MicroRNA: basic mechanisms and transcriptional regulatory networks for cell fate determination

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Characterization of regulatory mechanisms affecting microRNA (miRNA) expression and activity is providing novel clues for the identification of genes and complex regulatory circuits that determine cell and tissue specificity. Here, we review the molecular events leading to miRNA biogenesis and activity, focusing above all on endogenous and epigenetic transcriptional networks involving miRNA in early development, cellular lineage specification/differentiation of nervous, skeletal and cardiac muscle tissues and in haematopoiesis, as the de-regulation of such networks may be relevant to disease pathogenesis.

KEYWORDS
MicroRNAs; Transcription factors; Epigenetic gene regulation; Cell differentiation

1. Introduction

During both development and adult life, transcriptional regulators, DNA and chromatin modifiers play crucial roles to ensure that proper gene expression patterns are established and maintained in any given cell type. However, the control of gene expression also occurs at the RNA level. This includes alternative splicing, messenger RNA (mRNA) stability, and translational control, all of which appear to enhance the diversity and flexibility of gene expression regulation. Very recent findings in molecular biology have shown that the participation of small regulatory non-coding RNA is required for cellular diversity in a developing organism and during adulthood. These molecules act as sequence-specific post-transcriptional regulators in the expression of other RNA transcripts.\textsuperscript{1}

Small regulatory non-coding RNA molecules include microRNAs (miRNAs), small interfering RNAs (siRNAs) and repeat-associated siRNAs (rasiRNAs), which are unified by their association with the Argonaute (AGO) family of proteins and by their function. Indeed, all these RNAs direct the binding of protein complexes to specific nucleic acid sequences.\textsuperscript{1–6} SiRNAs and miRNAs repress the expression of target genes at the post-transcriptional level by interacting with the 3’ untranslated regions (UTRs) of specific mRNAs. However, siRNA also acts at the transcriptional gene level by affecting chromatin structure.\textsuperscript{7,8} RasiRNAs are produced as 29–30 nucleotide (nt) RNAs from a master gene locus composed of multiple transposons. These small RNAs differ from siRNAs and miRNAs in size and in their association with distinct protein complexes. In particular, rasiRNAs bind the Piwi (piRNAs) subfamily of AGO proteins, Aubergine, and AGO 3, and target homologous transposons scattered throughout the genome for their silencing.\textsuperscript{9–12}

The specialization and evolution of the core components of the RNA-mediated silencing machinery integrate RNA silencing in various cellular physiological regulatory networks, which, in addition to the regulation of gene expression by inhibition of translation initiation and elongation, co-translational protein degradation, and premature termination of translation (ribosome drop-off), include the survey against the proliferation of transposable elements and viruses, epigenetic changes in DNA and chromatin status, and accurate chromosome segregation during cell division.\textsuperscript{13,14}

Since the discovery of the first canonical miRNA genes, lin-4 and let-7, in the nematode \textit{Caenorhabditis elegans} (C. elegans), the role of miRNA activities appeared as a novel common theme in cell fate determination.\textsuperscript{15,16} To date, hundreds of miRNA genes have been identified in animals; these genes display either an ubiquitous expression or a strict cell/tissue expression pattern.\textsuperscript{17} miRNA biogenesis and activity is now regarded as a key regulatory mechanism in controlling developmental timing, tissue differentiation, and maintenance of tissue identity during embryogenesis and adult life.\textsuperscript{1,3}

Compelling evidence points to muscle and cardiovascular cell systems as powerful models for the understanding of the physiological regulatory networks and biological function of miRNAs during development and adult life. Moreover, recent findings indicate that miRNA expression/activity can be de-regulated in various diseases, including cardiovascular pathologies, thus offering new prospects for their therapeutic use.\textsuperscript{18}
In this review, we focused on the molecular events leading to miRNA biogenesis and activity, and the endogenous and epi-
genetic transcriptional networks involving miRNA in early
development, cellular lineage specification/differentiation of
skeletal and cardiac muscle tissues, nervous tissue and
haematopoiesis, as well as their potential relevance to
disease pathogenesis.

2. Biogenesis of mature microRNAs

2.1 Genomic distribution and gene transcription

miRNAs are non-coding single-strand RNAs (ssRNAs), of ~22
nt in length in their mature forms, that are individually
encoded by their own set of genes. Genes for miRNAs are
an integral component of the cell genetic program, many
of them also being evolutionarily conserved.1,3 Studies on
miRNA genes distribution throughout the genome, have
revealed their presence in clusters transcribed as poly-
cistronic primary transcripts, or within regions transcribed
as independent units, including intergenic regions, exon
sequences of non-coding transcription units or intronic
sequences of either protein coding or non-coding transcrip-
tion units19,20 (Figure 1A–C). Intronic miRNAs orientated in
the same direction as the surrounding genes are generally
transcribed coincidentally with their host genes and
excised by the splicing machinery from the larger transcript
in which they are embedded.21 Indeed, intronic miRNAs may
represent a simple way for a protein-coding gene to regulate
other protein-coding genes.

