After 72 hrs of enzymatic treatment with Rnase, cells showed a significant reduction in the number of TFCs. The TFC diameters decreased precipitately even though numerous perinuclear and cytoplasmic TFCs were still visible (Figure 3D).

Legend to Figure 4. Combined Rnase and Dnase treatment. As shown in Figure 4A, numerous TFCs are visible throughout the cytoplasmic and perinuclear areas (white arrows). However cells treated with the combination of Rnase and Dnase for 24 hours showed a significant reduction ($P > 0.001$) in the number of TFCs when compared to the control (Figure 4B, white arrow). Cell structure was intact and cellular micropathways for cell-to-cell communication were observable but were faint (Figure 4B, yellow arrows). Cells treated for both 24 and 48 hrs showed significant reduction in TFCs as compared to untreated controls but also exhibited very few prominent, larger irregular-shaped TFCs that were apparently more resistant, perhaps composed of G4-structures (yellow arrows). There was no change in cellular morphology (Figure 4B-C). P-body structures can also be visualized (red arrows). There were significantly less TFCs in 48 hrs vs 24 hrs of digestion. In contrast, after 72 hrs exposure to Rnase and Dnase, the enzymatic digestion showed a precipitous decrease in the number of TFCs ($P > 0.001$) (Figure 4D).

showed significant reduction in TFCs as compared to untreated controls but also exhibited a few prominent, larger TFCs that were apparently more resistant to the treatment due to their more compact nature. It is also possible that these larger, irregular-shaped TFCs are quadruplex nucleic acid structures [20]. There was no change in cellular morphology (Figure 4B-C). There was no significant difference between the 24-hrs versus 48-hrs enzymatic-treated cells, with regard to TFCs. In contrast, after 72 hrs exposure to Rnase and Dnase, the treated cells showed a precipitous decrease in the number of TFCs ($P > 0.001$) (Figure 4D).

3.1.4. Combined DNase + RNAse + S1 endonuclease treatment

Cells treated for 24 and 48 hrs with a combination of nucleases expressed significantly reduced TFCs ($P > 0.001$), as shown in Figures 3-4. After 72 hrs exposure, cells illustrated very few visible TFCs. There was no change in cellular morphology (Data not shown).

3.2. Inhibition of miRNA biosynthesis

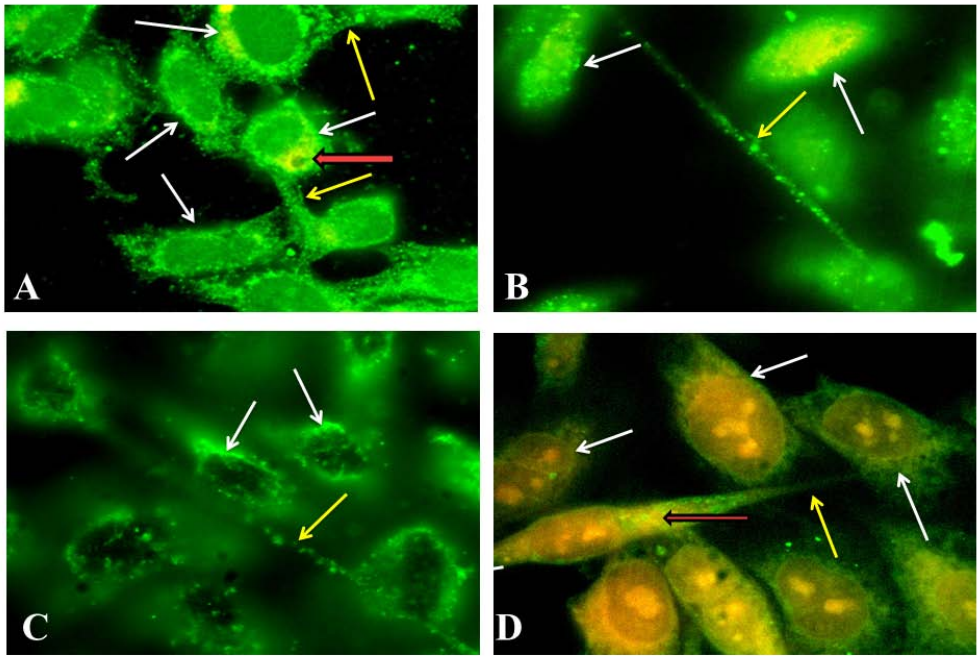
To determine whether miRNAs are involved in the formation of TFCs, experiments were carried out with two RNA polymerase II/III inhibitors. Cells were examined for the presence of TFCs by utilizing immunocytochemistry. HeLa cells were treated with RNA polymerase II/III inhibitors. α-Amanitin and Tagetin™ are two powerful specific Pol II and Pol III inhibitors, respectively [17-19]. A time-course analysis of Pol II/III inhibitors suggested that the miRNA biosynthesis gradually decreased in the presence of these Pol II/III inhibitors during the 24, 48 and 72 hrs of treatments [17].

