MICRORNAS: TISSUE EXPRESSION AND ROLE IN 3T3-L1 PRE-ADIPOCYTE DIFFERENTIATION

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MicroRNAs, which are endogenous small RNAs around 22 nucleotides, play important roles in many physiological processes. We investigated whether microRNAs regulate 3T3-L1 pre-adipocyte differentiation. The expression of microRNAs during 3T3-L1 pre-adipocyte differentiation was detected by microarrays and confirmed by northern blot and quantitative real time PCR. Several microRNAs, including *let-7*, were up-regulated at the late stage of 3T3-L1 adipogenesis. *let-7* expression specifically increased during the late stage of 3T3-L1 differentiation and ectopic introduction of *let-7* in 3T3-L1 cells before hormonal induction

inhibited 3T3-L1 adipogenesis. Both the mRNA and protein levels of HMGA2, a target of *let-7*, decreased with ectopic *let-7* presence in 3T3-L1 cells. Also, HMGA2 protein level was inversely correlated to *let-7* levels during 3T3-L1 adipogenesis. Knock-down of *Hmga2* or *E2f1* by siRNA inhibited 3T3-L1 pre-adipocyte differentiation. Our results suggest *let-7* can stop clonal expansion of 3T3-L1 cells and bring them to final growth arrest and terminal differentiation by targeting *Hmga2*.

In an effort to explore the role of microRNA, the expression of 111 microRNAs in 36 mouse tissues was detected by quantitative real time PCR. MicroRNAs either show universal expression in all tissues or specific expression in certain tissues, suggesting their roles in these tissues. Different isotypes of the same microRNAs or microRNAs transcribed from the same genomic location show similar expression pattern in mouse tissues. Hierarchical cluster analysis based on the expression of microRNA in tissues showed that tissues having similar physiologic functions or anatomic locations clustered together, suggesting the roles of microRNAs might be consistent with the functions of the tissues in which they are expressed. Comparison of the expression of microRNAs with that of nuclear receptors in mouse tissues showed positive correlations between a select number of nuclear receptors and microRNAs, but these relationships need to be verified by experimental data. In all, the expression profile of microRNAs in mouse tissues provides a useful tool for microRNA studies.

PRIOR PUBLICATIONS

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LIST OF ABBREVIATION

aP2	Fatty acid binding protein 4
APL	Acute promyelocytic leukemia
ASO	Antisense oligonucleotides
Ban	Bantam
BAT	Brown adipose tissue
BCKD	Branched chain alpha-ketoacid dehydrogenase
BMAL1	Brain and muscle Arnt-like protein-1
BMP	Bone morphogenetic proteins
C/EBP	CCAAT/enhancer-binding proteins
CAR	Constitutive androstane receptor
CHOP	C/EBP homologous protein
CNS	Central nervous system
CTGF	Connective tissue growth factor
Dlk1	Delta-like 1
DMEM	Dulbecco's Modified Eagle's Medium
DMI	Dexamethasone, IBMX and insulin
DRPLA	Dentatorubral-pallidoluysian atrophy
EB	Ethidium bromide
Ebf	Early B cell factor
EGF	Epidermal growth factor

EPAS1	Endothelial PAS domain protein 1
ERG	Ether-a-go-go related gene
ERK	Extracellular signal regulated kinase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FMR1	Fragile X mental retardation 1
Fstl1	Follistatin-like 1
FXR	Farnesoid X receptor
GJA1	Gap junction protein, alpha 1
GR	Glucocorticoids receptor
HDAC4	Histone deacetylase 4
Hh	Hedgehog
HIV	Human immunodeficiency virus
Hmga2	High mobitilty group AT-hook 2
HNF4	Hepatocyte nuclear factor-4
HRP	Horseradish Peroxidase
IBMX	3-isobutyl-1-methylxanthine
IGF1	Insulin-like growth factor 1
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IRAK1	IL-1 receptor-associated kinase 1

IRS	Insulin receptor substrate
KCNJ2	Potassium inwardly-rectifying channel, subfamily J, member 2
KLF	Krüppel-like Factor
Lck	Lymphocyte specific protein tyrosine kinase
LNA	Locked nucleic acid
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAPK	Mitogen activated protein kinases
MEF	Mouse embryonic fibroblasts
miRISC	MiRNA containing RNA induced silencing complex
miRNA	MicroRNA
MVEC	Microvascular endothelial cells
OATP2	Organic anion transporting polypeptide 2
PANK	Pantothenate kinase enzyme
PBS	Phosphate buffer solution
PDCD4	Programmed cell death 4
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
PFV-1	Primate foamy virus type 1
PI3K	Phosphatidylinositol-3 kinase
PKB	Protein kinase B
PPAR	Peroxisome proliferator-activated receptor

Pref-1	Preadipocyte factor 1
Pri-miRNA	Primary microRNA
PTB	Polypyrimidine tract binding protein 1
PTC	Patched
PXR	Pregnane X receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
Rb	Retinoblastoma
REST	RE1-silencing transcription factor
RNAi	RNA interference
RT	Reverse transcription
RXR	Retinoid X receptor
SCP1	Small C-terminal domain phosphatase 1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sens	Senseless
Shh	Sonic Hedgehog
SLITRK1	Slit and Trk-like 1
Smo	Smoothened
SOP	Sensory organ precursors
SREBP	Sterol regulatory element binding protein
SRF	Serum response factor

SV	Stromal-vascular
SV40	Simian virus 40
TACE	Tumor necrosis factor α converting enzyme
TBE	Tris-Borate-EDTA
TGF β	Transforming growth factor β
Thrap1	Thyroid hormone receptor-associated protein 1
TPM1	Tropomyosin 1
TRAF6	TNF receptor-associated factor 6
TRBP	HIV transactivating response RNA-binding protein
TS	Tourette's syndrome
Tsp1	Thrombospondin-1
TZD	Thiazolidinedione
UTR	Untranslated region
Utrn	Utrophin
WAT	White adipose tissue

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CHAPTER 1

General introduction

1.1 MicroRNA

1.1.1 Discovery of microRNA

The first microRNA (miRNA), *lin-4*, was found in *Caenorhabditis elegans* in 1993 (Lee et al., 1993). *lin-4* can regulate post embryonic *C. elelgans* development by negatively regulating *lin-14* protein through binding to the 3' untranslated region (3'UTR) of *lin-14* (Lee et al., 1993). In 2000, another microRNA, *let-7* was found in *C. elegans* (Reinhart et al., 2000). *let-7* can regulate *C. elegans* developmental timing by targeting *lin-14, lin-28, lin-41, lin-42*, and *daf-12* (Reinhart et al., 2000). The finding of *let-7* led to discovery of more microRNAs in worms, flies, and mammals (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). There are 154 microRNAs in *Caenorhabditis elegans*, 152 in *Drosophila melanogaster*, 472 in *Mus musculus,* and 678 in *Homo sapiens* in the miRBase (http://microrna.sanger.ac.uk/) as of July, 2008.

1.1.2 Methods to identify microRNAs

Forward genetics identifies genes that are responsible for certain phenotypes. It was the first method to find microRNAs such as *lin-4* and *let-7* in *Caenorhabditis elegans* (Lee et al., 1993; Reinhart et al., 2000). *bantam* was another microRNA found in *Drosophila* by genetic methods (Brennecke et al., 2003). Another way to discover microRNAs on a large scale is

cDNA cloning (Ambros et al., 2003b). Investigators clone small size RNAs, compare their sequences to genomic sequences, and use RNA folding predictions to check whether they belong to microRNA family. Then northern blot is used to check their expression (Ambros and Lee, 2004). The third method used to identify microRNA is using bioinformatics programs. These programs are based on the fact that the sequences of microRNA are evolutionally conserved and microRNA precursors have a stem loop structure. These bioinformatics programs are summarized in Table 1.1 (Kim and Nam, 2006).

1.1.3 Genomic location of microRNAs

It was thought that most microRNAs are made from independent transcripts (Lau et al., 2001). After annotating the sequences of some mammalian microRNAs with their genomes, researchers found 161 out of 232 microRNAs are located within the overlap of defined transcription units (Rodriguez et al., 2004). Among them, 90 out of 161 microRNAs are located in the intron of a protein encoding transcript, 27 are located in the intron of a non-protein encoding transcript, 30 are located in the exon of a non-protein encoding transcript (Rodriguez et al., 2004). Comparison of the expression pattern of microRNAs with their overlapping genes suggests that they may come from the same transcripts (Rodriguez et al., 2004). A single transcript may encode several microRNAs. For example, miR-35, miR-36, miR-37, miR-38, miR-39, miR-40, and miR-41 come from a single transcript in *C. elegans* (Lau et al., 2001). In the human genome, miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92a-1 are from a cluster on chromosome 13

(Lagos-Quintana et al., 2001; Mourelatos et al., 2002).

MicroRNAs may have their own promoters. For examples, the promoter of miR-155 (also known as BIC gene) was found by genetic methods (Tam, 2001). Also the temporal regulatory element of *let-7* promoter has been characterized (Johnson et al., 2003).

1.1.4 Regulation of microRNAs

MicroRNAs can be regulated by other genes. p53 can bind to the promoter of miR-34 and activate its expression, which will lead to cell apoptosis (Chang et al., 2007; Corney et al., 2007; He et al., 2007b; Hermeking, 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). c-Myc can promote the expression of the miR-17-92 cluster (O'Donnell et al., 2005). Furthermore, miR-1 is the direct transcriptional target of SRF (serum response factor), MyoD (myogenic differentiation 1) and Mef2 (myocyte enhancer factor-2) (Zhao et al., 2005).

1.1.5 MicroRNA biogenesis

The process of microRNA biogenesis is summarized in Fig. 1.1. Most microRNAs are transcribed by RNA polymerase II. It has been shown to bind to the promoters of microRNAs and inhibition of its activity leads to less primary microRNA products (Lee et al., 2004). The microRNA is transcribed as a large product called the primary microRNA (pri-miRNA), which is several kilo basepairs in length and contains 5’ caps and 3’ polyadenylated tails (Cai et al., 2004). Pri-microRNA then is processed by the nuclear

Table 1.1: Program for microRNA predication (Adapted from Kim and Nam, 2006)

Name of program	Methods	Prediction target	Nonconserved microRNA detection	Species	Sources
miRscan	Comparative analysis, stem-loop conservation	Pre-microRNA	No	Nematode	Lim et al, Genes Dev. (2003); 17(8):991-1008.
srnaloop	Sequential and structural properties	Pre-microRNA	No	Nematode	Grad et al, Mol. Cell (2003); 11:1253–1263
miRseeker	Comparative analysis, stem-loop conservation	Pre-microRNA	No	Fly	Lai et al, Genome Biol. (2003); 4:R4
	Sequential and structural properties, comparative analysis	Pre-microRNA	No	Arabidopsis	Wang et al, Genome Biol.(2004); 5:R65
ERPIN	Sequence or structural alignment	Pre-microRNA	No	Animal, plant	Legendre et al, Bioinformatics (2005); 21:841–845
findMicroRNA	Seed match, comparative analysis	Pre-microRNA and mature microRNA	No	Arabidopsis	Adai et al, Genome Res.(2005); 15:78–91
	Phylogenetic shadowing profile	Pre-microRNA	Yes	Human	Berezikov et al, Cell (2005); 120:21–24
	Seed match	Mature microRNA	Yes	Human	Xie, et al, Nature (2005); 434:338–345
miralign	Sequence or structural alignment	Pre-microRNA	No	Animal, plant	Wang, et al, Bioinformatics (2005); 21:3610–3614
ProMiR	Probabilistic model of pairwise sequences	Pre-microRNA and mature microRNA	Yes	Human	Nam et al, Nucleic Acids Res. (2005); 33:3570–3581
PalGrade	Sequential and structural properties	Pre-microRNA	Yes	Human	Bentwich et al, Nat Genet (2005); 37:766–770

RNaseIII, Drosha, into pre-microRNA which is around 60-70 nucleotides and contains stem-loop structures (Lee et al., 2003b; Lee et al., 2002). Cleavage of pri-microRNA into pre-microRNA by Drosha requires double stranded RNA binding protein DGCR8 (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). Pre-microRNA is exported from the nucleus into the cytoplasm by Exportin-5 in a Ran-GTPase dependent manner (Lund et al., 2004; Yi et al., 2003).

After pre-microRNA is exported into the cytoplasm, it is cut into a 22nt duplex by another RNase III endonuclease called Dicer (Lee et al., 2003b). Dicer is known to play a role in the RNA interference (RNAi) process (Bernstein et al., 2001) and was later found to be involved in microRNA maturation (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). Dicer can cut the pre-microRNA duplex like the way it does during the RNAi process (reviewed by Bartel, 2004). Double stranded RNA binding protein Loquacious (its homolog is human immunodeficiency virus (HIV) transactivating response RNA-binding protein, TRBP) helps Dicer to cleave pre-microRNA into mature microRNA (Forstemann et al., 2005; Leuschner et al., 2005; Saito et al., 2005; Chendrimada et al., 2005). After Dicer cleavage, only the strand with low stability at its 5' end of the duplex will remain in the microRNA containing RNA induced silencing complex (miRISC) while the other strand disappears quickly (Khvorova et al., 2003; Schwarz et al., 2003). miRISC contains RNA helicase Gemin 3, Gemin 4 and eIF2C2, which is a human homolog of Argonaute (Mourelatos et al., 2002). Once the RISC complex forms, microRNA can guide the complex by binding to the 3' UTR or open reading frame of its target by imprecise base pairing (reviewed by Pillai, 2005).

1.1.6 Mechanism of microRNA action

MicroRNAs can post-transcriptionally regulate their targets by repressing protein translation or accelerating mRNA decay (reviewed by Eulalio et al., 2008; Wu and Belasco, 2008).

MicroRNAs can repress protein translation of their targets in several ways. Firstly, they can inhibit protein translation initiation. Ago2 in the RISC complex has a motif similar to the m(7)G cap-binding domain of eIF4E, which is important for translation initiation. RISC complex can compete with eIF4E for m(7)G cap binding (Kiriakidou et al., 2007). Another possible mechanism for repressing protein translation after cap recognition is through preventing 80S ribosome association. RISC complex contains eIF6, which is an anti-association factor. It can prevent the association of small and large ribosomal subunits (Chendrimada et al., 2007). MicroRNAs also can accelerate nascent polypeptide chain degradation co-translationally on their targets (Nottrott et al., 2006). Finally, microRNAs may inhibit translation elongation by causing ribosome dissociation prematurely (Petersen et al., 2006).

MicroRNAs can also promote mRNA degradation. Some microRNAs in plants have perfect or nearly perfect sequence complementarity to their targets and they can directly cleave their targets in a way similar to siRNA (Rhoades et al., 2002). In animals, most microRNAs regulate their targets by translation repression, but some microRNAs can cause cleavage of their targets. For example, the miR-196 sequence is a nearly perfect match with the sequence of the 3' UTR of the HOXB8 and can cause the cleavage of HOXB8 transcript (Yekta et al., 2004). Argonaute protein in the RISC complex is responsible for microRNA

-dependent mRNA cleavage (Liu et al., 2004b; Meister et al., 2004; Okamura et al., 2004).

For those microRNAs whose sequences have only partial complementary to their targets, they may accelerate mRNA degradation by directing removal of 3' poly (A) tail and 5' cap from the mRNA they targeted (Behm-Ansmant et al., 2006; Eulalio et al., 2007c; Giraldez et al., 2006; Wu et al., 2006). Messenger RNA degradation by microRNA requires Argonaute protein and P-body component GW182. The deadenylation is carried out by CAF1-CCR4-NOT deadenylase complex (Behm-Ansmant et al., 2006). The deadenylation and loss of poly (A) binding protein leads to the decapping by Dcp2 complex, which includes several decapping activators such as DCP1, EDC3, Ge-1, and RNA helicase RCK/p54. Then mRNA is subject to 5' to 3' exonucleolytic degradation by Xrn1 (Eulalio et al., 2007c).

A large number of mRNAs that undergo translation repression or degradation are concentrated in cytoplasmic foci that are called processing bodies (P bodies). P bodies contain Argonate proteins, GW182, CAF1-CCR4-NOT deadenylase complex, DCP2 decapping enzyme complex, and other RNA degradation enzymes (reviewed by Eulalio et al., 2007a). Although enzymes in P body are important for microRNA-induced mRNA silencing and degradation, P body is a consequence of silencing instead of a cause (Eulalio et al., 2007b).

1.1.7 Distinctions between siRNA and microRNA

MicroRNAs are small noncoding RNAs around 22 nt that can be detected by northern blot or size-fractionated cDNA cloning. microRNAs are generated by Dicer from one arm

of precursors, which are around 60-80nt with stem-loop structures. Their sequences match genomic sequences and are evolutionary conserved (Ambros et al., 2003a). microRNA and siRNA share a lot of similarities such as small size, the same biochemical composition, indistinguishable function and being generated by dicer (Ambros et al., 2003a). The difference between microRNA and siRNA is their origin. microRNAs are from endogenous transcripts of the genome and siRNAs are from exogenous or endogenous dsRNA. microRNAs are cut from one arm of precursors with hairpin structures and siRNAs are cut from dsRNA. Moreover, each microRNA precursor can only yield one copy of a microRNA molecule, but each dsRNA can produce a lot of siRNA molecules. Furthermore, the sequences of microRNAs are generally conserved in different species, while siRNA sequences are not (Ambros et al., 2003a; Bartel, 2004).

1.1.8 Target identification for microRNA

MicroRNAs bind to the 3'UTR of their targets. Number 2-8 nucleotides of microRNAs were found to be crucial for binding to mRNA after analysis of the sequences of microRNAs and their targets. Positions 2-8 of the microRNA, called the 'Seed Region', are usually in perfect Watson-Crick base pairing with their mRNA binding sites (Lewis et al., 2003). The sequences of binding sites in the 3' UTR of mRNAs that are perfectly paired with the 'Seed Region' of a microRNA are conserved between different species and usually flanked with adenosines (Lewis et al., 2005). Some microRNA targets donot have perfect Watson-Crick base pairings with the 5' of microRNAs in their binding sites, but they have

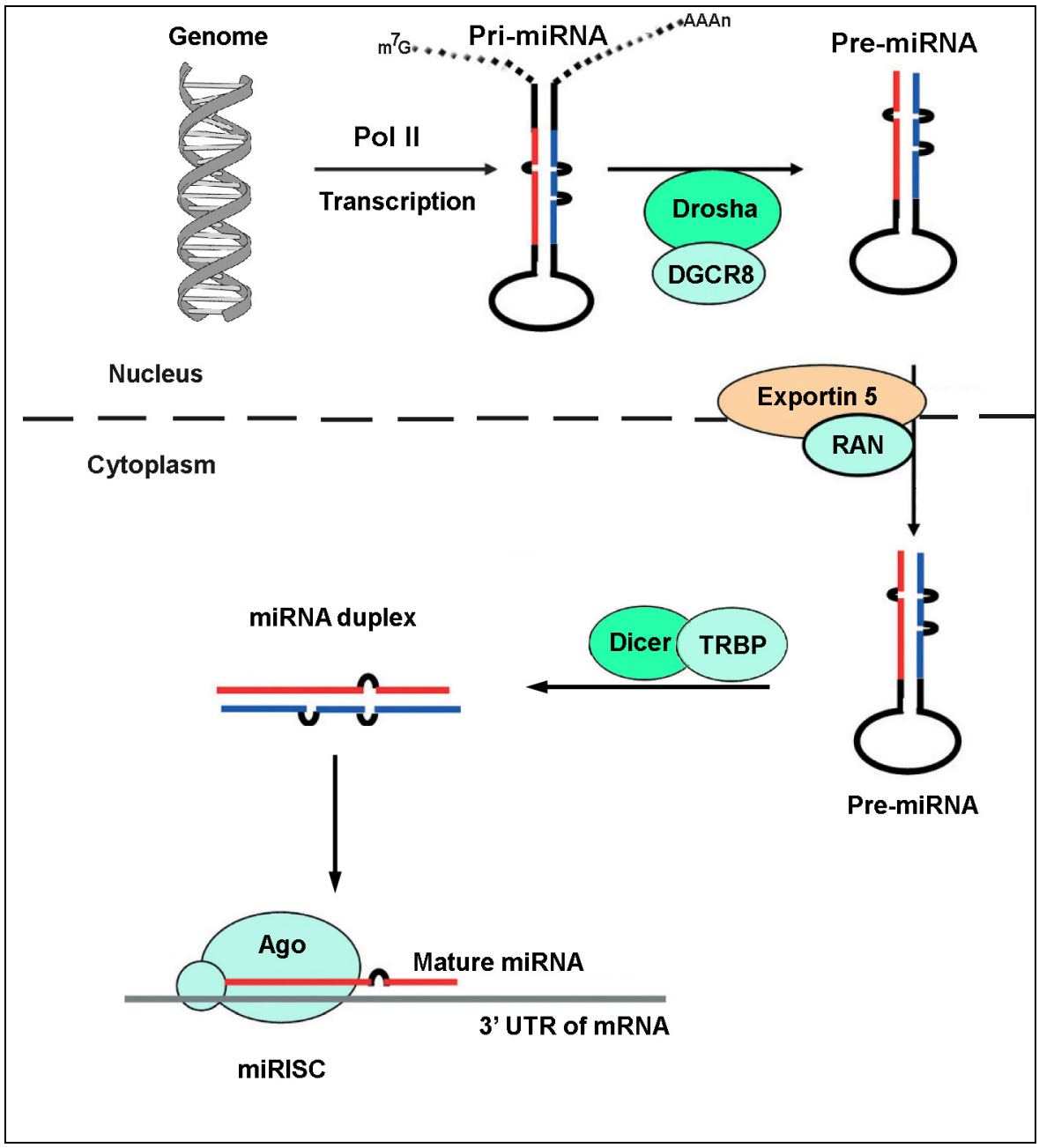


Fig. 1.1: Biogenesis of microRNA

(Adapted from Willams, 2008)

significant complementarity with the 3' sequences of microRNAs (reviewed by Maziere and Enright, 2007; Rajewsky, 2006).

Several bioinformatics tools and databases have been developed to predict microRNA targets in animals. Table 1.2 summarizes online methods and resources available for target prediction (reviewed by Maziere and Enright, 2007; Rajewsky, 2006).

Experimentally there are several ways to identify mRNA targets for microRNAs. Genetic studies found that *lin-14* is the target of *lin-4* (Lee et al., 1993). Since some microRNAs can regulate their targets at the mRNA level, over-expression of a certain microRNA can down-regulate its targets at the mRNA level, which can be detected by microarray (Lim et al., 2005). Inactivation of Drosha in *Drosophila* abolished microRNA production and led to accumulation of some targets for microRNAs, which can also be detected by microarray (Rehwinkel et al., 2006). Knock-out of a certain microRNA genes as well as inhibition of microRNAs by antisense oligonucleotides (ASO) or locked nucleic acids (LNA) also can increase the mRNA level of some microRNA targets (Elmen et al., 2008; Esau et al., 2006; Krutzfeldt et al., 2005). At the protein level, a reporter containing potential microRNA binding sites in the 3'UTR of a luciferase gene can be used to check whether it is a target for a certain microRNA (reviewed by Krutzfeldt et al., 2006).

Table 1.2: Online methods and resources for microRNA target predication

Name	Website
microRNA target predictions at EMBL	www.russell.embl-heidelberg.de/microRNAs/
miRanda	www.microrna.org/microrna/home.do
mirBase	microrna.sanger.ac.uk/targets/v2/
PicTar	pictar.bio.nyu.edu
TargetScan, TargetScanS	genes.mit.edu/targetscan
Chan et al, 2005	tavazoielab.princeton.edu/microRNAs/
miTarget	cbit.snu.ac.kr/~miTarget/
miRDB	mirdb.org/miRDB/index.html
RNA hybrid	bibiserv.techfak.uni-bielefeld.de/rnahybrid/
DIANA-MicroT	diana.pcbi.upenn.edu/DIANA-microT
RNA22	cbcsrv.watson.ibm.com/rna22.html
Tarbase	www.diana.pcbi.upenn.edu/tarbase.html
Argonaute	www.ma.uni-heidelberg.de/apps/zmf/argonaute/interface
microRNAMAP	microRNAmap.mbc.nctu.edu.tw/

(Adapted from Rajewsky, 2006; Mazière and Enright, 2007)

1.1.9 Functions of microRNAs

Despite the small size of microRNAs, they can play very important roles in many physiologic processes and diseases, such as development, metabolism, cell proliferation and differentiation, apoptosis, and cancer.

1.1.9.1 MicroRNA in Cancer

MicroRNAs may act either as oncogenes or tumor suppressors. The miR-17-92 cluster, which includes miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1, can promote cell proliferation, inhibit cancer cell apoptosis, and induce tumor angiogenesis

(reviewed by Mendell, 2008). Proto-onco gene c-Myc can activate the miR-17-92 cluster and E2F1. E2F1 in turn can be negatively regulated by miR-17-5p and miR-20a (O'Donnell et al., 2005). Amplification of products from the miR-17-92 locus was found in human B cell lymphoma and over-expression of the miR-17-92 cluster accelerated tumor progression in c-Myc-induced mouse B cell lymphoma model (He et al., 2005b). The miR-17-92 cluster can also target anti-angiogenic thrombospondin-1 (Tsp1) and connective tissue growth factor (CTGF), promoting tumor angiogenesis in a Myc-induced tumor phenotype (Dews et al., 2006).

miR-155 has been found to be involved in Burkitt's lymphoma, Hodgkin lymphoma and lung cancer (reviewed by Williams, 2008). In transgenic mice, miR-155 can promote mouse pre-B cell proliferation (Costinean et al., 2006).

miR-372 and miR-373 have been found working as oncogenes in testicular germ cell tumors (Voorhoeve et al., 2006). These microRNAs can target tumor suppressor LATS2 and abolish p53-mediated CDK inhibition (Voorhoeve et al., 2006).

miR-21 is highly over-expressed in human brain tumors (glioblastomas) (Chan et al., 2005). Several targets for miR-21 have been identified, such as tropomyosin 1 (TPM1), PTEN, programmed cell death 4 (PDCD4), and maspin (Asangani et al., 2008; Chan et al., 2005; Lu et al., 2008; Meng et al., 2007a; Zhu et al., 2007; Zhu et al., 2008).

miR-221, miR-222, and miR-146 are highly expressed in papillary thyroid carcinoma (He et al., 2005a). miR-221 and miR-222 can target the cell cycle inhibitor p27(Kip1) and promote cancer cell proliferation (Galardi et al., 2007; Gillies and Lorimer, 2007; le Sage et al., 2007; Visone et al., 2007).

The expression of several microRNAs is reduced in cancer. miR-143 and miR-145 are lower in colorectal neoplasia (Michael et al., 2003). In human breast cancer, miR-125b, miR-145, miR-21, and miR-155 are lower than in normal tissue (Iorio et al., 2005).

Some microRNAs act as tumor suppressors. Chromosome 13q14, where miR-15 and miR-16 are located, was often found to be deleted in B cell chronic lymphocytic leukemias (Calin et al., 2002). miR-15 and miR-16 can bind to the 3' UTR of Bcl2 and negatively regulate its function. Over-expression of miR-15 and miR-16 can induce apoptosis (Cimmino et al., 2005).

Tumor suppressor p53 can bind to the promoter of miR-34a and activate its expression, which contributes to p53-mediated apoptosis (Chang et al., 2007; He et al., 2007b; Hermeking, 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). miR-34a induced growth arrest and apoptosis may be due to targeting E2F3 (Tazawa et al., 2007; Welch et al., 2007).

miR-29 and miR-181 can target oncogene Tcl1 in chronic lymphocytic leukemia (Pekarsky et al., 2006). miR-29 was also reported to target Mcl1 and regulate cell apoptosis (Mott et al., 2007).

Hmga2 (high mobitilty group AT-hook 2) is repressed by *let-7* and disruption of this repression caused by chromosome translocation at 12q5 will lead to tumor formation (Lee and Dutta, 2007; Mayr et al., 2007). *let-7* can also down-regulate other oncogenes, such as RAS and Myc (Johnson et al., 2005; Sampson et al., 2007). *let-7* has been reported to be involved in many cancers, including breast cancer, ovarian cancer, lung cancer, and colon cancer (Akao et al., 2006; Esquela-Kerscher et al., 2008; Kumar et al., 2008; Lu et al., 2007;

Park et al., 2007; Yu et al., 2007).

1.1.9.2 MicroRNA in metabolism

miR-375 is highly expressed in pancreas and it regulates insulin secretion. Over-expression of miR-375 inhibits insulin secretion and inhibition of miR-375 can enhance insulin exocytosis (Poy et al., 2004). The action of miR-375 may be through targeting myotrophin (Poy et al., 2004). High expression of miR-9 in pancreatic beta cells will reduce the expression of the transcription factor Onecut-2 and result in increasing granuphilin/Slp4 and reducing insulin exocytosis (Plaisance et al., 2006). miR-96 can increase both the mRNA and protein of granuphilin and inhibit insulin secretion (Lovis et al., 2008). Another microRNA, miR-124a, can modulate the expression of proteins involved in insulin exocytosis and regulate insulin secretion in pancreatic beta-cells (Lovis et al., 2008). miR-143 is up-regulated during pre-adipocytes differentiation and inhibition of miR-143 blocks adipogenesis (Esau et al., 2004). miR-29 is highly up-regulated in diabetic rats and leads to insulin resistance in adipocytes (He et al., 2007a). miR-29 can also target the dihydrolipoamide branched chain acyltransferase component of a branch chain amino acid catabolism enzyme complex, branched chain alpha-ketoacid dehydrogenase (BCKD) complex and regulate amino acid metabolism (Mersey et al., 2005).

Drosophila lacking miR-278 are insulin resistant and lean. The effect of miR-278 may be through targeting *expanded* (Teleman et al., 2006). Flies with miR-14 deletion have increased levels of triacylglycerol and diacylglycerol (Xu et al., 2003).

Liver miR-122 can regulate cholesterol and fatty acid metabolism. Inhibition of miR-122 resulted in a decrease of hepatic fatty acid and cholesterol synthesis and plasma cholesterol level and an increase of hepatic fatty acid oxidation (Esau et al., 2006).

1.1.9.3 MicroRNA in muscle function and development

MicroRNAs can regulate cardiac and skeletal muscle differentiation (reviewed by Bushati and Cohen, 2007; Callis and Wang, 2008; Williams, 2008). miR-1 is specifically expressed in cardiac and skeletal muscle cells at a high level and its expression is under the control of several muscle transcription factors such as serum response factor (SRF), MyoD, and Mef2 (Zhao et al., 2005). Over-expression of miR-1 in heart causes defects in ventricular cardiomyocytes proliferation (Zhao et al., 2005). miR-1 can target Hand2, a transcription factor that promotes cardiomyocytes differentiation (Zhao et al., 2005). miR-1-2 knock out mice have nearly 50% lethality by the age of weaning and there are defects in hearts, revealing the role of miR-1-2 in cardiac morphogenesis, electrical conduction, and cell-cycle control (Zhao et al., 2007). miR-1 is over-expressed in coronary artery disease patients and over-expression of miR-1 exacerbates arrhythmogenesis in a rat model (Yang et al., 2007). Two molecules important for heart function, KCNJ2 (potassium inwardly-rectifying channel, subfamily J, member 2) and GJA1 (gap junction protein, alpha 1, 43kDa) are targets for miR-1 (Yang et al., 2007). miR-1 can also target histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression and promotes myogenesis (Chen et al., 2006).

miR-133 is transcribed as a single polycistronic transcript along with miR-1. miR-133 can promote myoblast proliferation by targeting serum response factor (SRF) (Chen et al., 2006). miR-133 can also down-regulate the protein level of alternative splicing factor nPTB during muscle development, leading to increased inclusion of a group of exons silenced by nPTB (Boutz et al., 2007). miR-133 is over-expressed in a rabbit model of diabetes and it can repress ether-a-go-go related gene (ERG) at the protein level, contributing to long QT syndrome and arrhythmias (Xiao et al., 2007). The expression of miR-133 and miR-1 is lower in cardiac hypertrophy, in agreement with the results that inhibition of miR-133 *in vivo* by antagomir causes cardiac hypertrophy. RhoA, Cdc42 and Nelf-A/WHSC2, which are important regulators in cardiac hypertrophy, are targets of miR-133 (Care et al., 2007).

miR-208 is encoded in an intron of α-MHC gene and miR-208 null mice failed to show cardiac hypertrophy and induction of β-MHC in cardiac hypertrophy model. These effects may be due to inhibition of thyroid hormone receptor-associated protein 1 (Thrap1) by miR-208 at the protein level (van Rooij et al., 2007).

miR-206 is highly expressed in skeletal muscle and plays an important role in myogenesis (reviewed by McCarthy, 2008). MyoD can activate miR-206, which in turn targets follistatin-like 1 (Fstl1) and utrophin (Utrn) (Rosenberg et al., 2006). During skeletal muscle development, miR-206 regulates connexin43 expression (Anderson et al., 2006).

1.1.9.4 MicroRNA in Neurogenesis

miR-124 is highly and specifically expressed in differentiated neurons. miR-124 can

directly target polypyrimidine tract binding protein 1 (PTB or PTBP1), which is a general repressor of alternative mRNA slicing (Makeyev et al., 2007). One exon of nPTB (neuronal PTB or PTBP2), which is a nervous system-enriched PTB homolog, is repressed by PTB and this repression leads to premature stop of nPTB mRNA. During neuronal differentiation, the switch from PTB to nPTB triggers a wide range of nervous system specific mRNA alternative splicing patterns. miR-124 can lower PTB levels and lead to accumulation of nPTB, which will repress non-neuronal gene expression and increase neuronal-specific gene expression (Makeyev et al., 2007).

Besides PTB, miR-124 can target anti-neural REST/SCP1 pathway during embryonic CNS development to regulate neural specific gene expression (Visvanathan et al., 2007). REST (RE1-silencing transcription factor, also known as NRSF) is a transcription repressor that can inhibit the expression of neuronal-specific genes in non-neuronal cells. SCP1 (Small C-terminal domain phosphatase 1) is an anti-neuronal factor that is recruited by REST to bind to neuronal specific genes. miR-124 is repressed by REST in non-neuronal cells and neural progenitors (Conaco et al., 2006). On the contrary, miR-124 can bind to the 3'UTR of SCP1 and down-regulate SCP1 levels, leading to expression of neuronal specific genes that are repressed by REST/SCP1 (Visvanathan et al., 2007).

miR-134 is expressed in hippocampal neurons and it inhibits the growth of dendritic spines (Schratt et al., 2006). The effect is mediated through inhibition of Limk1, a protein kinase that controls spine development (Schratt et al., 2006).

1.1.9.5 MicroRNA in immune system

miR-181a is highly up-regulated in CD4 and CD8 double positive (DP) T-lymphocytes and lower in mature T-lymphocytes, suggesting its role in the process of positive and negative selection (Li et al., 2007). miR-181a increases the sensitivity of DP cells to peptide antigens and inhibition of miR-181 impairs both positive and negative selections. miR-181 can lower the threshold of T-cell receptor activation in immature T-lymphocytes by down-regulating several phosphatases, leading to increase of basal levels of Lck (Lymphocyte specific protein tyrosine kinase) and ERK (Extracellular signal regulated kinase) (Li et al., 2007).

miR-155 plays an important role in immune functions. miR-155 knock-out mice were immunodeficiency and showed increased lung airway remodeling (Rodriguez et al., 2007). miR-155 can target the transcription factor c-Maf and loss of miR-155 results in up-regulation of c-Maf, which will increase cytokine IL-4 and T helper-2 cell number (Rodriguez et al., 2007). miR-155 null mice also showed decreased B-lymphocytes in the germinal centers, where B-lymphocytes differentiated into plasma cells (Thai et al., 2007). T-cell dependent antibody production is impaired in miR-155 null mice (Thai et al., 2007).

miR-146 can negatively regulate NF-κB pathway by targeting TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1). Since miR-146 is up-regulated upon stimulation, it is proposed that miR-146 works as a negative feedback signal in innate immune response (Taganov et al., 2006).

1.1.9.6 MicroRNA in Haematopoiesis

Human granulocytic differentiation is regulated by miR-223 (Fazi et al., 2005). Two transcription factors, NFI-A and C/EBPα, compete for binding to the miR-223 promoter. NFI-A maintains a low expression of miR-223 while C/EBPα up-regulates miR-223 expression upon retinoic acid treatment. miR-223 in turn represses NFI-A translation (Fazi et al., 2005). Over-expression of miR-223 in acute promyelocytic leukemia (APL) cells enhance differentiation, while knock-down of miR-223 inhibits retinoic acid induced differentiation (Fazi et al., 2005).

1.1.9.7 MicroRNA in Human diseases

MicroRNAs were reported to be involved in several human diseases (reviewed by Bushati and Cohen, 2007). For example, in the neuropsychiatric disorder Tourette's syndrome (TS), there are mutations in the miR-189 binding site of the 3'UTR of Slit and Trk-like 1 (SLITRK1) gene. Also SLITRK1 and miR-189 show co-expression pattern in the brain regions commonly implicated in Tourette's syndrome. It is suggested miR-189 may be involved in Tourette's syndrome (Abelson et al., 2005).

A mutation in the 3'UTR of myostatin (GDF8) gene yields a binding site for miR-1 and miR-206. This mutation causes translation inhibition of myostatin and leads to muscular hypertrophy in Texel sheep (Clop et al., 2006).

Fragile X syndrome is a common form of inherited mental retardation. It is caused by methylation induced gene silence of the fragile X mental retardation 1 (FMR1) gene as the result of a CGG repeat expansion in its 5'UTR (reviewed by O'Donnell and Warren, 2002).

Fragile X mental retardation protein (FMRP) is a selective RNA-binding protein and it interacts with microRNAs, RISC complex components dicer and argonaute 1 (Jin et al., 2004). Genetic evidence showed loss of Ago1 suppressed apoptosis and rough eye phenotype caused by over-expression of FMR1 in *Drosophila* (Jin et al., 2004). Pronounced synaptic overgrowth at neuromuscular junctions was found in loss-of-function dFmr1 mutants and it is exacerbated by heterozygous loss of Ago1 (Jin et al., 2004). These results suggest FMRP mediates microRNA-dependent translation repression and defects in this process may contribute to Fragile X syndrome disease.

Polyglutamine (polyQ) expansion of ataxin 3 causes cell toxicity and results in neuronal degeneration. Deleption of Dicer1, the enzyme responsible for microRNA processing, caused dramatic enhancement of polyQ toxicity in *Drosophila* and human cells (Bilen et al., 2006). MicroRNA *bantam* (*ban*) can prevent neuronal degeneration in flies (Bilen et al., 2006). miR-8 can directly target atrophin in *Drosophila* and elevated atrophin activity in miR-8 mutant results in elevated apoptosis in the brain and behavioral defects (Karres et al., 2007). miR-8 and atrophin orthologs are conserved in mammals and it implicates miR-8 may function in neurodegenerative disorder DRPLA (Dentatorubral-pallidoluysian atrophy) (Karres et al., 2007).

