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In situ detection of mature microRNAs by labeled extension on ultramer templates

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Abstract

We describe a new method for the in situ detection of a mature microRNA (miRNA) in formalin-fixed, paraffin-embedded tissues. The method involves the labeled extension of miRNA hybridized to an approximately 100-nucleotide-long ultramer template containing the complementary sequence of the miRNA at its 3' terminus. Pretreatment of the tissue involves incubation with protease to expose the genomic DNA to DNase digestion, thereby eliminating the ultramer-independent DNA synthesis process inherent in paraffin-embedded tissue. By direct comparison with real-time reverse transcriptase (RT)-PCR, RT in situ PCR, and standard in situ hybridization using a locked nucleic acid (LNA) probe, it was evident that the ultramer extension method detects only the mature miRNA, is easier to optimize, results generally in a stronger signal, and is much less expensive than the LNA probe method currently used.

Keywords

microRNA; in situ hybridization; in situ PCR; LNA probe

Introduction

MicroRNAs (miRNAs) are small, noncoding sequences of ~20–23 nucleotides that regulate cell processes by binding to the 3' untranslated region (UTR) of mRNAs resulting in its translation repression; it is predicted that they regulate thousands of human genes (1–3). miRNAs are transcribed as long primary precursor molecules (pri-miRNA) that are subsequently processed by the nuclear enzyme Drosha to the precursor miRNA (pre-miRNA). The pre-miRNA, in turn, is processed by Dicer to generate the mature miRNA (4). miRNA expression is critical in oncogenesis (2,5–7). Increased miRNA expression in cancers is associated with the down-regulation of tumor suppressors while miRNAs that are reduced in cancer may normally suppress oncogenes (2,5–7).

miRNA expression analyses have used real-time RT-PCR (8–10), Northern blot analysis (1, 4), or microarrays (2) that can detect either the precursor (8–10) or, more commonly, the mature

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form of the molecule (10). Relatively few studies on miRNA expression have used in situ-based techniques (5,11–14). Thus, there is evidence lacking to show that, in cancers, only the malignant cells—and not the adjacent normal tissue—are expressing the miRNA of interest. RT in situ PCR can be used to detect the miRNA precursors: since it is very sensitive, a negative result would effectively rule out the production of miRNA precursors (5,14,15). The in situ detection of miRNAs has been assisted by the use of locked nucleic acid (LNA)-modified nucleotides (13,16). The LNA nucleotides are much more rigid in three-dimensional space, which results in a substantial increase in the T_m of the small LNA-modified oligonucleotide probe hybridized to its target miRNA (17). It has been demonstrated by some that the LNA-modified probe can detect either the miRNA precursors or mature miRNA depending on which specific sequence in the pre-miRNA is targeted (18,19).

We have developed a novel method for the in situ detection of the mature miRNA based on the extension of the molecule after its hybridization to an ultramer template. This method, like RNA-primed, array-based, Klenow enzyme (RAKE) analysis, is based on the RNA molecule acting as a primer to initiate the reaction (12). However, unlike RAKE, which is only effective in solution phase reactions, our system allows for the in situ analysis of the mature miRNA. This novel method—along with the two established in situ methods of RT in situ PCR and in situ hybridization with LNA probes—were used to study the spatial patterns of different microRNAs in a variety of malignant cell lines, as well as in normal and cancerous tissues.

Materials and methods

Solution phase extension of miR-376a

Mature miR-376a oligoribonucleotide was synthesized by IDT (Coralville, IA, USA) and gel-purified. miR-376a oligomer (1 μ g) was end-labeled with γ - P^{32} ATP and T4 kinase. The labeled miR-376a oligomer was used to prime a miR-376a ultramer, and the oligos were extended using a variety of enzymes including rTth reverse transcriptase (Applied Biosystems, Foster City, CA, USA), *Taq* DNA polymerase (Promega, Madison, WI, USA), MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), MultiScribe reverse transcriptase (Applied Biosystems), and T7 RNA polymerase (Epicentre, Madison, WI, USA), according to each company's protocol. The extension products were verified on a 12% polyacrylamide, 6 M urea denaturing gel.

Cell lines and tissue samples

Tissue samples (from the files of G.J.N.) and cell preparations (HL-60 and Jurkat cell lines, provided by colleagues at Ohio State University Medical Center) were immediately fixed in 10% buffered formalin for 4–12 h at room temperature; buffered formalin is the optimal fixative for RNA in situ hybridization (15). All tissues were obtained via an approved protocol from the Ohio State University Medical Center Internal Review Board. We focused on cardiac and central nervous system (CNS) tissues, as these have been well associated with particular miRNAs [miR-1 (20), and miR-128a (21) (S. Lawler, unpublished data)], respectively. Furthermore, since mature miR-128a is decreased in the malignant counterpart (glioblastoma multiforme) (S. Lawler, unpublished data), this allowed us to study the in situ correlates of mature miRNA down-regulation in these cancerous tissues.

Ultramer extension method

General principle—The ultramer is at least 100 nucleotides in size. At its 5' end, it consists of a series of four 20-nucleotide repeats (GACCCCTTAATGCGTCTAAA), ending at the 3' end with the complementary sequence of the miRNA of interest. A DNA or RNA polymerase will extend the miRNA using the ultramer as the template (see Figure 1, panel A with miR-1 as the example). As the miRNA is being extended, the newly synthesized DNA incorporates

the reporter nucleotide digoxigenin (Figure 1A). The repetitive sequence allows the use of a complementary oligonucleotide (TTTAGACGCATTAAGGGGTC) that can be added at the onset of the reaction that will inhibit the extension of the miRNA. This serves as an additional negative control to document the specificity of the reaction.