3.2.1. Tagetin treatment

As shown in Figure 5A untreated cells exhibited clear evidence of cytoplasmic and perinuclear perinuclear TFCs. One of the most notable aspects of Tagetin treatment was the clear visibility of long-micropathways that carry TFCs (Figures 5A and 5B: yellow arrows). The TFC-loaded micro-transportations system may be one of the vesicles that cells use to move TFCs from cell-to-cell that are in close proximity. There was evidence of P-bodies at the root areas of the micropathways (red arrows). The intensity of staining differed from cell to cell perhaps due to different stages of cell division or cycle phase. There appear to be multiple exosomes and apparent P-bodies at the root or polar areas from which the micropathways appear to be originating (Figure 5A and 5D, Red arrow). As shown in Figure 5B, treatment with Tagetin for 24 hrs showed a significant reduction in the TFC number. The most remarkable observation was the significant reduction in cytoplasmic TFCs but there was a marked increase in nuclear TFCs, suggesting accumulation of TFCs in the nuclear areas, perhaps due to interference in the exportin-5 shuttling system or hindrance in some yet-to-be-discovered miRNA export mechanism. It appeared that TFCs were accumulating at the intranuclear area around the nuclear double membrane (white arrows). However, there was no significant reduction in the cell proliferation and growth rates. We observed a significant decrease ($P > 0.05$) in intracellular communication network, and the cell-to-cell connecting pathways as well as the number of TFCs inside the micropathway tracks was markedly declined (Figure 5B). As shown in Figure 5C, treatment with Tagetin for 48 hrs resulted in significant distortion in cellular morphology, and a decrease in cell proliferation ($P > 0.05$). We observed a significant increase ($P > 0.05$) in the cell-to-cell communication micropathways but the numbers of TFCs were significantly lower as compared to that of untreated cells ($P > 0.001$). The most striking observation was almost-absence of nuclear TFCs, but there was accumulation of TFCs in the nuclear double membrane, suggesting inhibition of miRNA biosynthesis as well as export of miRNA in the cytoplasmic areas. There was also evidence of cytopathic effects and degeneration of nuclear structure (visible as yellow staining). We also noted absence of TFCs in the nucleus and significantly smaller granules in the cytoplasm, suggesting a termination in TFC formation. Decreases in cell-to-cell pathways were also observed. The appearance of bulging at cell junction areas (red arrow) indicated the termination or breakdown of cell-to-cell junctions. Notably, there were apparent P-body structures visible (red arrow: Figure 5D).

3.2.2. α-Amanitin treatment

As shown in Figure 6A, similar to what we observed in Figure 5A, over 90-95% of cells exhibited nuclear, perinuclear and cytoplasmic TFCs.