Most of DiGeorge syndrome patients have a deletion in chromosomal region 22q11, where DGCR8 (involved in Drosha function) is located (Gregory et al., 2004; Lindsay, 2001), but the exact microRNA involved in DiGeorge remains to be elucidated.

1.1.9.8 MicroRNA and Virus

Some nuclear DNA viruses can express microRNAs to help them live in host cells. Simian virus 40 (SV40) can express microRNAs that target viral T antigens at a late stage of infection, reducing exposure of host cells to cytotoxic T-lymphocytes (Sullivan et al., 2005). Reciprocally, host cells may use microRNAs to defend against viruses. miR-32 can effectively restrict the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells through targeting viral mRNAs (Lecellier et al., 2005).

1.1.9.9 MicroRNA in C. elegans development

lin-4 was the first microRNA discovered in *C. elegans* and it can regulate L1-L2 development by binding to the 3'UTR of *lin-14* (Lee et al., 1993; Wightman et al., 1993). Without *lin-4*, worms cannot develop from the L1 to L2 stage due to differentiation defects. Over-expression of *lin-4* results in complete loss of cell division of larval stem cells. In both situations, the worms were stuck at L1 stage (reviewed by Williams, 2008).

L2-L3 transition is regulated by miR-48, miR-84, and miR-241. They may bind to the 3'UTR of *hbl-1* and down-regulate *hbl-1* activity (Abbott et al., 2005). *let-7* regulates the larval to adult transition by targeting *lin-41* (Reinhart et al., 2000). *let-7* and miR-84 were also reported to be involved in the vulval development (Johnson et al., 2005). *let-7* mutants have defects in the vulva. It may due to loss of *let-7*'s inhibition on *let-60/RAS*, which can promote the vulva formation (Johnson et al., 2005).

MicroRNA *lsy-6* and miR-273 act in a double negative feedback loop controlling

neuronal asymmetry in *C. elegans* (reviewed by Bushati and Cohen, 2007). MicroRNA *lsy-6* binds to the 3'UTR of homeobox gene *cog-1* and regulates its expression. Loss of *lsy-6* resulted in loss of asymmetry of bilateral taste receptor neurons known as ASE left and ASE right, only developing ASE right neuron (Johnston and Hobert, 2003). *Die-1* can activate *lsy-6* expression in ASE left neuron and *Die-1* itself is negatively regulated by another microRNA miR-273 in ASE right neuron (Chang et al., 2004). miR-273 is activated by *cog-1* in ASE right neuron (Johnston et al., 2005). Thus, two transcription factors *Die-1* and *cog-1* and two microRNAs work together to control the development of ASE neurons.

1.1.9.10 MicroRNA in Drosophila Development

Expression of the microRNA *bantam* is temporally and spatially regulated during patterning in *Drosophila*. *bantam* can promote cell proliferation by targeting the pro-apoptosis factor *hid* (Brennecke et al., 2003).

Loss of function studies using 2'O-methyl antisense oligo ribonucleotides injected into embryos reveal that the miR-2 family, which includes miR-2, miR-6, miR-11, miR-13, and miR-308, plays a role in suppressing embryonic apoptosis by targeting *hid, grim, reaper*, and *sickle* (Leaman et al., 2005). Loss of miR-31 in embryos results in severe segmentation defects (Leaman et al., 2005). Block of the miR-310/311/312/313/92 family in embryos shows morphogenetic defects (Leaman et al., 2005).

Embryos with miR-9 inhibitor injection rarely form any cuticle and have no internal differentiation (Leaman et al., 2005). miR-9a can also control the formation of sensory

organ precursors (SOPs) in the adult wing imaginal disc through targeting *senseless* (*sens*). Over-expression of miR-9a results in a severe loss of SOPs while loss of miR-9a in *Drosophila* peripheral nervous system leads to ectopic production of SOPs (Li et al., 2006).

miR-1 regulates the expansion and differentiation of cardiac and muscle progenitor cells in *Drosophila* through targeting transcripts encoding the Notch ligand *Delta* (Kwon et al., 2005).

Drosophila microRNA *iab-4*, which is homologous to miR-196 in vertebrates, can directly inhibit *Ubx* activity and cause a dominant homeotic transformation of halteres to wings (Ronshaugen et al., 2005)

miR-7 regulates photoreceptor cell differentiation (Li and Carthew, 2005). Transcription factor Yan can bind to the promoter of miR-7 and suppresses its expression in progenitor cells. Yan is phosphorylated in EGF (epidermal growth factor) signalling pathway and phosphorylated Yan activates miR-7 expression during the differentiation of progenitor cells into photoreceptor cells. Furthermore, miR-7 can down-regulate *Yan* through binding to its 3' UTR (Li and Carthew, 2005).

1.1.9.11 MicroRNA in Vertebrate Development

Dicer is required for generating mature microRNAs and knock-out of Dicer reveals the role of microRNAs in vertebrate development (reviewed by Williams, 2008). Dicer-deficient mice are embryonic lethal with lack of detectable multipotent stem cells (Murchison et al., 2005). Dicer-null mouse embryonic stem (ES) cells have severe defects

in differentiation (Kanellopoulou et al., 2005).

Using Dicer null conditional knock-out mice as a model, it was shown that Dicer is involved in the development of many tissues. In mice with epidermal specific Dicer deletion, hair follicles were stunted and hypoproliferative, suggesting Dicer is important for hair follicle morphogenesis (Andl et al., 2006).

Dicer conditional knock-out driven by the Sonic Hedgehog (Shh) promoter in the mouse lung epithelium showed branching arrests in the mutant lungs (Harris et al., 2006). Also the expression of Fgf10, a key factor that may work as a chemoattractant for the outgrowth of epithelial branches, is up-regulated and expanded in the mesenchyme cells of Dicer mutant lungs (Harris et al., 2006).

Dicer is also involved in angiogenesis. Dicer null mice have defects in blood vessel and yolk sacs. The expression of some important angiogenic regulators such as vegf, flt1, kdr, and tie1 is changed in the mutant embryos (Yang et al., 2005). These results may be due to up-regulation of some microRNA targets that are crucial for angiogenesis in dicer null mice.

Deletion of Dicer at an early stage of T cell development reduced the survival of αβ T cells, but the numbers of γδ-expressing thymocytes were not affected (Cobb et al., 2005).

Conditional knock-out of Dicer in limb mesoderm resulted in much smaller limbs due to massive cell death in limbs, but there is no defect in patterning and differentiation of the mouse limbs (Harfe et al., 2005). Furthermore, miR-196 has been shown to act upstream of Hoxb8 and Shh during limb development (Hornstein et al., 2005). Retinoic acid (RA) can induce the expression of the transcription factor Hoxb8 and consequently up-regulates the expression of Sonic Hedgehog (Shh) in the forelimb, but not in the hindlimb. However, in

Dicer conditional knock out mice, RA can induce expression of Hoxb8 in hindlimb. This result suggest that a microRNA negatively regultes Hoxb8 in hindlimb. miR-196 is highly expressed in hindlimb and it can down-regulte Hoxb8 and Shh level in hindlimb (Hornstein et al., 2005). The role of miR-196 is safeguarding excessive Hoxb8 level during normal limb development (Hornstein et al., 2005).

1.2 Adipogenesis

Adipogenesis is the process of preadipocyte differentiation into a mature fat cell. Adipose tissue is not only a structural component of the body and a place for energy storage, but also an important regulator of energy homeostasis through secreting signal molecules such as leptin and adipsin (reviewed by Rosen and Spiegelman, 2000). Investigation of adipogenesis is important to understand human diseases such as obesity and type II diabetes; both are frequent in modern society.

There are two types of adipocytes, white adipocytes and brown adipocytes. White adipocyte differentiation is intensively studied *in vitro* in several cell lines. Some preadipocytes cell lines can be differentiated into mature adipocytes through hormonal induction. Mature adipocytes have many characteristics different from preadipocytes such as morphological changes, cell growth arrest, increased lipid transportation and synthesis, extensive lipid accumulation, insulin sensitivity, and secretion of adipocyte specific proteins (Rosen and Spiegelman, 2000).

1.2.1 Differentiation program

There are several *in vitro* models for the study of adipogenesis. Embryonic stem (ES) cells can be differentiated into adipocytes upon retinoic acid and pro-adipogenic hormone treatment. Also mouse embryonic fibroblasts (MEFs) at E12-14 can be induced into adipocytes, although it is less than 100 percent efficient, after hormone treatment. Most immortalized MEFs that were generated through serial passaging or SV40 large T antigen introduction or chemical treatment can not differentiate into adipocytes without introduction of transcription factors such as PPARγ or C/EBPα. However, two cell lines 3T3-L1 and 3T3-F442A can be differentiated into mature adipocytes upon addition of an hormonal cocktail containing dexamethasone, cAMP phosphodiesterase inhibitor, and insulin treatment. The C3H10T1/2 cell line is an immortalized mouse cell line isolated from mesenchymal cells in bone marrow and it can also be differentiated into adipocytes (reviewed by Rosen and MacDougald, 2006).

3T3-L1 and 3T3-F442A cells are from Swiss 3T3 cells that are morphologically indistinguishable from mouse fibroblasts, but they are capable of differentiating into adipocytes in 4-6 days upon hormonal induction (Green and Meuth, 1974). This process has been intensively studied. The first stage of most pre-adipocyte cell lines differentiation is growth arrest upon contact inhibition. Then, cells enter the second phase called clonal expansion in which the cell cycle goes one or two rounds upon treatment of a pro-differentiative cocktail containing insulin, dexamethasone, and cAMP agonist. After clonal expansion, cells enter final and permanent growth arrest. After that, cells go to terminal differentiation into mature adipocytes (Rosen and Spiegelman, 2000).

The pre-adipocyte differentiation program is characterized by the expression of different genes. After hormonal inducer treatment, there is a rapid and transient induction of C/EBPβ and C/EBPδ (reviewed by Darlington et al., 1998). Following that, two key transcription factors, PPARγ and C/EBPα, are expressed right at the end of the clonal expansion stage and may help to stop cell cycles. During the final cell growth arrest, the expression of the catalytic subunit of the serine-threonine phosphatase PP2A decreases, which results in up-regulation of the phosphorylated form of DP-1. DP-1 is the binding partner of E2F family members that can control cell cycles. Phosophorylation of DP-1 decreases E2F/DP-1 binding to DNA (Altiok et al., 1997). E2Fs can regulate adipocyte differentiation. Loss of E2F1 impairs adipogenesis, while deletion of E2F4 causes MEFs to undergo spontaneous differentiation (Fajas et al., 2002). Retinoblastoma (Rb) protein can bind to some E2Fs and inhibit their transcription activity (reviewed by Harbour and Dean, 2000). Rb-null MEFs lost their ability to differentiate into adipocytes (Chen et al., 1996). The expression changes of several cyclin-dependent kinase inhibitors p18, p21, and p27 directly couple to differentiation stages during 3T3-L1 differentiation. PPARγ can increase the expression of p18 and p21 in NIH-3T3 fibroblasts (Morrison and Farmer, 1999). C/EBPα also can up-regulate p21 through increasing p21 gene expression and by post-translational stabilization of p21 protein (Timchenko et al., 1996). Increased expression of PPARγ and C/EBPα will lead to expression of adipocyte specific genes that characterize the mature adipocyte. These genes are involved in insulin sensitivity, such as insulin receptor and glucose transporter glut4, and lipid accumulation, such as fatty acid synthase (FAS), lipoprotein lipase (LPL), acetyl CoA carboxylase and fatty acid binding protein (aP2). Also

there are secreted products such as leptin, adipsin, and adiponectin (reviewed by Rosen, 2005).

1.2.2 Transcription factors regulating adipogenesis

1.2.2.1 PPARγ

Pre-adipocyte differentiation is regulated by many transcription factors both in positive and negative ways. PPARγ and C/EBP family members are critical for adipocyte differentiation. PPARγ is a nuclear receptor family member and it heterodimerizes with retinoid X receptor (RXR) (Kliewer et al., 1994). There are two isoforms of PPARγ, PPARγ1, and PPARγ2, produced by differential splicing and promoter usage. PPARγ1 is expressed in adipocytes and other cells, while PPARγ2 is extensively expressed in adipocytes. Ectopic expression of PPARγ2 has been shown to stimulate adipose differentiation of cultured fibroblasts (Tontonoz et al., 1994). Also, PPARγ is required for development of adipocytes both *in vivo* and *in vitro* (Barak et al., 1999; Rosen et al., 1999). Both isoforms of PPARγ can activate adipogenesis in PPARγ null fibroblasts, while PPARγ1 has stronger activity than PPARγ2 (Mueller et al., 2002). PPARγ2 knock out mice lack normal white adipose tissue and MEF cells lose the ability to differentiate into adipocytes (Zhang et al., 2004a). Another study showed there is normal adipose tissue, but insulin resistance is developed in PPARγ2 null mice (Medina-Gomez et al., 2005).

PPARγ is important not only for adipocyte differentiation, but also for maintaining adipocyte phenotype. Transfection of mature 3T3-L1 adipocyte with a dominant negative

form of PPARγ will reverse the adipocyte phenotype with decreased triglyceride content and adipocyte-specific genes (Tamori et al., 2002). Also *in vivo* experiments showed that mature PPARγ-null white and brown adipocytes die within a few days after induction of PPARγ deletion (Imai et al., 2004).

Thiazolidinedione (TZD) is a class of antidiabetic drugs that can bind to and potently activate PPARγ (Lehmann et al., 1995). Also, 15-deoxy-delta 12,14-PGJ2 (Prostaglandins J2) and some polyunsaturated fatty acids can activate PPARγ (Forman et al., 1997; Forman et al., 1995; Kliewer et al., 1995; Kliewer et al., 1997).

PPARγ is a key factor in adipogenesis. Other pro-adipogenic factors such as C/EBPs and several Krüppel-like factors can induce, while some anti-adipogenic factors such as GATA factors can repress, PPARγ expression (reviewed by Rosen and MacDougald, 2006).

1.2.2.2 C/EBP family

CCAAT/enhancer-binding proteins (C/EBP) family members C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPζ are expressed in adipocytes. The expression of C/EBPβ and C/EBPδ is up-regulated during the early stage and dereased during the late stage of adipogenesis. The accumulation of C/EBPβ and C/EBPδ leads to induction of C/EBPα in a transcription cascade. These C/EBPs can promote adipogenesis (reviewed by Rosen and MacDougald, 2006).

Ectopic expression of C/EBPβ in 3T3-L1 cells enables them to differentiate into mature adipocyte in the absence of hormonal inducers. C/EBPβ expression converts NIH-3T3 cells into pre-adipocytes. Over-expression of dominant negative forms of C/EBPβ inhibits

3T3-L1 differentiation (Yeh et al., 1995). MEFs from C/EBPβ knock-out mice significantly lose their ability to differentiate into mature adipocytes compared to that from wild type mice, while C/EBPδ knock-out MEFs only have a slight reduction in differentiation potential. MEFs that lack both C/EBPβ and C/EBPδ almost totally lose their ability to differentiate, suggesting C/EBPβ and C/EBPδ have synergic effects on pre-adipocyte differentiation (Tanaka et al., 1997). Approximately 85% of newborn C/EBPβ and C/EBPδ double knock out mice die at an early stage. For those that survive, they have less lipid accumulation in the brown adipose tissue and their white adipose tissue is significantly smaller than wild type (Tanaka et al., 1997).

In vitro and *in vivo* evidence has shown C/EBPα can promote adipogenesis. Ectopic expression of C/EBPα in a variety of mouse fibroblasts efficiently induces adipogenic differentiation, while repression of C/EBPα by RNAi blocks 3T3-L1 differentiation (Freytag et al., 1994; Lin and Lane, 1992; Lin and Lane, 1994). C/EBPα knock-out mice die right after birth due to defects in gluconeogenesis in the liver. Rescue of C/EBPα in the liver with a transgene in C/EBPα knock-out mice will improve survival. In these mice, white adipose tissue, not brown adipose tissue, is severely impaired (Linhart et al., 2001). Replacement of C/EBPα gene with C/EBPβ gene in mice resulted in a significant reduction of fat storage in white adipose tissue (Chen et al., 2000). C/EBPα-induced adipogenesis relys on PPARγ. PPARγ can induce adipogenesis in C/EBPα deficient cells while the reverse is not true (Rosen et al., 2002). In PPARγ-deficient MEFs, C/EBPβ is not able to induce C/EBPα expression and adipogenesis (Zuo et al., 2006).

C/EBPζ (also known as CHOP, C/EBP homologous protein) can dimerize with C/EBPα

and C/EBPβ and inhibit their function, markedly inhibiting preadipocyte differentiation (Batchvarova et al., 1995).

1.2.2.3 Kruppel-like Factor family

The Krüppel-like Factor (KLF) family are zinc-finger transcription factors that are important regulators of cell differentiation. KLFs can either play a positive role or a negative role in adipogenesis.

Krüppel-like factor 4 (KLF4) is an essential early regulator of adipogenesis. KLF4 is specifically induced by cAMP within 30 min after DMI (dexamethasone, IBMX and insulin) treatment in 3T3-L1 cells. KLF4 directly binds to the C/EBPβ promoter and activates its expression. Knock-down of KLF4 down-regulates C/EBPβ levels and inhibits 3T3-L1 cell adipogenesis. Normally, C/EBPβ suppresses Krox20 and KLF4 expression and C/EBPβ knock-down increases the expression of KLF4 and Krox20. Thus, KLF4 and C/EBPβ form a negative feedback loop (Birsoy et al., 2008).

C/EBPβ and C/EBPδ can bind to the KLF5 promoter and activate its expression. KLF5, in turn, binds to the PPARγ2 promoter and activates its expression along with C/EBPs. Over-expression of KLF5 induces adipocyte differentiation in the absence of hormonal stimulation while over-expression of a dominant-negative form of KLF5 inhibits adipocyte differentiation. MEFs from KLF5 heterozygous mice show reduced adipocyte differentiation, which is consistent with the fact that heterozygous KLF5 knockout mice have a marked deficiency in white adipose tissue development (Mori et al., 2005).

KLF6 has been shown to be a repressor of the Delta-like 1 (Dlk1) gene, which inhibits adipocyte differentiation. Down-regulation of KLF6 by small interfering RNA inhibits 3T3-L1 cells adipogenesis (Li et al., 2005).

KLF15 can activate PPARγ2 expression and promote adipocyte differentiation (Mori et al., 2005). KLF15 also can activate the expression of the glucose transporter GLUT4 in adipose (Gray et al., 2002).

KLF3 knockout mice have less white adipose tissue and the adipocytes are smaller and fewer in their fat pads, indicating KLF3 regulates adipocyte differentiation (Sue et al., 2008).

However, some KLFs can inhibit preadipocytes differentiation. KLF2 is highly expressed in preadipocytes but not in mature adipocytes. KLF2 inhibits adipogenesis by binding to the PPARγ promoter and repressing its expression (Banerjee et al., 2003; Wu et al., 2005). Over-expression of KLF7 also reduces 3T3-L1 cell differentiation (Kanazawa et al., 2005a).

1.2.2.4 Other factors

Other transcription factors also regulate adipogenesis. Krox20 is a zinc finger-containing transcription factor that is up-regulated during the early stage of differentiation. It activates C/EBPβ expression and promotes 3T3-L1 differentiation (Chen et al., 2005b).

The early B cell factor (Ebf) family of helix-loop-helix transcription factors can also promote adipogenesis. PPARγ1 and C/EBPα can be activated by Ebf1, which is induced by

C/EBPβ and C/EBPδ. siRNA against Ebf1 and Ebf2 blocks 3T3-L1 differentiation (Jimenez et al., 2007). SREBP1c (sterol regulatory element binding protein 1c) is important for lipid metabolism and it also regulates adipocyte differentiation. A dominant-negative form of SREBP1c represses 3T3-L1 cells' differentiation (Kim and Spiegelman, 1996). SREBP1c maybe responsible for producing an endogenous ligand for PPARγ and activates its activity (Kim et al., 1998).

Ectopic expression of STAT5A promotes adipogenesis in two non-precursor fibroblast cell lines by regulating PPARγ expression (Floyd and Stephens, 2003; Nanbu-Wakao et al., 2002). The Active form of CREB can promote adipogenesis by binding to C/EBPβ promoter and inducing its expression (Zhang et al., 2004b). MEFs from BMAL1 (Brain and muscle Arnt-like protein-1) knock-out mice lose their ability to differentiate into adipocytes, suggesting BMAL is required for adipogenesis (Shimba et al., 2005). Also EPAS1(endothelial PAS domain protein 1, also known as hypoxia-inducible factor 2alpha) has been shown to promote adipose differentiation in 3T3-L1 cells (Shimba et al., 2004).

GATA-2 and GATA-3 are specifically expressed in white adipocyte precursors, but not in mature adipocytes. Constitutive expression of GATA-2 and GATA-3 keeps preadipocytes from differentiating into adipocytes, while depletion of GATA-3 increases adipocyte differentiation (Tong et al., 2000). Inhibition of adipogenesis by GATA may be through repression of PPARγ expression or binding to C/EBPα and C/EBPβ (Tong et al., 2000; Tong et al., 2005).

1.2.3 Signalling pathway regulating adipogenesis

1.2.3.1 Insulin and IGF1 pathway

Insulin and insulin-like growth factor 1 (IGF1) are very important regulators of adipogenesis. IGF1 from fetal calf serum works together with insulin to activate IGF receptor, which is more abundant than insulin receptors in pre-adipocytes. The number of insulin receptors increases during preadipocytes differentiation (Smith et al., 1988). The signal passes along insulin receptor substrate (IRS), phosphatidylinositol-3 kinase (PI3K), and activates AKT/PKB (protein kinase B) (reviewed by Rosen and MacDougald, 2006). Insulin signaling pathway may activate CREB to promote adipogenesis (Klemm et al., 2001). Also insulin signalling can cause phosphorylation and nuclear exportation of adipogenic inhibitors FOXA2 and FOXO1 (Nakae et al., 2003; Wolfrum et al., 2003). In the insulin signalling pathway, GATA2, which is an adipogenesis inhibitor, is also phosphorylated and inactivated (Menghini et al., 2005). Moreover, insulin signaling pathway can repress the expression of necdin, which in turn releases the repression of PPARγ (Tseng et al., 2005).

1.2.3.2 Glucocorticoid Receptor

Dexamethasone is the most commonly used glucocorticoid to stimulate preadipocyte differentiation. The action of dexamethasone is through the nuclear receptor glucocorticoid receptor (GR) (Rosen and Spiegelman, 2000). Glucocorticoids may have a direct role on C/EBPδ induction, which will further activate PPARγ along with C/EBPβ (Cao et al., 1991;

Wu et al., 1996). Also glucocorticoids can repress the expression of the anti-adipogenic gene pref-1 (Smas et al., 1999).

1.2.3.3 FGF family

Fibroblast growth factor 1 (FGF-1) secreted by adipose-derived microvascular endothelial cells (MVECs) can promote proliferation and differentiation of human pre-adipocytes, which lack FGF-1 expression. FGF-1 is expressed in 3T3-L1 cells and treatment of 3T3-L1 cells with FGF-1 antibody decreases their adipogenic potential (Hutley et al., 2004; Newell et al., 2006). FGF-2 (also called basic fibroblast growth factor) enhances adipogenic differentiation of mesenchymal stem cells and adipose-derived stem cells (Kakudo et al., 2007; Neubauer et al., 2004). FGF10 signalling induces the expression of C/EBPβ and the subsequent adipogenic differentiation in preadipocytes. In FGF10 knock-out mice, the expression of C/EBPβ is reduced and the development of white adipose tissue is impaired (Sakaue et al., 2002).

1.2.3.4 MAPK pathway

The mitogen activated protein kinases (MAPKs), which include ERK, p38 and JNK, play both positive and negative roles in regulating adipogenesis (reviewed by Bost et al., 2005). MEF cells from ERK1 knock-out mice or 3T3-L1 cells lacking ERK1 have impaired adipogenic potential, suggesting ERK1 is required for adipogenesis. On the other hand, ERK1 can phosphorylate PPARγ and decrease its activity (Camp and Tafuri, 1997; Hu et al.,

1996).

Inhibitors of p38 MAP kinase prevent 3T3-L1 fibroblasts and C3H10T1/2 cells from differentiation into adipocytes, suggesting p38 MAP kinases are required for adipogenic differentiation of these preadipocyte cell lines (Engelman et al., 1998; Hata et al., 2003). However, p38 null embryonic stem cells have stronger potential to differentiate into adipocyte than wild type ES cells, indicating it has a negative role in adipogenesis (Aouadi et al., 2006). These controversial results may be due to MAP kinases playing varied roles at different differential stages or in different cell types.

1.2.3.5 TGFβ family

Transforming growth factor β (TGFβ) family members, including TGFβ and bone morphogenetic proteins (BMPs), play a role in regulating adipogenesis. Ligand binding induces dimerization and phosphorylation of TGFβ recetprs, which further phosphorylates R-Smad (from Smad1 to Smad5). Phosphorylated R-Smad binds to Smad4 and translocates into the nucleus (reviewed by Roelen and Dijke, 2003). TGF-ß inhibits adipocyte differentiation in preadipocyte cell lines, which is in agreement with *in vivo* data showing that adipocyte differentiation is significantly inhibited in TGF-ß transgenic mice (Choy et al., 2000; Clouthier et al., 1997). Smad3, which is a component of the TGF-ß pathway, inhibits adipogenesis by interacting with C/EBPβ and C/EBPδ and repressing their activity (Choy and Derynck, 2003).

Low concentration of BMP2 promotes adipocyte development, while high concentration

of BMP2 induces chondrocytes and osteoblasts differentiation (Wang et al., 1993). BMP-2 stimulation may lead to nuclear translocation of Schnurri-2 (Shn-2), which will activate PPARγ2 expression through direct interaction with both Smad1/4 and C/EBPα (Jin et al., 2006). Another BMP member, BMP4 has been shown to be involved in preadipocytes determination (reviewed by Bowers and Lane, 2007).

1.2.3.6 Wnt signalling

The Wnt family, which is an evolutionarily conserved family of secreted glycoproteins, plays a big role in regulating adipogenesis. Wnt10b, Wnt10a, and Wnt6 are expressed in preadipocytes. They can bind to transmembrane frizzled receptors and activate the Wnt/β-catenin pathway, which will lead to inhibition of GSK3β and cause hypophosphorylation and stabilization of β-catenin in the cytoplasm. Following that, β-Catenin is translocated into the nucleus and binds to TCF/LEF transcription factors to inhibit preadipocyte differentiation (reviewed by Prestwich and Macdougald, 2007). FABP4-Wnt10b transgenic mice have approximately 50% less total body fat compared to wild type (Longo et al., 2004). Conditional deletion of β-catenin in the myometrium converts it to adipose tissue (Arango et al., 2005). Wnt5b is different from other Wnt members. It is transiently up-regulated during adipogenesis and promotes preadipocyte differentiation (Kanazawa et al., 2005b). Other signals may utilize the Wnt/β-catenin pathway to carry out their functions. For example, testosterone induces androgen receptor binding to beta-catenin and inhibits adipogenesis in 3T3-L1 cells (Singh et al., 2006).

1.2.3.7 Hedgehog signalling pathway

Hedgehog (Hh) proteins are lipid containing secreted proteins that also regulate adipogenesis. Secreted Hh proteins bind to the cell surface receptor Patched (PTC) and release Smoothened (Smo). In turn, Smo regulates gene expression through the GLI family of transcription factors (reviewed by Hooper and Scott, 2005). In flies, activation of Hh signaling in the fat body inhibits fat formation, while inhibition of Hh in the fat body stimulates fat formation (Suh et al., 2006). *In vitro*, Sonic Hedgehog can inhibit 3T3-L1 cell and C3H10T1/2 cell adipogenesis (Spinella-Jaegle et al., 2001; Zehentner et al., 2000). The inhibitory effects of Hh may be due to induction of anti-adipogenic transcription factors such as Gata2 (Suh et al., 2006).

1.2.3.8 Pref1 signalling

Preadipocyte factor 1 (Pref-1, also named DLK1) is an epidermal growth factor (EGF) like repeat containing protein that can be activated through proteolytic cleavage by tumor necrosis factor α converting enzyme (TACE, also called ADAM 17) (reviewed by Wang et al., 2006). Pref-1 is highly expressed in preadipocytes and its expression diminishes when 3T3-L1 preadipocytes differentiate into adipocytes. Moreover, constitutive expression of pref-1 significantly inhibits adipogenic differentiation in preadipocytes (Smas and Sul, 1993). Pref-1 knock-out mice display obesity and increased serum lipid metabolites, while Pref-1 transgenic mice have a dramatic reduction in adipose tissue (Lee et al., 2003a; Moon et al., 2002).

CHAPTER 2

The role of microRNA in 3T3-L1 cell differentiation

2.1 INTRODUCTION

The 3T3-L1 preadipocyte cell line is a well established *in vitro* model to study adipogenesis (Green and Meuth, 1974). Upon a cocktail of cyclic AMP, insulin, and glucocorticoids treatment, 3T3-L1 preadipocytes undergo differentiation into mature adipocytes over 4-6 days period (Student et al., 1980). 3T3-L1 cells first reach growth arrest by contact inhibition. Then, cell division occurs for one or two rounds during clonal expansion. Finally, the cell cycle stops again and proceeds into final differentiation (Rosen and Spiegelman, 2000). We wanted to investigate whether microRNAs (miRNAs) play a role in this process.

MicroRNAs, which are endogenous small non-coding RNAs around 22 nt, play very important roles in many processes such as development, proliferation, apoptosis, metabolism and human diseases (Bushati and Cohen, 2007; Bartel, 2004). MicroRNAs bind to the 3' untranslated region (3'UTR) of their targets and negatively regulate them through translational repression or mRNA decay (reviewed by Eulalio et al., 2008).

miR-143 was reported to regulate adipocyte differentiation by targeting ERK5 (Esau et al., 2004), but another group reported that inhibition of some up-regulated microRNAs didnot affect 3T3-L1 differentiation (Kajimoto et al., 2006). These controversial views promoted us to investigate the role of microRNAs in pre-adipocyte differentiation.

MicroRNA *let-7* was first reported to regulate *C. elegans* developmental timing

(Reinhart et al., 2000). *let-7* can also suppress the oncogene Ras (Johnson et al., 2005). *let-7* was reported to be involved in cancer (Park et al., 2007), including breast cancer (Yu et al., 2007), ovarian cancer (Lu et al., 2007), non-small cell lung cancer (Kumar et al., 2008), lung cancer (Esquela-Kerscher et al., 2008), and colon cancer (Akao et al., 2006).

Other targets for *let-7* were also reported such as neurofibromatosis 2 (Meng et al., 2007b), Toll-like receptor 4 (Chen et al., 2007), CdK6, and Cdc25a (Johnson et al., 2007), and MYC (Sampson et al., 2007).

let-7 can also target High mobility group AT hook 2 (*Hmga2*) (Mayr et al., 2007; Lee and Dutta, 2007). *Hmga2* and *Hmga1* belong to the High Mobility Group A (*Hmga*) family, which encodes architectural transcription factors that bind to minor groove of AT-rich DNA and cause conformational changes in chromatin. HMGA2 protein contains an AT-hook domain that is responsible for DNA binding, a linker, and an acidic tail. *Hmga2* is involved in many physiologic process and diseases (reviewed by Young and Narita, 2007). Gene rearrangement was found at the *Hmga2* region in lipomas (Ashar et al., 1995) and mesenchymal tumors (Schoenmakers et al., 1995). Inactivation of the *Hmga2* gene in mice yields a pygmy phenotype due to lack of fat tissue (Zhou et al., 1995). Mice with heterozygous or null *Hmga2* gene are resistant to diet induced obesity (Anand and Chada, 2000). Over-expression of the truncated *Hmga2* gene produces a giant mouse with massive fat tissue (Battista et al., 1999). These results suggest the *Hmga2* gene plays a role in adipogenesis.

E2F family members also regulate adipogenesis. E2F family members are important transcription factors that regulate cell cycles. There are eight known E2Fs from E2F1 to

E2F8 in this family. E2F1 through E2F6 can dimerize with DP proteins (DP1, DP2/3, DP4). Tumor suppressor retinoblastoma protein (pRB) and other pocket proteins p107 and p130 can regulate the transcriptional activity of E2F1-E2F5 (reviewed by DeGregori and Johnson, 2006). HMGA2 can acetylate and activate E2F1 (Fedele et al., 2006). Lack of E2F1 impairs, while loss of E2F4 promotes, adipogenesis (Fajas et al., 2002).

Since microRNAs have been implicated in playing a role in regulating adipogenesis, in this work we attempted to identify microRNAs that play a role in 3T3-L1 cell differentiation and characterize the regulatory mechanism by which they work.

2.2 RESULTS

2.2.1 Expression of *let-7* and other miRNAs during adipogenesis

To investigate whether miRNAs are involved in adipocyte differentiation, we examined the expression of 386 miRNAs during 3T3-L1 differentiation using microarray analysis. Post-confluent 3T3-L1 cells were induced to differentiate using a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine and insulin (DMI). RNA was prepared from cells at 0, 1, 4 and 7 days after adipogenic induction (Fig. 2.1A) and small RNAs were purified for use in microarray analysis (Fig. 2.1B). Among the 386 miRNAs examined, 23 were either increased or decreased >1.5 fold during 3T3-L1 differentiation (Table 2.1). Induction of several of these, including *let-7*, miR-103, miR-143, miR-193 and miR-210, was confirmed by northern blot analysis (Fig. 2.2A). The expression of all these miRNAs was up-regulated after 2 days of differentiation and maintained at a high level in mature adipocytes. Consistent with our *in vitro* findings, *let-7*, miR-103, miR-143, miR-193 and miR-210 were all expressed in murine white adipose tissue (WAT) (Fig. 2.2B). Additional profiling studies revealed that each of these miRNAs was expressed in multiple tissues including brown adipose tissue (Fig. 2.2B).

We chose to focus further experiments on *let-7* given its recently established role in regulating cell fate decisions in *C. elegans* and *Drosophila* (Caygill and Johnston, 2008; Reinhart et al., 2000; Sokol et al., 2008). *let-7* expression was increased in 3T3-L1 cells differentiated by treatment with either the DMI cocktail or the PPAR agonist, rosiglitazone (Fig. 2.3A). Using a third independent assay, *let-7* levels were also increased during

insulin-induced differentiation of 3T3-F442A cells into adipocytes (Fig. 2.3A). In agreement with these findings, *let-7* was abundant in mature adipocytes isolated from mice but barely detectable in preadipocytes (Fig. 2.3C). *let-7* was not induced by DMI treatment of NIH3T3 cells, which do not differentiate into adipocytes, nor was it induced during differentiation of C2C12 cells into myotubes (Fig. 2.3B). These data show that *let-7* induction is not invariably associated with either DMI treatment or differentiation processes.

There are several *let-7* isoforms in the mouse genome that differ in only 1–2 nucleotides (Table 2.2). Since these isoforms cannot be distinguished by northern blot analysis, we quantified their levels by RT-qPCR. Consistent with the microarray and northern blot data presented above, the most abundant *let-7* isoforms, including *let-7a*, *let-7b* and *let-7d*, were all up-regulated during 3T3-L1 adipogenesis (Fig. 2.3D). Interestingly, with the exception of *let-7b*, all the *let-7* isoforms decreased from day 0 to day 1 and then increased. We speculate that this transient dip in *let-7* expression may be permissive for clonal expansion (see below).

2.2.2 *let-7* inhibits 3T3-L1 differentiation

To test whether *let-7* plays a role in 3T3-L1 adipogenesis, pre-*let-7a* oligonucleotide was transfected into 3T3-L1 cells, where it was efficiently converted into mature *let-7a* as confirmed by northern blot analysis (Fig. 2.4). Introduction of ectopic *let-7a* in 3T3-L1 cells prior to DMI treatment inhibited their differentiation into adipocytes as measured by Oil

Red O staining (Fig. 2.5) and triglyceride content (Fig. 2.6C) on day 6 of differentiation. mRNA levels of *Pparγ* and *C/ebpα*, two transcription factors whose induction is important for adipocyte differentiation, were significantly lower in cells transfected with *let-7a* compared to cells transfected with control oligonucleotide (Fig. 2.6B). Likewise, mRNA levels of the mature adipocyte markers, fatty acid binding protein 4 (aP2) and adipsin, were decreased by *let-7a* transfection (Fig. 2.6B). In contrast, *C/ebpβ* and *C/ebpδ*, two genes whose expression is important during the early stages of adipogenesis, were not significantly affected by *let-7a* at the 6 day time point.

2.2.3 Overexpression of *let-7* impairs clonal expansion of 3T3-L1 cells

The treatment of 3T3-L1 cells with an adipogenic stimulus initiates a complex sequence of events including clonal expansion, cell cycle exit and terminal differentiation (Rosen and Spiegelman, 2000). Since *let-7* controls exit from the cell cycle in *C. elegans* and *D. melanogaster* (Caygill and Johnston, 2008; Reinhart et al., 2000; Sokol et al., 2008), we postulated that its up-regulation on day 1 of adipogenesis might play an analogous role in promoting terminal differentiation of adipocytes. To test this hypothesis, 3T3-L1 cells were transfected with either *let-7a* or control oligonucleotides and then allowed to reach confluence, at which point the adipogenic program was initiated with the DMI cocktail. As shown in Fig. 2.7, cell number was significantly reduced in cultures transfected with *let-7a* compared to control oligonucleotide. These data support a role for *let-7* in blocking clonal expansion during adipogenesis.

2.2.4 Microarray analysis showed *let-7* down-regulated some genes at mRNA level.

In an effort to identify genes through which *let-7* mediates its effect on adipocyte differentiation, microarray analysis was performed using mRNA prepared from 3T3-L1 cells harvested 24 hours after transfection with either *let-7a* or control oligonucleotide. Genes whose expression was down-regulated >2 fold by *let-7a* are listed in Table 2.3. Several of these genes, including *Hmga2*, *E2f6*, *Cdc34* and insulin-like growth factor 2 mRNA binding protein 1 (*Igf2bp1*), have been previously shown to be targets of *let-7* (Boyerinas et al., 2008; Johnson et al., 2007; Lee and Dutta, 2007; Mayr et al., 2007).

E2F family members have been reported to be involved in adipogenesis. Knock-out of *E2f1* impairs, while deletion of *E2f4* promotes adipogenesis (Fajas et al., 2002). Since the *E2f6* mRNA level was down-regulated by *let-7* as detected by microarray, we checked whether *let-7* regulates adipogenesis through the E2F family. Using online bioinformatics tools, it was found that several members of the E2F family, including *E2f2*, *E2f3*, *E2f5*, and *E2f6*, contain potential *let-7* binding sites in their 3'UTR (http://cbio.mskcc.org/cgi-bin/microRNAviewer/microRNAviewer.pl). These potential *let-7* binding sites in the 3' UTR of *E2f2*, *E2f3*, *E2f5*, and *E2f6* were cloned and put at the 3' end of a luciferase reporter. The reporter activity was checked in the presence of *let-7* or control oligo in F9 cells, which lack endogenous *let-7* (Mayr et al., 2007). The activity of reporter containing 3' UTR of *E2f5* or *E2f6* is lower than negative control reporter, while that of *E2f2* or *E2f3* was comparable to control reporter (Fig. 2.8a). Mutation of two nucleotides from "C" to "A" in the binding sites of 3' UTR of *E2f5* or *E2f6*, which abolishes the paring of *let-7* with the binding sites, relieved the repression of reporter activity by *let-7* (Fig. 2.8c).