Both the RT in situ PCR and ultramer extension methods are optimized for formalin-fixed, paraffin-embedded tissue. Heating the paraffin to 65°C during the embedding process induces nicks and short gaps in the genomic DNA, whereas formalin fixation causes DNA- and RNA-protein crosslinks (Figure 1, B1) (15). Any DNA polymerase will utilize these nicks and short gaps to synthesize DNA that will incorporate the reporter nucleotide, either through gap repair or—if the polymerase has 5′–3′ exonuclease activity—via nick translation (Figure 1, B2). This will result in a nonspecific nuclear signal as previously shown with in situ PCR in paraffin tissue sections (5,14–16). In order to prevent this, it is necessary to degrade the genomic DNA by DNase pre-treatment to the point where it will no longer support this nonspecific DNA synthesis pathway (Figure 1, B3). Efficient DNase pre-treatment requires the removal by protease digestion of the formalin-induced DNA-protein adducts in the genomic DNA, which may sterically interfere with DNase activity. Optimal protease digestion is defined by the generation of an intense nuclear signal in the extension/labeling reaction of a test tissue section or cell preparation, reflecting loss of the steric interference of polymerase activity by the DNA-protein adducts (5,14–16). If the protease digestion is not sufficient, residual DNA-protein crosslinks will allow the persistence of sufficiently intact DNA not degraded by DNase, resulting in a nonspecific nuclear signal (Figure 1, B4). If protease digestion is too strong, then the morphology of the tissue or cell preparation is lost and thus the results are not interpretable (15). The key components of the ultramer extension method are as follows:

Protease digestion—Cells or deparaffinized tissue sections are digested in protease (2 mg/mL pepsin in RNase-free water) at room temperature. The optimal protease time is defined as the number of minutes of digestion that will elicit an intense nuclear-based signal in all cell types, in a test tissue section or cell preparation with direct incorporation of the reporter nucleotide in an extension/labeling reaction. This signal is completely eliminated by overnight digestion with RNase-free DNase (5,15).

DNase digestion—After optimal protease digestion, approximately 200 U of RNase-free DNase I (Roche Diagnostics, Indianapolis, IN, USA) is applied to each tissue section or cell preparation and incubated for 15 h at 37°C (15,16). The DNase is removed by successive washes in RNase-free water and 100% ethanol.

miRNA extension solution—The ultramer extension solution consisted of the following: 10 µl rTth buffer (Applied Biosystems), 1.6 µl of each of the four dNTPS (10 mM), 1.6 µl of 2% BSA, 12.4 µl of 10 mM manganese acetate, 2 µl of the ultramer (500 pmol), 0.6 µl of 1 mM digoxigenin dUTP, 2 µl of rTth DNA polymerase, and 15.0 µl of RNase free water.

In situ microRNA extension—After DNase digestion, the tissue section or cell preparation is covered with the miRNA extension solution and then with a polypropylene coverslip and mineral oil to prevent evaporation. The extension reaction proceeds for 30–60 min at 60°C. The incorporated digoxigenin is detected with an alkaline phosphatase conjugated to an antidigoxigenin antibody (75 U/100 µl; Roche Diagnostics) diluted 1:150 with Tris buffer (pH 7.0). As with the RT in situ PCR method, the chromogen is nitroblue tetrazolium (NBT; 300 µg/mL) and bromochloroindolyl phosphate (BCIP; 200 µg/mL) and the counterstain is nuclear fast red (Enzo Life Sciences, Farmingdale, NY).

An alternative method can be used to confirm the specificity of the ultramer-extended miRNA. The ultramer extension can proceed as described above, but minus the reporter nucleotide. The

ultramer can be end-tailed with biotin (17) and used as a probe to detect the unlabeled extended miRNA using the method described below, under “In situ hybridization with LNA-DNA probes.”

Interpretation—A successful reaction with direct incorporation of the reporter nucleotide is defined as a strong nuclear signal in the no-DNase control, and its complete elimination in the DNase control in which either no ultramer or (for miR-128a) a scrambled ultramer was used. The latter negative control section should be on the same slide as the test section in order to assure uniformity of variables such as protease digestion. As with RT in situ PCR, these two results indicate optimal protease digestion and successful degradation of the genomic DNA to the point that it can no longer support detectable DNA synthesis (Figure 1B, 2 and 3). If a signal is seen in the DNase negative control (no ultramer), which is nuclear-based, the reaction must be redone with increased protease digestion time. If the tissue morphology has been destroyed and, hence, there is no signal, the reaction must be redone with decreased protease digestion time (17).

In situ hybridization with LNA-DNA probes

Our protocol for detection of miRNAs by in situ hybridization has been previously published (13,16). In brief, tissue sections or cells were digested in pepsin (2 mg/mL) for 30 min at room temperature. The sequences of the different miRNAs studied by in situ hybridization with the LNA modified probes are provided in Table 1. The probes (250 pmol) were made by Exiqon (Woburn, MA, USA), and include multiple modified LNA nucleotides dispersed throughout the sequence. The probes were labeled with the 3' oligonucleotide tailing kit (Enzo Diagnostics, Farmingdale, NY) using biotin as the reporter nucleotide. The labeled probe was diluted in the in situ hybridization buffer (Enzo Life Sciences), added to the slide and denatured at 60°C for 5 min, and then hybridized at 37°C for 15 h. After a 10-min wash in 0.2× salt sodium citrate (SSC)/2% bovine serum albumin (BSA) at 5°C, the probe-target complex is visualized by the action of alkaline phosphatase (as part of the streptavidin complex; Enzo Life Sciences) on the chromogen NBT/BCIP. Nuclear fast red served as the counterstain. The negative controls included omission of the probe and the use of a scrambled LNA probe (for miR-128a; see Table 1). Furthermore, miR-96 was chosen as an additional negative control for each analysis as this miRNA is not expressed in most normal tissues, including brain, heart, and placenta (22). As with the ultramer extension method and RT in situ PCR, the negative control with the LNA in situ method was done on the same slide as the test section, since at least 2 sections (4 microns thick) were placed on each slide.