Tagetin-induced inhibition of TFC

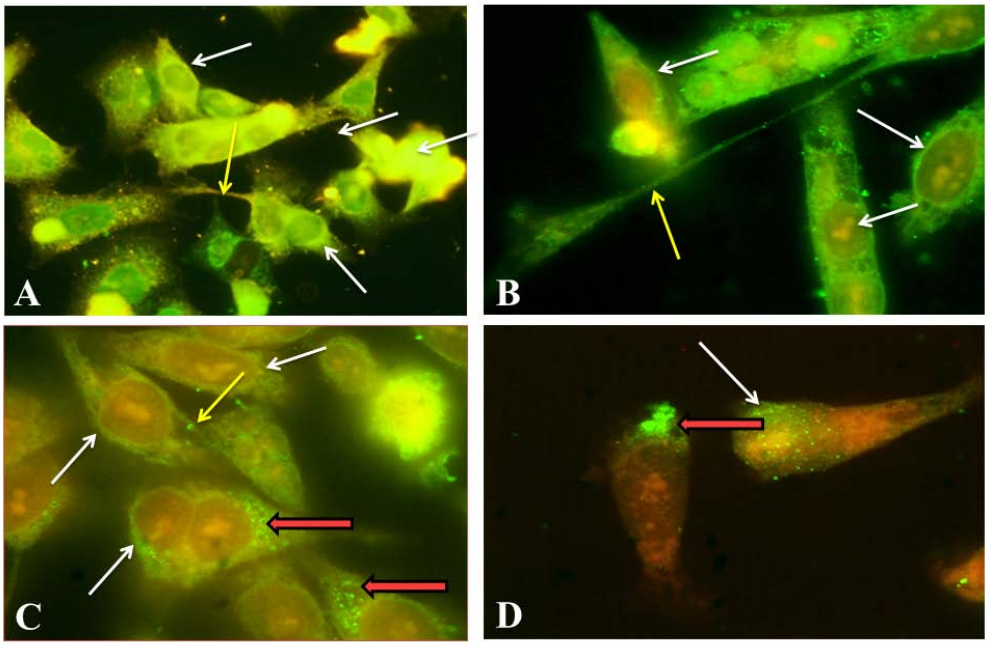
Figure 5. Tagetin treatment. As shown in Figure 5A untreated cells exhibited clear evidence of cytoplasmic and perinuclear TFCs. One of the most notable aspects of Tagetin treatment was the clear visibility of long-micropathways that carry TFCs (Figures 5A and 5B: yellow arrows). The TFC-loaded micro-transportations system may be one of the vesicles that cells use to move TFCs between cells that are in close proximity. There was evidence of P-bodies at the root areas of the micropathways (red arrows). The intensity of staining differed from cell to cell perhaps due to them being in different stages of cell division or cycle phase. There appear to be multiple exosomes and apparent P-bodies at the root or polar areas where the micropathways appear to be originating from (Figure 5A and 5D, Red arrow). As shown in Figure 5B, treatment with Tagetin for 24 hrs showed a significant reduction in the TFC number. The most remarkable observation was the significant reduction in cytoplasmic TFCs but there was a marked increase in nuclear TFCs, suggesting accumulation of TFCs in the nuclear areas, perhaps due to interference in the exportin-5 shuttling system or hindrance in some yet-to-be-discovered miRNA export mechanism. It appeared that TFCs were accumulating at the intranuclear area around the nuclear double membrane (white arrows). However, there was no significant reduction in the cell proliferation and growth rates. We observed a significant decrease ($P > 0.05$) in intracellular communication network, and the cell-to-cell connecting pathways as well as the number of TFCs inside the micropathway tracks was markedly declined (Figure 5B). As shown in Figure 5C, treatment with Tagetin for 48 hrs resulted in significant distortion in cellular morphology, and a decrease in cell proliferation ($P > 0.05$). We observed a significant increase ($P > 0.05$) in the cell-to-cell communication micropathways but the numbers of TFCs were significantly lower as compared to that of untreated cells ($P > 0.001$). The most striking observation was the near-absence of nuclear TFCs. But, there was accumulation of TFCs inside the double nuclear membrane structures, suggesting inhibition of miRNA biosynthesis as well as export of miRNA in the cytoplasmic areas. There was also evidence of cytopathic effects and degeneration of nuclear structure (visible as yellow staining). We also noted absence of TFCs in the nucleus and significantly smaller granules in the cytoplasm, suggesting a termination in TFC formation. Decreases in cell-to-cell pathways were also observed. The appearance of bulging at cell junction areas (red arrow) indicated the termination or breakdown of cell-to-cell junctions. Notably, there were apparent P-body structures visible (red arrow: Figure 5D).

There was evidence of cell-to-cell communication micropathways (yellow arrow). The TFCs were very dense and were highly concentrated around the perinuclear areas and in the cytoplasmic areas. We also observed multiple exosome-like structures, emerging from various cellular membranes, many of which still appeared to be attached to glycocalyx. These structures are loaded with TFCs (Figure 6A).

As shown in Figure 6B, after 24 hrs of treatment with the Pol II inhibitor, α-Amanitin, there was a significant (P > 0.001) decrease in nuclear TFCs, with a dramatic cytopathic effect, rounding of nuclear bodies, and disturbance in cellular morphology. A polarization of TFC around one side of the cytoplasmic areas was noted in ~50% of the cells (arrow). There was reduction of TFCs in the micropathways. After 48 hrs of treatment with α-Amanitin, we observed accumulation of TFCs in what appears to be P-bodies (Figure 6C, red arrows). As shown in Figure 6D, the cells exposed to α-Amanitin for 72 hrs suffered significant cytopathic effects, and there were very few cells that survived. In the surviving cells the diameters of TFC-granules were significantly smaller than the ones seen in the controls. The absence of micropathways was also noted. Biosynthesis of both cytoplasmic and nuclear TFCs was significantly (P > 0.001) decreased. However, in very few cells, P-bodies (red arrow) were visible.

3.2.3. Tagetin + α-Amanitin treatment for 24 hrs

Treatment of HeLa-cells with both Tagetin and α-Amanitin for 24 hrs resulted in significant (P > 0.001) cytopathic effects and over 60% of the cells died after 12 hrs of combined treatment (data not shown).