These results suggest *let-7* can regulate *E2f5* and *E2f6* by binding to the 3' UTR of *E2f5* and *E2f6*, respectively. To check whether *let-7* regulate adipogenesis through *E2f5* or *E2f6*, small interference RNA against *E2f5* or *E2f6* was transfected into 3T3-L1 cells and the adipogenic potential of 3T3-L1 cells was checked after standard DMI induction. Either knock-down of E2F5 or E2F6 didn't inhibit 3T3-L1 differentiation (data not shown). These results suggest the effect of *let-7* on 3T3-L1 adipogenesis may not be mainly through E2F5 or E2F6.

2.2.5 *let-7* may regulate clonal expansion and differentiation by targeting HMGA2

Hmga2 was the gene whose expression was most affected by *let-7a* in 3T3-L1 cells (Table 2.3). Notably, mice lacking HMGA2 have a striking reduction in adipose tissue (Zhou et al., 1995). Conversely, transgenic overexpression of a truncated HMGA2 in mice resulted in a marked increase in fat tissue, adipose tissue inflammation and a high incidence of lipomas (Arlotta et al., 2000; Battista et al., 1999). Thus, HMGA2 was a strong candidate for being a *let-7* target in differentiating adipocytes. In agreement with the microarray data, ectopic *let-7* reduced HMGA2 protein concentrations >3-fold in 3T3-L1 cells (Fig. 2.9A). Interestingly, *Hmga2* mRNA was rapidly induced in 3T3-L1 cells during adipocyte differentiation, with levels peaking 4 hours after treatment with the DMI cocktail and returning to basal concentrations on day 2 (Fig. 2.9B). There was a subsequent increase in HMGA2 protein concentrations, with levels peaking at day 2 (Fig. 2.9C). HMGA2 and *let-7* expression were inversely correlated during adipocyte differentiation (compare Fig. 2.3A,

left panel, with Fig. 2.9A and 2.9B). Finally, siRNA knockdown of HMGA2 inhibited 3T3-L1 differentiation as measured by *aP2* and *Pparγ* mRNA levels (Fig. 2.9D). Taken together, these results strongly suggest that *let-7* regulates 3T3-L1 differentiation in part by targeting HMGA2.

2.2.7 *let-7* and Hmga2 play an import role in 3T3-L1 adipogenesis

3T3-L1 pre-adipocytes undergo growth arrest, clonal expansion, final growth arrest and terminal differentiation into mature adipocytes (Rosen and Spiegelman, 2000). Although the main transcription cascades regulating adipogenesis are known, the exact details of how 3T3-L1 cells undergo differentiation remains unknown. Our results show that the microRNA *let-7* specifically increases at the late stage of 3T3-L1 differentiation and Hmga2 is induced at the clonal expansion stage and decreases at final growth arrest and terminal differentiation stages (Fig 2.10). We suggest that *let-7* regulates 3T3-L1 cell differentiation by stopping clonal expansion and bringing it to final growth arrest and terminal differentiation by targeting Hmga2.

Fig. 2.1. Expression of microRNAs during 3T3-L1 differentiation.

A: Oil Red O staining showes lipid accumulation during 3T3-L1 differentiation; **B**: microRNA array expression data from 3T3-L1 cells cultured in differentiation medium for 0, 1, 4 or 7 day. Normalized log2 data are plotted as a heat map. Red denotes high expression and green denotes low expression relative to the median; only the microRNA that were changed by more than 1.5 fold in differentiated adipocytes are shown.

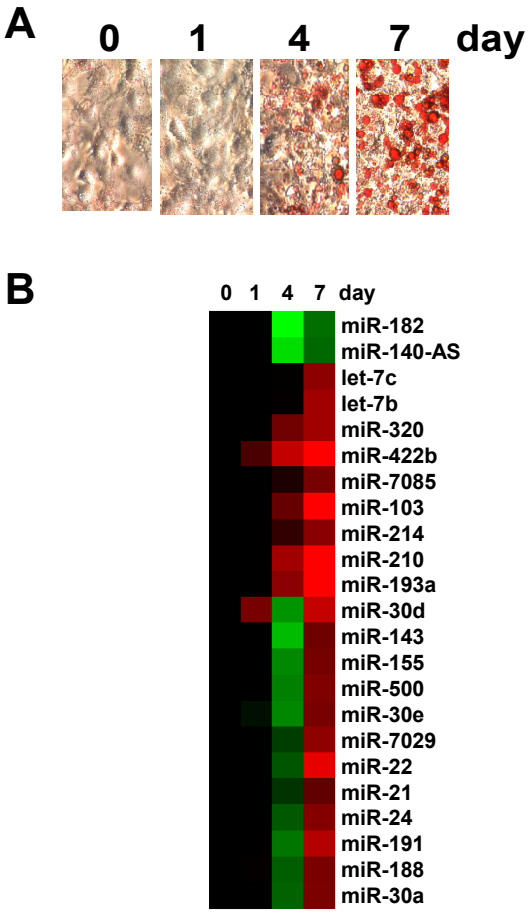

A
0
1
4
7
day
B
0 1 4 7 day
miR-182
miR-140-AS
let-7c
let-7b
miR-320
miR-422b
miR-7085
miR-103
miR-214
miR-210
miR-193a
miR-30d
miR-143
miR-155
miR-500
miR-30e
miR-7029
miR-22
miR-21
miR-24
miR-191
miR-188
miR-30a

Table 2.1 The expression changes of microRNAs during 3T3-L1 differentiation

Name	0 day	1 day	4 day	7 day
hsa_miR_422b	1	1.50	2.89	12.01
hsa_miR_210	1	1.08	2.42	11.93
hsa_miR_103	1	1.01	1.74	7.53
hsa_miR_193a	1	1.02	2.14	5.55
hsa_miR_22	1	1.07	0.62	3.55
hsa_miR_30d	1	1.92	0.44	2.91
hsa_miR_191	1	1.00	0.52	2.68
hsa_let_7b	1	1.13	1.03	2.41
hsa_miR_320	1	1.21	1.85	2.40
ambi_miR_7029	1	1.02	0.71	2.18
hsa_let_7c	1	1.21	1.01	2.14
hsa_miR_214	1	1.32	1.31	2.12
hsa_miR_24	1	1.24	0.61	2.06
hsa_miR_500	1	1.09	0.49	2.01
hsa_miR_30a_5p	1	0.98	0.58	1.95
hsa_miR_188	1	1.03	0.59	1.94
hsa_miR_30e_3p	1	0.91	0.47	1.92
ambi_miR_7085	1	1.02	1.17	1.91
mmu_miR_155	1	1.01	0.47	1.88
hsa_miR_143	1	1.00	0.35	1.83
hsa_miR_21	1	1.15	0.75	1.69
mmu_miR_140_AS	1	1.23	0.29	0.56
hsa_miR_182	1	1.01	0.16	0.54

Fig. 2.2. Up-regulation of a subset of microRNAs (*let-7*, miR-103, miR-143, miR-193, and miR-210) was confirmed by northern blot during 3T3-L1 differentiation.

Northern blot analysis of microRNA expression from different time points as indicated during 3T3-L1 differentiation as well as in 10 different adult mouse tissues. U6 snRNA was used as a loading control.

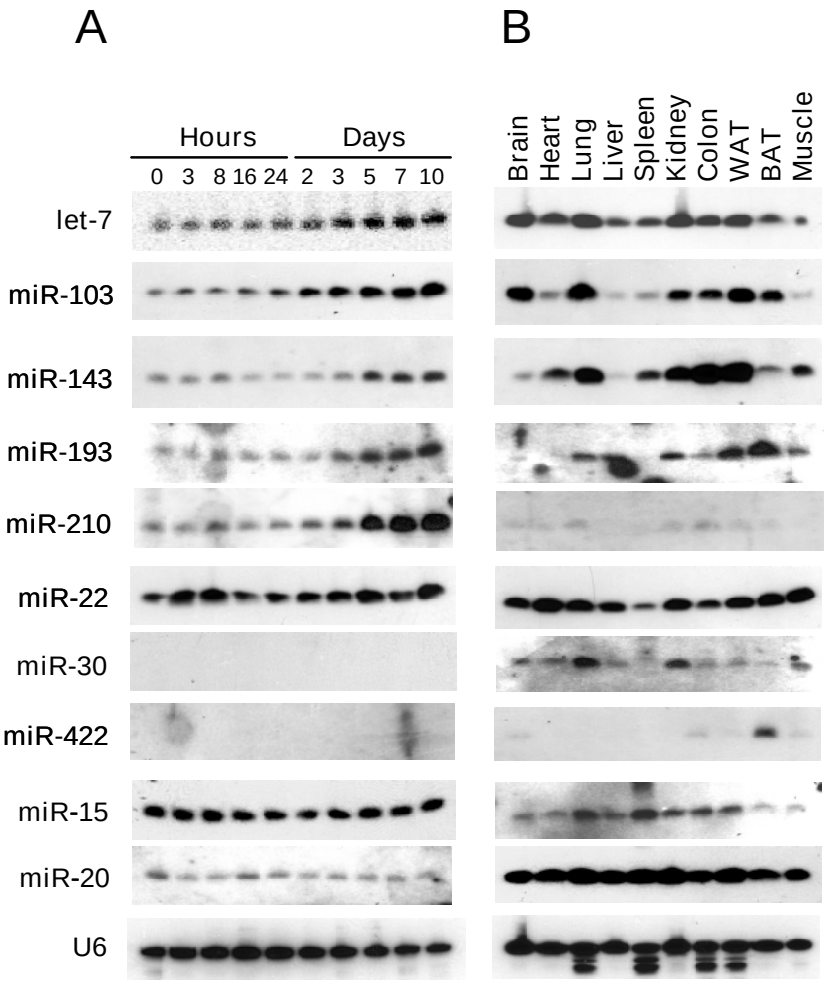

A
B
Hours
Days
0 3 8 16 24 2 3 5 7 10
Brain
Heart
Lung
Liver
Spleen
Kidney
Colon
WAT
BAT
Muscle
let-7
miR-103
miR-143
miR-193
miR-210
miR-22
miR-30
miR-422
miR-15
miR-20
U6

Fig. 2.3. *let-7* induction is specific for adipogenesis.

A: post-confluent 3T3-L1 cells were induced to differentiate by incubation with DMI cocktails or Rosiglitazone (Rosi.), and 3T3-F442A cells were induced to differentiate by insulin. RNA was isolated at serial time points as indicated and northern blot was performed using a *let-7a* probe. *let-7* precursor and mature *let-7* are shown. The values are normalized by loading control and represented as fold-changes compared to the level at 0-day **B:** C2C12 cells were induced to differentiate into myotubes by incubation with differentiation medium, and NIH3T3 cells were treated with the same inducers as 3T3-L1 differentiation. Northern blots were used to detect *let-7*. The values were normalized by U6 and represented as fold changes compared to that of 0-day. **C:** Pre-adipocytes (SV fraction) and mature adipocytes (Ad) were isolated from adipose tissue of mice. RNA was extracted and northern blot was performed as described in materials and methods. The image of ethidium bromide (EB) stained gel served as a loading control. **D**: RNA was isolated at different time points as indicated during 3T3-L1 differentiation. Individual isoforms of *let-7* family were quantified by specific ABI Taqman Q-PCR probe and primer sets. Error bar represent SDEV from triplicate wells.

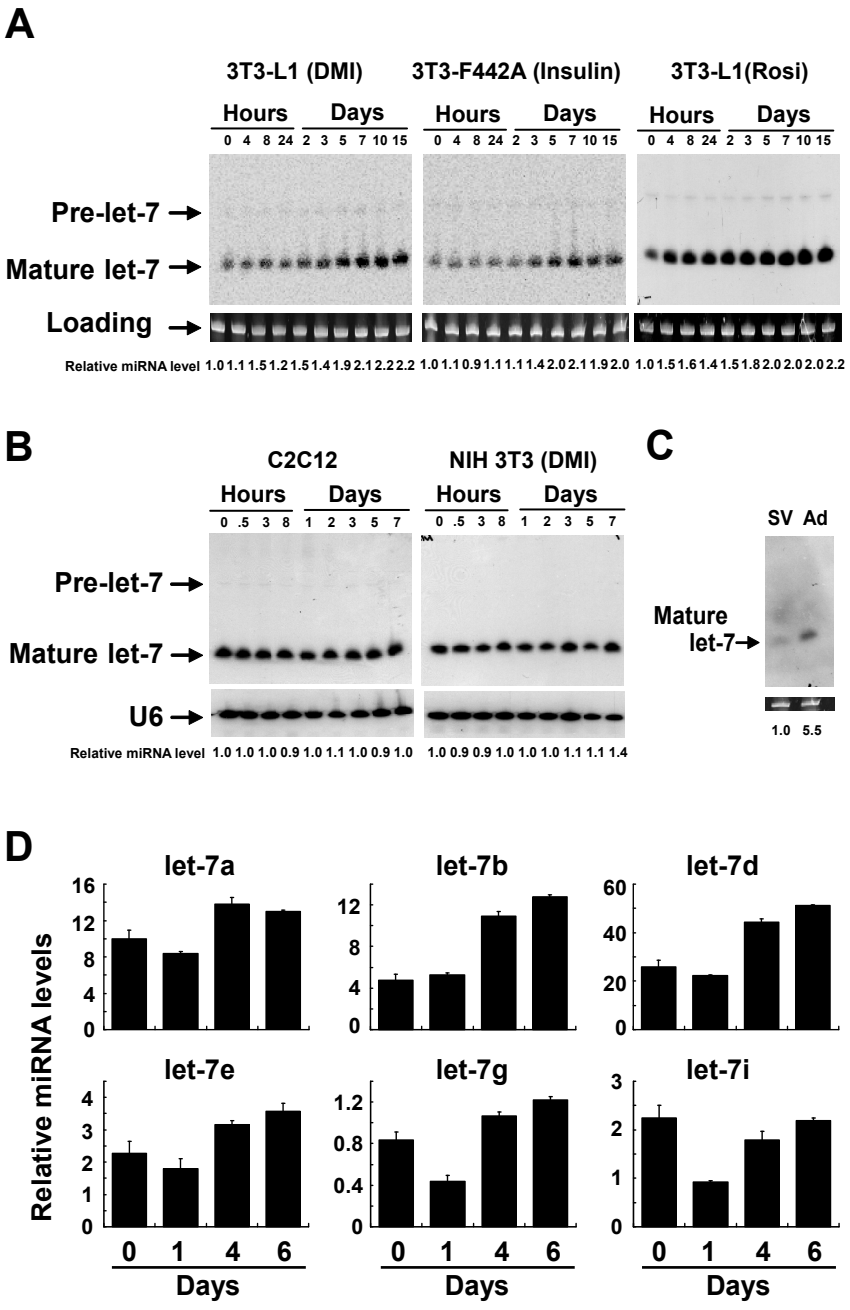

A
3T3-L1 (DMI)
3T3-F442A (Insulin)
3T3-L1(Rosi)
Hours
Days
0 4 8 24 2 3 5 7 10 15
Pre-let-7
Mature let-7
Loading
Relative miRNA level 1.0 1.1 1.5 1.2 1.5 1.4 1.9 2.1 2.2 2.2 1.0 1.1 0.9 1.1 1.1 1.4 2.0 2.1 1.9 2.0 1.0 1.5 1.6 1.4 1.5 1.8 2.0 2.0 2.0 2.2
B
C2C12
NIH 3T3 (DMI)
0 .5 3 8 1 2 3 5 7
U6
Relative miRNA level 1.0 1.0 1.0 0.9 1.0 1.1 1.0 0.9 1.0 1.0 0.9 0.9 1.0 1.0 1.0 1.1 1.1 1.4
C
SV Ad
Mature let-7
1.0 5.5
D
let-7a
let-7b
let-7d
let-7e
let-7g
let-7i
Relative miRNA levels
0 1 4 6
Days

Table 2.2 *let-7* isotypes in mice

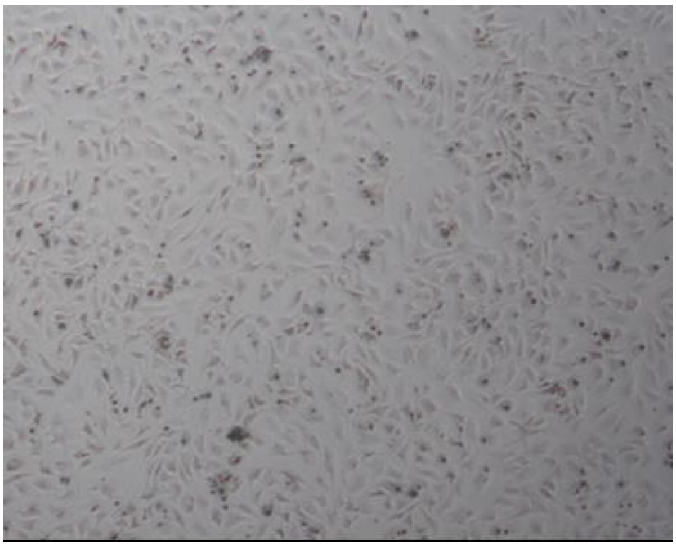
Name	Chromosome	Sequence
mmu-let-7-a1	**13**	**ugagguaguagguuguauaguu**
mmu-let-7-a2	**9**	**ugagguaguagguuguauaguu**
mmu-let-7-b	**15**	**ugagguaguagguuguugugguu**
mmu-let-7-c1	**16**	**ugagguaguagguuguaugguu**
mmu-let-7-c2	**15**	**ugagguaguagguuguaugguu**
mmu-let-7-d	**13**	**agagguaguagguugcauaguu**
mmu-let-7-e	**17**	**ugagguaggagguuguauaguu**
mmu-let-7-f1	**13**	**ugagguaguagauuguauaguu**
mmu-let-7-f2	**x**	**ugagguaguagauuguauaguu**
mmu-let-7-g	**9**	**ugagguaguaguuuguacaguu**
mmu-let-7-i	**10**	**ugagguaguaguuugugcuguu**

(Data from sanger miRbase)

Fig. 2.4. Pre-*let-7* can be transfected and converted into its mature form in 3T3-L1 cells.

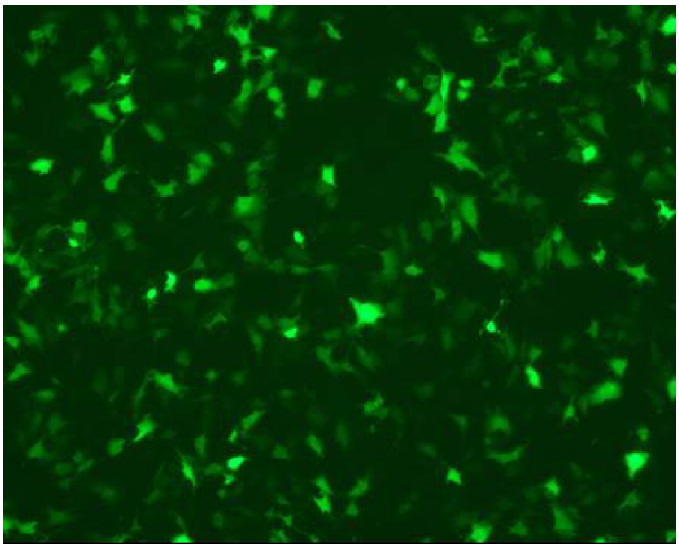
Bright field view (**A**) or GFP fluorescence (**B**) of 3T3-L1 cells transfected with plasmid expressing GFP. **C**: 3T3-L1 cells were transfected with *let-7*a precursor, control oligo, or GFP by electroporation. RNA was isolated 24 h after transfection and northern blot was performed using *let-7*a probe. The same membrane was re-probed with U6 as a loading control.

A



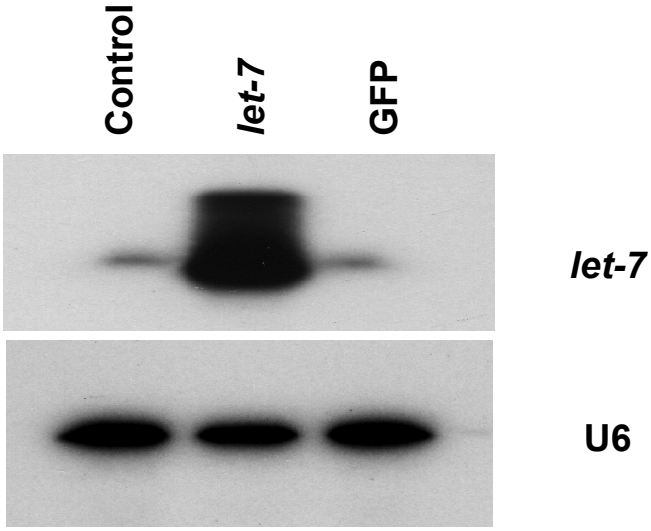
(100X)

B

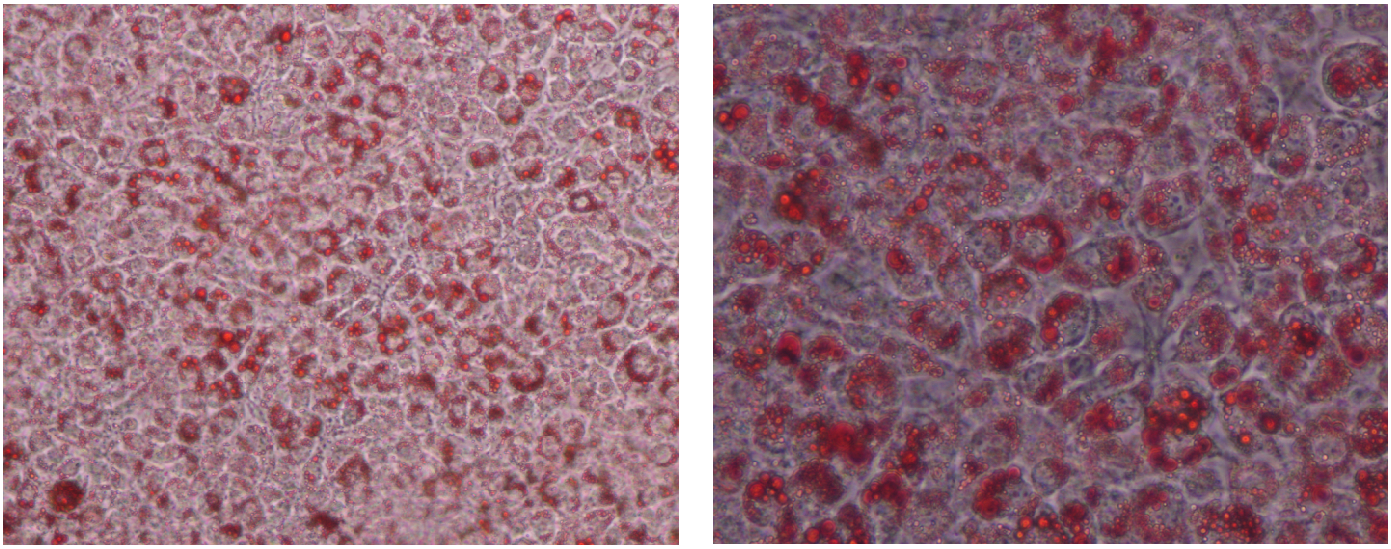


(100X)

C



A Control oligo, 100X Control oligo, 200X



B *let-7*, 100X *let-7*, 200X

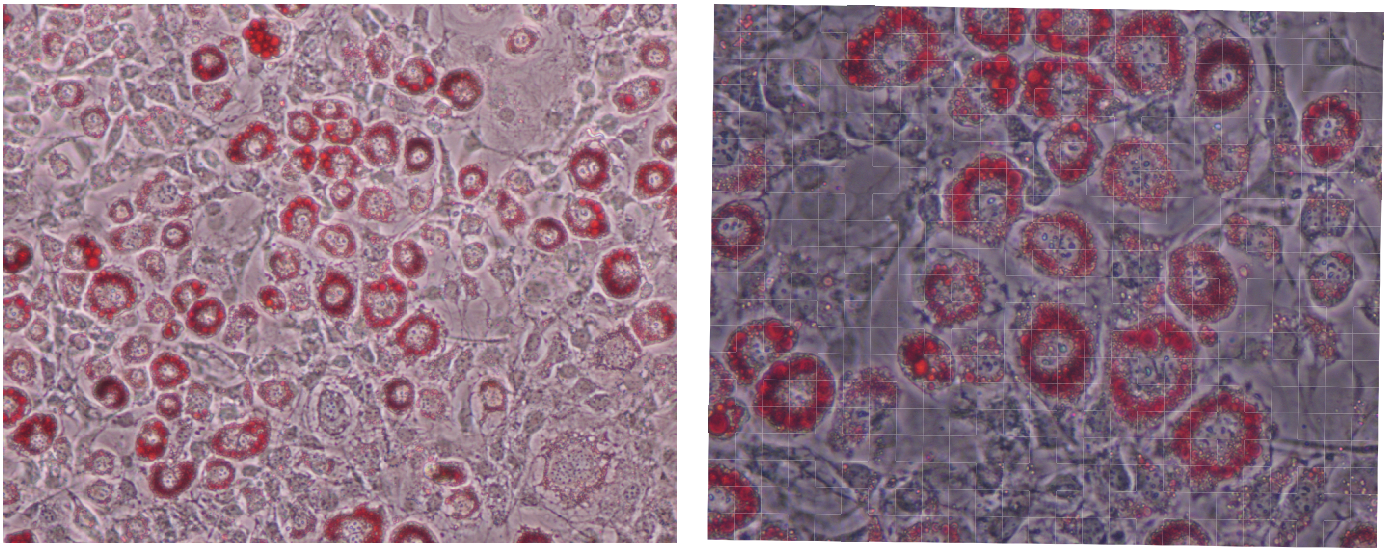
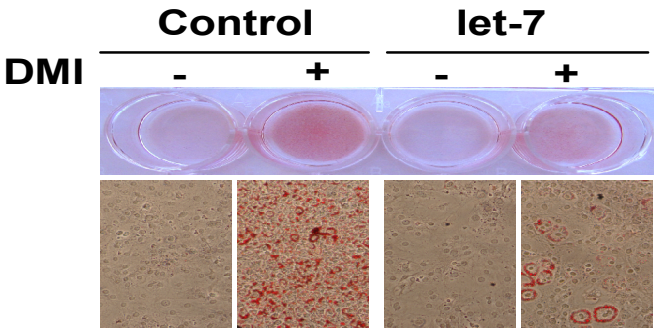


Figure 2.5 Ectopic introduction of *let-7* inhibits 3T3-L1 cell differentiation. Oil-Red-O stain of 3T3-L1 cells transfected with control oligo (A) or *let-7* (B) and differentiated into mature adipocytes. The number of magnification was shown.

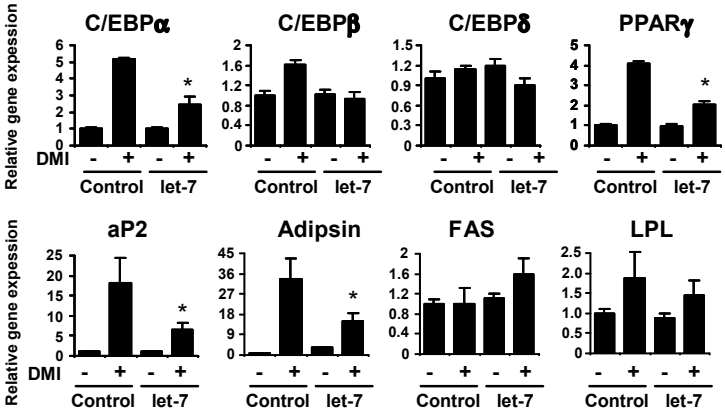
Fig. 2.6. Ectopic presence of *let-7* inhibits 3T3-L1 adipogenesis.

3T3-L1 cells were transfected with *let-7a* precursor or control oligo. After confluency, transfected cells were either continually incubated with growth medium or induced into differentiation with DMI cocktail. After 6 days, the cells were stained by Oil Red O (magnification 200X) (**A**). **B**: The gene expression was analyzed by Q-PCR. (n=3$\pm$SEM, *P<0.01). **C**: Triglyceride content was measured by fluorescence assay described in material and methods. (n=6$\pm$SEM, *P<0.01).

A


Control
let-7
DMI
-
+
-
+

B


C/EBPα
C/EBPβ
C/EBPδ
PPARγ
aP2
Adipsin
FAS
LPL
Relative gene expression
DMI
Control
let-7

C

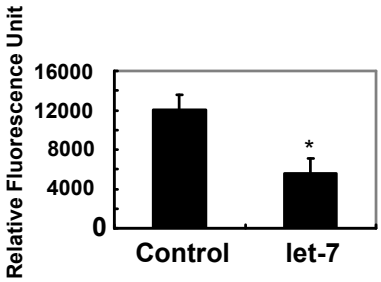

Relative Fluorescence Unit
16000
12000
8000
4000
0
Control
let-7

Fig. 2.7. Ectopic presence of *let-7* inhibits 3T3-L1 clonal expansion.

3T3-L1 cells were transfected with *let-7*a mimic or control oligo by electroporation and cultured in growth medium for 24 h after transfection. Then the cells were incubated with DMI-cocktail. On day 1, 2, and 3, the cell number was counted. (n=3$\pm$SEM, *P<0.01 vs control oligo).

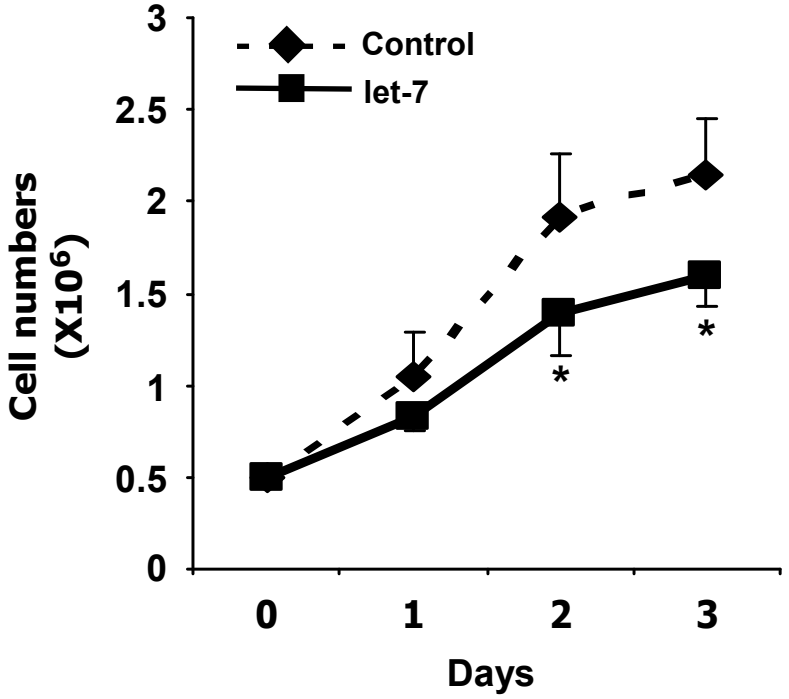

Control
let-7
Cell numbers (X10^6)
3
2.5
2
1.5
1
0.5
0
0
1
2
3
Days
*
*

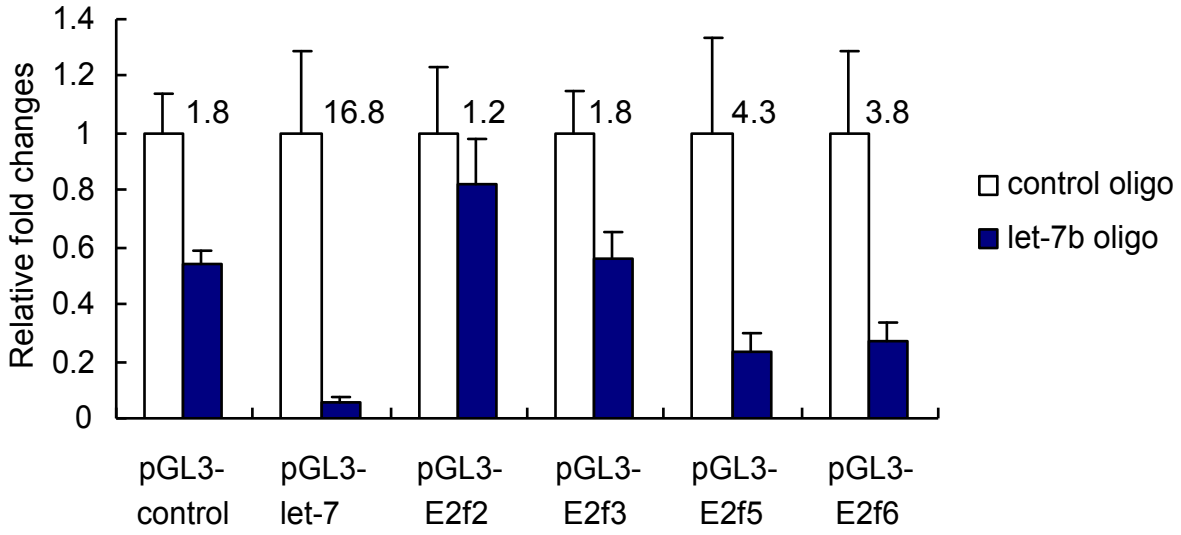
Table 2.3. Genes down-regulated by *let-7* in 3T3-L1 cells.

Gene	Access number	Fold change
Hmga2	NM_010441	3.58
Isg15	NM_015783	3.28
Nfib	NM_001113209	2.78
Nme4	NM_019731	2.70
Stat1	NM_009283	2.65
Parp12	NM_172893	2.65
Usp18	NM_011909	2.64
Rnf213	NM_001040005	2.62
Iigp2	NM_019440	2.61
Apol9a	XM_128064	2.60
Mx2	NM_013606	2.56
Vstm2a	NM_145967	2.54
Cdsn	NM_001008424	2.48
Lgals3bp	NM_011150	2.25
Apol9b	NM_173743	2.21
Arhgap20	NM_175535	2.20
Irgm	NM_008326	2.18
Igtp	NM_018738	2.17
Ube1l	NM_023738	2.17
Samd9l	XM_620286	2.17
E2f6	NM_033270	2.15
AI606181	XR_035116	2.11
Cdc34	NM_177613	2.09
Plagl2	NM_018807	2.07
Igf2bp1	NM_009951	2.00

Fig. 2.8. *let-7* can bind to the 3' UTR of *E2f5* and *E2f6* and regulate their expression.

A: The activity of pGL3-luc, pGL3-luc containing the exact complementary sequence for *let-7* (pGL3-*let-7*) or binding site from the 3' UTR of *E2f2*, *E2f3*, *E2f5* or *E2f6* (pGL3-E2F) was checked in the presence of control oligo or *let-7b* in F9 cells. The fold changes are shown. (n=3±SEM, RLU: relative luciferase unit) **B**: The paring of *let-7* with binding sites in the 3' UTR of *E2f5* or *E2f6*. The mutated sites are shown as underlined letters. **C**: The activity of pGL3 reporter containing wild-type or mutant binding sites for *let-7* from the 3'UTR of *E2f5* or *E2f6* was measured in the presence of *let-7* or control oligo. The fold changes are shown. (n=3±SEM)

A



B

```
12:5'   AA-CUUGGGACUAUUAUCUAAAUCU   3'   Mouse E2f5 UTR mutant
12:5'   AA-CUUGGGACUAUUAUCUACCUCU   3'   Mouse E2f5 UTR
        ||  |:   :  ||  |::   |||||||
 3'     UU.GG--UGUGUUGGAUGAUGGAGU   5'   mmu-let-7b

4310:5' ACCUA-GCGCUCU-CUAAAUCA      3'   Mouse E2f6 UTR mutant
4310:5' ACCUA-GCGCUCU-CUACCUCA      3'   Mouse E2f6 UTR
        |  ||| :|: :|| ||||||||
  3'    UUGAUAUGUUGGAUGAUGGAGU      5'   mmu-let-7a
```

C

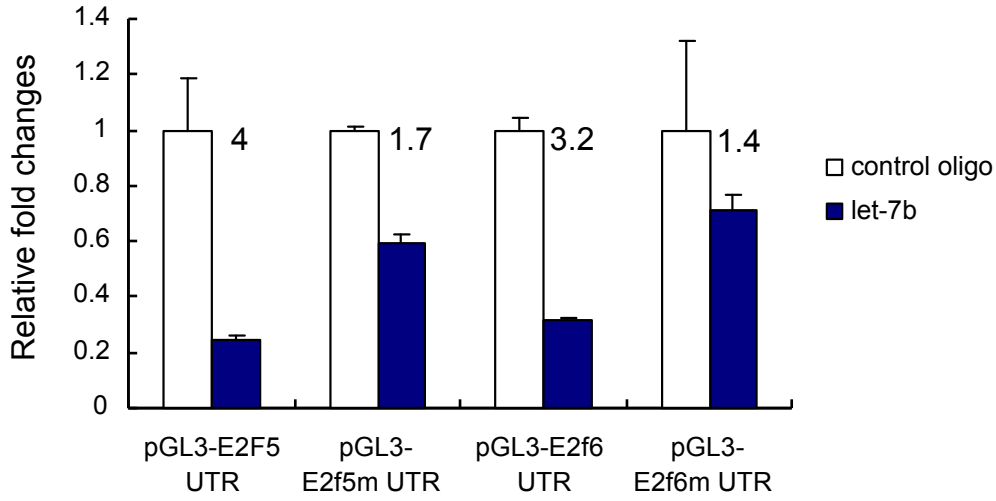
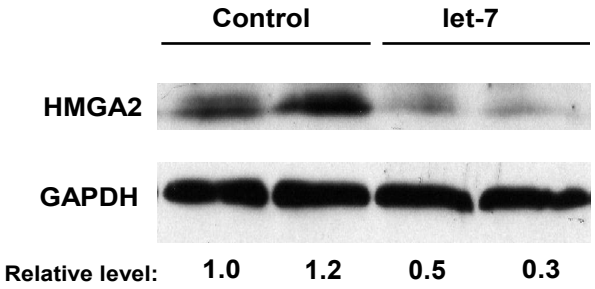


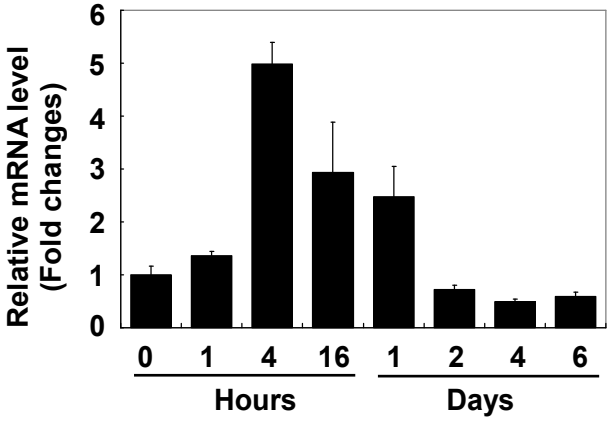
Fig. 2.9. *let-7* regulates 3T3-L1 cell differentiation through the HMGA2 pathway.