Generally, miRNAs are transcribed by the RNA polymerase
II (Pol II), as a primary transcript, which may be several
kilobases long, bearing hairpin-shaped structures called
pri-miRNAs that transiently receive a 5’ cap and a poly(A)
tail similar to that of the mRNAs.22 Recent studies,
however, have shown that the RNA Pol III drives miRNA tran-
scription from dense human clusters interspersed among
repetitive Alu elements.23

2.2 Initial processing and export from the nucleus

The processing of the pri-miRNA occurs in the nucleus, in
a stepwise fashion, through the activity of a double-stranded
specific endonuclease Rnase III (ds-RnaseIII), Drosha.
Drosha and its partner, the double-stranded RNA binding
domain protein DGCR8, cleave the pri-miRNA near the
hairpin base and release an ~70–90 nt pre-miRNA hairpin-
shaped stem-loop precursor.22,24 An alternative pathway
for pre-miRNA-like hairpin biogenesis has recently been
identified in flies, nematodes, and mammals; this
pathway generates new regulatory RNAs from intronic pre-
nmiRNA precursors called ‘mirtrons’, using the splicing
machinery and the lariat-de-branching enzyme to bypass
Drosha cleavage in initial maturation.25,26 Identification of
several well-conserved mirtrons in different species indi-
cates their relatively ancient incorporation into cellular
regulatory pathways and highlights their likely contribution
to animal evolution.25,26

The subsequent export of the pre-miRNA into the cyto-
plasm is mediated by the exportin-5, a Ran-GTP-dependent
nuclear transport receptor.27 Exportin-5 interacts with the
pre-miRNA ‘minihelix motif’ (consisting of an ~14 nt stem
and a short 3’ overhang), which also stabilizes the pre-
mRNA structure.27–29

2.3 Processing into mature form, activation, and
target recognition

In the cytoplasm, the pre-miRNA is processed by the cyto-
plasmic ds-Rnasell Dicer into an ~22 nt miRNA:miRNA
duplex with 2 nt overhanging its 3’ end.30 The RNA strand
with relatively unstable base pairs at the 5’ end is recruited
as a single-stranded molecule into the RNA-induced silencing
complex (RISC) and is assembled through processes that are
dependent on Dicer, TAR (HIV) RNA binding protein (TRBP),
and dsRNA-binding proteins of the AGO family and by other
factors including helicases, nucleases and RNA binding
proteins.2,6,31–33 When assembled into the RISC complex,
the mature miRNA sequence targets its complementary
mRNA and negatively regulates gene expression by cau-
sing either mRNA translational repression or degradation.
miRNAs recognize their target mRNAs mainly through a limited base-pairing interaction between the 5' end ‘seed’ region (2–8 nt from the 5' end) and the complementary sequences present in the 3'UTRs of phylogenetically conserved target mRNAs. Few animal miRNAs have enough complementarities to mRNAs to allow mRNA cleavage (slicing). The majority of animal miRNAs imprecisely match their targets, thereby causing target mRNA destabilization through ‘non-slicer’ mechanisms, including de-adenylation and other forms of translational repression (e.g. decapping). When targeted for silencing by miRNAs, mRNAs can be concentrated, sequestered from translational machinery, degraded or stored for subsequent use in large macroscopic cytoplasmic foci, named processing bodies (P-bodies). The P-bodies contain a wide range of enzymes involved in RNA turnover, including de-capping enzymes, de-adenylases and exonucleases (Figure 1).

The imprecise matching between animal miRNAs and their targets implies that any given miRNA can bind different mRNAs, which bestows an enormous regulatory potential. The regulatory potential of each miRNA is rendered even more complex by recent evidence indicating that miRNAs can also bind to the 5'-UTR of mRNAs and that translation up-regulation can, in specific conditions, result from their binding to mRNA. In addition, miRNAs with a specific nuclear localization sequence can also be re-localized into the nucleus. In the nucleus, miRNAs can interact with, and regulate, the expression of transcripts transcribed in the upstream regions encoding a particular set of ncRNAs, whose expression is altered in human cancer. These findings point to the possible involvement of miRNA in the transcriptional control of gene expression, as has already been demonstrated for gene regulation by endogenous siRNAs, piRNAs, and antisense RNAs.