α-Amanitin-induced inhibition of TFC

Figure 6. α-Amanitin treatment. As shown in Figure 6A, similar to what we observed in Figure 5A, over 90-95% cells exhibited nuclear, perinuclear and cytoplasmic TFCs. There was evidence of cell-to-cell communication micropathways (yellow arrow). The TFCs were very dense and were highly concentrated around the perinuclear areas and in the cytoplasmic areas. We also observed multiple exosome-like structures, loaded with TFCs, emerging from various cellular membranes, some of them still being attached to glycocalyx (Figure 6A). As shown in Figure 6B, after 24 hrs of treatment with the Pol II inhibitor, α-Amanitin, there was a significant (P > 0.001) decrease in nuclear TFCs, with a dramatic cytopathic effect, and rounding of nuclear bodies, and disturbance in cellular morphology. A polarization of TFC around one side of the cytoplasmic areas was noted in ~50% of the cells (arrow). There was reduction of TFCs in the micropathways. After 48 hrs of treatment with α-Amanitin, we observed accumulation of TFCs in what appears to be P-bodies (Figure 6C, red arrows). As shown in Figure 6D, the cells exposed to α-Amanitin for 72 hrs suffered significant cytopathic effects, and there were very few cells that survived. In the surviving cells the diameters of TFC-granules were significantly smaller than the ones seen in the controls. The absence of micropathways was also noted. Biosynthesis of both cytoplasmic and nuclear TFCs was significantly (P > 0.001) decreased. However, P-bodies (red arrow) were visible in very few cells.

3.2.4. Actinomycin D

Treatment of HeLa cells with Actinomycin-D caused severe cytotoxic effects even in the 12-hr treatment. There was a total absence of TFCs and cell-to-cell communication micropathways (data not shown).

3.3. Evidence of cell-cell communication micropathways

We investigated the unique cell-cell communication micropathways by analyzing micropathways under high-magnification microscope at 2,000x in cells that were treated with α-Amanitin for 24, 48 and 72 hrs.

As shown in Figure 7A, the control HeLa cells showed the presence of large number of TFCs in the cytoplasm as well as in the cell-cell communication micropathways. At the junction of presumably a miRNA donor cell (red arrow) one can observe what appears to be the TFC storage area or P-body (red arrow). After blocking Pol II synthesis using α-Amanitin for 24-48 hrs, there was a clear decrease in the number of TFCs in the nuclear and perinuclear areas (Figure 7B-C). Thinning of micropathways was also noted(yellow arrow). After 72 hrs of incubation with the Pol II blocking agent there was a precipitous decrease in TFCs (Figure 7D) and clear evidence of cytopathic effects. There were very few TFCs that were visible and yellowing of nuclear areas indicated cell death, with non-specific adsorption of secondary antibodies. One can also observe the remnants of P-bodies (yellow arrow).

3.4. Evidence of TFCs in miRNA-expressing cell lines

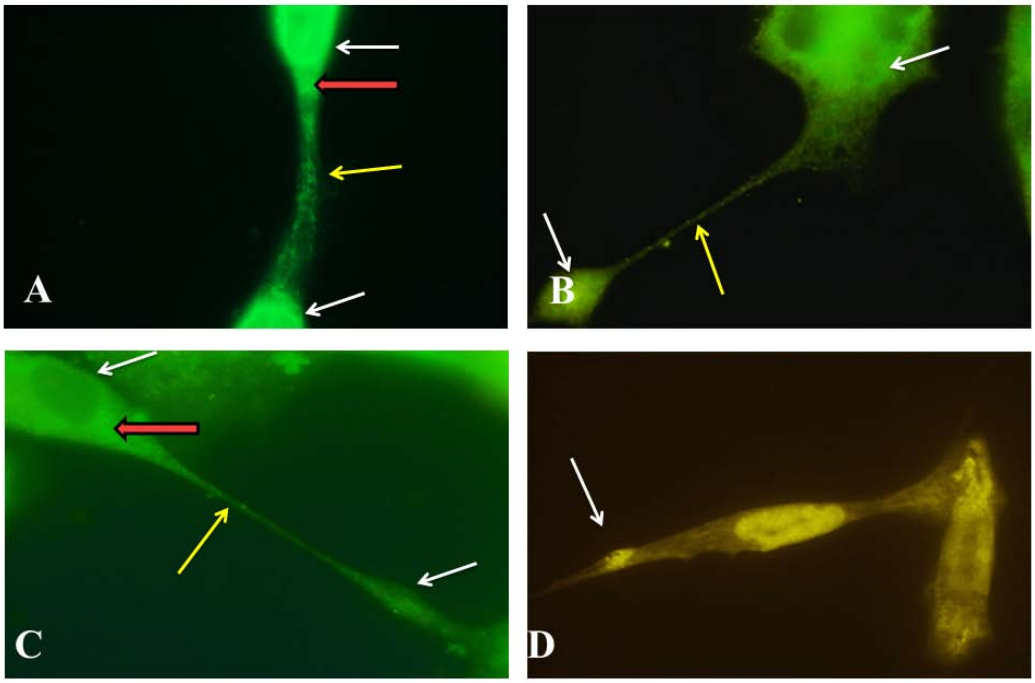
We transfected the HeLa cell line with two different human miRNA, i.e., *hsa-miR- Let-7i* and *hsa-miR-195* and examined the distribution of TFCs in transiently transfected cell lines. Soon after transient transfection, the cells were transferred into 8-well glass slides and after 48 hrs of incubation, the cells were fixed and the locations and distributions of TFCs were examined by immunostaining utilizing triplex-specific miRNAs [13].

