Title

Quantitative Real-Time PCR Analysis of MicroRNAs and Their Precursors Regulated by TGF-β Signaling

Authors

Kang, Hara

Hata, Akiko

Publication Date

2016

DOI

10.1007/978-1-4939-2966-5_20

Peer reviewed

Chapter 20

Quantitative Real-Time PCR Analysis of MicroRNAs and Their Precursors Regulated by TGF-β Signaling

Hara Kang and Akiko Hata

Abstract

The signaling pathway of TGF-β and its family member BMP has been implicated in vascular development and maintenance of homeostasis by modulating expression of small noncoding microRNAs (miRNAs). MiRNAs repress target genes, which play a critical role in regulating vascular smooth muscle cell (VSMC) growth, phenotype, and function. To understand the mechanisms by which specific miRNAs control the TGF-β and BMP signaling pathway in VSMC, it is essential to quantitate levels of specific miRNAs and their precursors whose expression are controlled by TGF-β/BMP signaling. Here, we describe a real-time quantization method for accurate and sensitive detection of miRNAs and their precursors, such as primary transcripts of miRNAs (pri-miRNAs) and precursor miRNAs (pre-miRNAs). This method requires two steps; synthesis of single-stranded complementary DNAs (cDNAs) from total RNA samples and quantization of specific pri-, pre-, or mature miRNAs by quantitative polymerase chain reaction (PCR) using a real-time PCR machine.

Key words MicroRNA, TGF-β, BMP, Quantitative real-time PCR

1 Introduction

The transforming growth factor-β (TGF-β) family of growth factors is evolutionarily conserved. These growth factors are critical modulators of various biological processes such as cell fate determination, cell differentiation, proliferation, and migration in embryos and adults from insects to mammals [1], including vascular development and the maintenance of homeostasis. The TGF-β family of growth factors, such as TGF-β and BMP, has been shown to promote differentiation of VSMC by upregulating the expression of contractile genes and inhibiting VSMC growth and migration [2, 3]. In recent studies, several miRNAs were found to be expressed in the vascular cells and deregulation of these miRNAs is involved in vascular diseases [4, 5]. Moreover, Smad proteins, as signal transducers of the TGF-β family of growth factors, have been demonstrated to regulate generation of subset of miRNAs at

Xin-Hua Feng et al. (eds.), *TGF-β Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 1344,
DOI 10.1007/978-1-4939-2966-5_20,

the transcriptional and/or posttranscriptional level [6, 7]. In this chapter we summarize the mechanism of regulation of miRNA biogenesis by the TGF-β family of growth factors and techniques to quantitate the levels of miRNAs and their precursors upon stimulation with the TGF-β family of growth factors.

1.1 Biogenesis of miRNAs

miRNAs are transcribed by RNA polymerase II as long primary transcripts known as pri-miRNAs which contain a hairpin structure. Although mature form of miRNAs is ~22 nucleotides (nt) in length, pri-miRNAs can be as long as a few kilo nt long, and are cleaved sequentially by two ribonuclease enzymes; Drosha and Dicer [8–11]. After the first processing by Drosha in the nucleus, pre-miRNAs, which are ~60–100 nt hairpins, are exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, the pre-miRNAs associate with Dicer, which cleaves the pre-miRNA into an approximately 22 nt miRNA. The miRNA duplex is then loaded into Argonaute (Ago) proteins, which select one strand and present it to the RNA-induced silencing complex (RISC) for recognizing target mRNAs. The mature miRNAs regulate expression of protein coding genes by promoting degradation of mRNAs or repressing their translation [12] (Fig. 1a). Although miRNAs represent a relatively abundant class of RNAs, their expression levels are found to vary greatly in different cell types, tissues, and developmental stages. To elucidate a critical role of miRNA during normal physiological and developmental processes, as well as pathogenesis of various disorders, sensitive, efficient, and accurate quantitation of miRNA levels is essential.

1.2 Control of miRNA Biogenesis by Smads and Quantitative Analysis of Pri-, Pre-, and Mature miRNAs

One of the best-characterized steps of miRNA biogenesis is at the point of pri-to-pre-miRNA processing by the Drosha microprocessor complex. It was discovered that when pulmonary artery smooth muscle cells (PASMCs) are stimulated with ligands of the TGF-β family, such as TGF-β and bone morphogenetic protein 4 (BMP4), pri-miR-21 levels are unchanged while mature miR-21 expression increases significantly [3, 6]. This result suggested a transcription-independent mechanism of miR-21 regulation, which was confirmed by the induction of miR-21 even in the presence of the transcriptional inhibitor α-amanitin [3, 6]. Biochemical analysis showed that the increase in mature miR-21 is caused by faster turnover in the biogenesis of miR-21 mediated by the nuclear translocation of Smad proteins [3, 6].