RT in situ PCR

The protocol we used has been previously described (5,15). The miRNA precursors tested were miR-1, and -128a using the same primers employed for solution phase RT-PCR analysis, as recently described (14). The controls for the RNA-based signal included omission of the primers, as lack of signal demonstrated loss of the nonspecific DNA synthesis pathway in the intact cells (5,15; see Figure 1B).

Nuclear and cytoplasmic fractionization and real-time RT-PCR

HL60 and Jurkat cells were collected with a packed cell volume of 20 µl and nuclear, and cytoplasmic fractions were isolated using NE-PER Nuclear and Cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Total RNA was isolated from both fractions using Trizol LS reagent (Invitrogen, Carlsbad, CA), and RNA pellets were dissolved in an equal volume of water. One microliter of RNA from the nuclear or cytoplasmic fraction was reverse transcribed using the protocol described in the TaqMan assay (Applied Biosystems), and mature and miR-150 precursor expression was measured by

real-time RT PCR as previously described (14). The mean of triplicate cDNAs was determined and the data are presented as 2^{-C_t} , as described (20).

Results

Solution phase extension of mature miRNAs

Before testing the ultramer extension method in situ, we used an ultramer to miR-376 [chosen because of prior work with this miRNA in pancreatic cancer (5)] to demonstrate that the mature form of this miRNA could be extended in solution by a variety of polymerases. As evident from Figure 2, rTth RT/DNA polymerase, *Taq* DNA polymerase, MMLV RT, Multiscribe RT, and T7 RNA polymerase were each able to extend the mature miR-376. The increased size of the *Taq* DNA polymerase product was likely due to the template-independent terminal transferase activity of the polymerase that adds a single deoxyadenosine to the 3' ends of PCR products.

Cell lines: correlation of real-time RT-PCR with in situ-based data in cell lines

It should be noted that the primers designed to anneal to the miRNA hairpin for real-time RT-PCR and RT in situ PCR will amplify both the pri- and pre-microRNA since the hairpin is contained within each (14). For this reason, we shall consider the amplification of miRNA precursors to represent both the pri- and pre-miRNA. We analyzed the cancer cell lines HL60 and Jurkat for miR-150 expression (precursor and mature forms) by real-time RT-PCR (Figure 3) based on prior work with this miRNA that showed it was variably expressed in different malignant cell lines (14). Note that both cell lines had high levels of the miR-150 precursors (Figure 3A). However, the mature miR-150 was barely detectable in the HL60 cell line whereas it was present at a high level in the Jurkat cell line (Figure 3B). This data allowed us to test whether the ultramer extension system would detect only the mature miR-150 in these two cell lines. No signal was seen using the ultramer extension method for miR-150 in the HL60 cells (Figure 4B) whereas a strong, primarily cytoplasmic signal was seen in the Jurkat cells (Figure 4A). In situ hybridization with an LNA probe for miR-150 yielded an intense, primarily cytoplasmic signal for the Jurkat cell line (Figure 4C). Using the same hybridization conditions, no signal was seen in the HL60 cells (Figure 4D). Using RT in situ PCR for miR-150 precursors, the Jurkat cells showed a cytoplasmic signal (Figure 4F) whereas the HL60 cells showed a signal present in the entire cell (Figure 4E). The in situ data was then compared with cell fractionation data using real-time RT-PCR to compare the miRNA copy numbers in the nucleus versus the cytoplasm. As seen in Figure 3C, mature miR-150 had increased expression in the cytoplasm of the Jurkat cells compared with the nuclear fraction and was undetectable in either compartment in the HL60 cells; this is consistent with the LNA and ultramer extension data. The miR-150 precursors were present in both the nucleus and cytoplasm of the Jurkat cells (Figure 3C), predominating in the latter, which is consistent with the RT in situ PCR data. However, the miR-150 precursors were mostly cytoplasmic in the HL60 cells (Figure 3C) whereas the RT in situ PCR demonstrated a nuclear- and cytoplasmic-based signal (Figure 4E).

miR-128a and miR-1

miR-128a is highly expressed in normal brain (21,22). As demonstrated through real-time RT-PCR, normal brain tissue expressed a high copy number of mature and miR-128a precursors (Figure 5B). In comparison, whereas glioblastoma multiforme had equivalent amounts of miR-128a precursors, this malignant tumor did not express detectable mature miR-128a (Figure 5, A and B). Since the mature sequence of miR-128a is located at the 3' end of the precursor, the premiR-128a could theoretically prime the ultramer and be extended. Thus, normal versus malignant brain tissue allowed us to examine the specificity of the in situ ultramer extension method for the mature miRNA in this type of situation. We examined five normal brain tissues and three glioblastoma multiformes for mature and precursor miR-128a.

Using an LNA probe for miR-128a on normal brain tissue, a strong cytoplasmic signal was seen in neurons, but not other cell types such as endothelium and microglial cells (Figure 5C). The negative controls (scrambled probes or miR-96 probe) gave no signal (data not shown). Also, no signal was seen in the majority of cells in the glioblastoma samples with the LNA probe (Figure 5D). Rare glioblastoma cells showed a nuclear signal with the LNA probe that was slightly above background; the significance of this signal is unclear (data not shown). The ultramer extension method for miR-128a demonstrated a predominantly cytoplasmic signal in neurons in each of the five normal brain tissues (Figure 5E), but not in the three cases of glioblastoma multiforme (Figure 5F). RT in situ PCR for miR-128a precursors in normal brain tissue showed a predominantly cytoplasmic signal in cells with the cytologic features of neurons (Figure 5G), while in glioblastoma cells it showed a mostly nuclear signal (Figure 5H). Thus, the normal brain/glioblastoma data provided further evidence that the ultramer extension method is capable of only detecting the mature form of the miRNA and that extension of the pre-miRNA on the ultramer template is not occurring.