A: HMGA2 protein level in *let-7* or control oligo transfected 3T3-L1 cells. GAPDH is shown as a loading control. **B**: Relative *Hmga2* mRNA levels at indicated time points during 3T3-L1 cell differentiation detected by Q-PCR (SYBR green assay). (n=3$\pm$SDEV) **C**: Western blots showing HMGA2 protein levels at indicated days during 3T3-L1 cell differentiation. GAPDH is shown as a loading control. **D**: Relative mRNA level of *aP2* and Pparγ in *Hmga2* siRNA, *E2f1* siRNA, or control siRNA transfected 3T3-L1 cells after differentiation. (n=3$\pm$SEM, *P<0.05, **P<0.01)

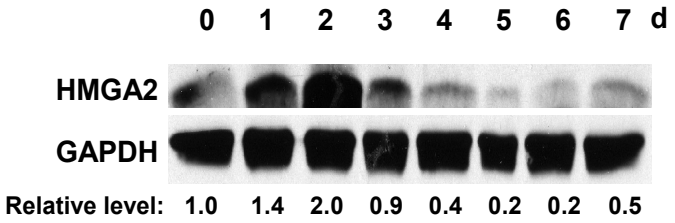
A


Control
let-7
HMGA2
GAPDH
Relative level: 1.0 1.2 0.5 0.3

B


Relative mRNA level (Fold changes)
0 1 2 3 4 5 6
0 1 4 16 1 2 4 6
Hours
Days

C


0 1 2 3 4 5 6 7 d
HMGA2
GAPDH
Relative level: 1.0 1.4 2.0 0.9 0.4 0.2 0.2 0.5

D

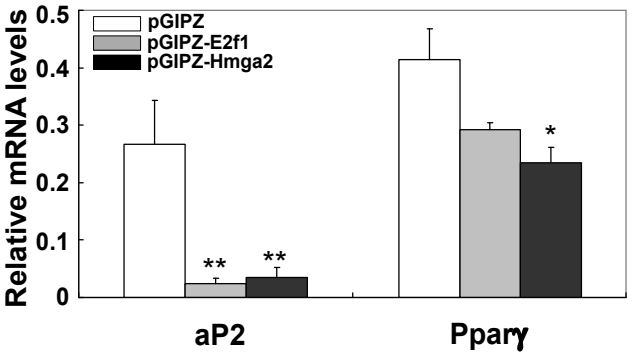
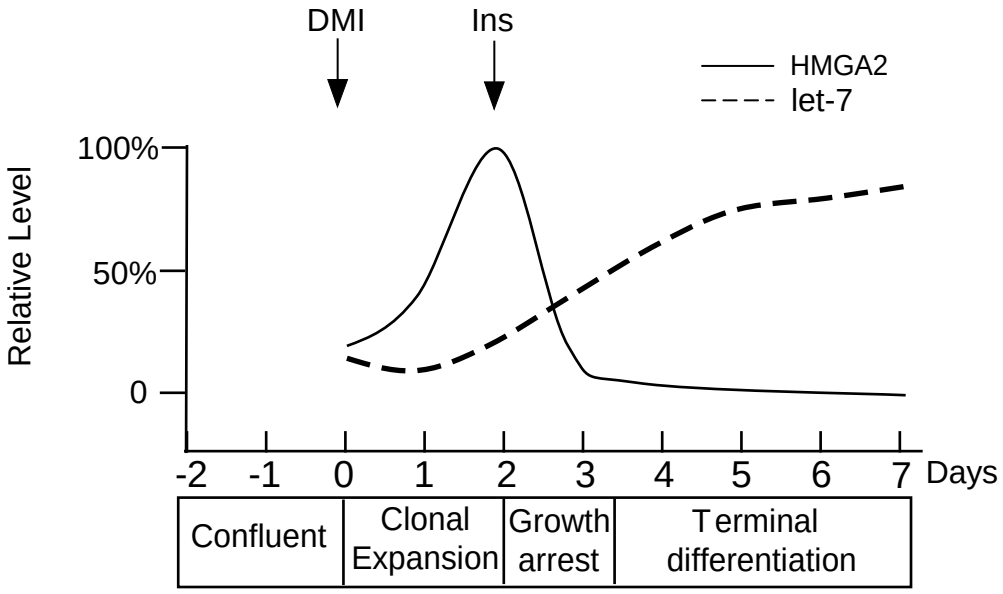

pGIPZ
pGIPZ-E2f1
pGIPZ-Hmga2
Relative mRNA levels
0 0.1 0.2 0.3 0.4 0.5
**
**
*
aP2
Pparγ

Fig. 2.10. Schematic of *let-7* and HMGA2 level during 3T3-L1 cell differentiation. Dash line shows *let-7* expression and solid line indicates HMGA2 protein level.


DMI
Ins
HMGA2
let-7
Relative Level
100%
50%
0
-2
-1
0
1
2
3
4
5
6
7
Days
Confluent
Clonal Expansion
Growth arrest
Terminal differentiation

2.3 DISCUSSION

The differentiation of preadipocytes into mature fat cells requires a highly orchestrated series of changes in gene expression. Although a transcription factor cascade has been identified that regulates adipocyte differentiation, the molecular mechanisms that coordinate the different phases of adipogenesis are not yet completely understood. In this report, we have identified the miRNA *let-7* as an important regulator of adipogenesis in 3T3-L1 cells. *Let-7* is up-regulated following induction of adipogenesis by either the standard DMI cocktail or the combination of rosiglitazone and insulin. Consistent with these *in vitro* findings, *let-7* is much more abundant in mature adipocytes than preadipocytes derived from mouse epididymal adipose. Notably, introduction of ectopic *let-7* blocks 3T3-L1 cell growth during the clonal expansion stage and completely blocks terminal differentiation as measured by both the expression of marker genes and lipid accumulation.

How does *let-7* block adipocyte differentiation? In microarray experiments performed with RNA from 3T3-L1 cells transfected with *let-7a*, *Hmga2* was the most strongly down-regulated RNA, and there was a corresponding decrease in HMGA2 protein concentrations. Previous studies have demonstrated that *let-7* represses HMGA2 expression by binding to six different sites in the *Hmga2* 3'-untranslated region (Mayr et al., 2007). HMGA2 is an architectural transcription factor that alters chromatin structure. Rearrangements of the *Hmga2* gene are frequently observed in benign tumors of mesenchymal origin, including lipomas (Ashar et al., 1995; Schoenmakers et al., 1995). Interestingly, both gain-of-function and loss-of-function experiments in mice implicate

HMGA2 in adipogenesis. Mice lacking HMGA2 have marked reductions in adipose tissue (Zhou et al., 1995). Conversely, transgenic mice overexpressing either full-length or truncated derivatives of HMGA2 develop lipomatosis (Arlotta et al., 2000; Battista et al., 1999; Fedele et al., 2002). These findings, together with our data showing that siRNA-mediated knockdown of HMGA2 blocks 3T3-L1 adipogenesis, suggest that HMGA2 is an important target for the effects of *let-7* on adipocyte differentiation. However, since *let-7* regulates numerous genes, its effects on adipocyte differentiation are likely to be complex and involve regulation of additional genes. In this regard, it is interesting that *let-7* reduced mRNA levels of *E2f6* and *Stat1*. Other members of the E2F family are known to regulate adipogenesis (Fajas et al., 2002), and STAT1 has been shown to be up-regulated during adipogenesis and to regulate gene expression in mature adipocytes (Hogan and Stephens, 2001; Hogan and Stephens, 2003; Stephens et al., 1996).

The regulation of miRNA expression during adipocyte differentiation has been examined by several groups. In a survey of miRNA regulation during 3T3-L1 cell differentiation, Kajimoto et al. observed increased expression of the *let-7b* isoform, which was robustly induced in our study (Kajimoto et al., 2006). In a study of human adipocyte differentiation, Esau et al. saw increased expression of *let-7a* and *let-7c* (Esau et al., 2004). These findings support a role for *let-7* in regulating fat cell differentiation in both humans and mice. Surprisingly, there is relatively little overlap in the miRNAs regulated in our study and that of Kajimoto et al., with only *let-7b*, miR-143, miR-182 and miR-422b showing similar patterns. The basis for this difference is not known, but it does not appear to be due to marked differences in the differentiation protocols.

While we were able to observe a strong gain-of-function phenotype in 3T3-L1 adipocytes with *let-7a*, we did not observe a reciprocal phenotype in knockdown experiments performed with 2'-O-methyl oligoribonucleotide inhibitors against the various *let-7* isoforms (data not shown). Functional redundancy amongst miRNAs is well documented (Miska et al., 2007) and thus the presence of many *let-7* isoforms is a likely explanation for the lack of an effect we seen in these experiments. In this regard, we note that transfection of *let-7b* had the same effect as *let-7a* on 3T3-L1 cell differentiation (data not shown).

In summary, we provide evidence that *let-7* regulates adipocyte differentiation. We propose that *let-7* does this in part by targeting the transcription factor HMGA2, thereby promoting the transition of preadipocytes from clonal expansion to terminal differentiation (Fig. 2.10). The role of *let-7* in mediating this transition switch is reminiscent of its developmental role in *C. elegans*, where *let-7* regulates the transition from the larva to adult (Johnson et al., 2005; Reinhart et al., 2000). Our findings suggest that *let-7* may have important implications in obesity and other forms of metabolic disease in which there are alterations in the amount and/or function of adipose tissue.

2.4 SUMMARY

3T3-L1 pre-adipocytes undergo growth arrest, clonal expansion, final growth arrest and terminal differentiation into mature adipocytes. We investigated whether microRNAs, endogenous small RNAs, play a role in this process. Microarrays were performed to detect the expression of microRNAs during 3T3-L1 pre-adipocyte differentiation at day 0, day 1, day 4, and day 7. Several microRNAs including *let-7* were up-regulated at the late stage of 3T3-L1 adipogenesis. Microarray results were confirmed by northern blot and quantitative real time PCR. *let-7* expression specifically increased during the late stage of 3T3-L1 differentiation. Over presence of *let-7* in 3T3-L1 cells before DMI induction inhibits 3T3-L1 adipogenesis. Both the mRNA and protein levels of *Hmga2*, a target for *let-7*, decreased after ectopic introduction of *let-7* in 3T3-L1 cells. HMGA2 protein level is inversely correlated to *let-7* levels during 3T3-L1 adipogenesis. Knock-down of *Hmga2* or *E2f1* by siRNA also inhibits 3T3L1 pre-adipocyte differentiation. Our results suggest *let-7* can stop clonal expansion of 3T3-L1 cells and bring them to final growth arrest and terminal differentiation by targeting *Hmga2*.

CHAPTER 3

Profiles of microRNA Expression in Normal Mouse Tissues Detected by Quantitative Real Time PCR

3.1 INTRODUCTION

Genes encoding microRNAs are located in intergenic regions or in the introns or exons of other genes on chromosomes. They either have their own promoters or are controlled by the promoters of other genes. The expression of microRNAs in different tissues should offer some clues into their function. Also, the expression level of microRNA in different normal tissues can be used as a reference for that of physiologic or pathologic status.

There are several ways to detect expression of microRNAs. Northern analysis is commonly used for detecting microRNA expression. For example, researchers have used northern analysis to detect the expression of 119 microRNAs in mouse and human tissues (Sempere et al., 2004). This strategy has some shortcomings. It is labor-intensive and can not distinguish microRNAs with very similar sequences.

Microarrays have the advantage of being high-throughput and have been used for detecting the profile of microRNA expression (Barad et al., 2004; Liu et al., 2004a; Miska et al., 2004; Nelson et al., 2004; Sun et al., 2004; Thomson et al., 2004). However, microarrays cannot differentiate microRNAs with similar sequences, and can not detect microRNAs with very low expression.

In an effort to improve the specificity of microarray for microRNAs, locked nucleic acid

(LNA)-modified capture probes have been used in microarrays. LNA probe sets were designed for uniform, high-affinity hybridizations that can produce highly accurate signals and are able to discriminate single nucleotide differences due to their unique biophysical properties (Castoldi et al., 2007; Castoldi et al., 2008; Castoldi et al., 2006).

Another group used bead-based flow-cytometric microRNA expression profiling to perform expression analysis of 217 mammalian microRNAs from 334 samples, including multiple human cancer samples (Lu et al., 2005). It is claimed that bead-based hybridization is superior to glass array hybridization in specificity since it is solution based chemistry. This method also is fast, high-throughput, and low cost (Lu et al., 2005).

To achieve the goals of high specificity and sensitivity, a novel microRNA quantification method has been developed using stem-loop revese transcription (RT) followed by TaqMan PCR analysis (Chen et al., 2005a). Stem-loop RT primers are more efficient and specific than conventional RT primers. TaqMan microRNA assays can specifically detect mature microRNAs and are not affected by genomic DNA, pri-microRNAs, or pre-microRNAs. This assay can discriminate microRNAs with similar sequences that only differ by a single nucleotide. The Taqman PCR process amplifies targets and can detect microRNAs of very low abundance (Chen et al., 2005a). This method has been widely used for detecting microRNA expression in cell lines, tissues, and tumors (Chen and Stallings, 2007; Gaur et al., 2007; Jongen-Lavrencic et al., 2008; Lee et al., 2008; Liang et al., 2007).

In an effort to explore the role of microRNAs in different tissues, we analyzed the expression profiles of 111 microRNAs in 36 mouse tissues using an Applied Biosystem

microRNA Taqman assay kit. These resultant profiles provide hints toward the function of different microRNAs. Furthermore, a comparison of microRNA expression with nuclear receptor expression was performed and may provide useful information on nuclear receptor regulation of microRNA expression, and vice versa.

3.2 RESULTS AND DISCUSSION

3.2.1 MicroRNAs show different expression patterns in mouse tissues.

Total RNA was extracted from 36 tissues from C57BL/6J mice using a standard protocol (Bookout et al., 2006). These tissues include eight from CNS (eye, brain stem, cerebellum, cerebrum, olfactory bulb, spinal cord, hypothalamus, and pituitary gland), three belong to endocrine tissues (adrenal gland, pancreas, and thyroid gland), seven of them are from metabolic tissues (duodenum, jejunum, ileum, colon, gall bladder, liver, and kidney), two are from adipose tissues (WAT and BAT), two from immune system (spleen and thymus gland), eight of them are from reproductive tissues (ovary, uterus, epididymus, preputial gland, prostate, seminal vesicles, testis, and vas deferens), three of them are from the respiratory system (aorta, lung, and heart), and three are from structural tissues (muscle, skin, and bone).

The RNA was reverse transcribed using microRNA-specific stem-loop RT primers. Then the cDNA was amplified in a Taqman PCR reaction using microRNA-specific PCR primers and Taqman probes. The data were analyzed using the standard curve assay (Chen et al., 2005a).

To check data accuracy, northern analysis was performed on select microRNAs. Northern analysis confirmed the result (Fig. 3.1). In addition, our Q-PCR results are consistent with previous publications (Esau et al., 2006).

3.2.1.1 Low versus high expression level

The expression of different microRNAs varied considerably. Some showed high expression in different tissues, while others were very low to undetectable (Fig. 3.3). For example, miR-30c and miR-16 were highly expressed in most tissues (Fig. 3.2; 3.4). On the contrary, microRNAs like miR-190 were barely detectale.

3.2.1.2 Specific expression pattern

A subset of microRNAs exhibited exclusive expression in mouse tissues. For example, miR-122a was exclusively expressed in liver, in agreement with other reports (Esau et al., 2006). miR-133 was highly expressed in heart and skeletal muscle, with low expression in skin, bone and eye. The expression level of miR-133a and miR-133b was increased during C2C12 myocyte differentiation (Fig. 3.5). These results are consistent with its role in muscle development (reviewed by Callis and Wang, 2008). miR-194 was highly expressed in intestinal organs such as duodenum, jejunum, ileum, and colon, as well as at a lower levels in liver (Fig. 3.6). miR-23a and miR-23b had relatively higher expression in colon, and lower expression in heart, lung, skin and muscle (Fig. 3.7). miR-203 and miR-205 are highly expressed in preputial gland and skin. miR-205 was also expressed at low levels in reproductive tissues such as epididymus, prostate and seminal vesicles (Fig. 3.8). miR-213 was highly expressed in thymus. The expression of miR-148a was higher in pancreas than other tissues (Fig. 3.9). In white adipose tissue, the expression of miR-335 was high. It was also expressed in pituitary (Fig. 3.4). miR-140 had high expression in aorta and bone.

miR-142-3p was also mainly expressed in bone (Fig. 3.10). miR-182, miR-183, and miR-184 were mainly expressed in eyes (Fig. 3.11; 3.12).

Some microRNAs was exclusively or mainly expressed in the central nerve system (CNS). For example, miR-124 was exclusively expressed in the CNS (Fig. 3.13) and has been reported to play a role in neuronal development (Makeyev et al., 2007; Visvanathan et al., 2007). miR-9, miR-9*, miR-29, miR-128, miR-132，miR-204, miR-218, and miR-219 are highly expressed in the CNS (Fig. 3.14; 3.15; 3.16; 3.17; 3.18). miR-127, miR-129, miR-134, miR-137, miR-154, miR-323, and miR-370 were mainly expressed in the CNS, but at a much lower level (Fig. 3.19, 3.20, 3.21).

The expression data of other microRNAs in this study was shown in (Fig. 3.27-3.43).

3.2.2 Hierarchical clustering of microRNAs based in mouse tissues

Hierarchical clustering of microRNAs expression from 36 mouse tissues in these tissues showed high correlation between tissues from the same anatomic location (Fig. 3.22). Tissues from GI tract, including duodenum, jejunum, ileum, and colon are clustered together. Also tissues from the CNS, such as brain stem, cerebellum, cerebrum, olfactory bulb, spinal cord, and hypothalamus clustered together. Epididymus, vas deferens, and prostate belong to the male productive organs and they clustered together. Tissues with similar physiologic functions also showed high correlation in the cluster analysis. For example, heart and skeletal muscle are clustered together; Spleen and thymus clustered together. These results suggest that the role of a microRNA is consistent with the function of the tissue in which it is expressed.

Liang et al. provided expression data of 345 microRNAs from 40 normal human tissues. In that study, the cluster analysis of human tissues based on their microRNA expression drew similar conclusions to our study (Liang et al., 2007).

Hierarchical clustering of microRNAs based on their expression in different tissues showed different isotypes of microRNAs clustered together (Fig. 3.23). Two isoforms of *let-7*, *let-7*a, and *let-7*d, clustered together, indicating their similar expression (Fig. 3.24). miR-133a and miR-133b also clustered together. Although these different isoforms of microRNA may come from different locations of chromosomes, the similar expression pattern is consistent with their redundant roles in tissues.

MicroRNAs from a single transcript clustered together (Fig. 3.23). For example, the miR-17-92 cluster is located on chromosome 14 of the mouse genome. The expression of three of these microRNAs (miR-17-5p, miR-19a and miR-20) clustered together based on their expression in mouse tissues (Fig. 3.25). This result indicates microRNAs under the control of a common promoter also exhibited similar expression patterns.

3.2.3 Correlation between nuclear receptors and microRNAs based on their expression pattern in mouse tissues.

The expression pattern of microRNAs from this study was compared to that of nuclear receptors in different tissues (Bookout et al., 2006), to provide meaningful information on regulation of nuclear receptors and microRNAs. If a microRNA shows positive correlation with a nuclear receptor, it may indicate that that nuclear receptor regulates its expression. If the correlation is negative, it may suggest the nuclear receptor is regulated by that

microRNA. One point worth noting here is that these correlations are only based on microRNA and nuclear receptor tissue expression pattern. These predications will need to be validated by further experimentation.

Based on the expression of microRNAs and nuclear receptors in mouse tissues, the correlation coefficient was calculated using Pearson correlation. As shown in Table 3.1, there are 50 pairs of nuclear receptors and microRNAs that have positive correlation with coefficients greater than 0.8. None of them shows a negative correlation with an absolute value greater than 0.5. miR-184 is exclusively expressed in eyes and it has high correlation with the photoreceptor cell-specific nuclear receptor (PNR) (Fig. 3.11). miR-194 has high expression in intestinal tissues and it has high correlation with HNF4γ and PXR (Fig. 3.6).

To investigate whether miR-194 is a target of PXR, we checked whether miR-194 level is changed in wild type and PXR knock-out mice treated with pregnenolone-16α-carbonitrilepcn (PCN, a PXR ligand). CYP3a11 and OATP2 (organic anion transporting polypeptide 2) are target genes of PXR and their expression was up-regulated by PCN treatment in wild type but not PXR knock-out mice. However, miR-194 expression was unchanged in wild-type mice treated with PXR ligand. These results suggest miR-194 is not a direct target of PXR. Also the expression of miR-194 was not up-regulated by thyroid hormone T3, vitamin D3, or ligands for FXR, LXR, RXR, CAR, RAR, PPARα/β/δ/γ (Fig. 3.26).

Although the correlation between miR-194 and PXR predicated by clustering was disproved by experimental data, there are still many pairs of microRNAs and nuclear receptors remaining to be explored. For example, the relationship between miR-194 and

HNF4γ needs to be further investigated.

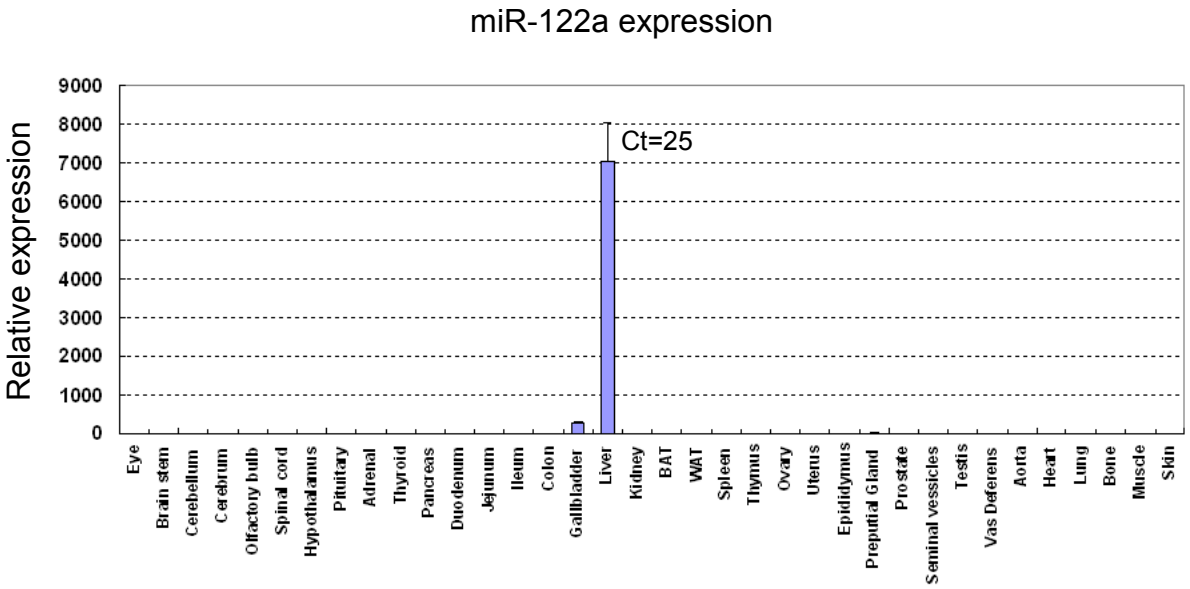
Another way to investigate the regulation between microRNA and nuclear receptor is using microarray. Comparing the expression of microRNAs before and after ligand treatment for nuclear receptors will help to identify microRNAs which are the targets of nuclear receptors.

3.3 SUMMARY

The expression of 111 microRNAs in 36 mouse tissues was detected by quantitative real time PCR. MicroRNAs have diverse expression patterns in different tissues. Some are universally expressed while others are specifically expressed in certain tissues, suggesting their roles in these tissues. The expression level of different microRNA varies from each other. MicroRNAs transcribed from the same genomic location have similar expression patterns in mouse tissues. Also different isotypes of microRNAs have similar expressions in mouse tissues despite that they may come from different locations of choromosomes. Hierarchical cluster analysis based on the expression of microRNA in tissues revealed that tissues having similar physiological functions or from the same anatomic location are clustered together, suggesting the roles of microRNAs are consistent with the function of the tissue in which they are expressed. Comparison of the expression of microRNAs with that of nuclear receptors in mouse tissues showed positive correlations between nuclear receptors and microRNAs. Whether these relationships reflect a functional relationship remains to be explored in the future. Taken together, the expression profile of microRNAs in mouse tissues provides a useful resource to the microRNA and nuclear receptor

communities. To that end, these data sets will be deposited at www.nursa.org.

A



B

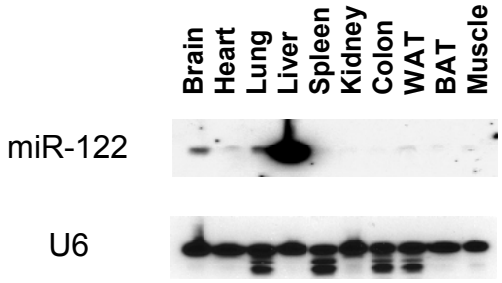


Figure 3.1 Quantitative real time PCR (A) and northern analysis (B) showed miR-122 is specifically expressed in liver.

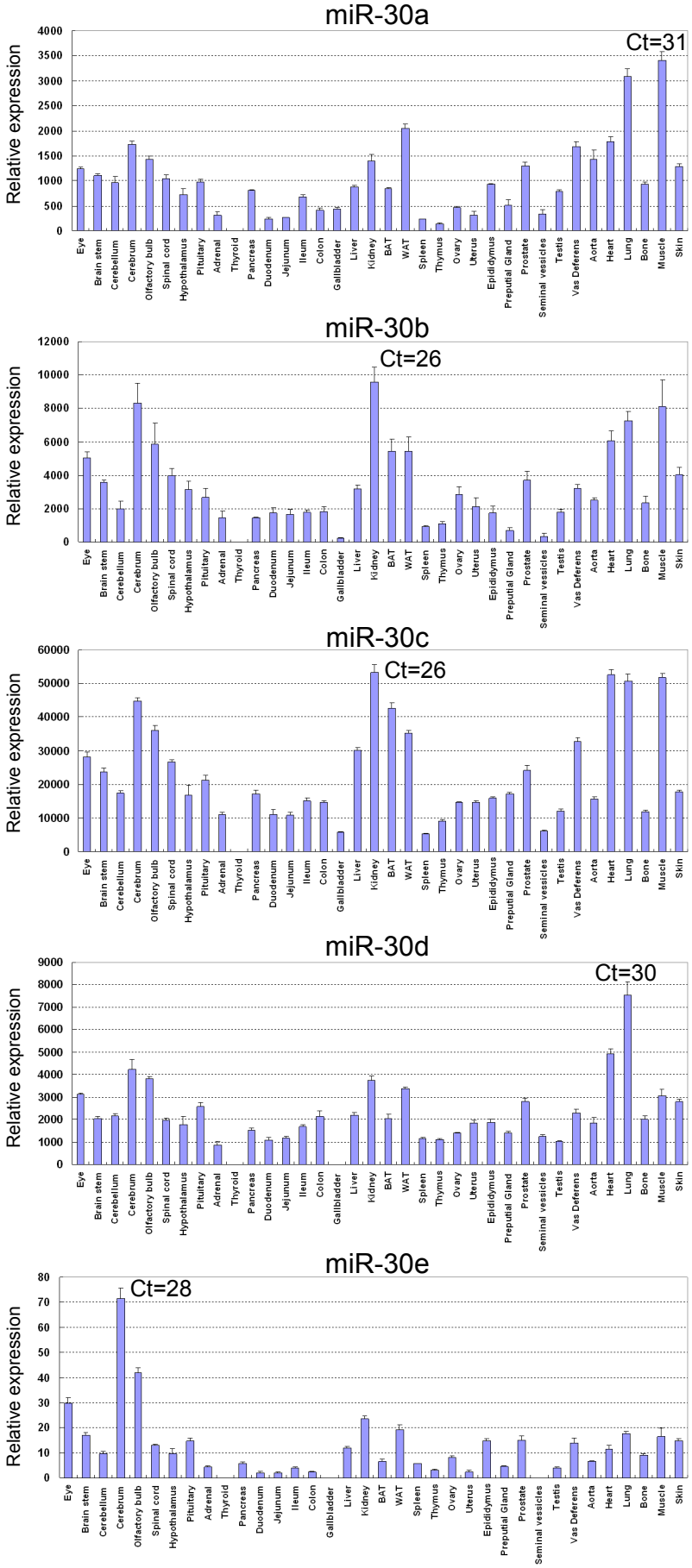
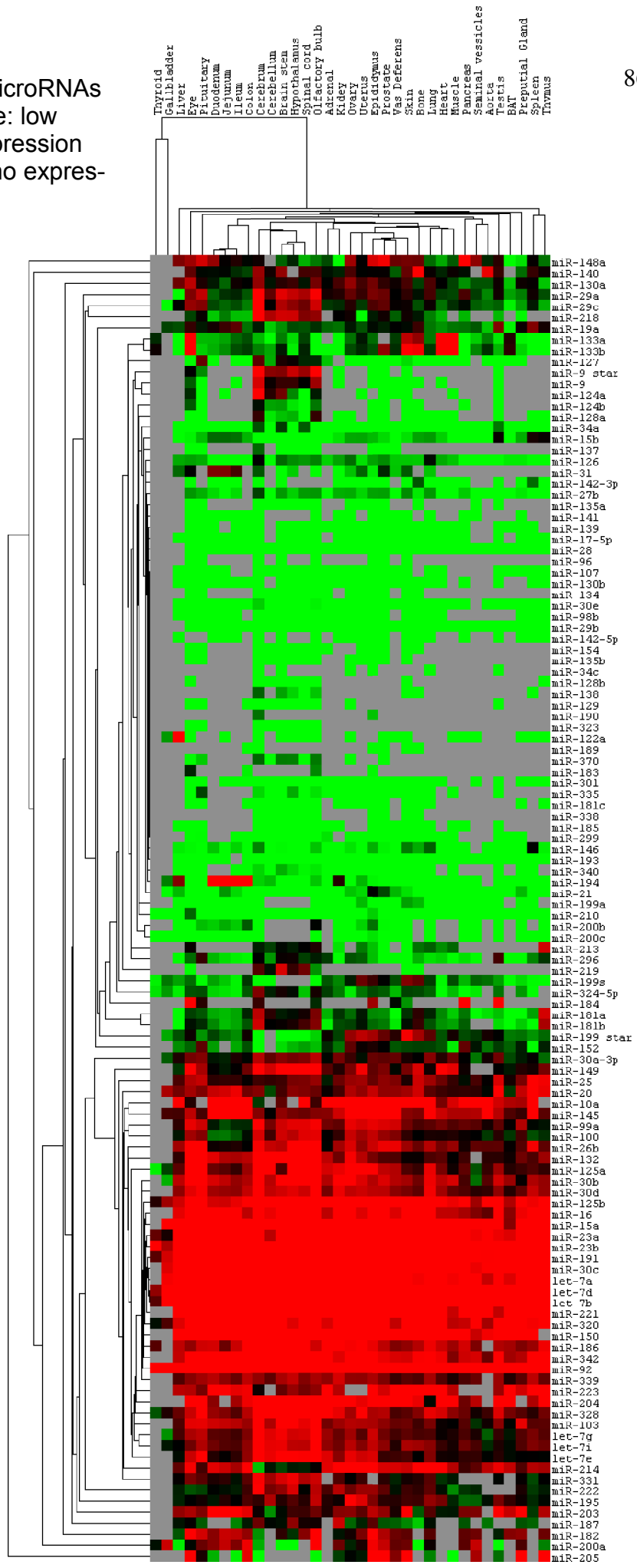


Figure 3.2 The expression of miR-30 in mouse tissues.

Figure 3.3 The expression of microRNAs in mouse tissues. Color scheme: low expression (green); median expression (black); high expression (red); no expression (grey).



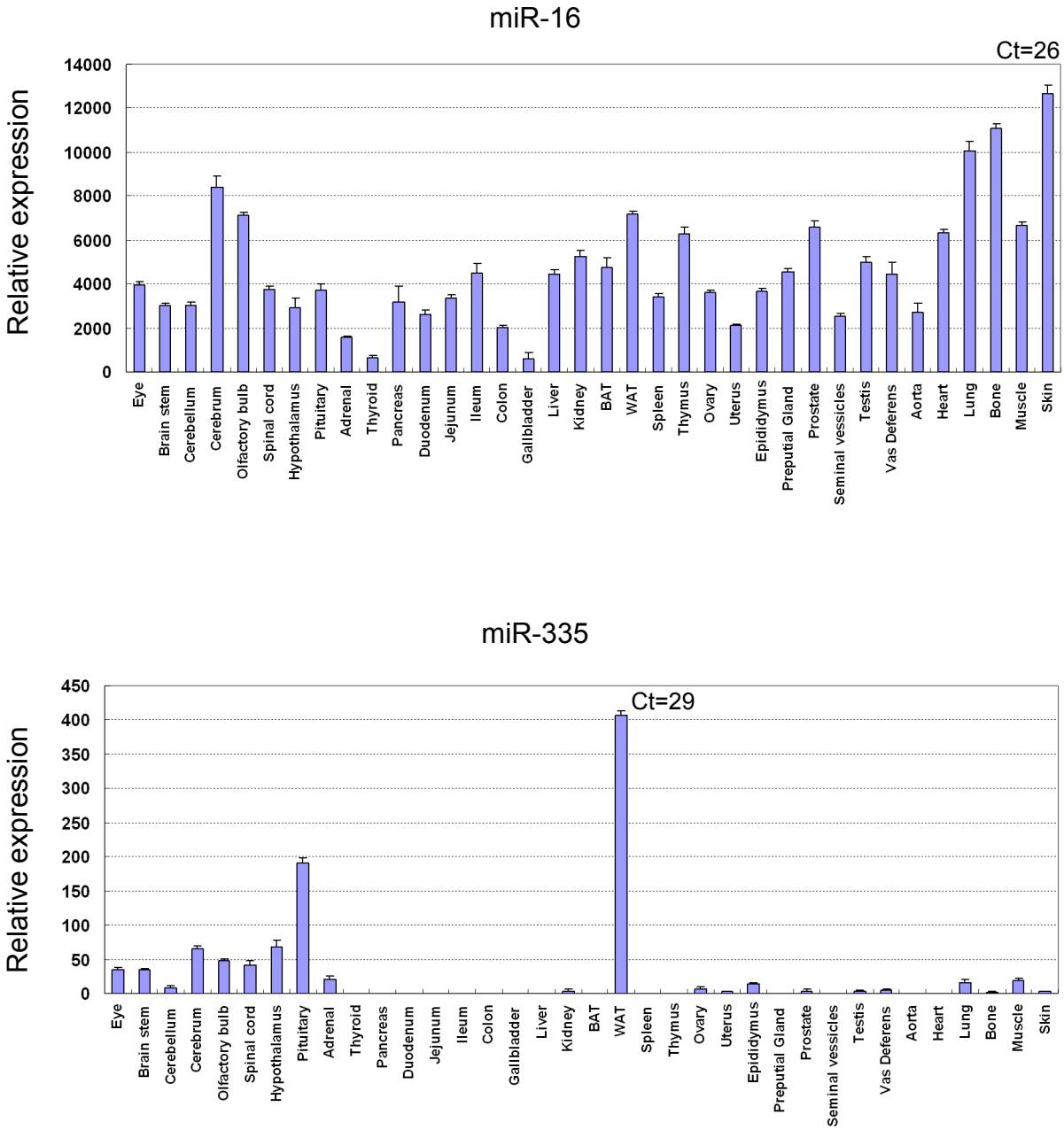


Figure 3.4 The expression of miR-16 and miR-335 in mouse tissues.

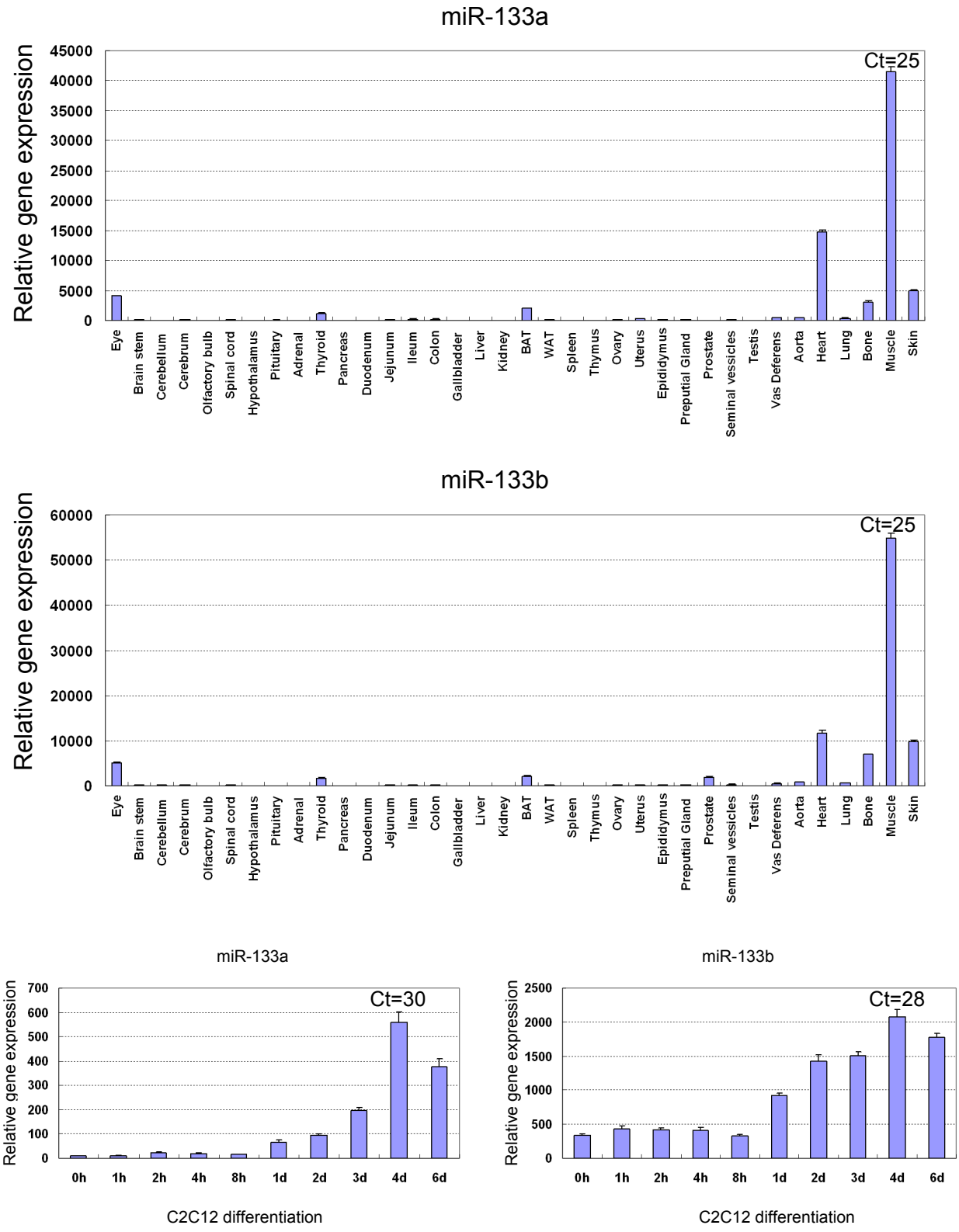


Figure 3.5 The expression of miR-133 in mouse tissues and during C2C12 differentiation.