Smads represent the primary signal-transduction molecules of the TGF-β family of growth factors. Receptor activation by TGF-β family ligands induces phosphorylation of several Smad proteins, collectively called the Receptor-specific Smads (R-Smads) [1]. Phosphorylated R-Smads form a complex with the common-Smad (co-Smad), Smad4, and translocate to the nucleus where they act as transcriptional regulators to promote or inhibit gene expression [1].

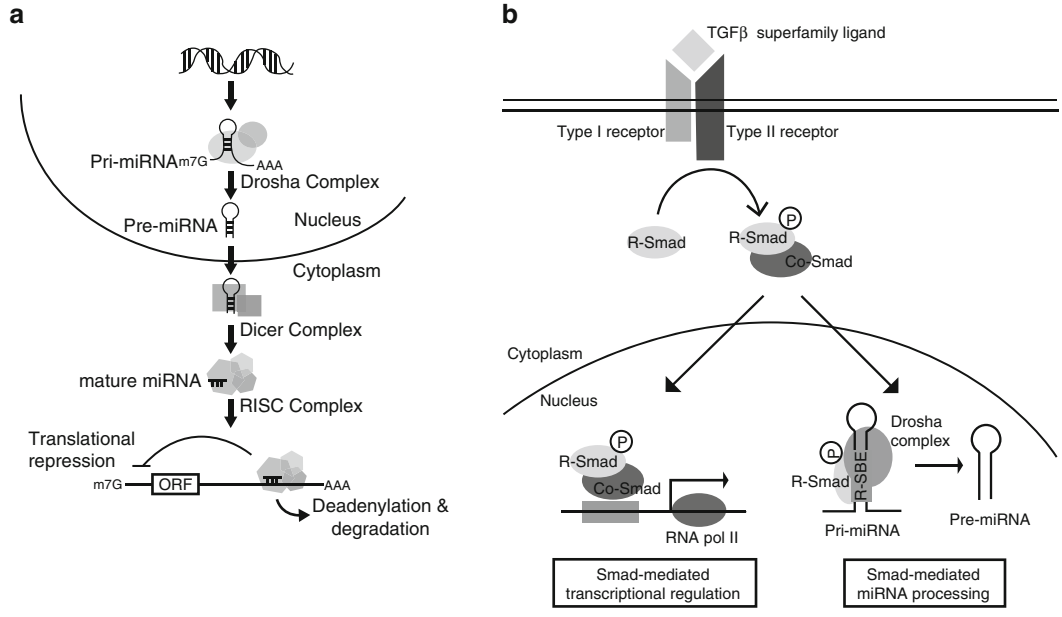

Fig. 1 Schematic diagram of miRNA biogenesis and its regulation by the TGF-β signaling pathway. (**a**) Pri-miRNAs are transcribed and processed into hairpin-structured pre-miRNAs by Drosha complex. The pre-miRNAs are exported from the nucleus to the cytoplasm and are cleaved by the Dicer complex into ~22 nt miRNAs. The mature miRNA is loaded into the RISC complex and mediates posttranscriptional repression of target mRNA by translational repression and/or deadenylation and degradation. (**b**) Activation of receptor Smads by TGF-β family ligands leads to their translocation into the nucleus. In the nucleus, R-Smads bind, independent of the transcriptionally necessary co-Smad, to a conserved sequence in pri-miRNAs which they recruit to the Drosha microprocessor complex and facilitate pri- to pre-miRNA maturation

R-Smad/co-Smad hetero-dimerization is required for the transcriptional regulation mediated by TGF-β signaling [1]. Surprisingly, Smad4 is dispensable for TGF-β mediated induction of miR-21 [3, 6]. Furthermore, stimulation with TGF-β ligands induced an association between Smads and the RNA helicase p68, indicating a direct recruitment of R-Smads to the Drosha microprocessor complex [3, 6].