Finally, we examined normal cardiac tissue for miR-1, as the mature form of this miR is abundantly expressed in this tissue (22,23). The miR-1 ultramer-based signal was intense in the heart tissue where it was present in over 95% of the myocytes and not evident in the surrounding cells (Figure 6C). The signal was evident in a banding-type pattern that corresponded to the A and H bands and the M line of the sarcomere (Figure 6C), providing internal control evidence on the extension's specificity. The ultramer-based signal was eliminated if the inhibitor was added at the start of the reaction (Figure 6D, inset) or if the ultramer was omitted (Figure 6D). A similar cytoplasmic localization for the miR-1 precursors using RT in situ PCR was noted in cardiac tissue (Figure 6A), which was lost if the primers were omitted (Figure 6B). The ultramer extension method was done on five heart tissues in which the reporter nucleotide digoxigenin dUTP was omitted during the miR extension process. After the ultramer extension process occurred, in situ hybridization was done using the 101-nucleotide ultramer labeled with biotin. After in situ hybridization, a strong signal was evident in the myofibrils of cardiac tissue and not the surrounding cell types (Figure 6E). This signal was lost if the ultramer extension was omitted (Figure 6F).

Direct comparison of the ultramer extension and LNA in situ methods for miRNA detection

To compare the relative signal intensity of the LNA versus the ultramer extension method, serial sections from normal placenta blocks were chosen and analyzed at the same time for miRNAs 128a, 29b, and 16. As detailed by Liang et al. (22), miR-128a is not expressed in placenta, miR-29b is expressed in low copy number, and the copy number of miR-16 is sixty times higher than miR-29b. The relative intensity of the signals was then scored for six different 400× fields per slide, blinded to the miRNA being tested using Photoshop CS3 (Adobe Systems, Inc., San Jose, CA, USA) and the eyedrop RGB function on the entire field. The mean scores (\pm standard error) were as follows: miR-128a (LNA: 10.0 ± 2.8 ; ultramer: 8.1 ± 2.4), miR-29b (LNA: 63.0 ± 6.6 , ultramer: 64.3 ± 7.6), miR-16 (LNA: 115.1 ± 6.9 , ultramer: 122.3 ± 5.9). There was a significant difference between the signal intensities of miR-128a compared with miR-29b ($P = 0.002$) and miR-16 ($P = 0.0001$) for both the LNA probes and ultramer extension methods. There was also a significant difference between the intensities for low copy (miR-29b) and high copy (miR-16) microRNA for each method ($P = 0.002$). However, although the ultramer extension method gave less background and a stronger signal in each class, the differences were not statistically significant. Finally, the signal intensities for the LNA method were highly dependent on stringency conditions, whereas this was not the case with the ultramer extension method. Specifically, increasing the post-hybridization wash to 50°C, which is typical with LNA probes and miRNA detection (11,18) eliminated the LNA-based signal for miR-29b (11.0 ± 2.2) but had no effect on the signal for miR-29b as detected by the ultramer extension method (ultramer -71.2 ± 5.6).

Discussion

We have described a new method for the in situ detection of mature microRNAs. The major advantages of the ultramer extension method over the LNA probe method include that it is specific for the mature, active form of the miRNA, it is easier to optimize with a broader window between signal and background, is less sensitive to changes in stringency, and it is much less expensive. For example, changes in stringency of around 50°C with the LNA probe method could result in the eradication of the signal, as shown in this study and by Obernosterer et al. (18), or the emergence of background (16). Under the same changes in stringency, the ultramer extension method signal is not affected. Our cost for the LNA-labeled probe (250 pmol) was \$40 per test. In comparison, the cost for the ultramer (6000 pmol) was \$4 per test, although there would be the additional charge for the reporter nucleotide and polymerase. The potential for nonspecific DNA synthesis underlines the importance of performing the negative control after DNase digestion for RT in situ PCR (no PCR primers) or the ultramer extension reaction (no ultramer) on the same slide as the test section, and assuring that it shows no nuclear signal. Similarly, we routinely perform the negative control for the LNA in situ hybridization on a section on the same slide as the test, which underscores the importance of placing multiple sections per slide. Another important internal control for the interpretation of the specificity of in situ-based miRNA analysis is the well-documented cell and tissue specificity associated with many miRNAs (22).

The ultramer extension method yielded a signal in those cells and tissues for which real-time RT-PCR or published Northern blot analysis (22) supported the fact that the cells contained the mature form of the given miRNA. A reason that we chose to study cardiac tissue and normal brain tissues was because the abundant production of mature miRNAs that occur in these tissues (miR-1 and -128a, respectively) is well-established (21,22). Conversely, the ultramer extension method did not show any signal in HL60 cells for miR-150 or glioblastoma multiforme tissues for miR-128a, where real-time RT-PCR showed that these samples did contain the miRNA precursors, but not the mature form of the specific miR. This provides further evidence that this method is specific for mature miRNA.