As shown in Figure 8A and 8B, the HeLa cells expressing miR-let-7i and miR-195, respectively, exhibited clear evidence of TFCs in their cytoplasms, concentrated mostly in the perinuclear areas as well as in the long cell-cell communication micropathways. Surprisingly, in both transiently transfected cell lines, the micropathways were long compared to those of untransfected cells and appeared to form highly distinct microconnections compared to untransfected cell lines observed in Figures 2-7. We also observed a large number of extracellular vesicles (i.e. exosomes). Apparent transmissions of TFCs were highly visible. We treated the transfected cell with Rnase as shown in Figure 3 for 24-48 hrs. As shown in Figure 8C-D, Rnase digestion significantly reduced the numbers of TFCs.

4. DISCUSSION

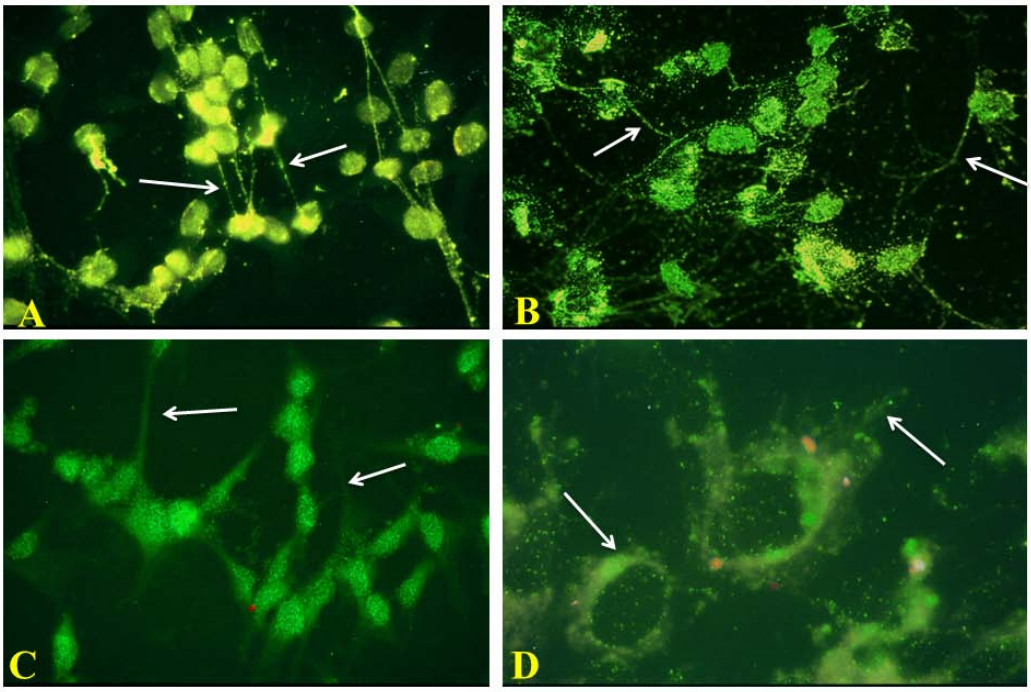
Since the discovery of miRNAs in early 1998 [21], these small molecules have been increasingly recognized as major players in the regulation of all biological functions [1-7, 22, 23]. Also, their essential role in many diverse human diseases has been acknowledged [23-27]. The well-established function of miRNAs is to target the 3' untranslated region (3' UTR) of cytoplasmic messenger RNA (mRNA) to post-transcriptionally regulate mRNA and protein synthesis. Additionally, miRNAs can also regulate gene expression by binding to its promoter regions or primary miRNA transcripts in the nucleus. miRNAs are ubiquitously expressed and regulate almost all biological functions; therefore, they orchestrate both physiological and pathological processes, such as development, cell differentiation, proliferation, neurogenesis, immune development, apoptosis and tumor growth [28, 29]. Recently, their pivotal role in oncogenesis has taken a special place in tumor research and therapeutics [23, 25, 26]. The number of miRNA genes is increasing and an alteration in the level of miRNAs is involved in the initiation, progression and metastases formation of several tumors [25].