More recent miRNA expression profiling experiments have revealed that stimulation of PASMCs with either TGF-β or the related BMP4 induces the expression of approximately 20 miRNAs [6]. Following stimulation, many of these miRNAs are bound directly by Smad proteins through a 5-base RNA sequence motif (R-SBE) that closely resembles the Smad DNA binding element (SBE; 5′-CAGAC-3′) in a region overlapping the encoded mature miRNA [6]. Thus, R-Smads associate with pri-miRNA in a sequence specific manner and facilitate the Drosha microprocessor activity to enhance mature miRNA expression (Fig. 1b). Interestingly, in silico analyses have found that pre-miRNA sequences encode a higher number of transcription factor biding

sites than would be predicted by chance [13]. These results suggest that the mechanism for Smad-dependent regulation of pri-miRNAs may represent a widespread, though under appreciated, strategy for controlling miRNA expression.

During the last decade, more than 30 miRNA quantitation methodologies have been developed, including northern blot [14], miRNA microarray [15], next generation sequencing [16], and quantitative real-time PCR (qPCR) [17, 18]. Of these methods, qPCR is most sensitive and easily applicable to validate the data obtained from the high-throughput approaches. Our group has found that it is difficult to detect small changes in miRNA expression upon growth factor stimulation using next generation sequence (A.H. unpublished observation). qPCR-based miRNA analysis is also convenient as it does not require the use of radioactive material. Because of the small size, detection of mature miRNAs by PCR is technically challenging. A number of specific qPCR techniques have been developed for the detection of miRNAs. Here, we present qPCR methods based upon reverse transcriptase reaction with a stem-loop primer followed by a TaqMan PCR analysis to detect changes in expression levels of miR-21 and miR-302c in the cell in response to stimulation with the TGF-β family of growth factors [7, 19] (Fig. 2).

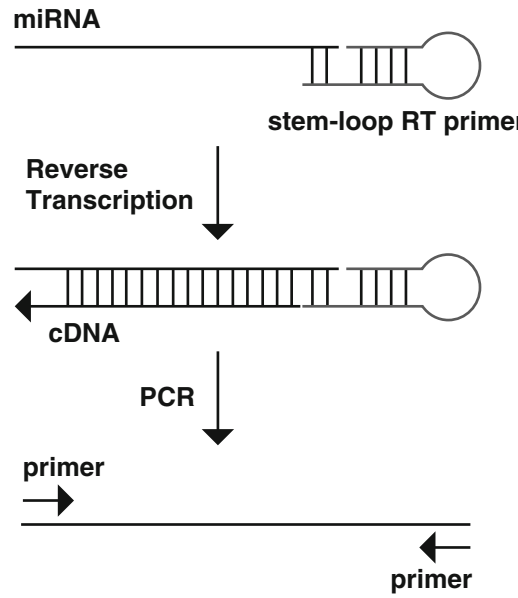

Fig. 2 Schematic description of miRNA detection by qPCR. Stem-loop RT primer binds to the 3′ end of miRNA and is reverse-transcribed. The synthesized cDNA is amplified using miRNA specific PCR primers

2 Materials

Cell culture reagents: Human pulmonary artery smooth muscle cells (PASMC) and SmGM-2 smooth muscle growth medium (CC-2581 and CC-3182) are purchased from Lonza. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin sulfate, 0.25 % Trypsin–EDTA, and phosphate buffered saline (PBS) are purchased from Invitrogen. For cytokine stimulation, human recombinant TGF-β1 and BMP4 are purchased from R&D systems.

RNA isolation components: TRIzol and glycogen are purchased from Invitrogen. Chloroform, ethanol, and isopropanol are purchased from Sigma.

Reverse-transcription (*RT*) *components*: To synthesize cDNA of mature miRNAs, TaqMan miRNA reverse transcription kit (PN 4366596) and TaqMan MicroRNA Assays for human U6 snRNA (#001973), hsa-miR-21 (#000397), and hsa-miR-302c (#000533) including miRNA-specific RT primers are purchased from Applied Biosystems (Table 1). To synthesize cDNA of pri- or pre-miRNA, iScriptcDNA synthesis kit is purchased from Bio-Rad. For RT reactions, S1000 Thermal cycler (Bio-Rad) is used.

qPCR components: Each TaqMan MicroRNA Assay contains a mix of a miRNA-specific forward PCR primer, a specific reverse PCR primer, and miRNA-specific TaqMan probe linked to FAM dye. AmpliTaq DNA polymerase with buffer I (N8080160) and 10 mM dNTP mix are purchased from Applied Biosystems. To detect pri- and pre-miRNA levels, primers are synthesized from IDT and iQ