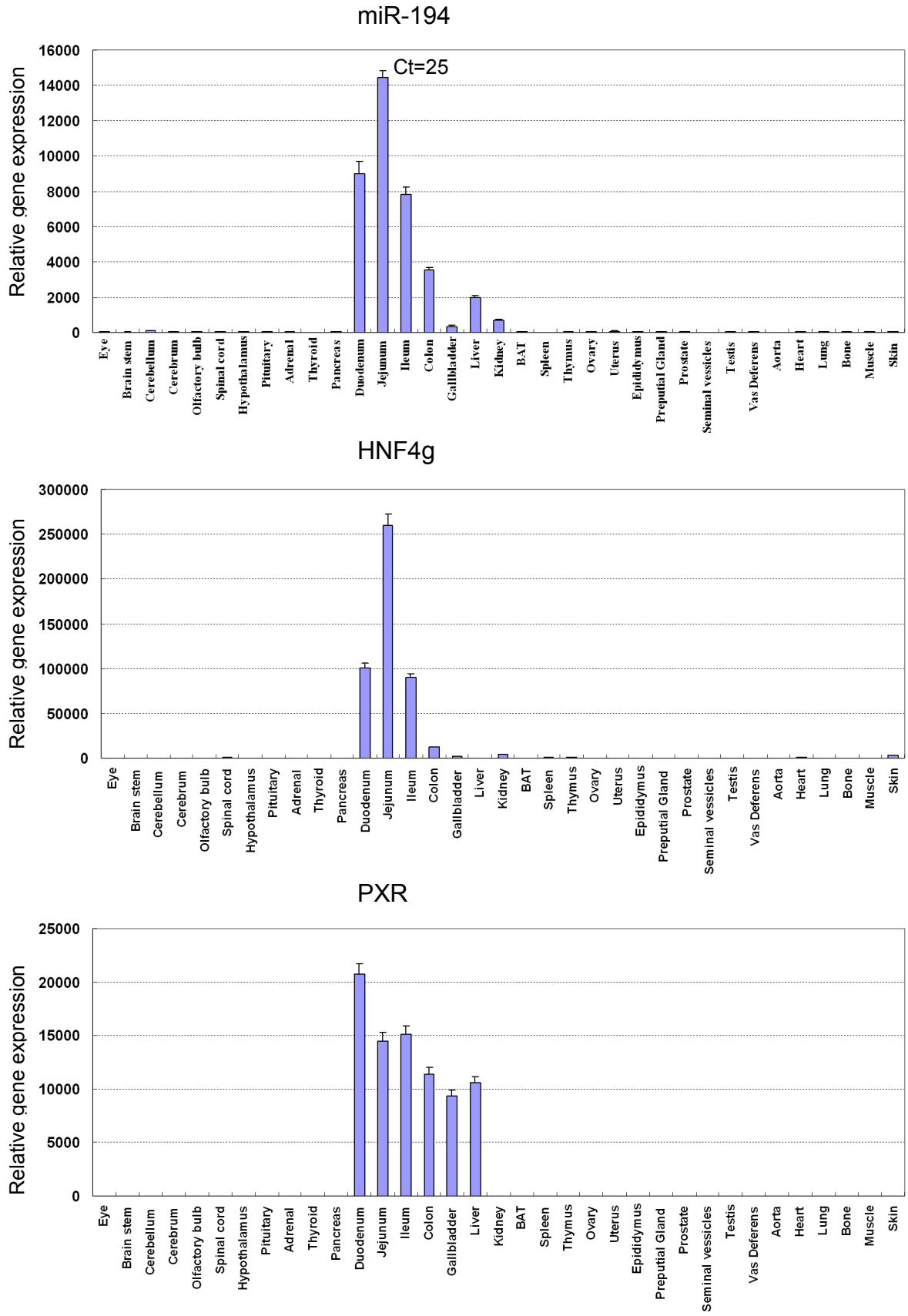


Figure 3.6 The expression of miR-194, HNF4g, and PXR in mouse tissues. The expression data of HNF4g and PXR are from (Bookout et al., 2006).

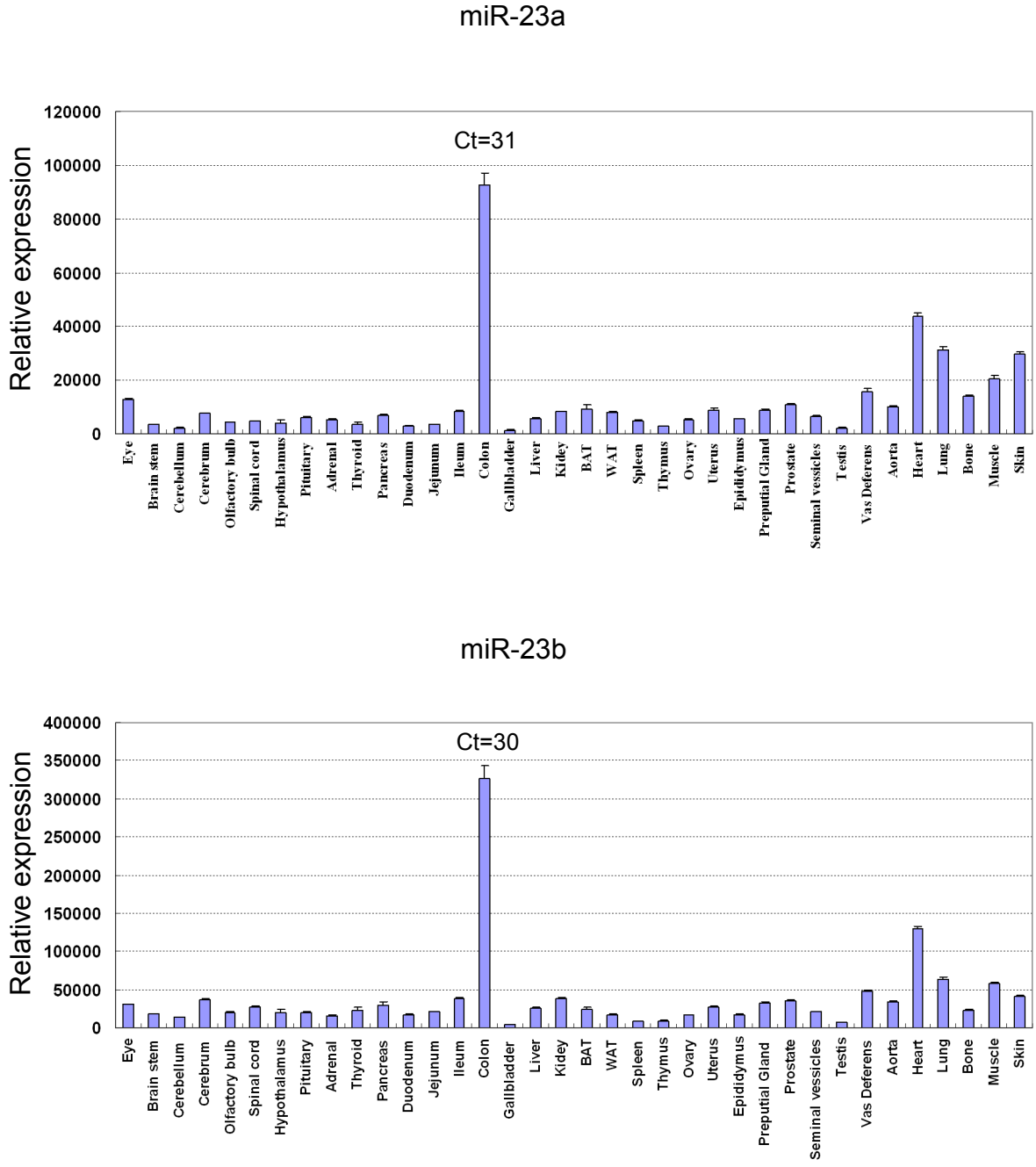


Figure 3.7 The expression of miR-23a and miR-23b in mouse tissues.

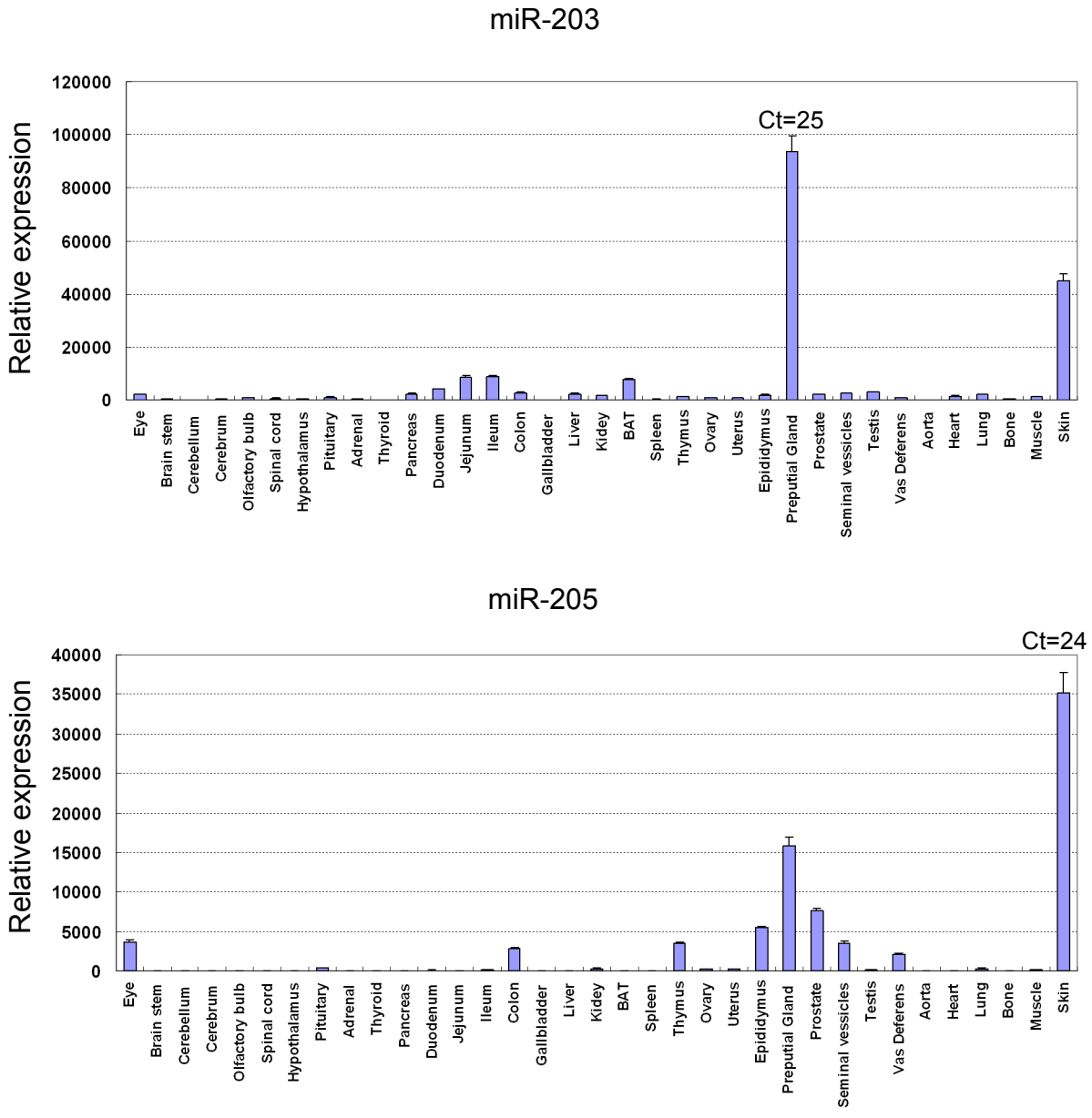


Figure 3.8 The expression of miR-203 and miR-205 in mouse tissues.

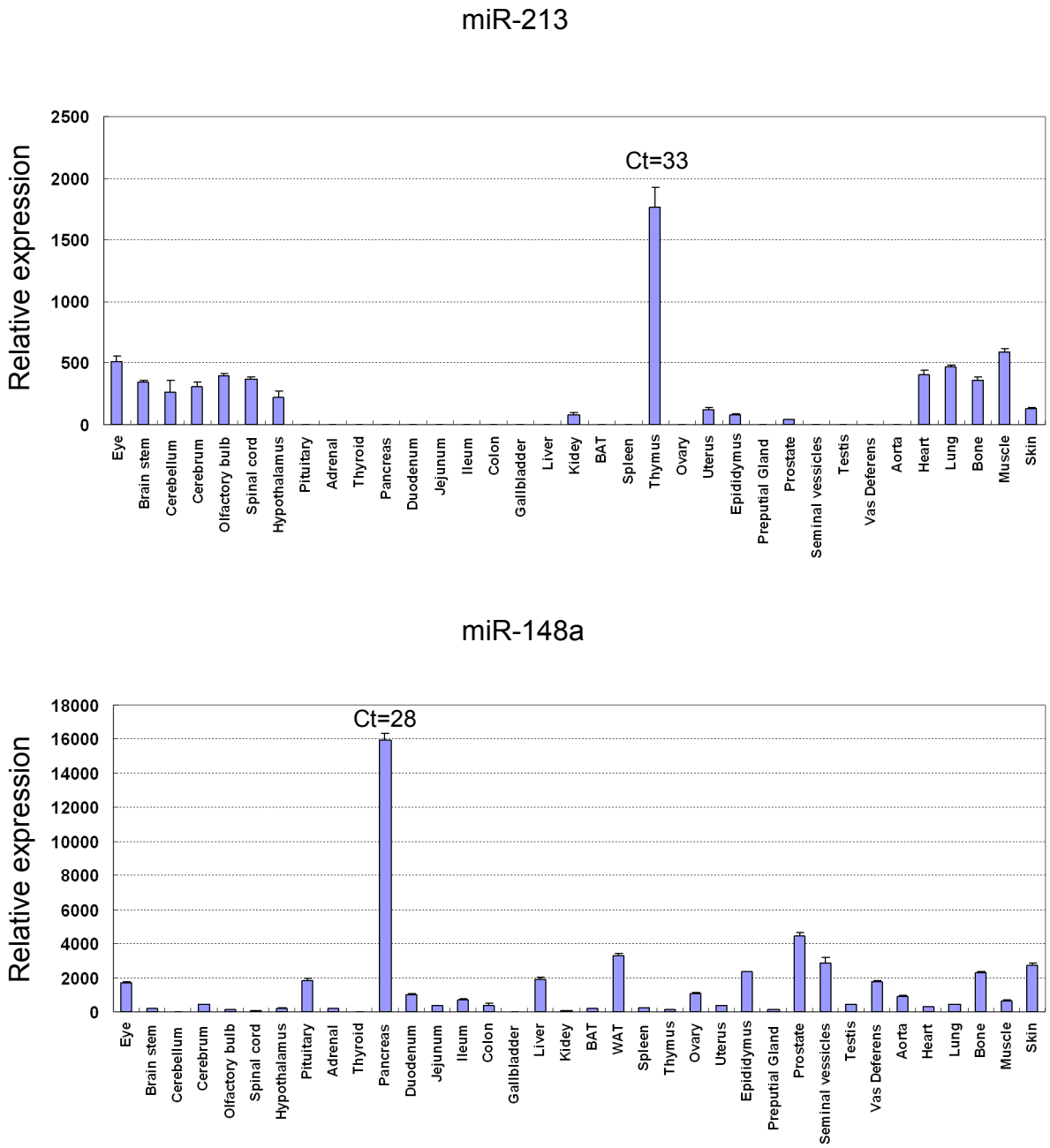


Figure 3.9 The expression of miR-213 and miR-148a in mouse tissues.

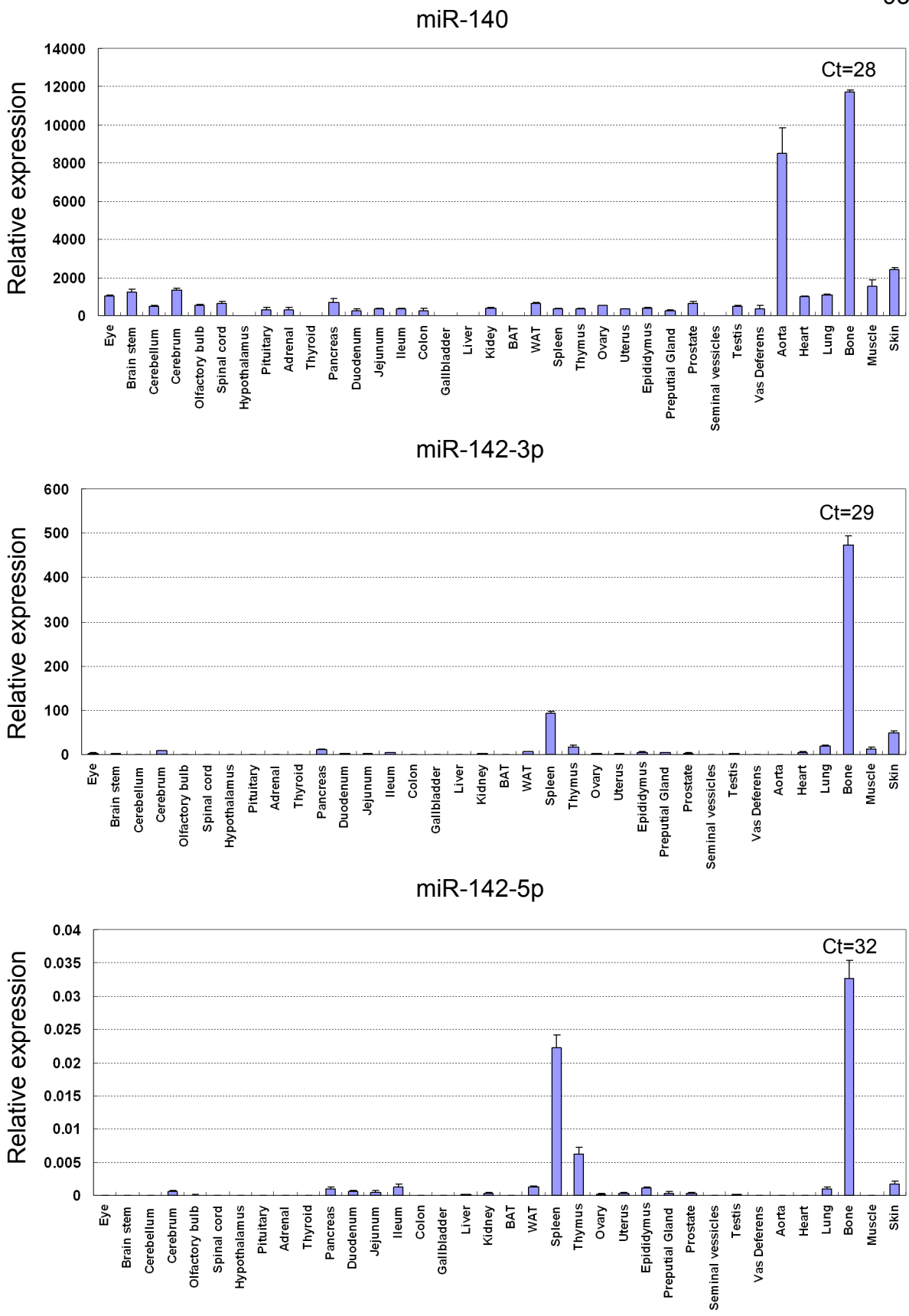


Figure 3.10 The expression of miR-140 and miR-142 in mouse tissues.

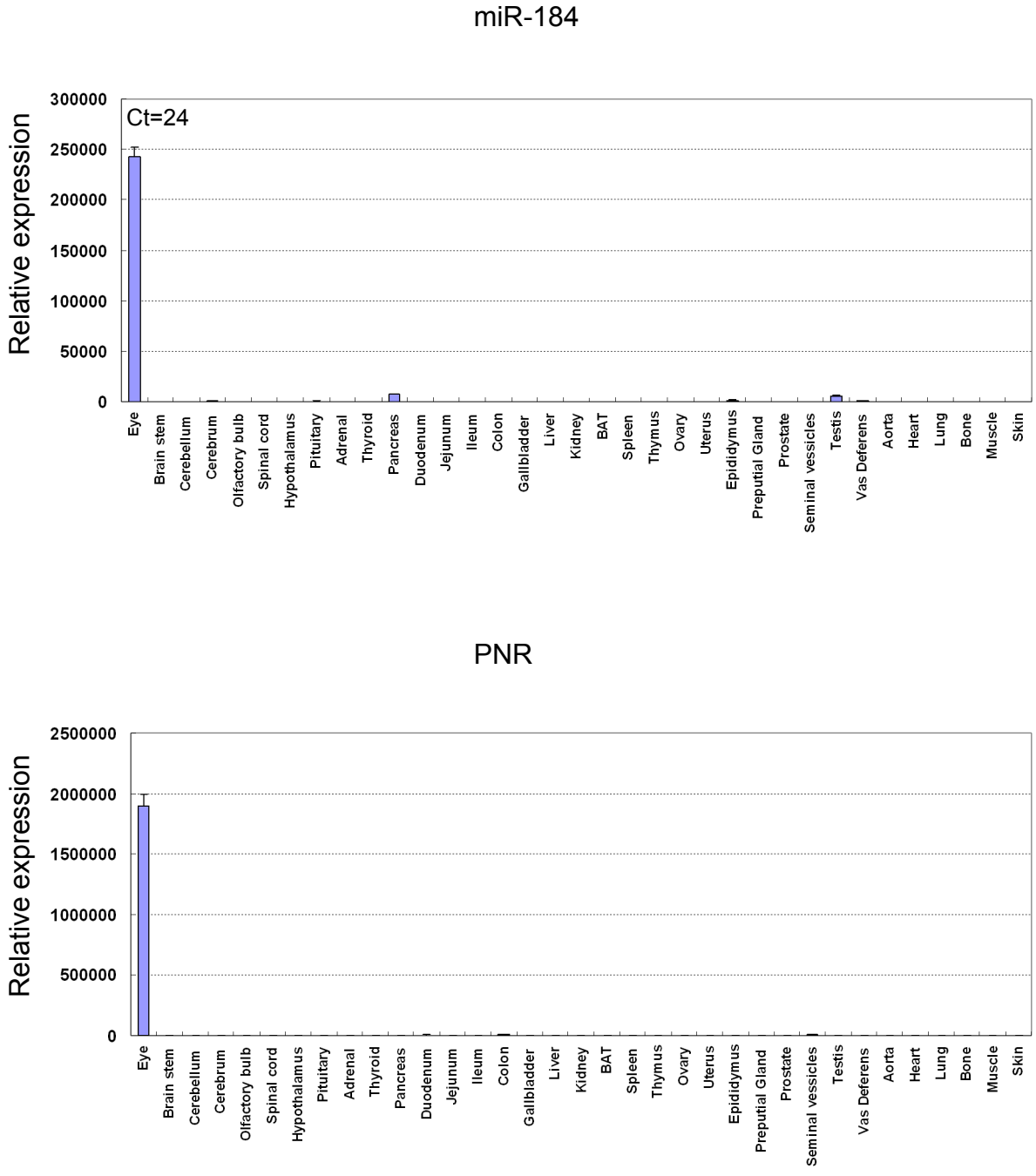


Figure 3.11 The expression of miR-184 and PNR (Bookout et al., 2006) in mouse tissues.

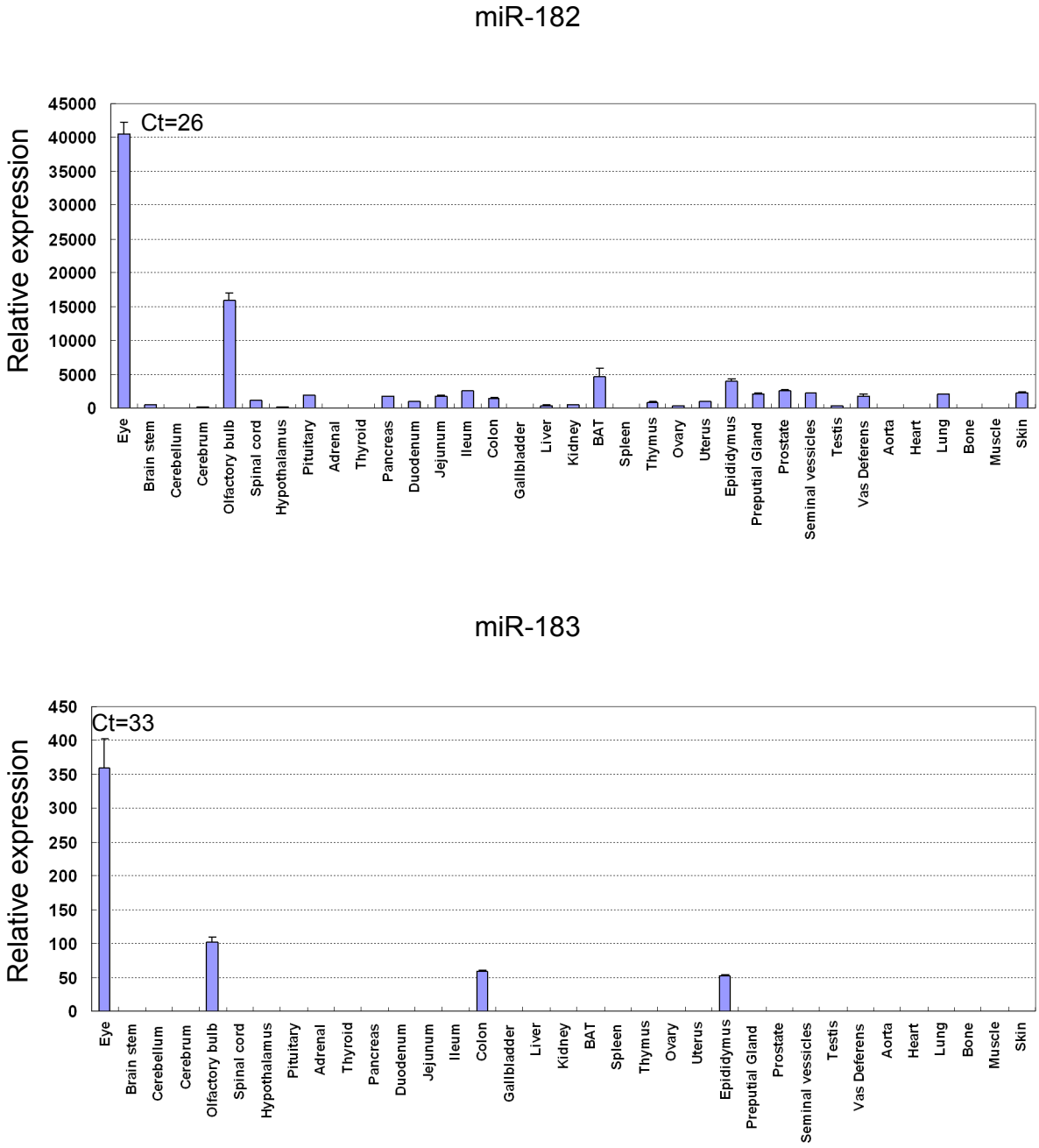


Figure 3.12 The expression of miR-182 and miR-183 in mouse tissues.

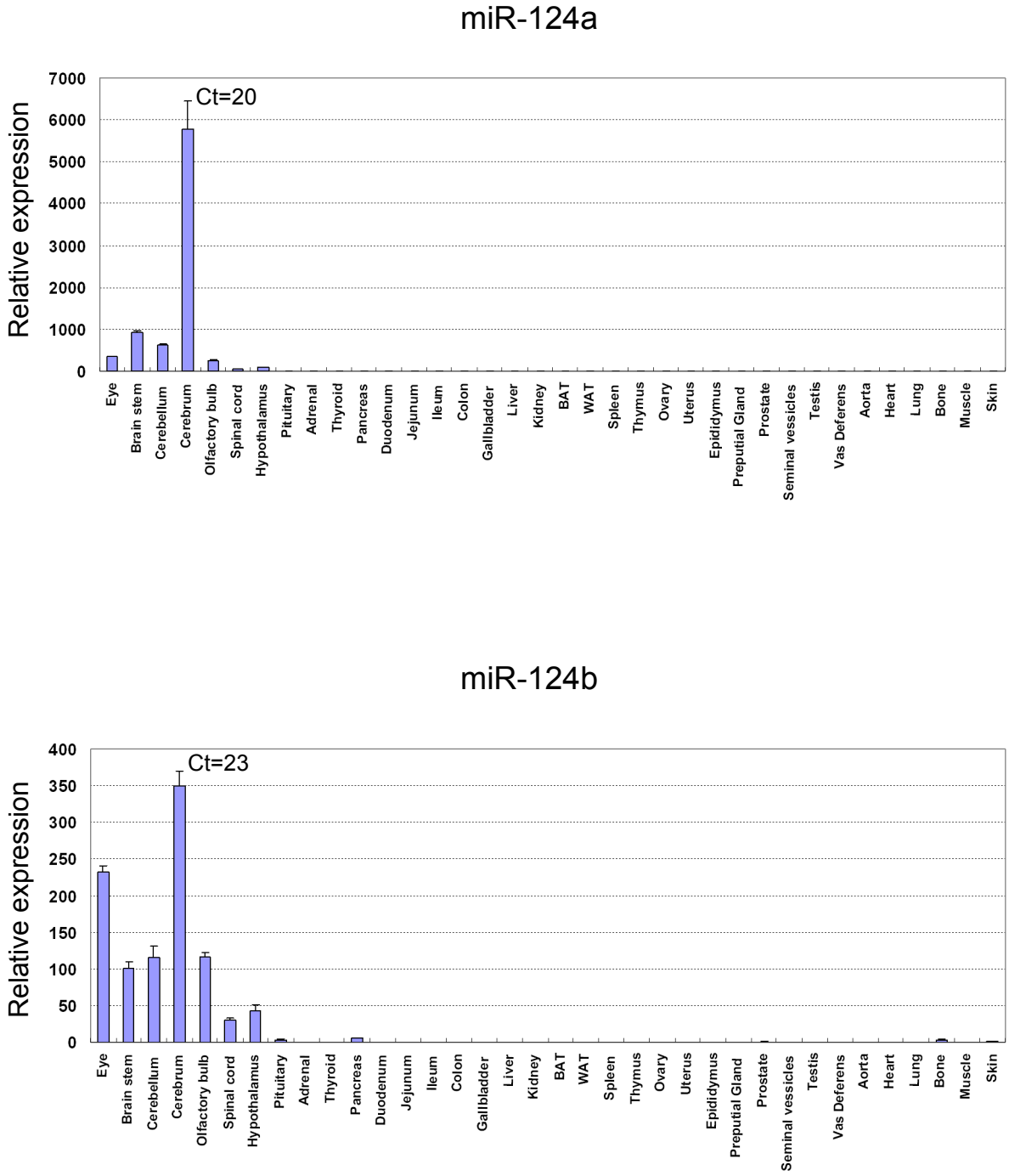


Figure 3.13 The expression of miR-124 in mouse tissues.

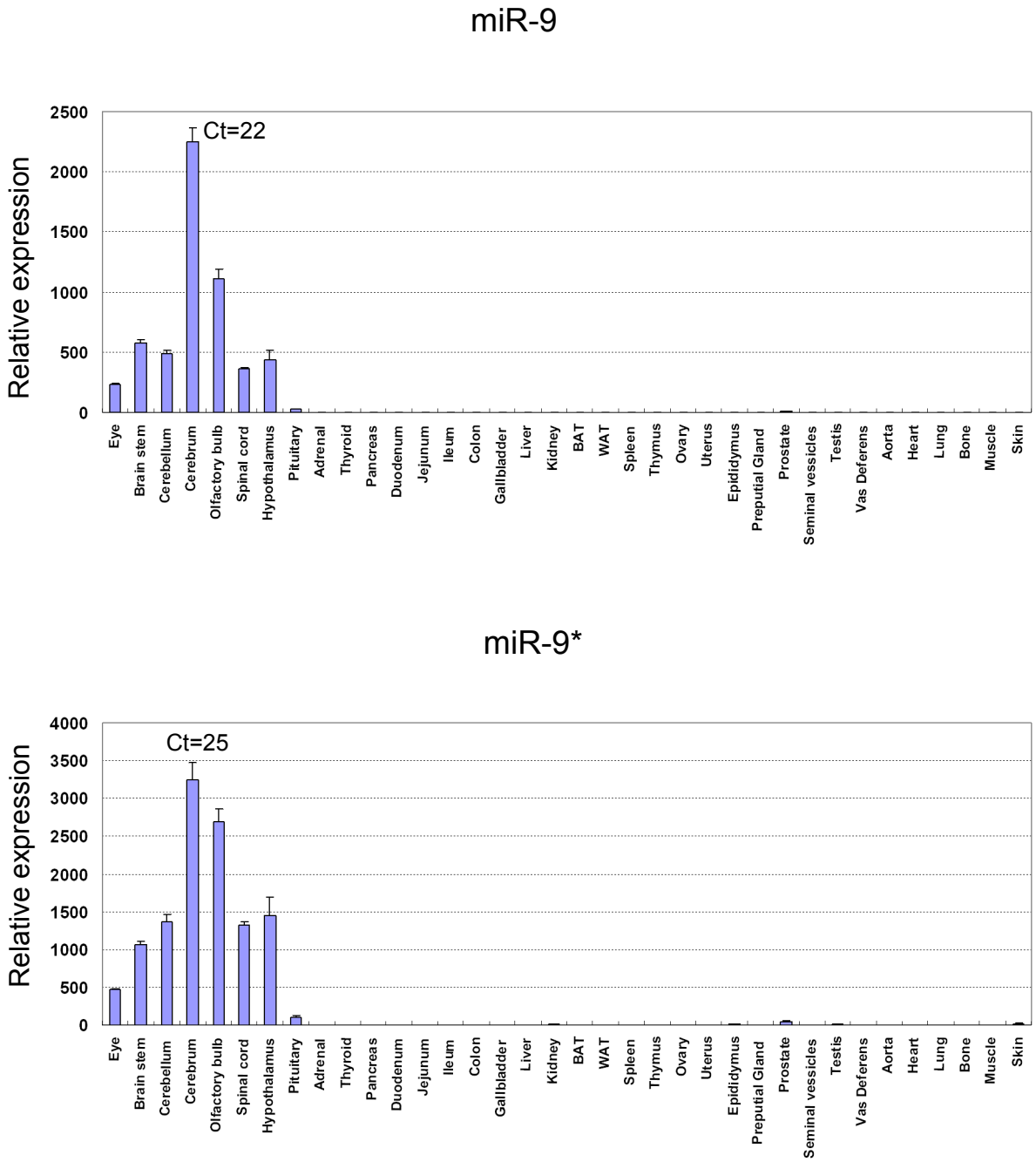


Figure 3.14 The expression of miR-9 in mouse tissues.

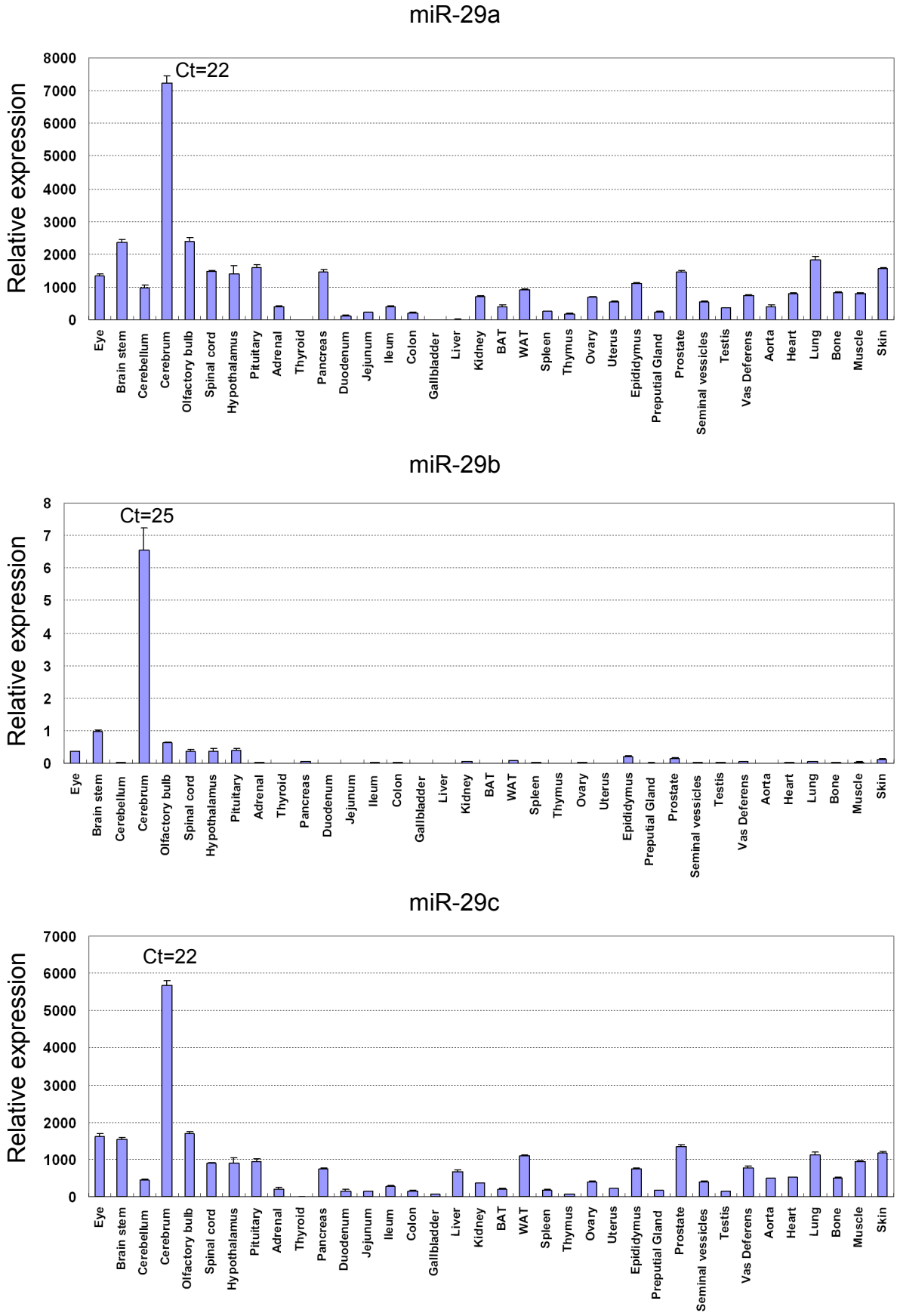


Figure 3.15 The expression of miR-29 in mouse tissues.