One of the promising areas of miRNA-omics involves the study of their potential therapeutic role in treatment of a variety of diseases [30, 31]. However, utilizing miRNAs properly in therapeutics, would require a thorough understanding not only of their epigenetic regulatory role but a clear understanding of their transport and survival mechanisms. One of the least investigated areas of miRNA research relates to how miRNA survive the degradation caused by endonucleases that are so pervasive in all intra- and extracellular compartments. Cell-to-cell communication can



α-Amanitin-induced inhibition of TFC in micropathways

Figure 7



Triplex formation and micropathways in miRNA-transfected cell lines

Figure 8

occur by several means, including chemical receptor-mediated events, direct cell-cell contact and cell-cell synapses. Emerging evidence shows that extracellular vesicles (EV)-associated miRNAs (i.e. exosomes) play important roles in regulating a wide range of normal physiologic and immunologic processes as well as in diseases, particularly in oncogenesis where they function through the regulation of protein expression of the important genes that make contributions toward development of tumors and tumor metastases [7, 24, 30-32].

In the present study, we provide evidence that miRNAs survive degradation by forming TFCs. In our previous studies we have shown that miRNAs form TFCs [13]. Here we provide evidence that structures of TFCs have evolved, as part of the protective mechanisms to avoid digestion by highly ubiquitous endonucleases. We show that TFCs structures provide protection against various endonucleases including Dnase, Rnase and S1 nucleases and combinations of all three major types of endonucleases. We also showed evidence that structures of TFCs are protected in intracellular as well as in extracellular environments. Therefore, TFC-formation protects uninterrupted direct communication *via* micropathways [13] as well as long-distance communication *via* EVs.

We also show that a significant amount of miRNAs can withstand the digestive effects of all three kinds of endonucleases for 24-48 hrs or longer. A majority of investigators have focused on EVs as one of the major sources of miRNA transport and generally, EV-based miRNA transport takes ~24 hrs or less to move the miRNA cargo from secreting-cells to the target cells *via* blood, cerebrospinal fluid (CSF) or other bodily secretions [7, 10-11]. It is well established that EVs (i.e. exosomes) contain numerous endonucleases [7, 10] and miRNAs must survive the voyage from the cells of origin to the target cells.

In order to further validate that TFCs are indeed miRNAs, we blocked the biosynthesis of miRNAs by using two Pol II/III inhibiters: Tagetin and α-Amanitin. Both these chemicals are powerful inhibitors of RNA polymerase II/III [15-19], respectively. The α-Amanitin blocks Pol lI activity by interacting with the bridge helix in RNA polymerase II enzyme and thereby interferes with the translocation of RNA and DNA. This process, empties the site for the next round of miRNA synthesis, thereby terminating the miRNA biosynthesis [16, 19]. The bridge helix has evolved to be flexible and its movement is required for translocation of polymerase II along the DNA backbone. Binding of α-Amanitin puts a constraint

Legend to Figure 7. Evidence of cell-cell communication micropathways. We investigated the unique cell-cell communication micropathways by analyzing micropathways in cells that were treated with α-Amanitin for 24, 48 and 72 hours using a high-magnification microscope at 2,000x. As shown in Figure 7A, the control HeLa cells showed the presence of a large number of TFCs in the cytoplasm as well as in the cell-cell communication micropathways. At the junction of what is presumably a miRNA donor cell (red arrow) one can observe what appears to be the TFC storage area or P-body (red arrow). After blocking Pol II synthesis using α-Amanitin for 24-48 hrs, there was a clear decrease in the number of TFCs in the nuclear and perinuclear areas (Figure 7B-C). Thinning of micropathways was also noted (yellow arrow). After 72 hrs of incubation with the Pol II blocking agent there was a precipitous decrease in TFCs (Figure 7D) and clear evidence of cytopathic effects. There were very few TFCs that were visible and yellowing of nuclear areas indicated cell death, with non-specific adsorption of secondary antibodies. One can also observe the remnants of P-bodies (yellow arrow).