Table 1
miRNA, control U6 snRNA, and primer sequences used for qPCR

Name	Sequence
Has-miR-21	UAGCUUAUCAGACUGAUGUUGA
Has-miR-302c	UAAGUGCUUCCAUGUUUAGG
Human U6 snRNA	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACA GAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAA TTCGTGAAGCGTTCCATATTTT
Human pri-miR-302c	TGAATCCAATTTACTTCTCCA and TCCTTTAACCTGTAACAAGC
Human pre-miR-302c	CCTTTGCTTTAACATGGGGG and CCTCCACTGAAACATGGAAG
Human pri-miR-21	TTTTGTTTTGCTTGGGAGGA and AGCAGACAGTCAGGCAGGAT
Human pre-miR-21	TGTCGGGTAGCTTATCAGAC and TGTCAGACAGCCCATCGACT
Human *GAPDH*	ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA

SYBR Green Supermix is purchased from Bio-Rad. Primer sequences are shown in Table 1. Hard-shell thin-wall 96-well skirted PCR plates and microseal "B" adhesive seals are purchased from Bio-Rad. For qPCR reactions, the MJ Research Opticon2 continuous fluorescence detection system is used.

3 Methods

3.1 Cell Culture and Stimulation with TGF-β Family Growth Factors

Human PASMC are maintained in SmGM-2 media containing 5 % fetal calf serum. Early passage (passage 6–8) PASMC are split into 6-well plates with 120,000 cells/well. To examine changes of miRNA expression levels upon TGF-β signals, cells are cultured in DMEM with 0.2 % FBS for 24 h before growth factor stimulation. TGF-β (400 pM) or BMP4 (3 nM) is treated under starvation conditions (DMEM with 0.2 % FBS) for 24 h. Cells are cultured at 37 °C in the presence of 5 % CO_2.

3.2 RNA Isolation

1. Rinse cells with PBS and add 500 μL of TRIzol directly to the cells in a well of the 6-well plate to isolate total RNA.
2. Incubate the plate on a rotator for 5 min at room temperature and lyse the cells directly by pipetting.
3. Collect and transfer the mixture of cells and TRIzol into an eppendorf tube (E-tube). Homogenized samples can be stored at room temperature for several hours or at −80 °C for at least 1 month.
4. Add 100 μL of chloroform, shake vigorously for 15 s, and spin at 15,000 × *g* for 15 min at 4 °C. The homogenate will separate into a clear upper aqueous layer containing RNA, an interphase, and a red lower organic layer containing the DNA and proteins.
5. Collect the upper aqueous layer and transfer it into a new E-tube and add 250 μL of isopropanol and 40 μg of glycogen to the sample. Incubate for 10 min at room temperature. RNA is precipitated from the aqueous layer by the isopropanol.
6. Centrifuge the sample at 15,000 × *g* for 15 min at 4 °C.
7. Decant the supernatant and wash the pellet with 1 mL of 75 % ethanol to remove impurities.
8. Spin at 15,000 × *g* for 5 min at 4 °C to remove any remaining ethanol. Air-dry the pellet for 5–10 min.
9. Dissolve the RNA pellet in 20 μL of nuclease-free water and incubate at 55 °C for 10 min. Isolated total RNA can be frozen and stored at −80 °C.

3.3 cDNA Synthesis for Detection of Mature miRNAs

cDNA is reverse-transcribed from the isolated total RNA using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan MicroRNA Reverse Transcription kit.

All experimental procedure should be done on ice. Vortex samples thoroughly between each step to be sure to mix.

1. Dilute the isolated total RNA to 10 ng/μL in nuclease-free water.
2. Prepare RT master mix. For each 15 μL RT reaction, RT master mix consists of 9.66 μL of nuclease-free water, 1.5 μL of 10× reverse transcription buffer, 0.19 μL of RNase inhibitor (20 U/μL), 0.15 μL of 100 mM dNTPs, and 1 μL of MultiScribe reverse transcriptase (50 U/μL).
3. Gently mix 1 μL of the diluted total RNA, 1.5 μL of specific miRNA primer with 12.5 μL of RT master mix.
4. Spin to bring solution to the bottom of the tube and incubate the tube on ice until a thermal cycler is ready.
5. To perform reverse transcription, program a thermal cycler. The reaction proceeds for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C.
6. Load the reaction tube into the thermal cycler and start the reverse transcription run.