Direct comparisons of in situ hybridization with an LNA-modified probe, RT in situ PCR, and the ultramer extension method provide insights into miRNA modulation. Specifically, the observation that miR-150 and miR-128a precursors tend to be nuclear when there is no detectable mature form (in HL 60 and malignant brain cells, respectively), but cytoplasmic in the corresponding cell types when the mature form is evident, suggests that precursor sequestration in the nucleus and its transport into the cytoplasm may play a role in the modulation of miRNA expression. We reported a similar phenomenon in various cancer cell lines (14). The data with miR-1, where the precursors are localized to the perimeter of myofibrils and the mature form was seen in the A/H bands of the myofibrils, provides further evidence that precursor transport to a specific compartment of the cell may be an important factor in the processing of the precursor by Dicer into the mature, active form.

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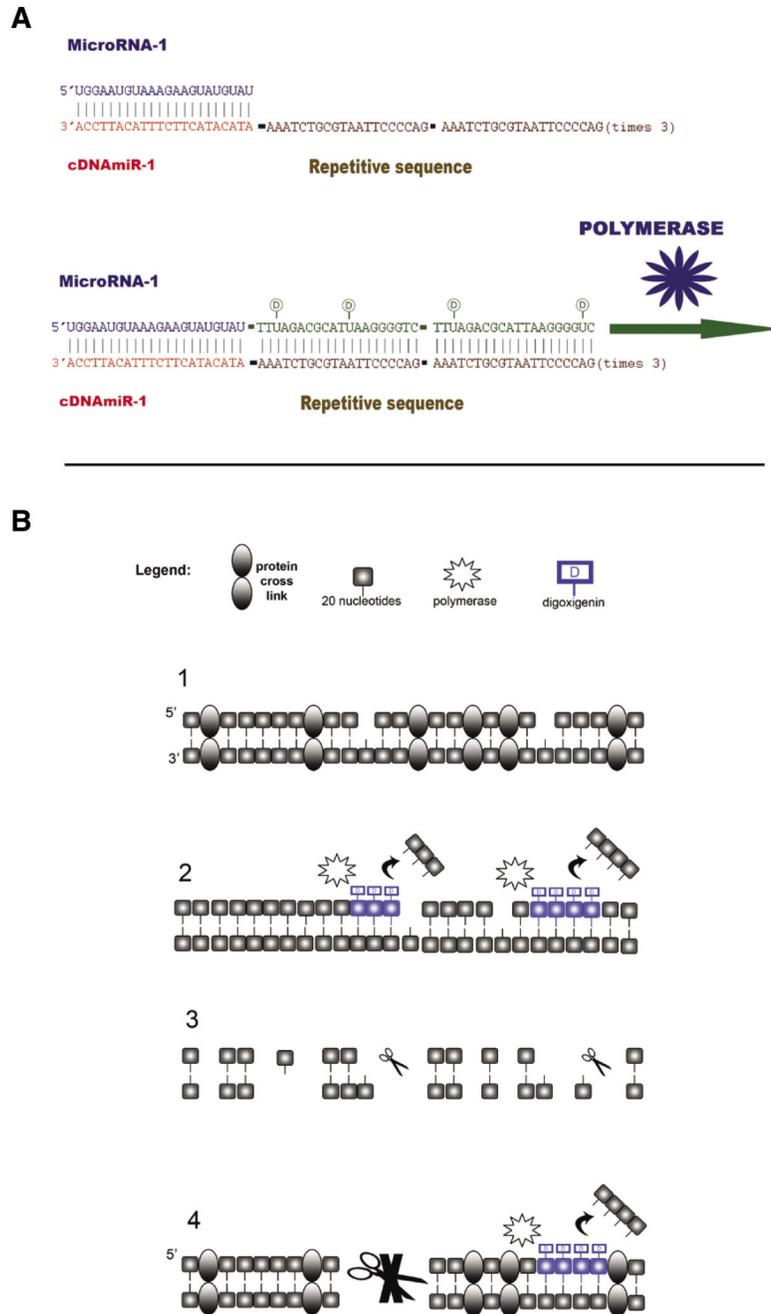


Figure 1. Schematic representation of (A) the ultramer extension method and (B) the non-specific in situ DNA synthesis process in paraffin-embedded, formalin-fixed tissues

(A) An ultramer that has the cDNA of the microRNA (upper panel; mature miR-1 is used in this example) at its 3' end preceded by a series of 20 nucleotide repeats (5'-GACCCCTTAATGCGTCTAAA-3') serves as the template for miR extension. As the miR is being extended, a reporter nucleotide such as digoxigenin-dUTP is incorporated into the synthesized DNA (lower panel) which can then be detected using an anti-digoxigenin antibody.

(B) Fixation in buffered formalin creates extensive crosslinking between proteins and nucleic acids in the cell. The high temperature (60–70°C) used in paraffin embedding the formalin-fixed tissues for tissue sections induces small nicks and short gaps in the DNA (part 1), which

are the initiation points for non-specific DNA synthesis by a DNA polymerase. This DNA repair-like process is facilitated by protease digestion, which removes the proteins crosslinked to the DNA (part 2). Optimal protease digestion also allows the genomic DNA to be completely digested by DNase (part 3) such that it can no longer support this nonspecific DNA synthesis pathway. If too many protein-DNA crosslinks persist due to inadequate protease digestion, then the DNase cannot eliminate this DNA repair-like pathway (part 4).



Figure 2. Solution phase extension of miR-376a ultramer

A miR-376a ultramer was primed by a ³²P-labeled miR-376a oligoribonucleotide. The oligos were extended using a variety of polymerases and the products were verified by gel electrophoresis. Each column represents either a positive or negative enzymatic reaction: 1, rTth polymerase; 2, no rTth polymerase control; 3, no *Taq* DNA polymerase control; 4, *Taq* DNA polymerase; 5, MMLV RT; 6, no MMLV RT control; 7, Multiscribe RT; 8, no Multiscribe RT control; 9, T7 RNA polymerase; 10, no T7 RNA polymerase control. The molecular size was estimated by comparing the migration to that of a 10-nucleotide (nt) RNA ladder.