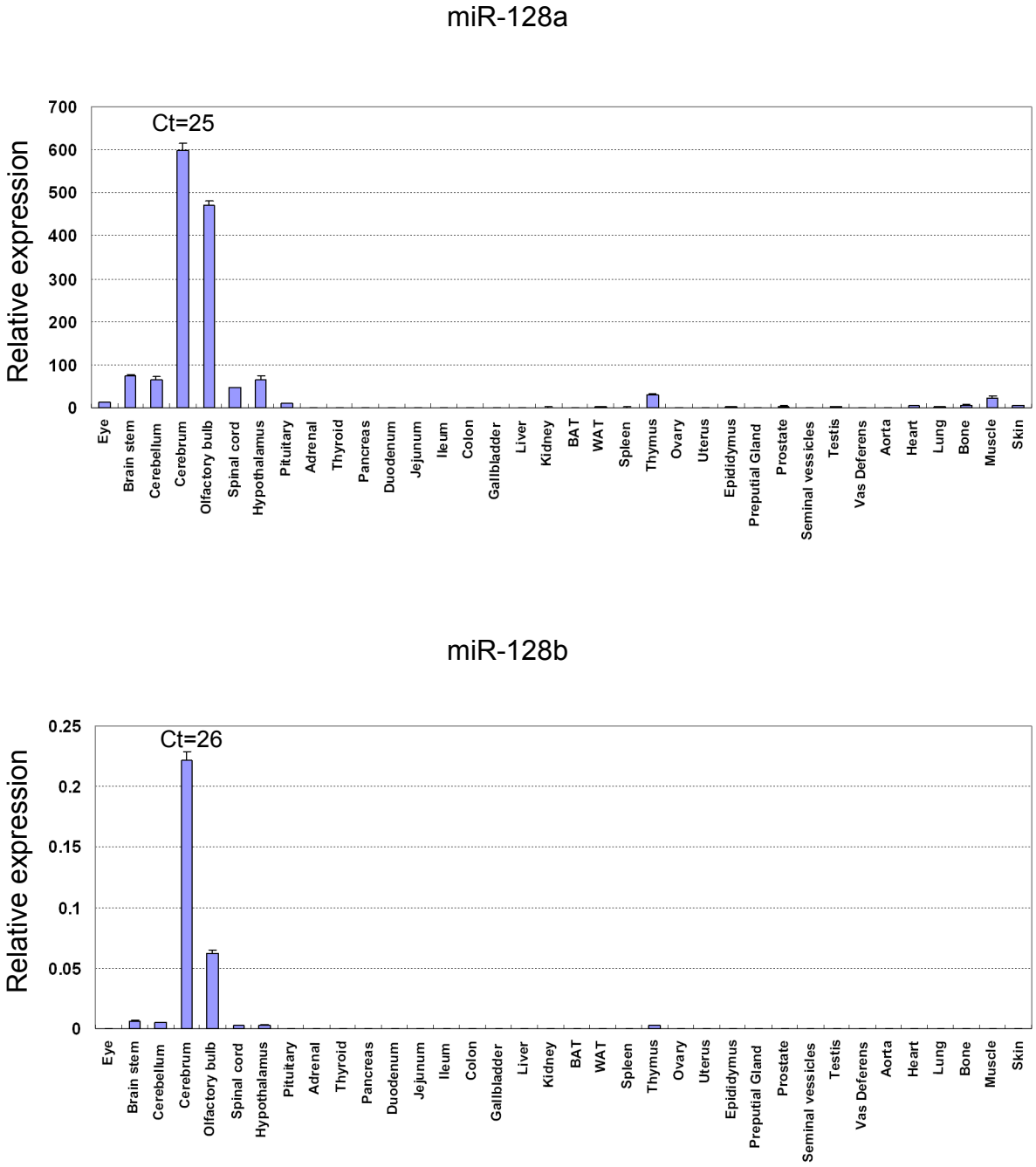


Figure 3.16 The expression of miR-128 in mouse tissues.

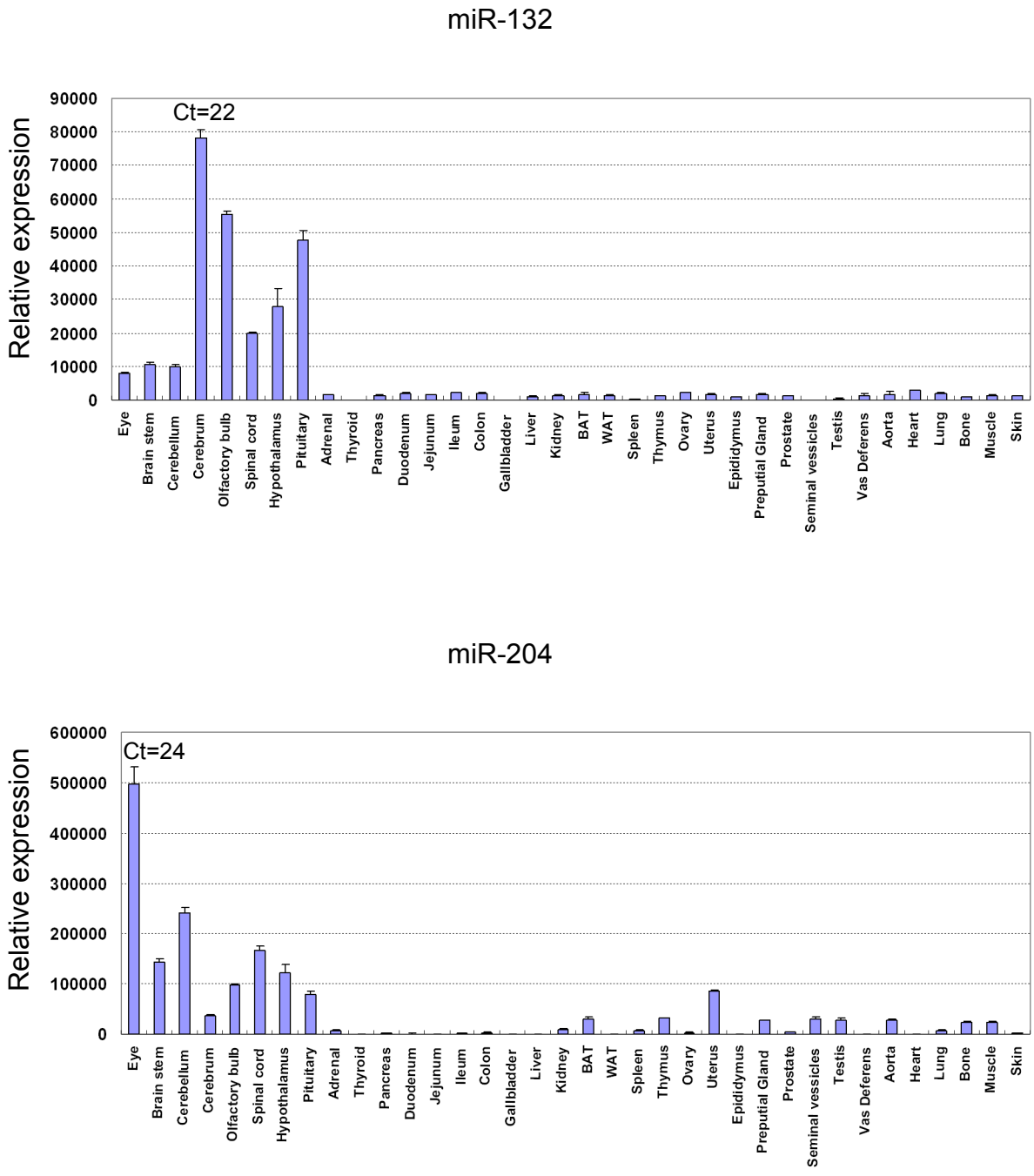


Figure 3.17 The expression of miR-132 and miR-204 in mouse tissues.

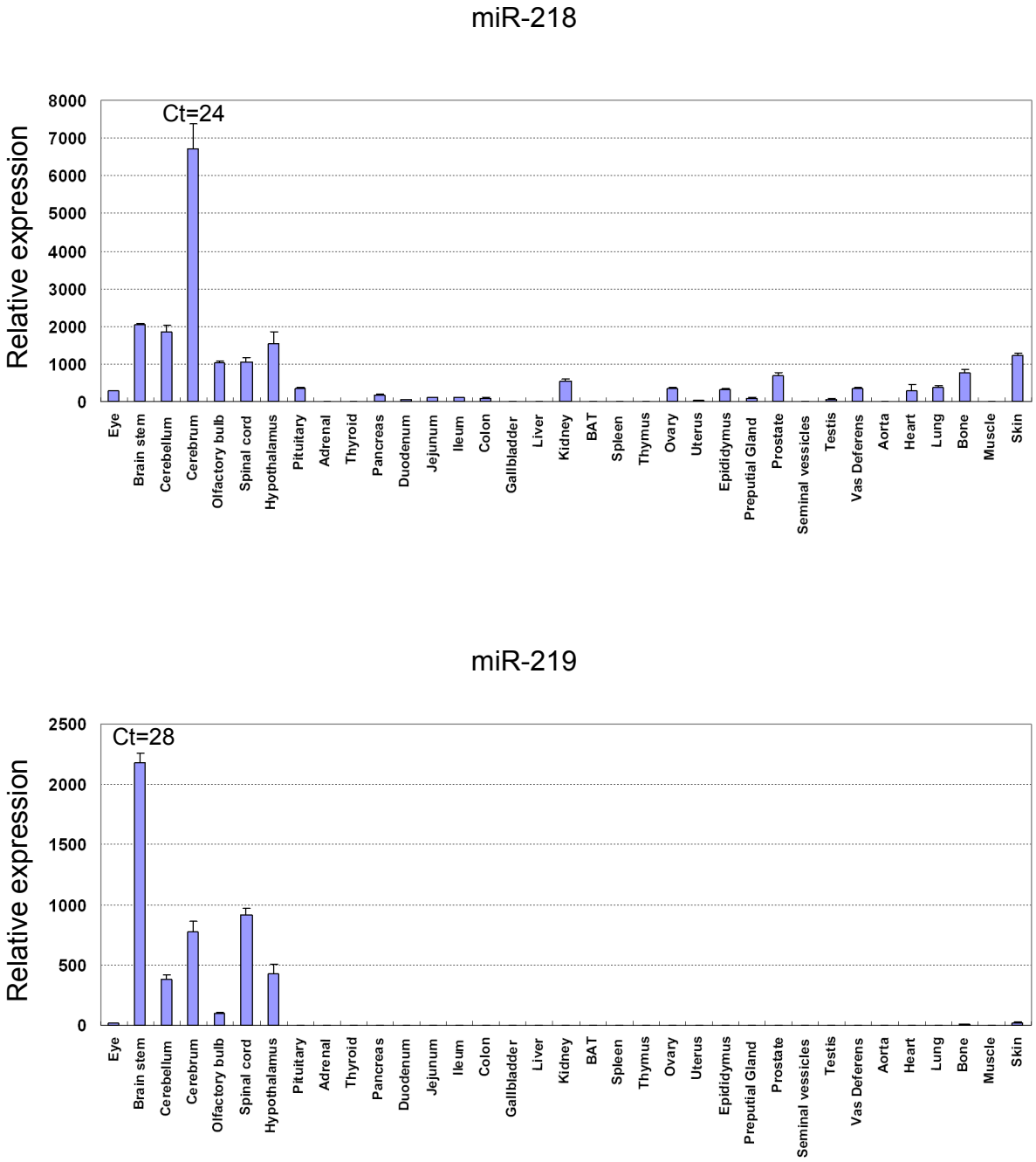


Figure 3.18 The expression of miR-218 and miR-219 in mouse tissues.

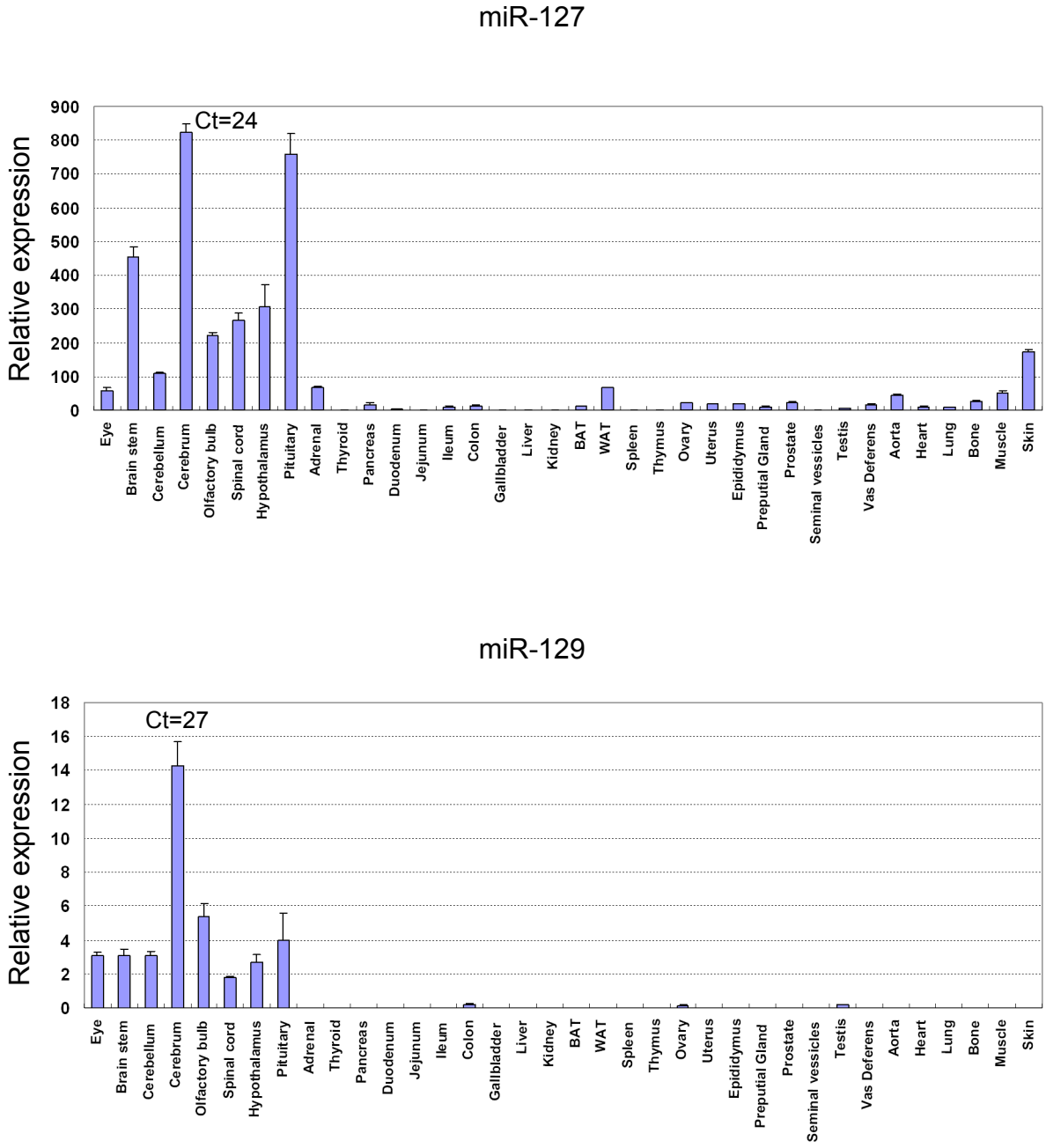


Figure 3.19 The expression of miR-127 and miR-129 in mouse tissues.

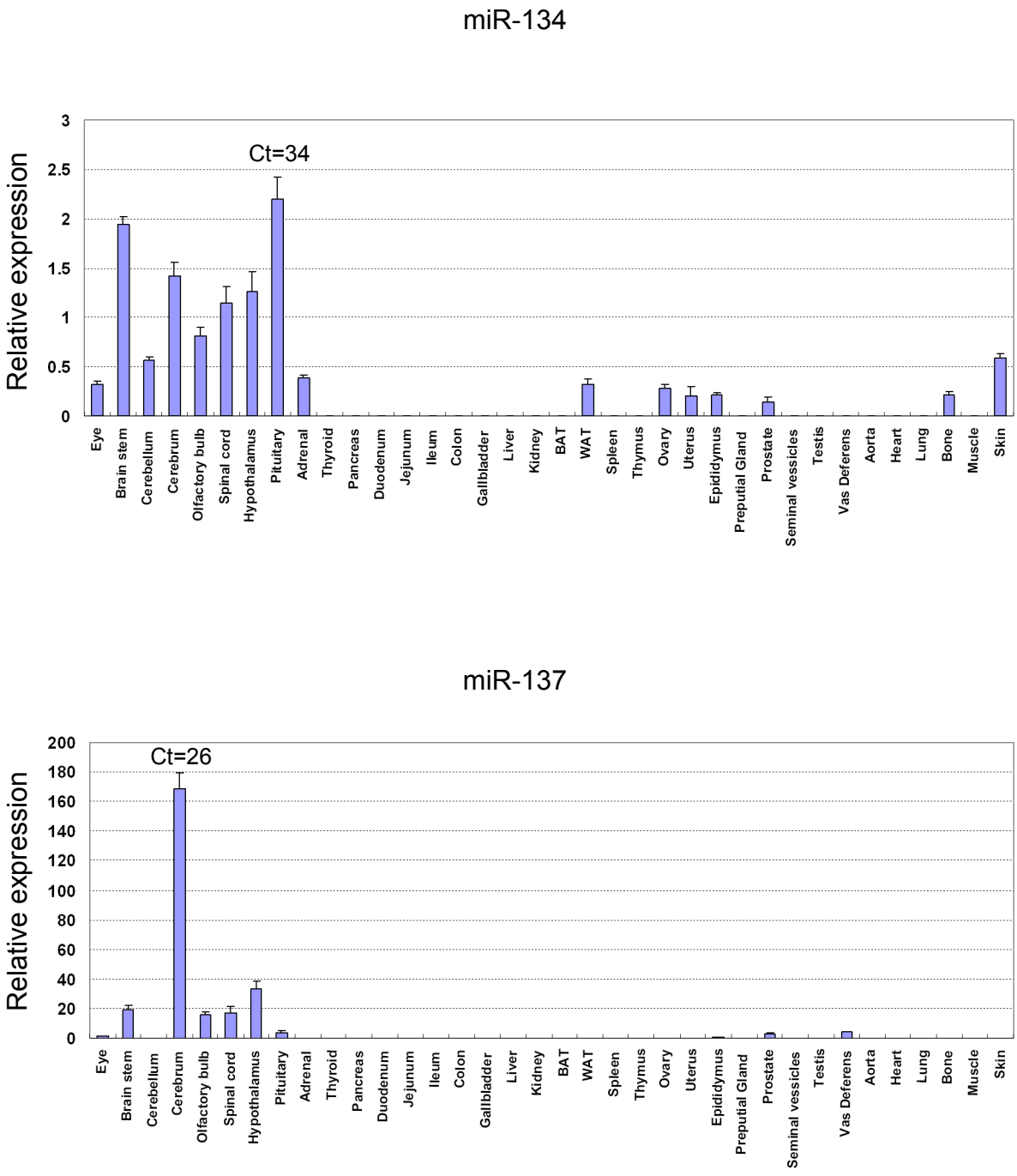


Figure 3.20 The expression of miR-134 and miR-137 in mouse tissues.

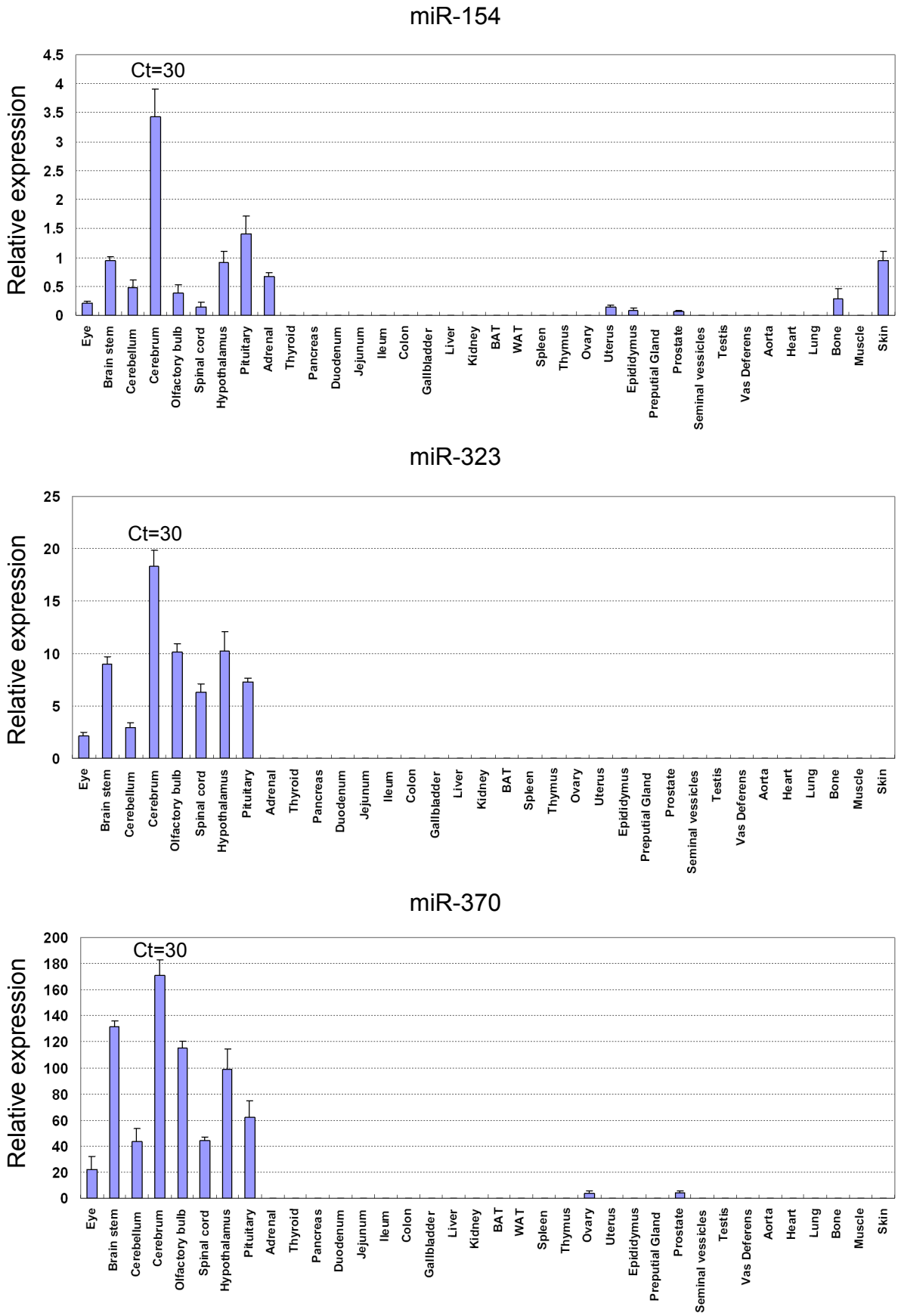


Figure 3.21 The expression of miR-154, miR-323, and miR-370 in mouse tissues.

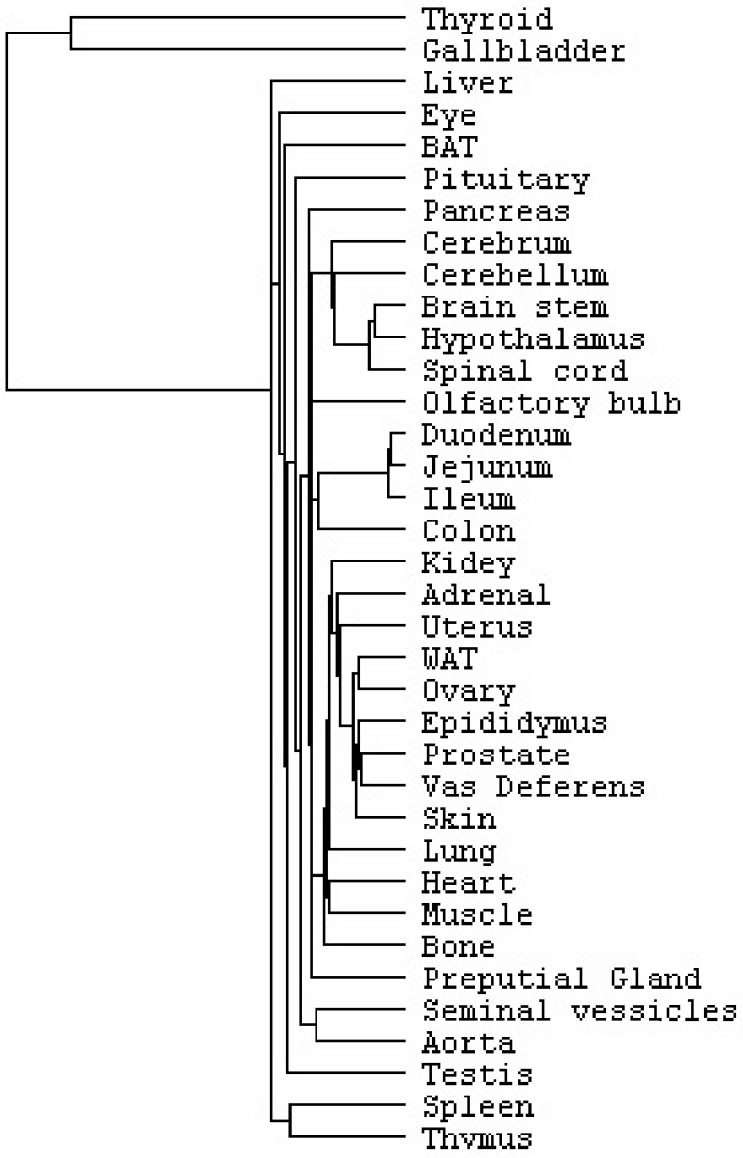
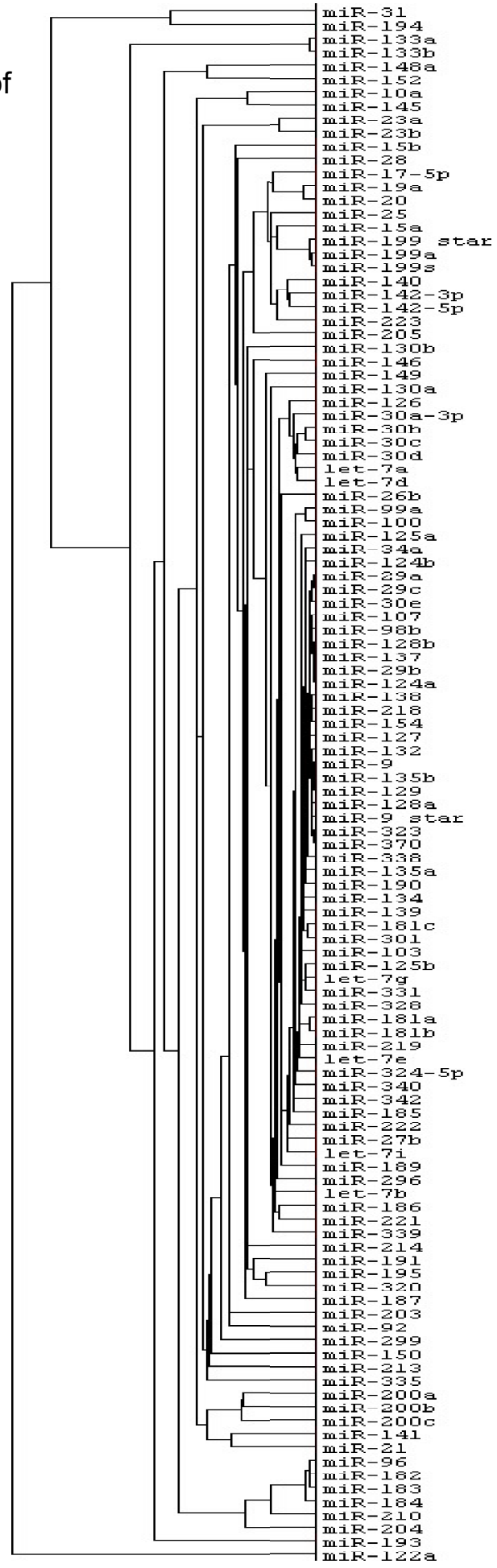


Figure 3.22 Cluster analysis of mouse tissues based on their microRNA expression.

Figure 3.23 Cluster analysis of microRNAs based on their expression in mouse tissues



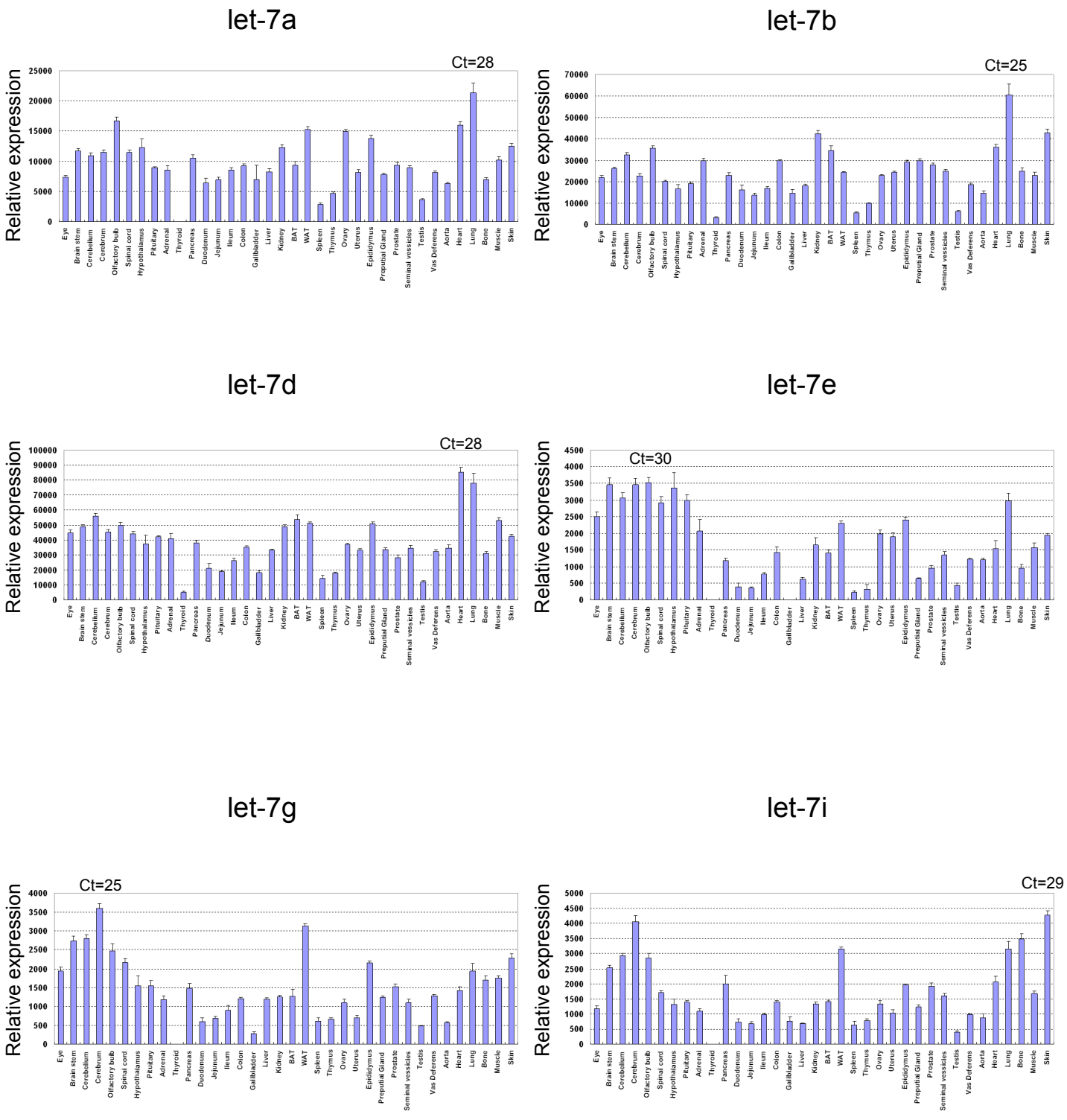


Figure 3.24 The expression of let-7 in mouse tissues.

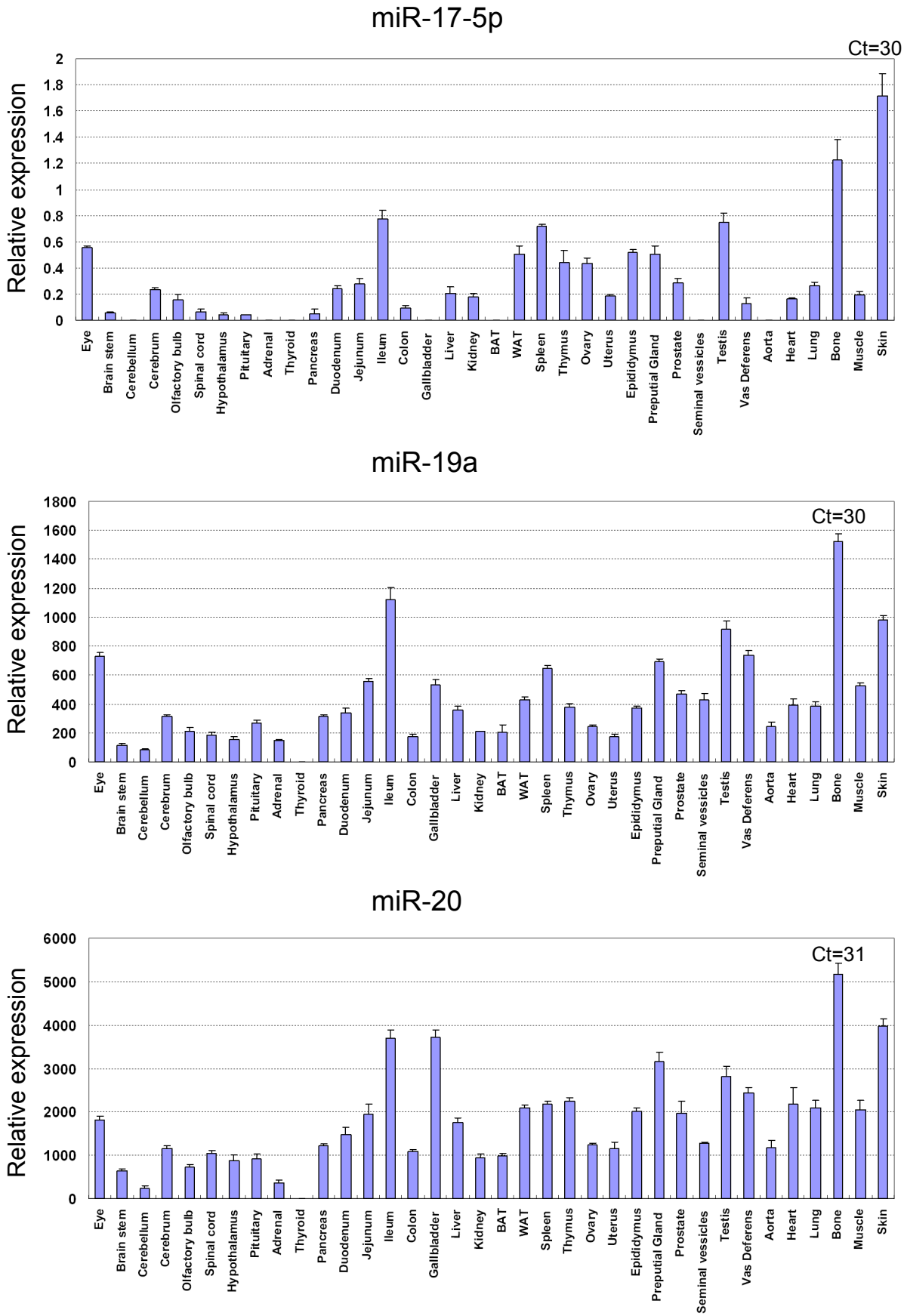


Figure 3.25 The expression of miR-17, miR-19a, and miR-20 in mouse tissues.