Legend to Figure 8. Evidence of TFCs and long micropathways in two HeLa cell lines transiently transfected with miRNAs. As shown in Figure 8A and 8B, the HeLa cells expressing miR-let-7i and miR-195, respectively, exhibited clear evidence of TFCs in their cytoplasms. These TFCs were mostly concentrated in the perinuclear areas and appeared to have been transported *via* the long cell-cell communication micropathways. In both transiently transfected cell lines the micropathways were long compared to those of untransfected cells and appeared to form highly distinct microconnections compared to the untransfected cell lines observed in Figures 2-7. We also observed a large number of extracellular vesicles (i.e. exosomes). Apparent transmissions of TFCs were highly visible. We treated the transfected cell with Rnase as shown in Figure 3 for 24 or 48 hrs. As shown in Figure 8C-D, Rnase digestion significantly reduced the number of TFCs.

on the polymerase, hence slowing down its translocation and the rate of synthesis of miRNA molecule [15-19]. At low concentration α-Amanitin blocks the activity of Pol II, the essential enzyme in miRNA synthesis [18]. At higher concentrations it also blocks Pol III [15-16].

Tagetin™ is a bacterial toxin that has been shown to preferentially inhibit eukaryotic RNA polymerase III elongation without significant effects on the transcriptional activities of other polymerases [15-19]. Tagetin™ RNA pol inhibitor is the only compound known to potently and selectively inhibit RNA polymerase III in a variety of eukaryotic organisms including mammalian cells, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Bombyx mori*, and *Xenopus laevis oocytes* [17]. Tagetin Inhibitor complements the activity of α-Amanitin, a potent and selective inhibitor of eukaryotic RNA polymerase II. Although the precise mechanism of Tagetin inhibition is not understood, studies with yeast nuclear extracts indicate that the effect is due to increased pausing of the elongation complex at discrete points on the DNA template [18].

Even though, Pol II is a well-established mediator of miRNA synthesis, the role of Pol III has also been implicated in transcriptions of numerous short and long RNAs [33]. For example, Pol III catalyzes the transcriptions of tRNAs, ribosomal 5 S rRNA, and U6 snRNAs. It also transcribes short interspersed elements, small RNA (i.e. some miRNAs) and repeated elements in the human genome [33]. There are reports that suggest both Pol II and III may be involved in different types of miRNA synthesis [15-19, 33, 34]. For example, initially it was believed RNA Pol III was mediating miRNA transcription because it transcribes most small RNAs such as tRNAs and U6 snRNA [15]. However, circumstantial evidence suggested otherwise. First, primary miRNA transcripts can be over several kilobases long and contain stretches of more than four 'U's, which would likely terminate the Pol III-initiated transcription. However, there is other evidence that suggests the involvement of Pol III in some types of miRNA biosyntheses [19, 34]. Therefore, we utilized both Pol II and Pol III inhibitors to validate the hypothesis that the usage of triplex-forming complexes may be a major gene-regulatory mechanism for miRNA to transport its miRNA-mediated signals from the cells-of-origin to the far-distant target sites [33-34].

Here we established that TFCs are the major structural forms that are immune to the destructive effects of highly ubiquitous endonucleases. Therefore, by adopting triplex-formation, miRNAs can be safely transported from cell to cell [1]. In addition, we also investigated a direct cell-cell-communication pathway that involves micropathways for cell-to-cell communication. These micropathways have not been described previously in detail [13]. As we have shown, this newly observed mechanism of cell-cell communication utilizes TFCs. Furthermore, we provide preliminary evidence that the P-body-like structures most likely serve as storage compartment for TFCs and are located near the origin of the micropathways. The cell-cell communication system transports TFCs directly from one cell to the other cell thereby increasing the complexity by which cells may communicate. This genetic communication between cells may be a common system for miRNA transport utilizing TFCs that are resistant to Dnase, Rnase and S1 endonucleases. It appears that miRNA transport in the form of a triplex complex is a safer way to transport miRNAs across longer distances *via* exosomes and other EVs, due to the resistance offered against endogenous nucleases found in the biological fluids. Transport of post-transcriptional gene silencing information between cells may be one of the most important signaling mechanisms not only for regulating genes but also for serving as a molecular immune system, where the virally infected cells may send miRNAs to arm the uninfected cells against the infectious agents [2]. Evolutionarily, the miRNA-based molecular immune system may pre-date the evolution of cellular, cytokine and lymphocyte-based innate immune defenses [2]. A study in *Caenorhabditis elegans* demonstrated that when a strong promoter is used to derive transgenes it leads to detectable silencing information between cells. However when a relatively weak, body wall muscle-specific promoter was used, gene silencing was only observed in body wall muscles [35, 36].