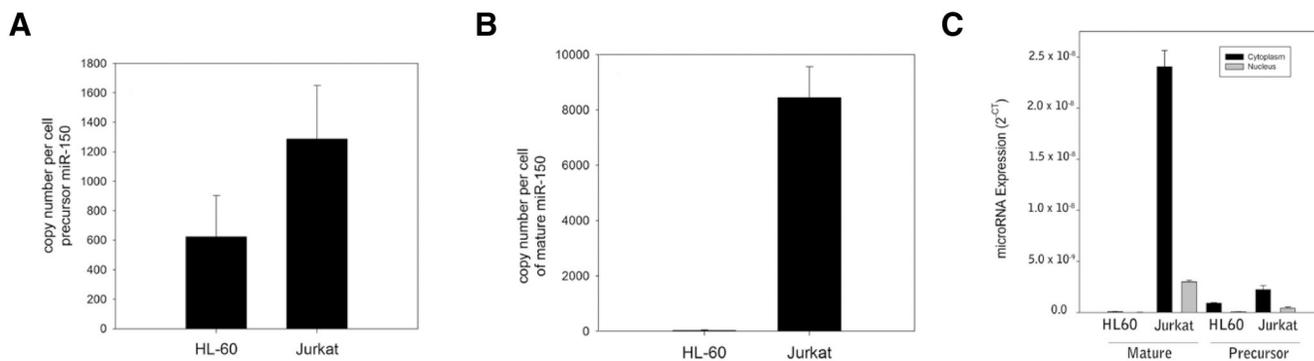


Figure 3. Real-time RT-PCR for miR-150 precursors and mature molecules in two cancer cell lines
 Real-time RT-PCR shows that miR-150 precursors were each present in the cell lines HL60 and Jurkat (A), while only the mature form of miR-150 was detectable in the Jurkat cell line (B). The cell fractionation experiments showed that the mature miR-150 was present in much higher amounts in the cytoplasm (black bar) than the nucleus (gray bar) in the Jurkat cells (C) and, thus, was consistent with the ultramer extension data (see Figure 4).