3.4 qPCR Analysis for Detection of Mature miRNAs

For qPCR, performed three replicates each of 10 μL PCR reactions (triplicate). All experimental procedure should be done on ice.

1. Prepare PCR master mix. For each 10 μL PCR reaction, PCR master mix consists of 7.2 μL of nuclease-free water, 1 μL of 10× PCR buffer, 0.2 μL of 10 mM dNTPs, and 0.1 μL of AmpliTaq DNA polymerase.
2. Gently mix the PCR master mix with 0.5 μL of the specific miRNA PCR primer from TaqMan MicroRNA Assay.
3. Add 1 μL of the synthesized cDNA from the RT reaction tube into the PCR reaction tube.
4. Mix and spin to bring the solution to the bottom of the tube and prepare the PCR reaction plate by transferring 10 μL of the complete PCR reaction mix into each well.
5. Seal the plate with an optical adhesive cover and centrifuge the plate at 220×*g* for 2 min at 4 °C to spin down the contents and eliminate any air bubbles.
6. Load the reaction plate into the MJ Research Opticon2 continuous fluorescence detection system and start the run. The reactions are incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

3.5 cDNA Synthesis for Detection of Pri- and Pre- miRNAs

We have found that the 5× iScript reaction mix generates some precipitation upon thawing occasionally. Mix thoroughly to resuspend the precipitate.

1. For each 20 μL RT reaction, mix 4 μL of 5× iScript reaction mix, 1 μL of iScript reverse transcriptase, and 1 μg of total RNA. Add nuclease-free water to make total volume of 20 μL.
2. Mix gently and spin to bring solution to the bottom of the tube.
3. To perform reverse transcription, incubate the reaction mix for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C in the thermal cycler.

3.6 qPCR Analysis for Detection of Pri- and Pre-miRNAs

To amplify pri-miRNAs, forward and reverse primers are designed to anneal to the outside stem portion of the hairpin sequence of the miRNA precursors. On the other hand, to amplify pre-miRNAs, primers are designed to be located within the hairpin sequence of the pre-miRNA. In theory, pre-miRNA primers are able to anneal and amplify pri-miRNAs, however, due to a secondary structure of long pri-miRNAs, pre-miRNA primers do not amplify pri-miRNAs efficiently under the PCR condition described below, and selectively measure the quantity of pre-miRNAs. Pre-miRNA sequences are obtained from the miRBase website (www.mirbase.org). The extended pre-miRNA sequences to design pri-miRNA primers are obtained from Ensembl Genome Browser (www.ensembl.org). Primers are designed using Primer3Plus (http://frodo.wi.mit.edu/). Primers are designed with a maximal Tm difference between both primers of less than 2 °C and a primer length between 18 and 24 nt. An ideal Tm of 55–60 °C is selected for the primers. Following design and purchase of primers from IDT (Table 1), qPCR is carried out as follows.

1. Thaw iQ SYBR Green supermix and store on ice protected from light.
2. Prepare PCR reaction mix. For each 10 μL PCR reaction, PCR master mix consists of 5 μL of iQ SYBR Green supermix, 0.5 μL of both 10 μM forward and reverse primers, 3.5 μL of nuclease-free water, and 0.5 μL of cDNA.
3. Mix gently and spin to bring the solution to the bottom of the tube and prepare the PCR reaction plate by transferring 10 μL of the complete PCR reaction mix into each well.
4. Seal the plate with an optical adhesive cover and centrifuge the plate at 220×*g* for 2 min at 4 °C to spin down the contents and eliminate any air bubbles.
5. Load the reaction plate into the MJ Research Opticon2 continuous fluorescence detection system and start the run. PCR cycling conditions are 94 °C for 3 min and 40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 40 s.

3.7 Analysis of qPCR Data

Data analysis is performed using the comparative C_T method in the accompanying MJ Opticon Monitor 3 software. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. An average of three experiments, each performed in triplicate with standard errors is presented. To normalize human miR-21 or miR-302c, the constitutively expressed endogenous control, human U6 snRNA, is used. Expression levels of pri- and pre-miRNAs are normalized to the endogenous expression level of human *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*.