Table 3.1 Correlations between nuclear receptors and microRNAs

miRNA	Nuclear Receptor	Correlation	P value
miR-184	PNR	0.99	6.24E-50
miR-96	PNR	0.98	6.23E-24
miR-34c	GCNF	0.98	7.37E-24
miR-34c	DAX	0.96	2.51E-21
miR-194	HNF4g	0.96	1.48E-20
miR-183	PNR	0.94	7.9E-18
miR-9*	COUP-TFI	0.93	3.18E-16
miR-9	TLX	0.92	9.89E-16
miR-135b	TLX	0.92	1.11E-15
miR-182	PNR	0.92	1.33E-15
miR-128a	TLX	0.92	2.35E-15
miR-129	TLX	0.91	2.38E-14
miR-34c	TR2	0.90	5.63E-14
miR-128b	TLX	0.90	6.44E-14
miR-9	COUP-TFI	0.89	2.25E-13
miR-34c	FXRb	0.89	3.05E-13
miR-98b	TLX	0.88	2.6E-12
miR-184	ERRb	0.87	3.07E-12
miR-137	TLX	0.87	3.5E-12
miR-9*	TRa	0.87	8.71E-12
miR-9*	TLX	0.87	9.24E-12
miR-107	TLX	0.86	2.0E-11
miR-129	NURR1	0.86	2.95E-11
miR-194	PXR	0.85	3.61E-11
miR-132	NURR1	0.85	3.93E-11
miR-132	TLX	0.85	4.13E-11
miR-129	COUP-TFI	0.85	4.18E-11
miR-124b	TLX	0.85	4.22E-11
miR-29b	TLX	0.85	4.3E-11
miR-29c	TLX	0.85	4.29E-11
miR-30e	TLX	0.85	4.68E-11
miR-96	ERRb	0.85	6.79E-11
miR-128a	NURR1	0.85	8.48E-11
miR-323	COUP-TFI	0.84	1.16E-10
miR-9	NURR1	0.84	1.48E-10
miR-29a	TLX	0.84	2.23E-10
miR-323	TLX	0.83	2.45E-10
miR-218	COUP-TFI	0.83	3.94E-10
miR-370	COUP-TFI	0.83	4.79E-10
miR-128a	COUP-TFI	0.82	1.15E-09
miR-135b	COUP-TFI	0.82	1.41E-09
miR-138	TLX	0.81	2.08E-09
miR-204	PNR	0.81	2.09E-09
miR-218	TLX	0.81	2.7E-09
miR-204	RORb	0.81	2.89E-09
miR-183	ERRb	0.81	3.18E-09
miR-128b	NURR1	0.80	3.68E-09
miR-9*	NURR1	0.80	4.79E-09
miR-135b	NURR1	0.80	6.42E-09

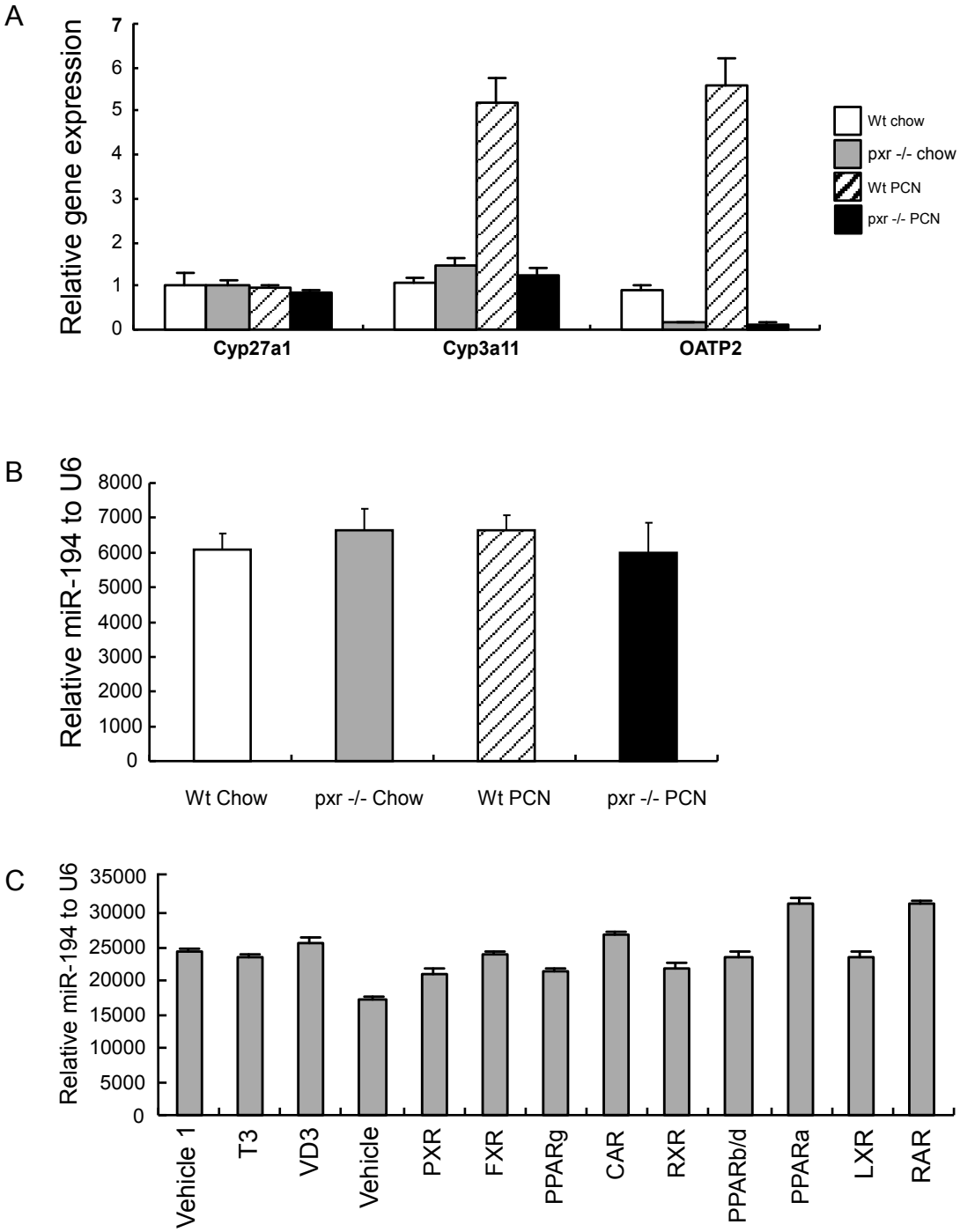


Figure 3.26 Test whether miR-194 is a direct transcriptional target of nuclear receptors. A. The expression of PXR target genes in wild-type or PXR knock-out mice treated with PCN. B. The expression of miR-194 in wild-type or PXR knock-out mice treated with PCN. C. The expression of miR-194 in the presence of different ligands for nuclear receptors.

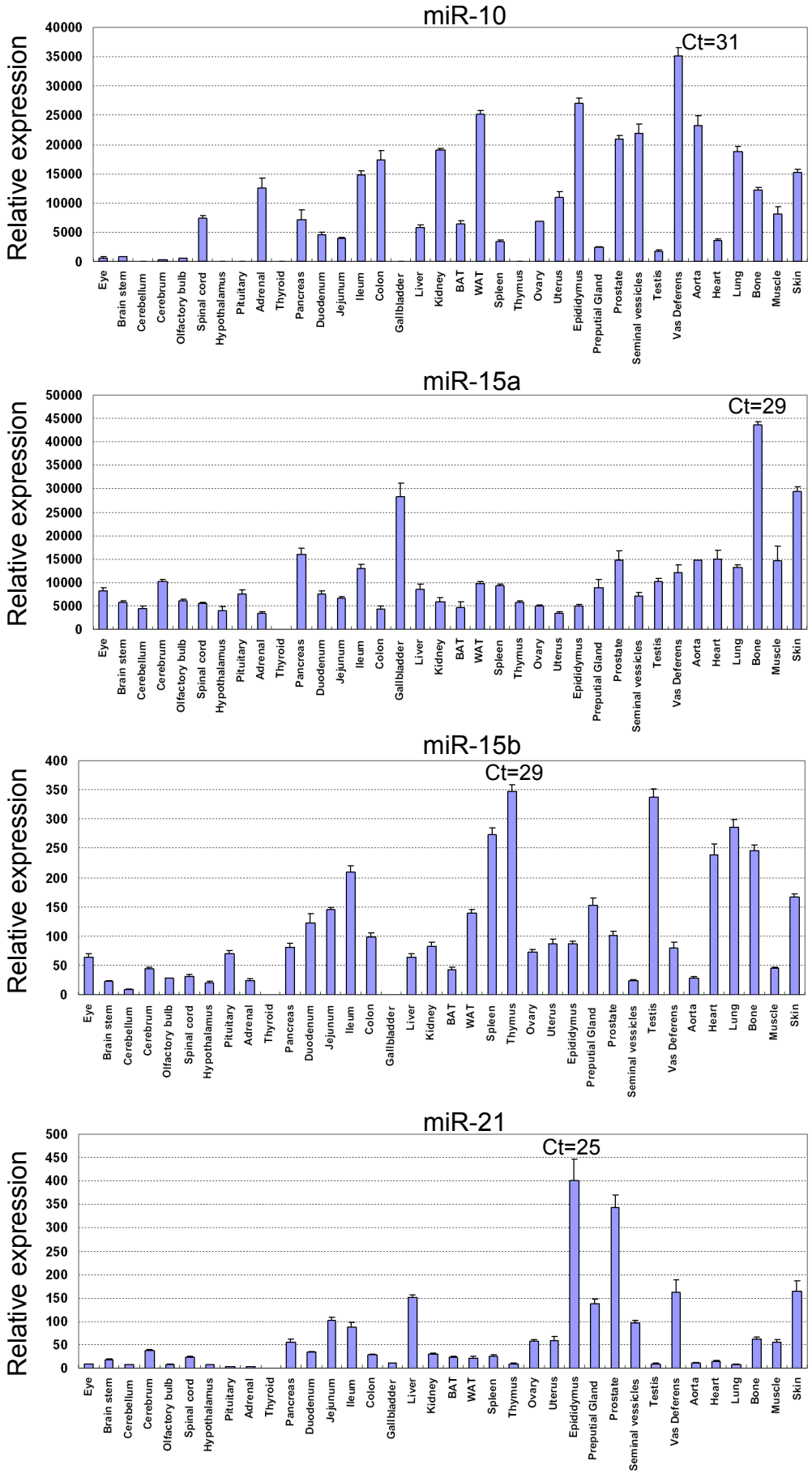


Figure 3.27 The expression of miR-10, miR-15, and miR-21 in mouse tissues.

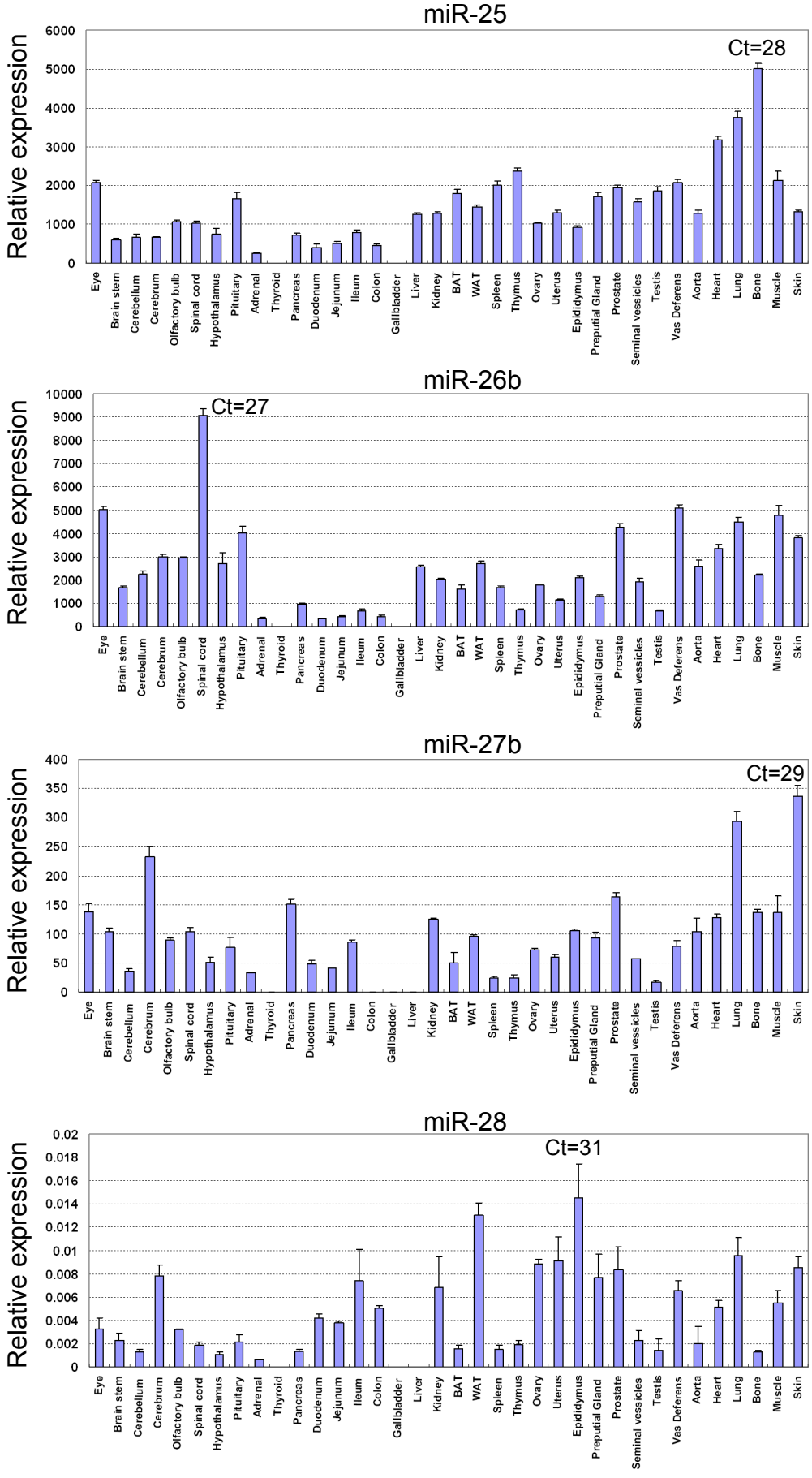


Figure 3.28 The expression of miR-25, miR-26b, miR-27b, and miR-28 in mouse tissues.

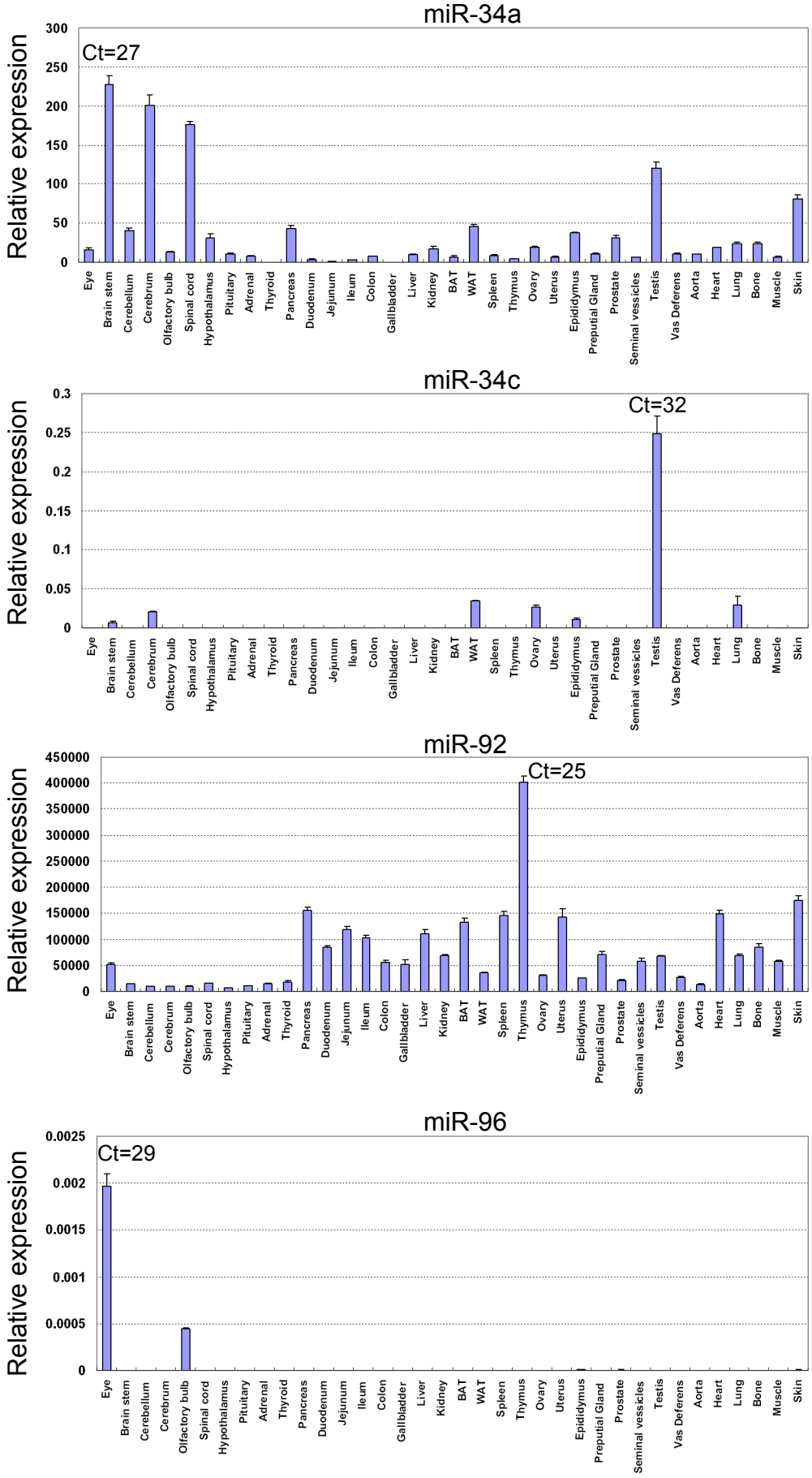


Figure 3.29 The expression of miR-34, miR-92, and miR-96 in mouse tissues.

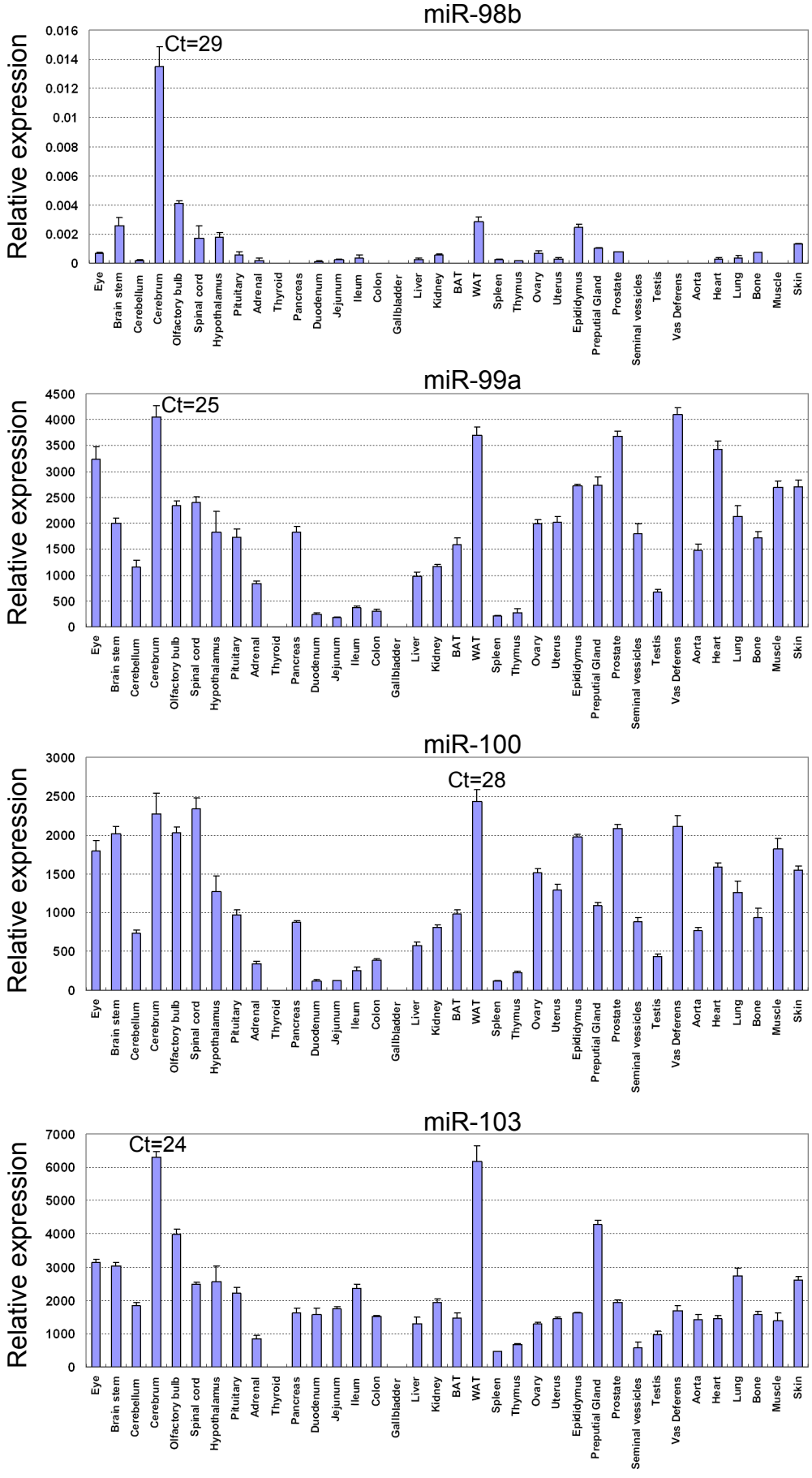


Figure 3.30 The expression of miR-98b, miR-99a, miR-100, and miR-103 in mouse tissues.

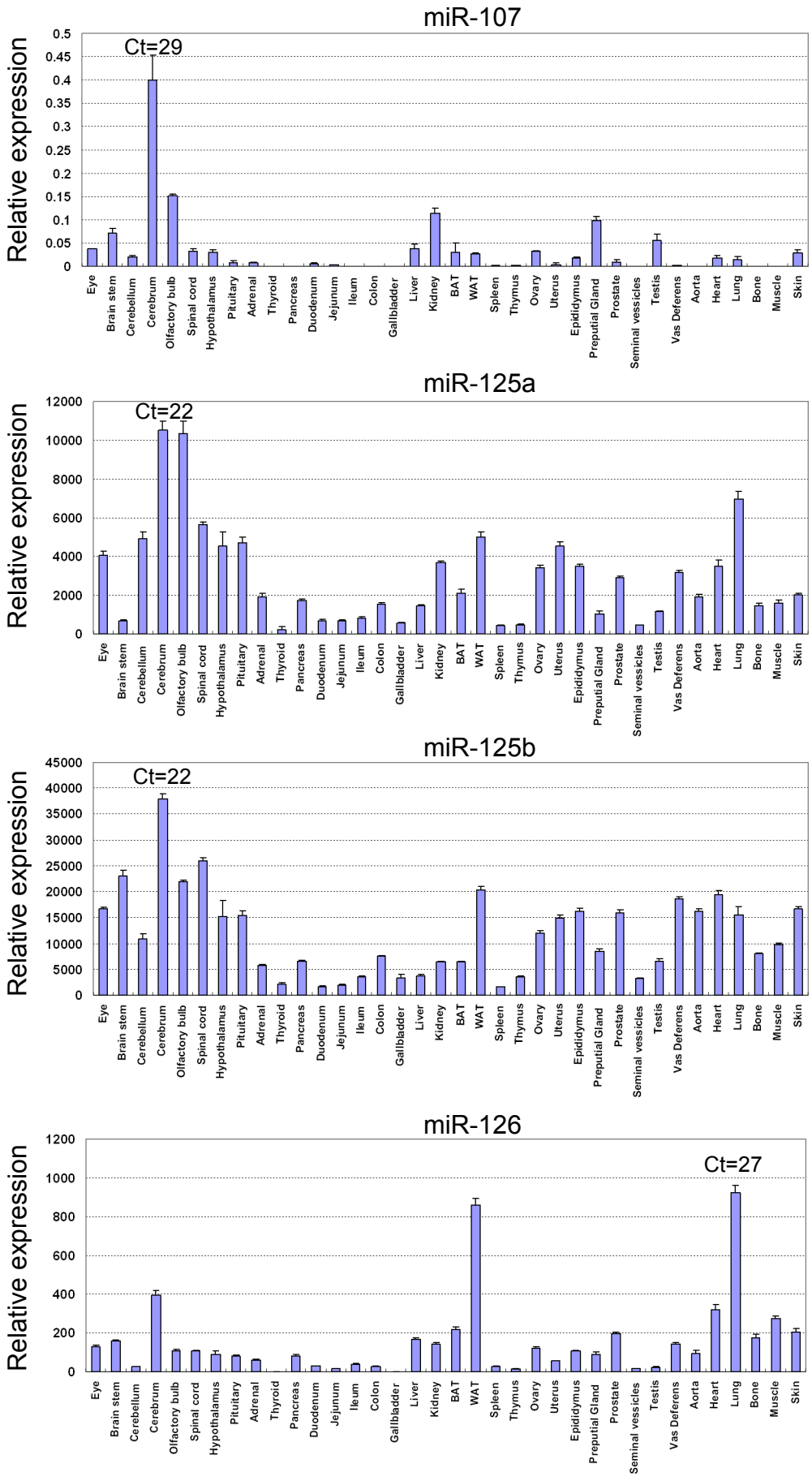


Figure 3.31 The expression of miR-107, miR-125a, miR-125b, and miR-126 in mouse tissues.

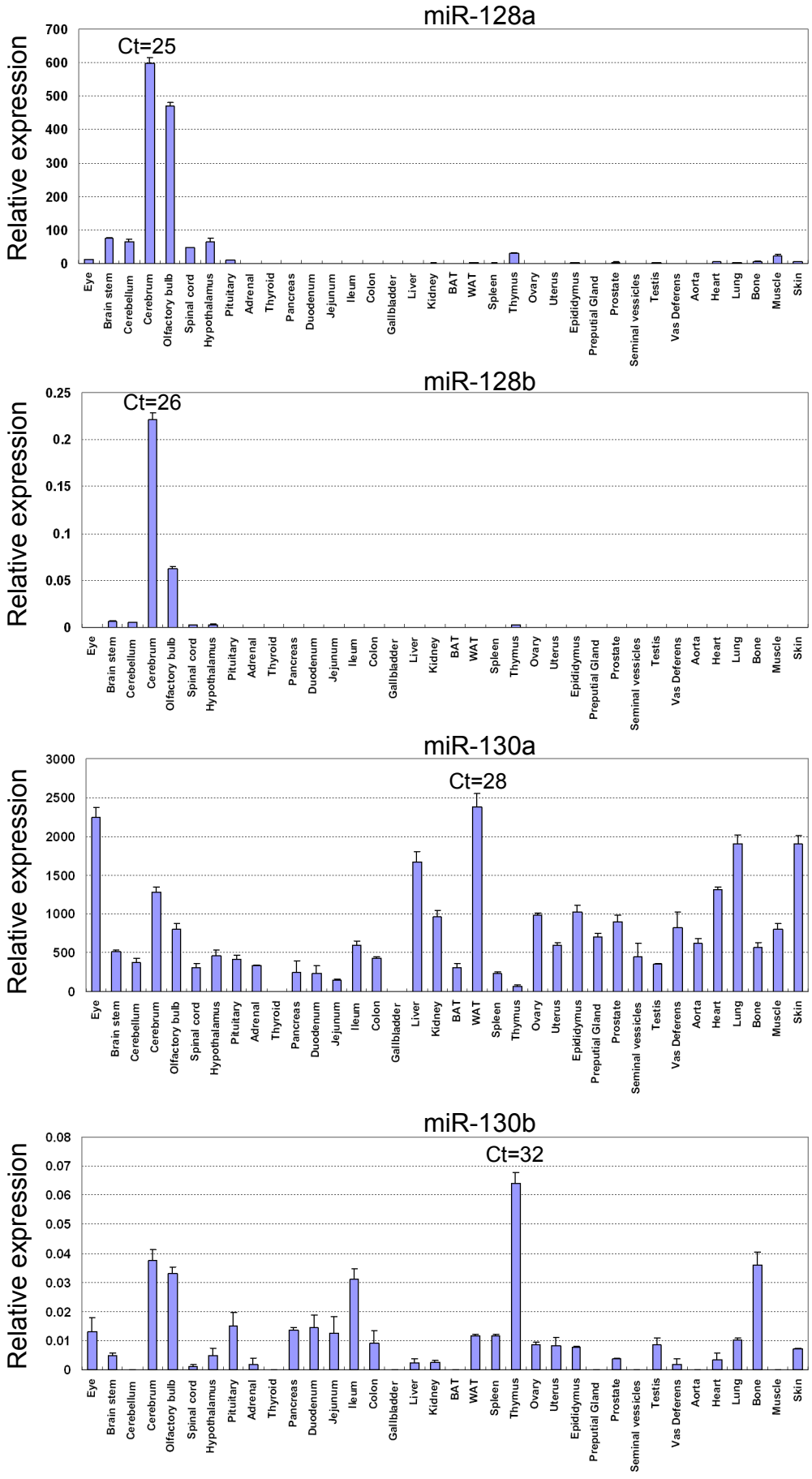


Figure 3.32 The expression of miR-128 and miR-130 in mouse tissues.

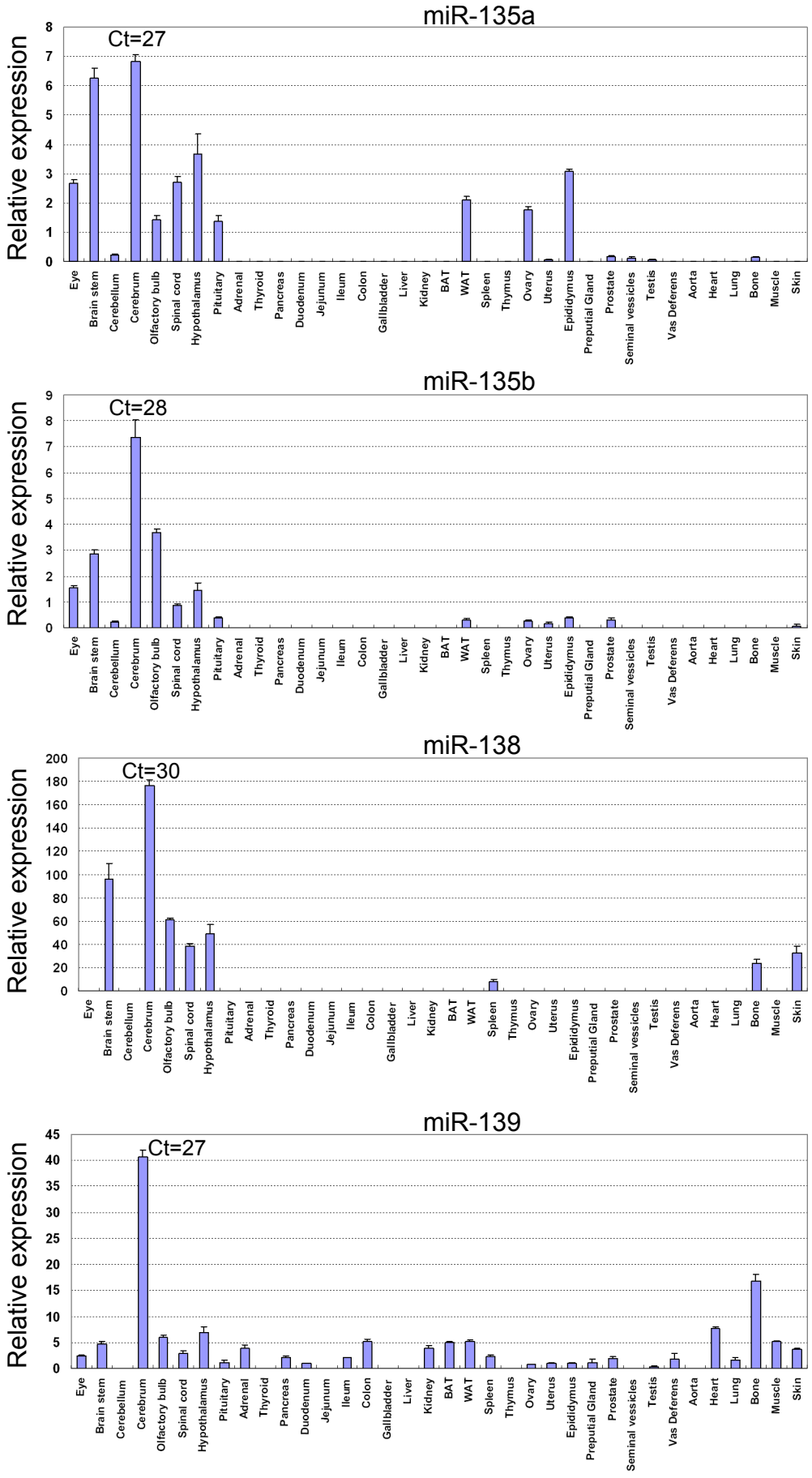


Figure 3.33 The expression of miR-135, miR-138, and miR-139 in mouse tissues.

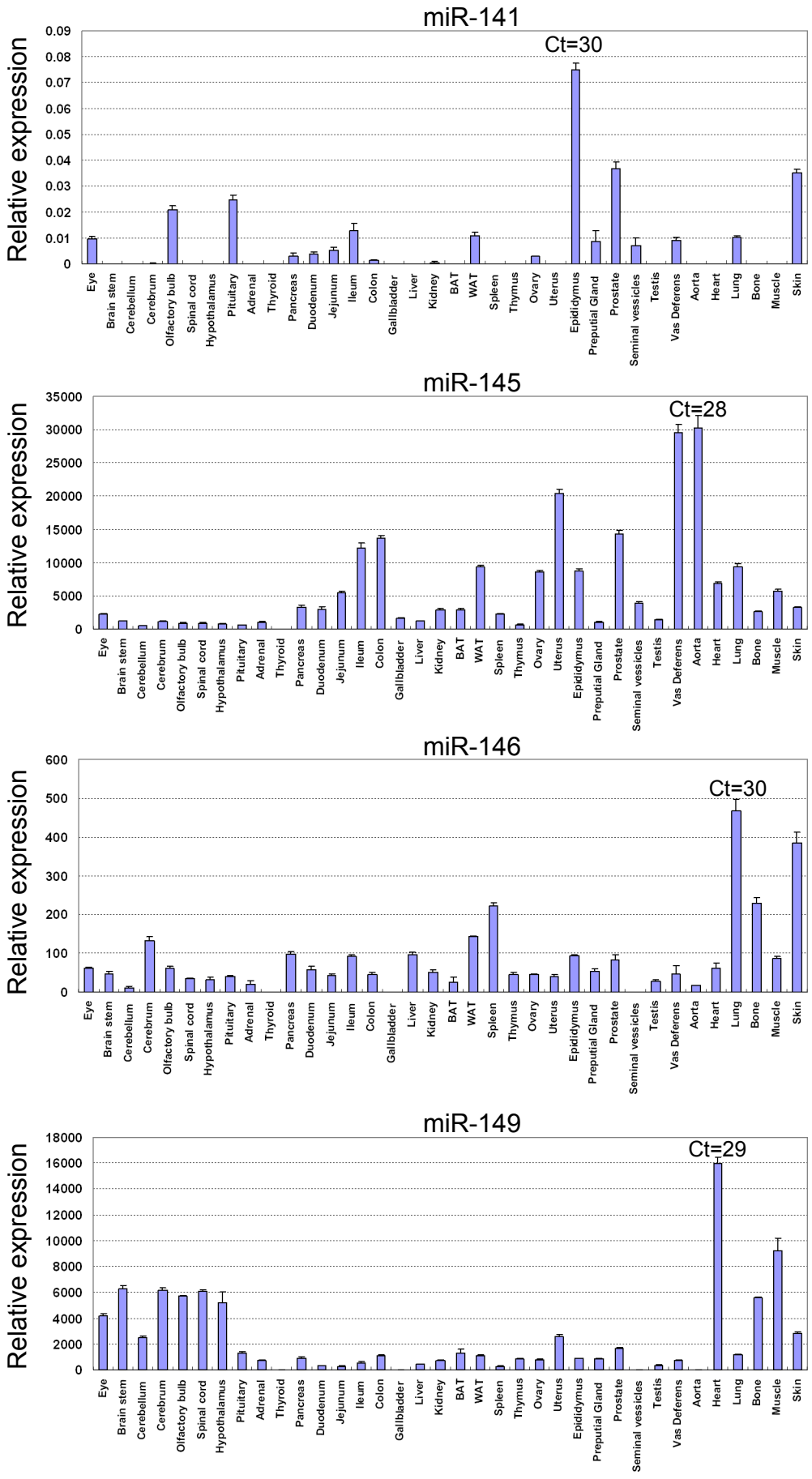


Figure 3.34 The expression of miR-141, miR-145, miR-146, and miR-149 in mouse tissues.

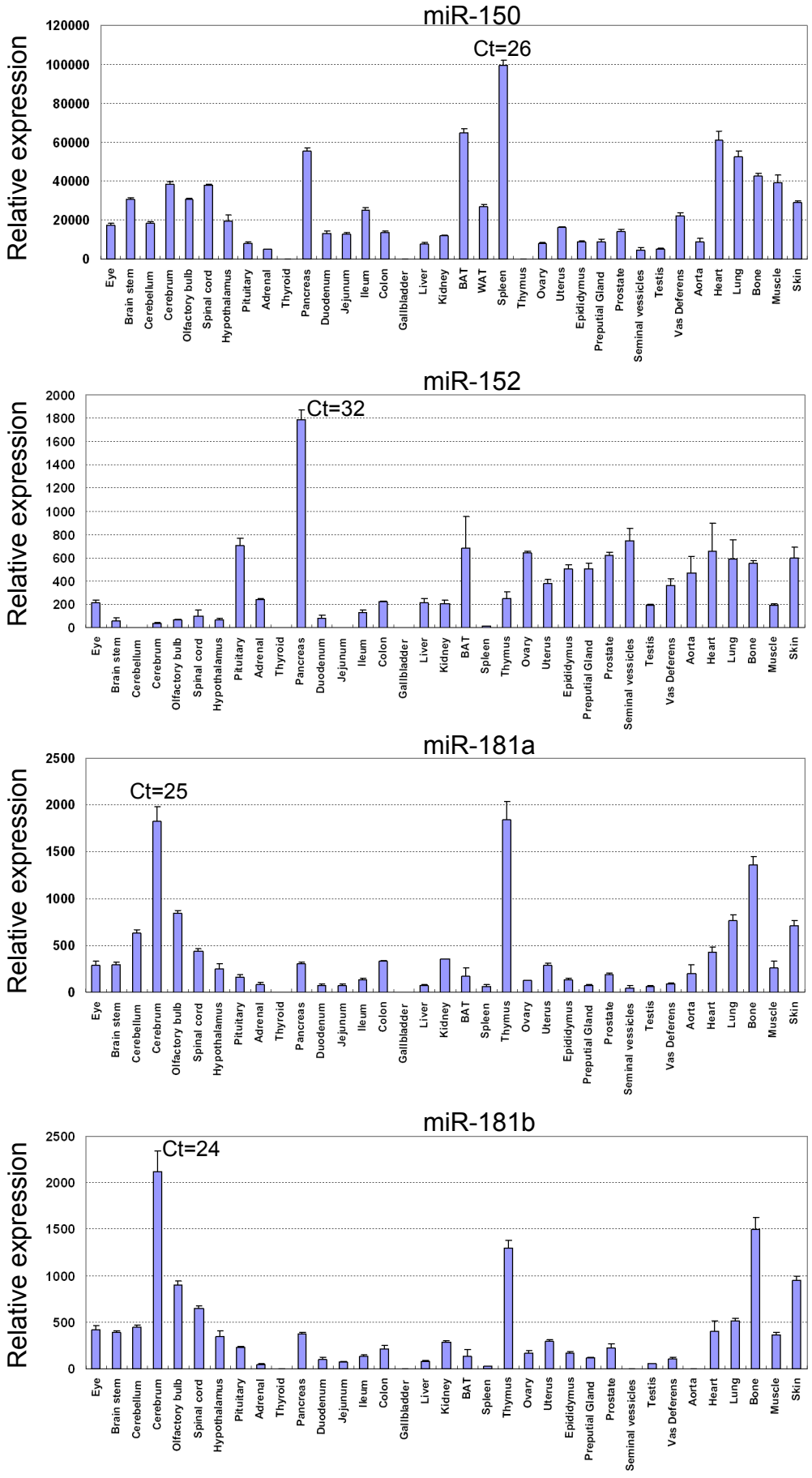


Figure 3.35 The expression of miR-150, miR-152, and miR-181 in mouse tissues.