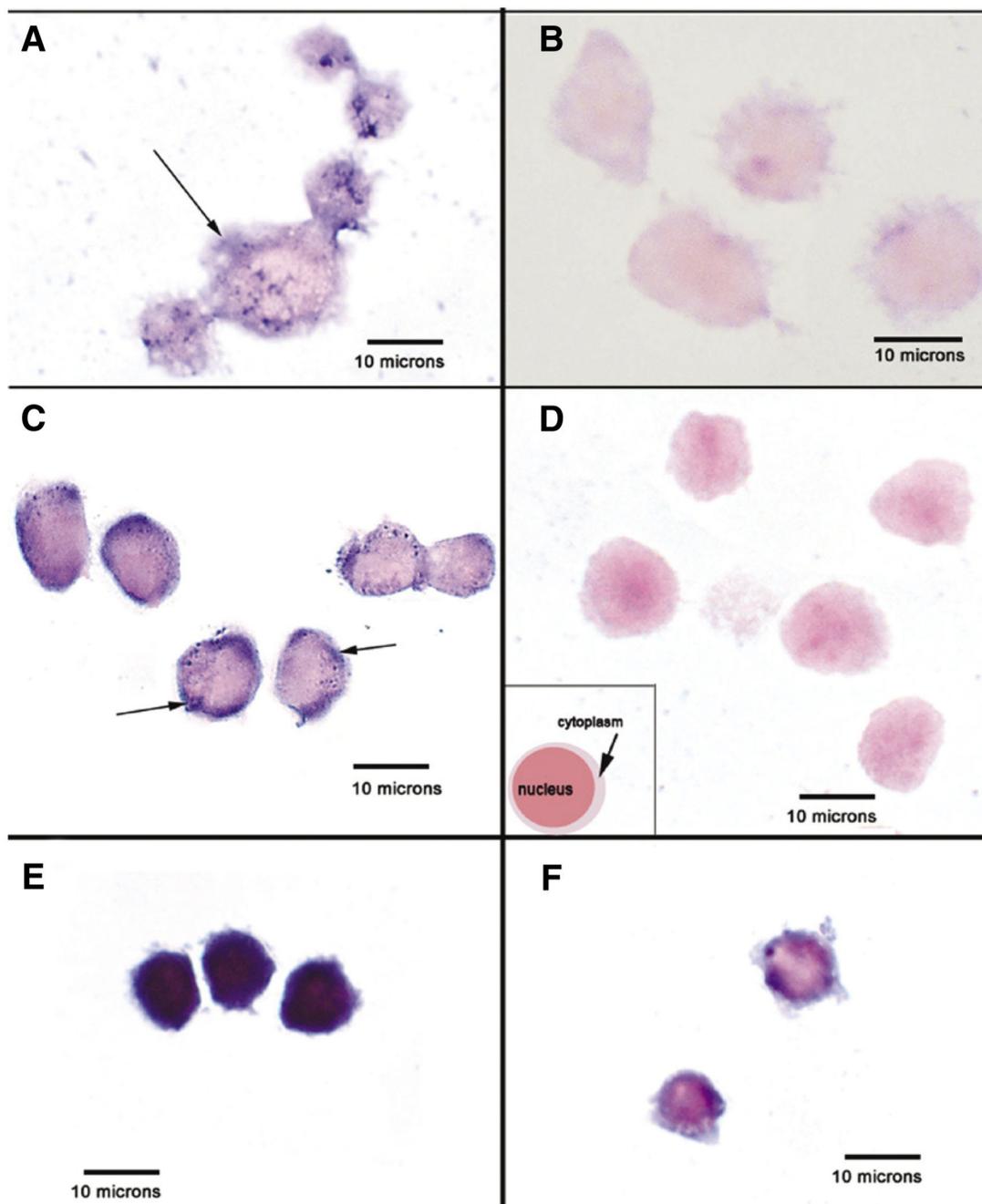


Figure 4. In situ detection of miR-150 precursors and mature molecules in two cancer cell lines
 The ultramer extension method for mature miR-150 yielded a strong cytoplasmic signal in the Jurkat cells (A, arrow) and no signal with the HL60 cells (B) under identical conditions. Using in situ hybridization with an LNA probe, a strong cytoplasmic signal was seen in the Jurkat cells (C, arrow) and no signal was seen in the majority of the HL 60 cells (D). The inset in D shows the relative sizes of the nuclear and cytoplasmic compartment in these cells. E and F show the results for RT in situ PCR using the primers for miR-150 precursors; note that the Jurkat signal (F) is primarily cytoplasmic. The signal for the miR-150 precursors in the HL60 cells (E) shows an intense signal that involves the entire cell. In such cases, it is not possible

to determine if the signal is cytoplasmic- and nuclear-based, or primarily nuclear-based (D, inset).

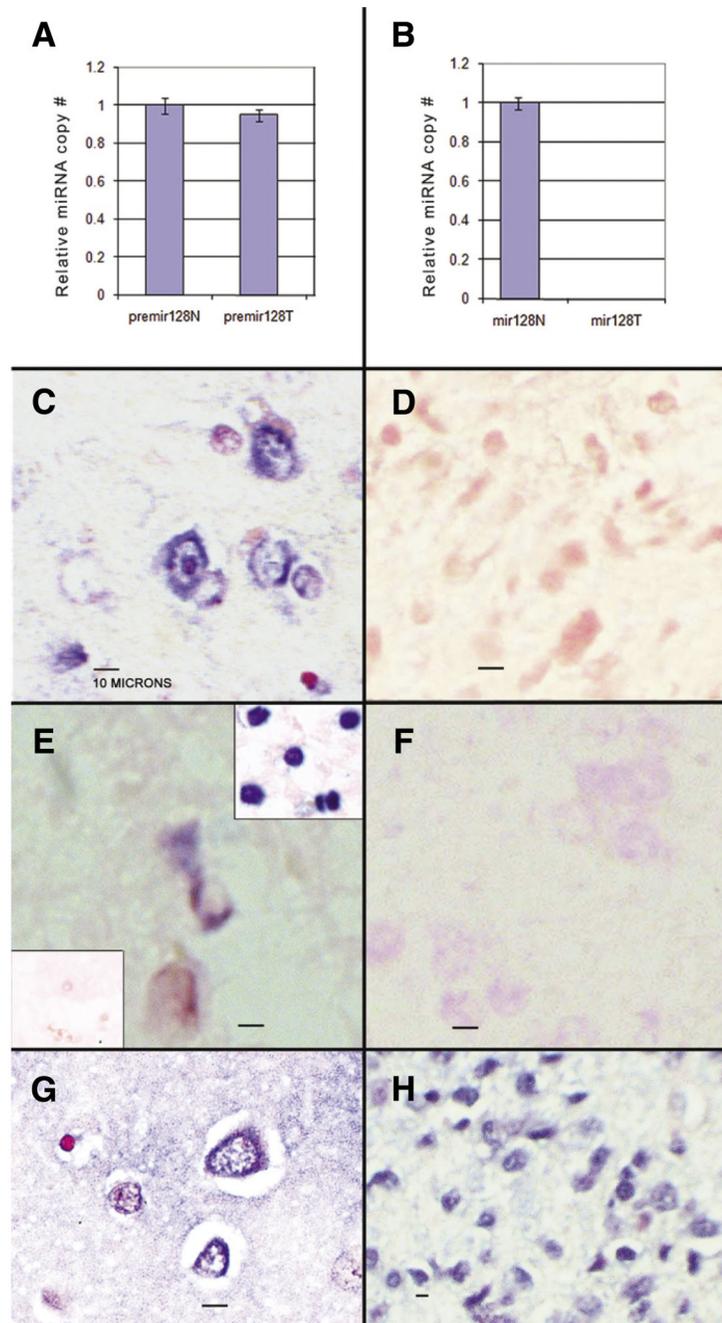


Figure 5. The in situ localization of miR-128a in normal versus malignant brain tissues (A) Equivalent copy numbers by qRT-PCR are shown for the miR-128a precursors in normal (premiR128N) versus malignant brain tissue (premiR128T), while the mature form of miR-128a was produced in normal brain tissue (miR128N) but not in the brain cancer cells (miR128T) (B). The LNA probe for miR-128a produced a strong cytoplasmic signal in neurons in normal brain (C) and no signal in the majority of glioblastoma multiforme cells (D). A cytoplasmic signal is seen after ultramer extension in normal brain, and it is localized to the same compartment of neurons as the LNA probe (E; the lower inset is the negative control of adding the inhibitor to the ultramer reaction and the upper inset is the control of no DNase). Malignant brain tissue showed the absence of any signal with the ultramer extension method

(F). G and H show the RT in situ PCR results for the miR-128a precursors in normal and malignant brain tissue, respectively; note the predominantly cytoplasmic signal seen in neurons (G) and the nuclear signal present in most malignant brain cells (H).