4 Notes

1. Use RNase ZAP spray, filter tip, and nuclease-free water throughout all the experimental procedures to avoid RNase contamination.
2. We find that the RNA is often invisible prior to centrifugation and forms a gel-like pellet on the side and bottom of the tube.
3. Do not allow the RNA to dry completely, because the RNA pellet will become difficult to solubilize. Purity of RNA samples should be examined by measuring the $OD_{260/280}$ ratio, which should be greater than 1.6.
4. RT reactions should include negative controls without addition of RNAs. All PCR reactions should include samples that are not treated with RT as negative controls.
5. Keep all TaqMan MicroRNA Assays protected from light during storage in the freezer until ready to be used. Excessive exposure to light affects the fluorescent probes and decreases the sensitivity of quantization during qPCR.
6. The RT primers which contain the stem-loop structure increase the specificity and sensitivity of the assay in comparison with regular linear RT-primers because of base stacking and spatial constraint of the stem-loop structure [17].
7. TaqMan miRNA assays are specific for mature miRNAs and discriminate between related miRNAs that differ by as little as one nucleotide. Furthermore, they are not affected by genomic DNA contamination.
8. This method uses commercially available miRNA detection kits and primer sets. It requires a unique probe and set of primers for each miRNA assay, and therefore is neither cost effective nor suitable for high-throughput analysis of miRNAs.

5 Concluding Remarks

Control of miRNA biogenesis is an integral component of cell biological activities. Deregulation of miRNA biogenesis results in aberrant expression of multiple mRNAs, which can lead to developmental defects and human diseases. Increasingly studies highlight the importance understanding transcriptional regulation as well as posttranscriptional regulation of miRNA biogenesis upon stimulation with different growth factors. We anticipate that in the future more accurate and easy methodology to quantitate miRNAs will be developed. In the meanwhile, the RT-PCR method described above provides a basis for the quantization of miRNAs and their precursors upon growth factor signaling and during pathogenesis of various human disorders.

Acknowledgement

We thank members of the Hata lab in particular Matt Blahna for critical reading of the manuscript. This work was supported by grants from the National Institute of Health: HL093154 and HL108317, the American Heart Association: 0940095N and the LeDucq foundation Transatlantic network grant to A.H. and the National Research Foundation of Korea (Basic Science Research Program; 2012R1A1A1042812) to H.K.

References

1. Massague J (2012) TGFbeta signalling in context. Nat Rev Mol Cell Biol 13:616–630
2. ten Dijke P, Arthur HM (2007) Extracellular control of TGFbeta signalling in vascular development and disease. Nat Rev Mol Cell Biol 8:857–869
3. Davis BN, Hilyard AC, Lagna G, Hata A (2008) SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454:56–61
4. Hata A. Functions of microRNAs in cardiovascular biology and disease. Annu Rev Physiol 75:69–93
5. Kang H, Hata A. (2012) MicroRNA regulation of smooth muscle gene expression and phenotype. Curr Opin Hematol 19:224–231
6. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. (2010) Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. Mol Cell 39:373–384
7. Kang H, Davis-Dusenbery BN, Nguyen PH, Lal A, Lieberman J et al. (2012) Bone morphogenetic protein 4 promotes vascular smooth muscle contractility by activating microRNA-21 (miR-21), which down-regulates expression of family of dedicator of cytokinesis (DOCK) proteins. J Biol Chem 287:3976–3986
8. Borchert GM, Lanier W, Davidson BL (2006) RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13:1097–1101
9. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the microprocessor complex. Nature 432:231–235
10. Han J, Lee Y, Yeom KH, Nam JW, Heo I et al (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125:887–901
11. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T et al (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293:834–838
12. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215–233

13. Piriyapongsa J, Jordan IK, Conley AB, Ronan T, Smalheiser NR (2011) Transcription factor binding sites are highly enriched within microRNA precursor sequences. Biol Direct 6:61
14. Valoczi A, Hornyik C, Varga N, Burgyan J, Kauppinen S et al (2004) Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res 32, e175
15. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A et al. (2010) Circulating microRNAs in patients with coronary artery disease. Circ Res 107:677–684
16. Wu Q, Lu Z, Li H, Lu J, Guo L et al (2011) Next-generation sequencing of microRNAs for breast cancer detection. J Biomed Biotechnol 2011: 597145
17. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33, e179
18. Benes V, Castoldi M. (2010) Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods 50:244–249
19. Kang H, Louie J, Weisman A, Sheu-Gruttadauria J, Davis-Dusenbery BN et al. (2012) Inhibition of microRNA-302 (miR-302) by bone morphogenetic protein 4 (BMP4) facilitates the BMP signaling pathway. J Biol Chem 287:38656–38664