By utilizing triplex-specific antibodies we demonstrated that miRNAs are protected by an evolutionarily conserved resistance mechanism [1, 2]. The discovery of direct communication

between cells suggests a unique mechanism that may be operative in cells that are in close proximity to each other. Moreover, it was apparent that triplex nucleic acid complexes were transmitted between cells *via* discrete extracellular miRNA micropathways [13]. Of note, triplex formation between dsDNA and ssDNA, dsRNA and ssRNA, dsRNA [i.e. miRNA] and ssDNA as well as triplex RNA has been described by numerous reports and is a well-established gene-regulatory mechanism [3, 13, 36-40]. In this report we show that triplex formation also serves as an endonuclease-resistant mechanism.

The triplex nature of miRNA was also confirmed by treating cells with a wide range of nucleases, most importantly, Rnases and analyzing the TFCs presence for an extended time period (i.e., 24, 48 and 72 hrs). The reduction in the number of TFCs after 48-72 hrs of incubation with endonuclease suggests vulnerability of TFC after 48 hrs of digestion. Indeed, we hypothesize that this may be an important regulatory mechanism. Generally, miRNA transport from cells-of-origin to the target organs or sites are complete within 24 hrs in an organism [7], and their extended presence in a organism may be more harmful than beneficial [8]. Our anecdotal evidence suggests that a minuscule percentage of TFCs may be in quadruplex formations. It is possible that quadruplex-forming complexes provide an additional layer of miRNA protection or represent a regulatory mechanism [33-34]. However, this observation will need further investigation.

The above observations that the triplexes are mostly composed of triple RNA (but not entirely miRNAs) were further confirmed by the Dnase digestion experiment that suggested that the miRNA transport system may be much more complex than a simplex-triplex formation [11]. The utilization of Pol II inhibitors further validated that TFCs contained miRNAs. The transient transfections of the HeLa cells with two miRNAs (i.e. miR-let-7i and miR-195), where miRNAs were overexpressed in the transfected cells, vividly brought about the transmission of TFCs *via* micropathways.

The prominent visualization of processing P-bodies that represent sites of mRNA degradation or miRNA storage in HeLa cells where most of the TFCs were degraded after the enzymatic digestion or inhibited by Pol II/III may suggest more unique mechanisms of TFC survival. Several lines of evidence indicate that mRNA degradation takes place in P-bodies [41-42]. Our observation of the presence of P-bodies at the origins of micropathways indicates that P-bodies may serve as the storage compartment for miRNAs. Of note, cells treated with α-Amanitin for 72 hrs showed relatively larger-sized P-bodies. Hence, the blocking of mRNA decay in cells results in larger observable-sized and more numerous P-bodies. At this juncture, we are uncertain whether these prominently visible P-bodies represent an evolutionarily advanced form of miRNAs that perhaps form quadruple structures or G4-structures with unique proteins that protect the TFCs for long-term storage [33-34] or whether they are garbage mRNAs that would be ready for exocytosis [43-44]. We favor the former viewpoint, since these P-bodies are located right at the miRNA transport micropathways, designed to be transported to other cells.

The precise site-specificity of triplex miRNAs makes them highly valuable biochemical, pharmacological and therapeutic agents that can act as epigenetic gene-regulators that may provide precise tumor or aberrant gene-silencing tools to quell oncogenesis, autoimmune or other disease pathogeneses. In order for these molecules to be utilized therapeutically, it is important to understand how to protect them from endonuclease-based degradation. Our study takes a step forward towards a better understanding of this process.

5. CONCLUSION

MiRNAs regulate a wide range of biological processes, including development, differentiation, proliferation, cell metabolism and programmed cell death, and immune defense [1, 2]. They are abundant in many mammalian cell types and estimated to target about 60% of the genes of humans and other mammals [1, 2]. Over 2654 mature miRNAs and 1917 precursor miRNAs have been identified in *Homo sapiens* and the numbers are growing [46]. Dysregulation of individual miRNAs or subsets of miRNAs can result in human diseases, such as cancer, cardiovascular disorders, viral infections and metabolic diseases [1-3]. miRNAs are evolutionarily conserved complex organisms that regulate numerous

cellular functions by transporting miRNAs to target organs and cells that are nearby and far away [8]. We hypothesized that miRNAs must have developed protective mechanisms to survive destruction caused by ubiquitous endogenous nucleases [11, 13].

We present extensive evidence that miRNAs adopt TFC-structures that make them immune to the destructive effects of endogenous nucleases and allows the safe transport of miRNAs to the target sites [8, 47, 48]. We believe that our current study opens a new way towards this approach. It is possible that many of the miRNA-based treatments have been unsuccessful or inconclusive due to the miRNA degradation process, since they were produced by *in vitro* synthesis and were unprotected from endonucleases. In the future this approach could be used to protect the therapeutic miRNAs by allowing them to form the TFCs before delivery for therapy.

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CONFLICT OF INTEREST STATEMENT

The investigators declare no conflict of interest.

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