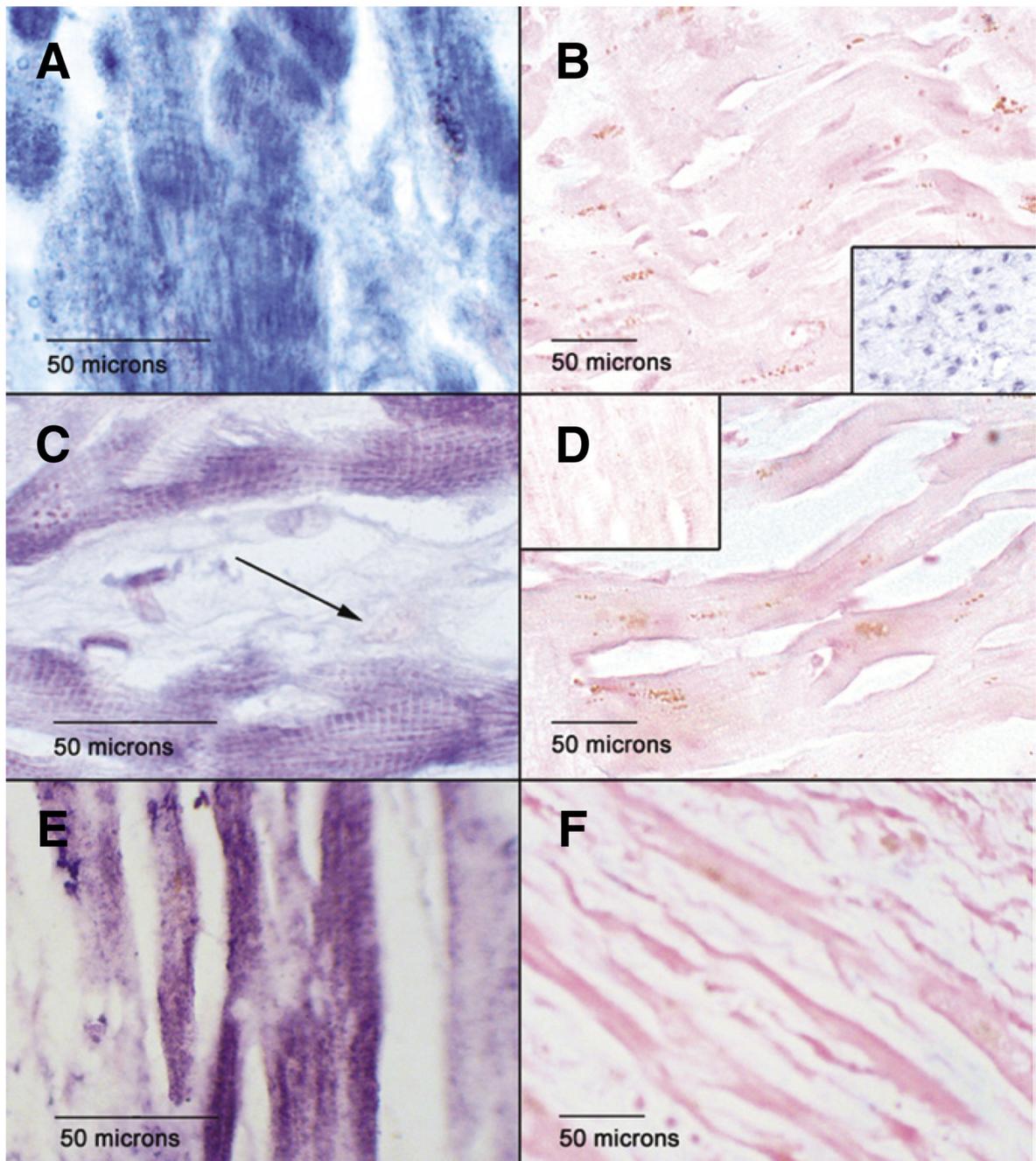


Figure 6. The in situ localization of miR-1

(A) Depiction of the miR-1 precursors in myocytes as detected by RT in situ PCR; note that the signal localizes to the periphery of each myofibril. The signal was lost if the primers were omitted (B); the insert shows the control of no DNase digestion where the signal is nuclear-based. With the ultramer extension method for the mature miR-1, the signal was seen in the myofibril, but now in the A and H bands (C); the arrow depicts a negative fibroblast which serves as an internal control. The ultramer extension signal was lost if the ultramer was omitted (D) or if the inhibitor DNA sequence was added at the onset of the extension (D, inset). E shows the results with the ultramer extension where the reporter nucleotide was omitted, and

the miR-1 extended product detected by in situ hybridization with a biotin labeled probe; the signal was lost if the ultramer was omitted during extension (F)

Table 1

Sequence of the LNA-Modified Probes and Ultramers Used in This Study

microRNA	Probe sequence
miRNA-1	TACATACTTCTTTACATTCCA
miR-16	TAGCAGCACGTAATATTGGCG
miR-29b	GAACACCAGGAGAAATCGGTCA
miR-96	AGCAAAAATGTGCTAGTGCCAAA
miRNA-128a	AAAGAGACCGGTTCACTGTGA
miRNA-150	CACTGGTACAAGGGTTGGGAGA
Scrambled probe (miR-128a)	CGTATAGGCCCAAGAATTAGG
Ultramer miR-1	GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA <u>TACATACTTCTTTACATTCCA</u>
Ultramer miR-16	GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA <u>TAGCAGCACGTAATATTGGCG</u>
Ultramer miR-29b	GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA <u>GAACACCAGGAGAAATCGGTCA</u>
Ultramer miR-128a	GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA <u>AAAGAGACCGGTTCACTGTGA</u>
Ultramer miR-150	GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA <u>CACTGGTACAAGGGTTGGGAGA</u>
Scrambled ultramer miR-128a-	GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAA CGTATAGGCCCAAGAATTAGG
Inhibitor	TTTAGACGCATTAAGGGGTC

The underlined sequence is complementary to the mature miRNA.