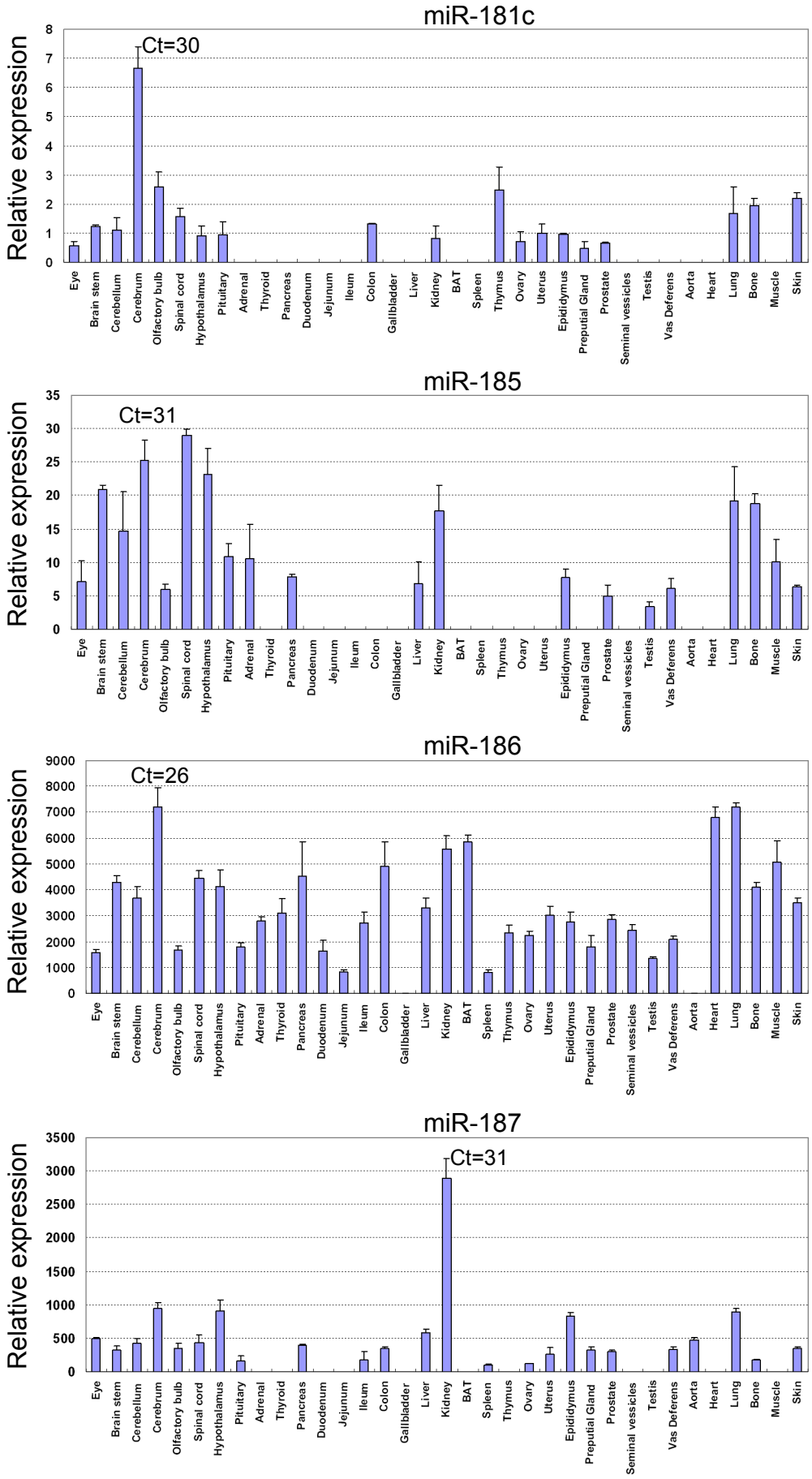


Figure 3.36 The expression of miR-181c, miR-185, miR-186, and miR-187 in mouse tissues.

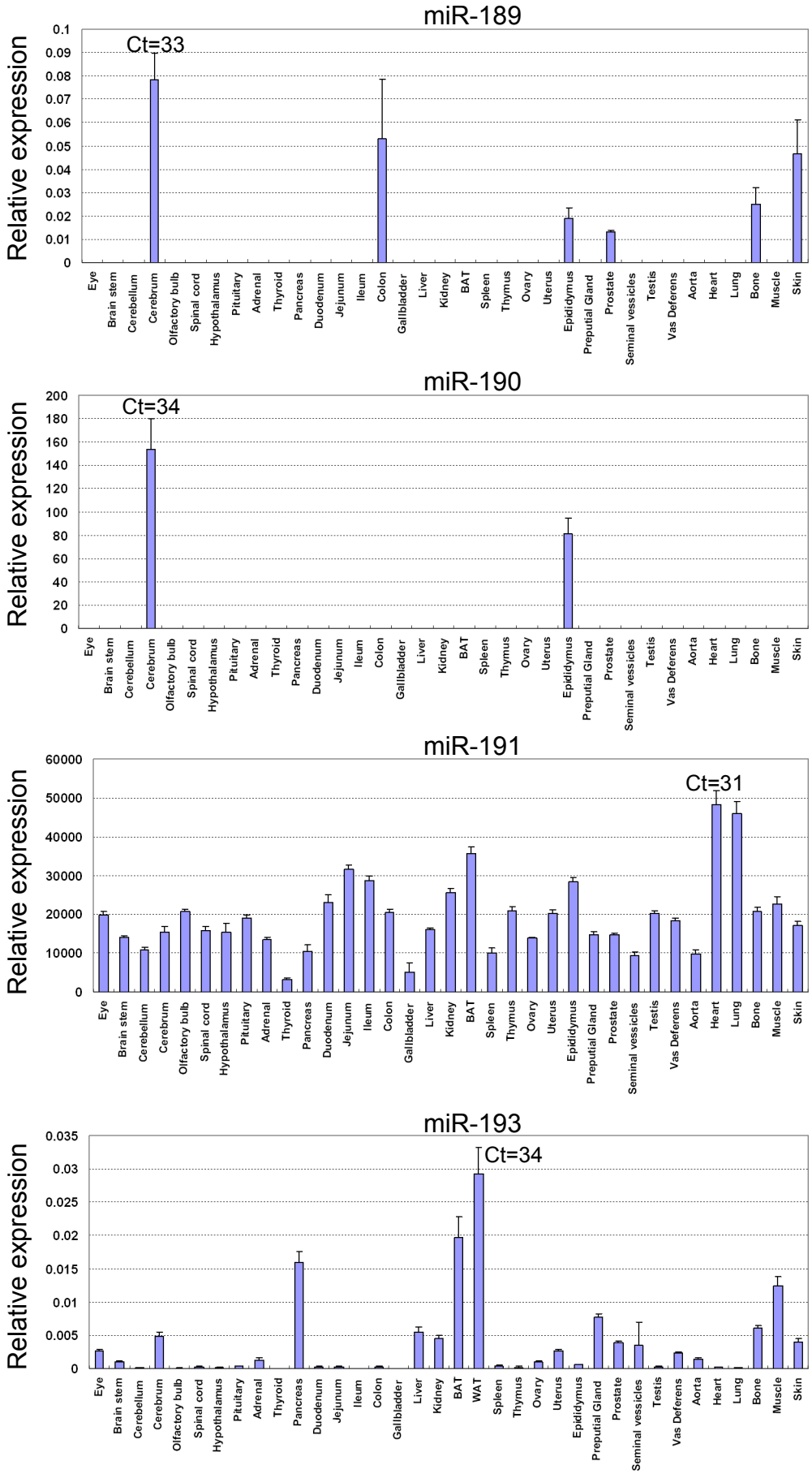


Figure 3.37 The expression of miR-189, miR-190, miR-191, and miR-193 in mouse tissues.

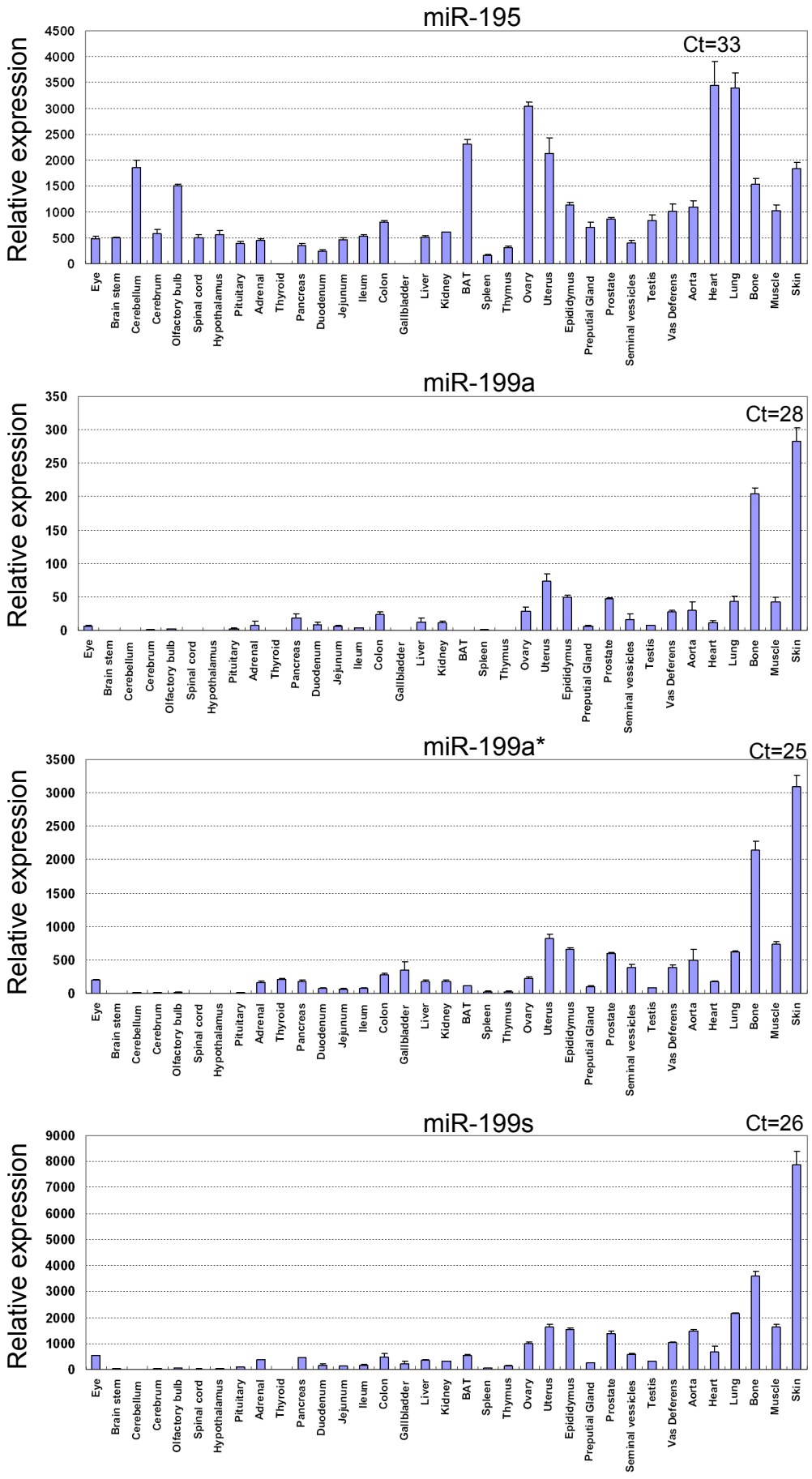


Figure 3.38 The expression of miR-195 and miR-199 in mouse tissues.

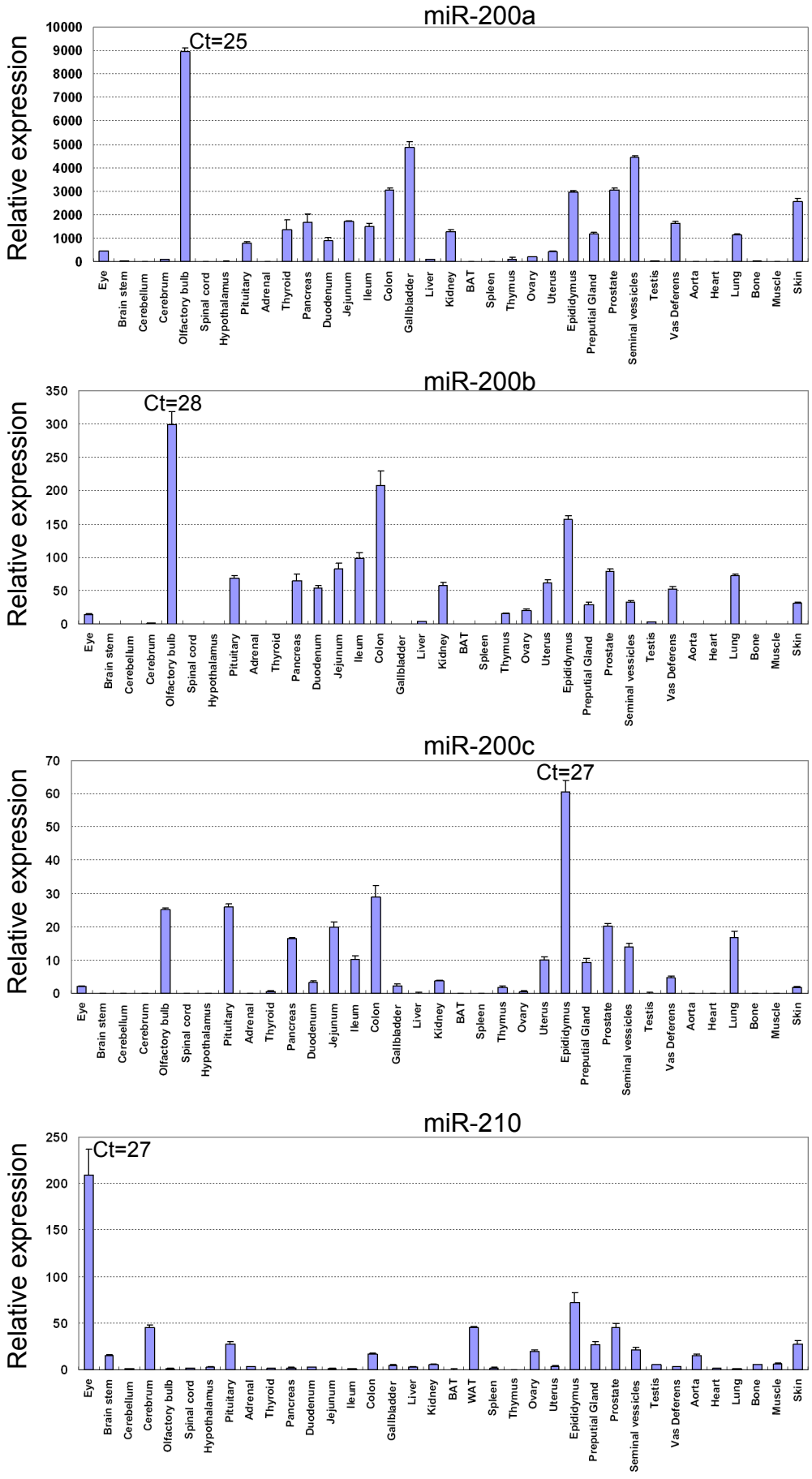


Figure 3.39 The expression of miR-200 and miR-210 in mouse tissues.

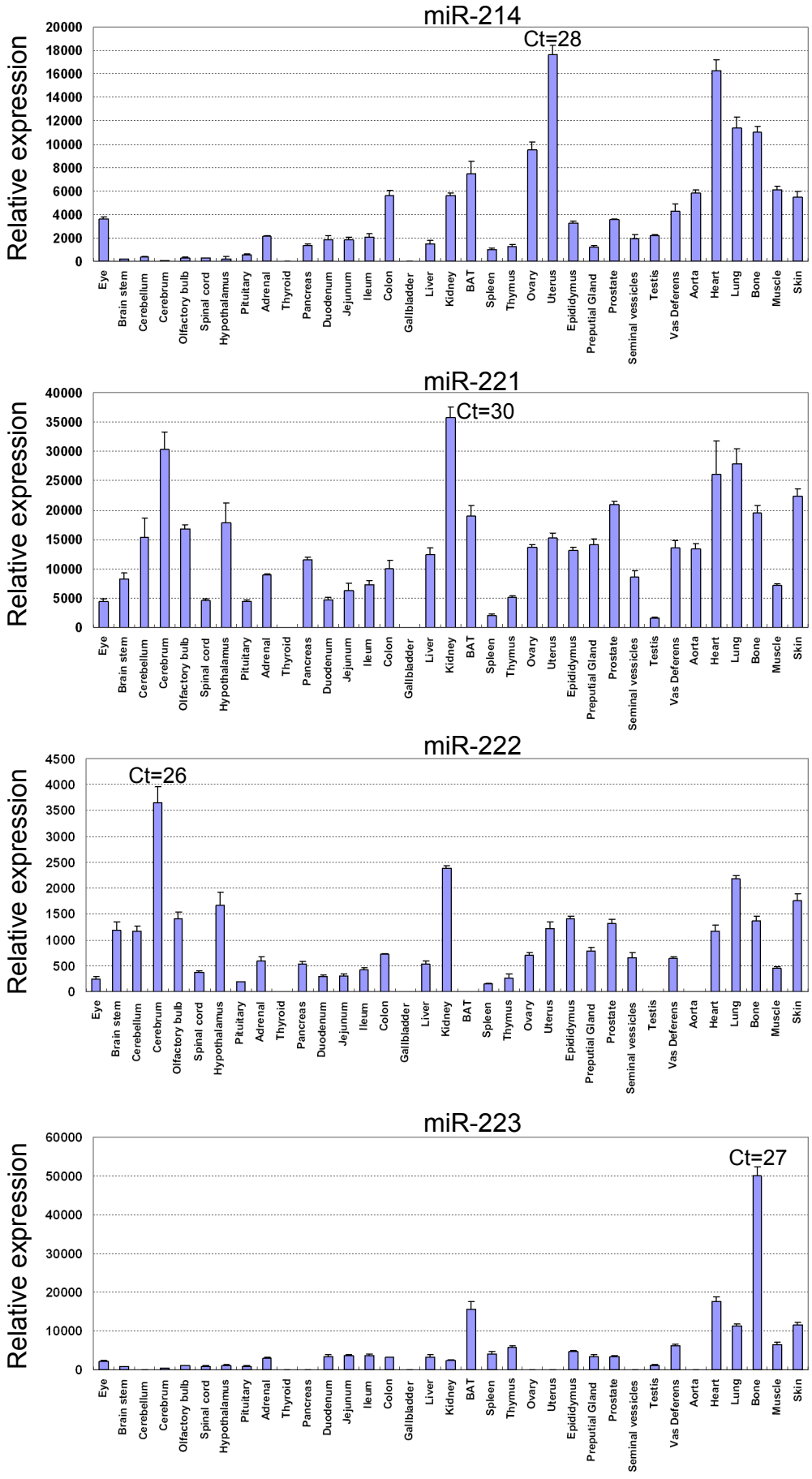


Figure 3.40 The expression of miR-214, miR-221, miR-222, and miR-223 in mouse tissues.

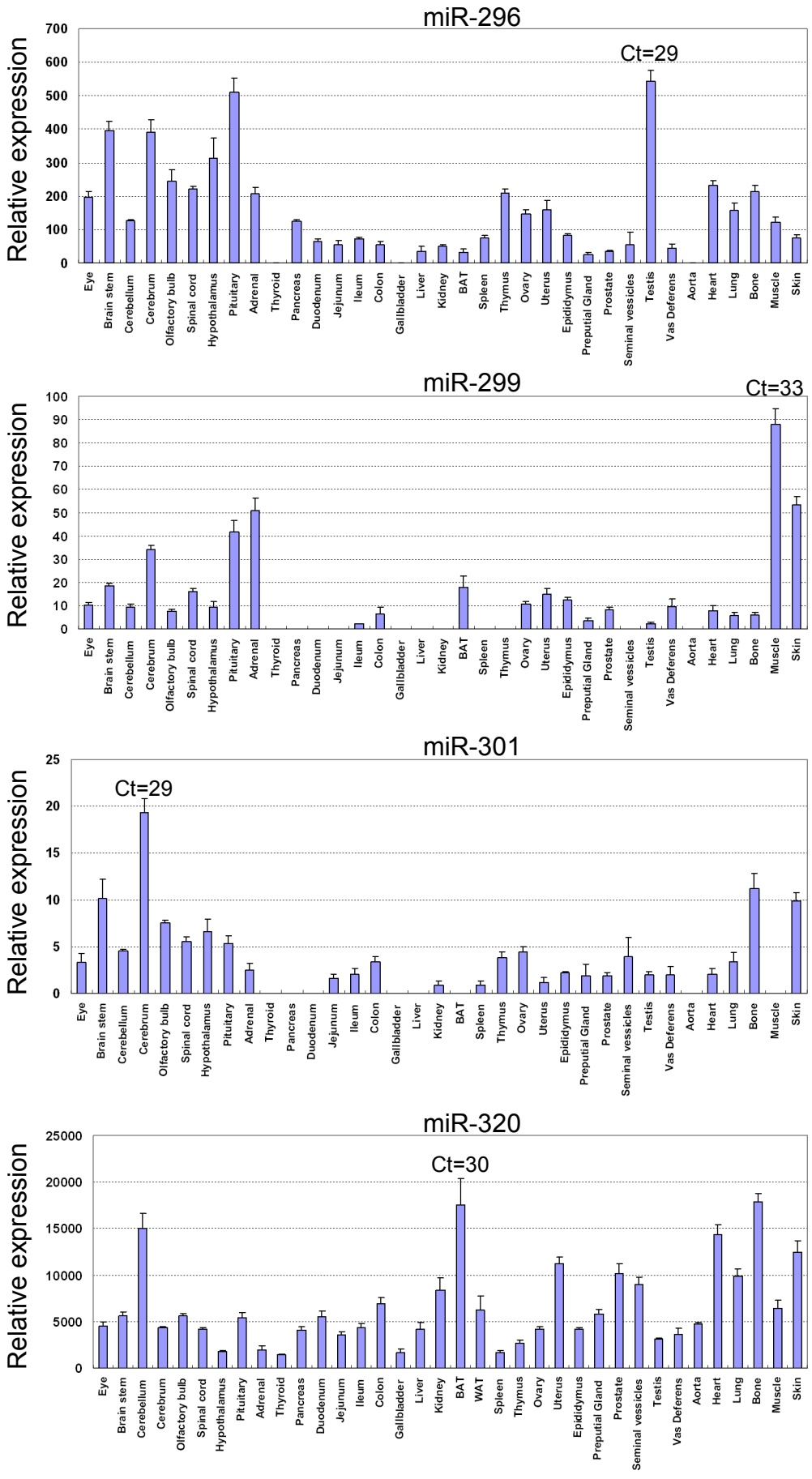


Figure 3.41 The expression of miR-296, miR-299, miR-301, and miR-320 in mouse tissues.

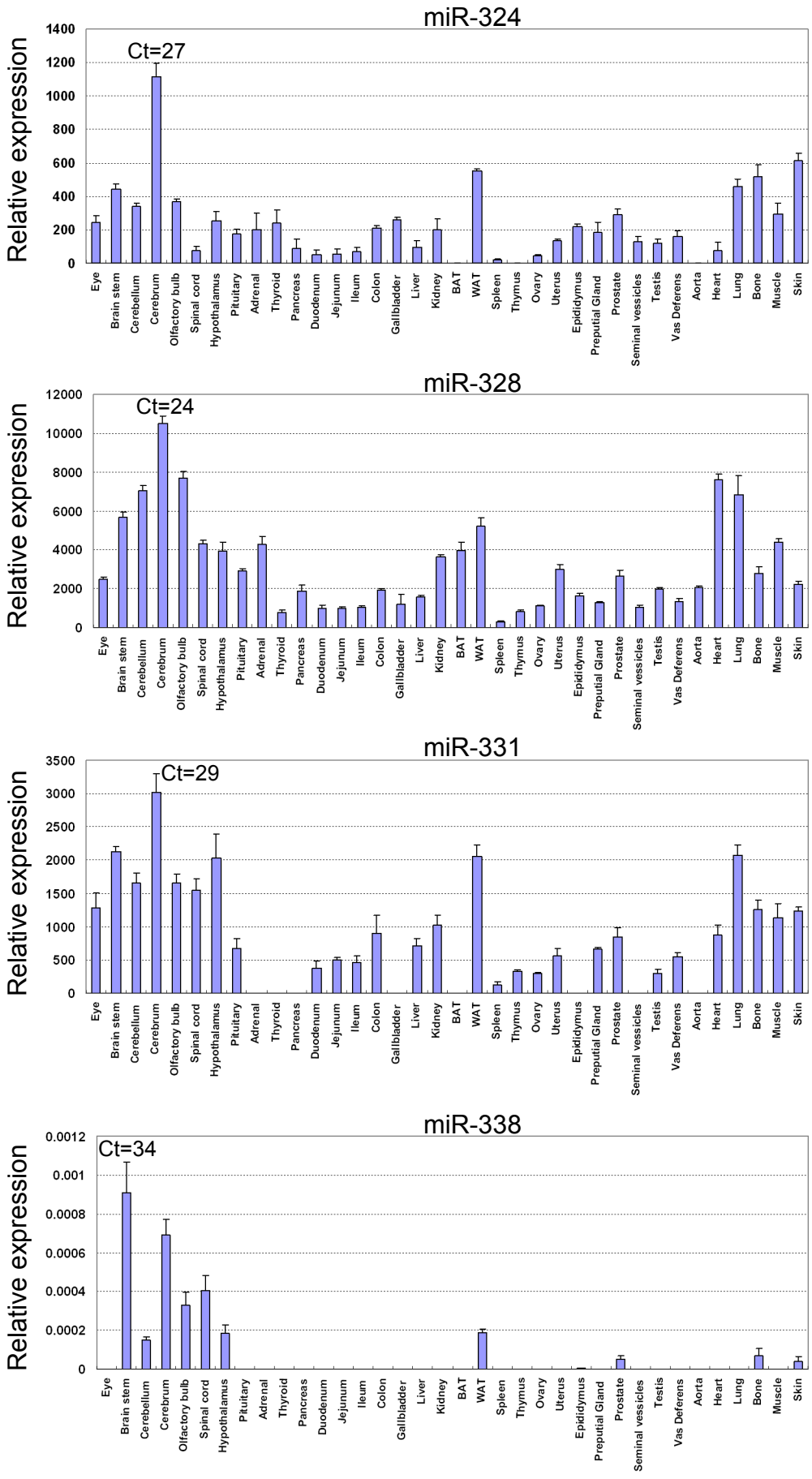


Figure 3.42 The expression of miR-324, miR-328, miR-331, and miR-338 in mouse tissue.

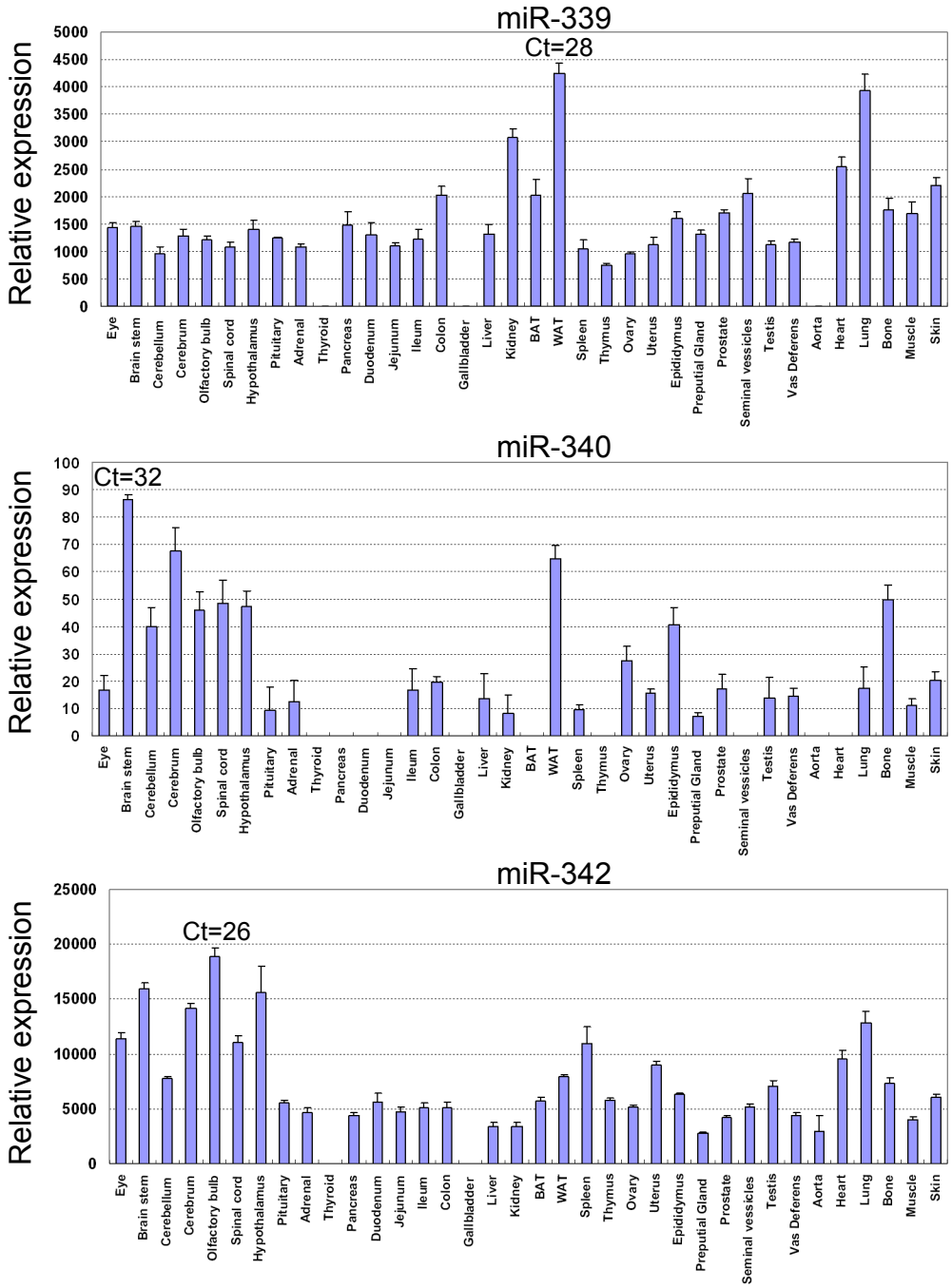


Figure 3.43 The expression of miR-339, miR-340, and miR-342 in mouse tissues.

CHAPTER 4

Material and Methods

4.1 Cell culture and differentiation

3T3-L1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with L-glutamine (Invitrogen, CA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100U/ml penicillin, 100mg/ml streptomycin, and incubated at 37°C in 5% CO2 humidified atmosphere. Differentiation with DMI: two days after confluence, a cocktail containing 1uM dexamethasone, 0.5mM 3-iso-butyl-1-methylxanthine (IBMX), and 5ug/ml insulin were added into culture medium. After culturing with DMI for 48 hours, culture medium was replaced every 48 hours with DMEM containing 10% FBS and 5ug/ml insulin until pre-adipocytes become mature adipocytes. Differentiation with Rosiglitazone: after 3T3-L1 preadipocytes reached confluence, cells were cultured in DMEM supplemented with 10% FBS and 5uM Rosiglitazone. Medium was replaced every 48 hours. 3T3-F442A differentiation: 3T3-F442A cells were cultured in DMEM high glucose with 10% FBS and 5ug/ml insulin was added to the culture medium to induce 3T3-F442A differentiation after confluence. C2C12 differentiation: C2C12 myocytes were cultured in DMEM supplemented with 20% FBS and differentiated into myotubes by culture in DMEM with 2% horse serum after confluence.

4.2 Northern blot for microRNAs

10ug of total RNA for each sample was mixed with 2x sample loading buffer and incubated at 65°C for 10 minutes, chilled on ice for 3 minutes and loaded on a 15% polyacrylamide gel containing 7M Urea (Invitrogene, CA). Electrophoresis was performed at 250V for 1 hour in 1X Tris-Borate-EDTA (TBE) buffer. RNA was transfered onto Hybond N+ membranes (Amersham Biosciences, NJ) at 20V for 45 minutes in 0.5X TBE. RNA was cross-linked to this membrane by UV-linker. Starfire probes for each microRNA were labeled with α-P^{32}-dATP (Amersham Biosciences, NJ) using the Starfire kit (Integrated DNA Technology, Coralville, IA). Labeled probe was separated from free α-P^{32}-dATP using Sephadex G-25 column (Roche, IN). Hybridization was done at 45°C in 0.2M Na_2HPO_4, 7% SDS buffer overnight. Membranes were washed with 2X SSC/0.1% SDS twice and exposed to X-ray film. Either ethidium bromide (EB) stained gel or U6 snRNA northern blot was used as a loading control.

4.3 RNA preparation and quantitative real time PCR

Total RNA was isolated from 3T3-L1 cells using RNA stat-60 (Tel-Test, TX) and precipitated with isopropanol. Messenger RNA was converted to cDNA and detected with ABI 7900HT sequence detection system using SYBR greener assay (Bookout and Mangelsdorf, 2003). Cyclophilin or 18S rRNA was used as loading control. The data analysis was done according to (Fu et al., 2005).

Primers for real time PCR (Fu et al., 2005):

Gene	Forward primer	Reverse primer
C/EBPα	gacatcagcgcctacatcga	tcggctgtgctggaagag
C/EBPß	atttctatgagaaaagaggcgtatgt	aaatgtcttcactttaatgctcgaa
C/EBPδ	ttccaacccctttccctgat	ctggagggtttgtgttttctgt
Adipsin	aggacgacctcattctttttaagc	acttctttgtcctcgtattgcaa
aP2	gcctgccactttccttgtg	gacatcagcgcctacatcga
PPARγ	caagaataccaaagtgcgatcaa	gagctgggtcttttcagaataataag
FAS	gctgcggaaacttcaggaaat	agagacgtgtcactcctggactt
LPL	ggccagattcatcaactggat	gctccaaggctgtaccctaag
E2F1	cccctcctgagacccaacta	gctcttaagggagatctgaaatgtc
Hmga2	aacctgtgagccctctcctaag	gccgtttttctccaatggtc (Lin et al., 2007)

For microRNA quantitative real time PCR, Taqman MicroRNA assay kit (Applied Biosystems, CA) was used. Reverse transcription (RT) of each microRNA with microRNA specific stem loop RT primer and quantitative real time PCR with Taqman probe were done according to product manual to detect mature microRNA. Universal RNAs from mouse tissues were used to set up a standard curve for each microRNAs. U6 snRNA level was used as a loading control.

4.4 Microarray

For microRNA microarray, microRNAs were isolated from total RNA using the FlashPAGE fractionator (Ambion, TX). 100ug of total RNA was loaded to FlashPAGE precast gel and small size RNA was collected after electrophoresis at 80V for 12 minutes. Then small size RNA was purified by FlashPAGE reaction clean up kit (Ambion, TX). 1ug of small size RNA was used for microarray on a custom prepared slide containing 386 probes for human, mouse, and rat microRNAs from mirVana microRNA Probe Set (Ambion, TX). Microarrays were done at UT Southwestern Medical Center University Microarray

Core Facility. After Labeling, hybridization, and washing, slides are scanned and analyzed using GeneTraffic software.

For mRNA microarray, Illumina beadarray (Illumina, CA) was used. Total RNA was extracted from control microRNA or *let-7* transfected 3T3-L1 cells 24 hours post transfection. After cDNA synthesis and purification, aRNA purification, hybridization, wash, staining, and scanning of slides, data was analyzed.

4.5 Western blot

3T3-L1 cells were scraped from culture dishes and lysed with M-Per mammalian protein extraction reagent (Pierce, IL) after phosphate buffer solution (PBS) washing. Proteins were separated with 15% SDS-PAGE and transferred to cellulose membranes. Membranes were blotted with 1:200 anti-Hmag2 rabbit antibody (Biocheck, CA). Then 1:5,000 anti rabbit HRP secondary antibody was blotted to the membrane. The same membrane was blotted with 1:10,000 anti-GAPDH HRP conjugated antibody (Sigma-aldrich, MO) as a loading control.

4.6 Oil Red O staining

Oil red O staining was done according to (Wu et al., 1998). Cells in culture dishes were fixed in 10% formaldehyde in PBS for 15min after two times of PBS wash. Cells then were stained in freshly made Oil Red O solution (60% of Oil Red O stock solution which consists of 0.5% Oil Red O in isopropanol, 40% of H_2O) for at least 1 hour. After staining, the cells

were washed with 60% isopropanol, then with H_2O until background staining was gone.

4.7 Glyceride content measurement

Glyceride content was measured using AdipoRed assay reagent (Cambrex, MD) according to manufactory protocol. In summary, 5ul of AdipoRed reagent was added to each well of a 96 well plate containing 200ul PBS after washing with PBS. After mixing and 10 minutes wait, the 96 well plate was measured in the Victor 1420 Multilabel Counter fluorimeter (PerkinElmer, Waltham, Massachusetts) at excitation wave at 485nm and emission wave at 572nm.

4.8 3T3-L1 cell transfection using Nucleofector

3T3-L1 cells were trypsinized and pelleted down at 90g for 10 minutes. After removal of supernatant, 100ul of buffer V and 2ug of *let-7* pre-mir (Ambion, TX) or plasmids were used to resuspend the pellet. 3T3-L1 cells were tranfected using Nucleofector (Amaxa, MD) in buffer V with program T-30. Cells were cultured in 6 well plates or 96 well plates after transfection.

4.9 Luciferase assay in F9 cells

F9 cells were cultured in 0.1% gelatin-coated 48 well plates with DMEM containing 10% FBS. Plasmids for luciferase reporter, control renilla luciferase reporter, and control or

let-7 oligo were transfected into F9 cells with X-tremeGENE siRNA transfection reagent (Roche, IN) according to the product manual. Cells were lysed and luciferase and renilla luciferase activity were measured using dual luciferease assay kits (Promega, WI) 24hours after transfection. Luciferase reporter activity was normalized to renilla luciferase activity.

4.10 Hierarchical cluster analysis

MicroRNAs or mouse tissues were subjected to unsupervised hierarchical cluster analysis based on the expression of microRNAs in different mouse tissues using Cluster 3.0 software (which was originally developed by Michael Eisen at Stanford University and later developed by M. J. L. de Hoon, S. Imoto, J. Nolan, and S. Miyano at the Human Genome Center of University of Tokyo). The log2 data was centered by median and hierarchical cluster was performed for microRNAs or tissues using a single linkage metric. The result was shown using Java TreeView 1.1.3 (Created by Alok Saldanha). The data was displayed using color code.

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