3. Regulation of microRNA expression by transcription factors and epigenetic mechanisms

During development, miRNA expression is tissue specific, which means that miRNA may play a role in establishing and maintaining cell type and tissue identity. Moreover, different expression profiling analyses have shown that most miRNAs are controlled by developmental or tissue-specific signalling. Although some miRNAs are controlled at the post-transcriptional level alone, several lines of evidence point to transcriptional regulation as the main step in the control of miRNA expression. However, the structure and the transcriptional regulation of miRNA genes are largely unknown. Computational approaches have yielded information on transcription and sequential processing of miRNAs in regulatory elements present in the upstream region of miRNA genes. These motifs are preferentially located within 1 kb upstream of the genomic region of protein-coding genes and are evolutionarily conserved. Recent work by Saini et al. has indicated regulatory binding sites for transcription factors located slightly upstream of the pre-miRNA gene regions (~60% of these sites are clustered within 1 kb). In addition, their distribution heavily overlaps that of the predicted transcription-starting site (TSS). The predicted TSS for intronic miRNAs may lie predominantly in the −2 to −6 kb region upstream of the pre-miRNA sequence, and may correspond to the annotated TSS of the host transcript. Interestingly, miRNA genes may possess more than one predicted TSS. Indeed, a significant fraction of human intergenic miRNAs are characterized by two separate, prominent peaks containing the predicted TSS, with one in the vicinity of the pre-miRNA sequences (within 2 kb) and the other at a distance of about −10 kb. Putative promoters for several human miRNA genes within 0.5 kb upstream of the pre-miRNA sequences (named ‘core promoter regions’) are believed to contain essential components for the regulation of gene transcription.

Indeed, a growing body of evidence indicates that transcription factors take part in specific cellular pathways of transcriptional regulation of miRNA genes. Generally, these factors activate or repress specific gene expression by interacting directly with their cis-regulatory motifs (8–15 nt long), which are often located in the genomic regions upstream of and near TSS. miRNA upstream regions usually contain a larger number of regulatory motifs if compared with the promoters of protein-coding genes. Moreover, miRNA activities have been found to be linked to transcription factor functions, being present in feedback loops in which they control each other’s function, as described in more detail later in this review.

However, transcription factors are not the only key regulators of lineage-specific gene expression. Epigenetic mechanisms, such as DNA methylation, post-translational modifications of the histone code, and nucleosomal remodelling, all contribute to the modulation of gene expression and to the determination of cell and tissue specificity. DNA methylation, a normal process used by mammalian cells to maintain normal expression patterns, is achieved by the addition of methyl groups to cytosines within CpG dinucleotides (CpGs), frequently gathered in clusters (Cpg Islands), by DNA methyltransferases (DNMTs). Newly methylated CpGs act as docking sites for the recruitment of the DNA-methyl CpG-binding proteins MeCPs (methyl CpG-binding protein 2) and MBDs (methyl binding domain proteins), which stabilize the methylation process for gene silencing. DNMTs, MeCPs, and MBDs are often present in protein complexes with other chromatin remodelling activities, including histone-deacetylases (HDACs), for the transcriptional regulation of gene expression. Recent evidence suggests that miRNA expression is regulated by epigenetic mechanisms. CpG islands are present both upstream and downstream of genomic miRNA coding sequences. A significant portion of these CpG islands (~40%) also overlaps predicted TSS sites. A potential oncogenic role of altered miRNA activities has been reported as a consequence of DNA hypermethylation and histone modification of miRNA genomic regions. This opens a largely unexplored area regarding the epigenetic regulation of miRNA gene expression in normal tissues, tumour development and progression.

4. Transcription factors, microRNAs and lineage specification/differentiation of eukaryotic cells

4.1 Early stages of embryonic development

The essential role of miRNA activity regulation during tissue development is supported by the consequences of the targeted inactivation of Dicer, which results in loss of processed miRNAs and accumulation of miRNA precursors. In zebrafish,
maternal-zygotic Dicer mutants display abnormal morphogenesis during gastrulation, brain formation, heart development, and somitogenesis. It is noteworthy that brain defects can be rescued by miRNA-430 injection.41 In mice, the elimination of Dicer activity causes embryo death before gastrulation. Dicer inactivation in mammalian limb, myogenic compartment, lung, and skin results in severe morphological defects and is associated with aberrant cell proliferation and apoptosis during tissue development.51-55

Interestingly, miRNA genes are among the components of the transcriptional regulatory circuitry believed to be regulated by Oct4, Sox2, and Nanog in human embryonic stem (ES) cells.56 Oct4, Sox2, and Nanog are the earliest-expressed set of genes that play essential roles in early development, maintenance of ES pluripotency and lineage specification.57-59 By genome scale localization analysis (chromatin immunoprecipitation coupled with DNA microarrays), Oct4, Sox2, and Nanog were found to co-occupy the promoters of a large population of genes, mainly encoding transcription factors implicated in developmental processes of human ES cells.56 Oct4, Sox2, and Nanog were also associated with the gene loci of 14 miRNAs and co-occupied the promoters of at least two miRNA genes (miRNA-137 and miRNA-301). Moreover, increased miRNA-134 levels directed mouse ES cell differentiation towards ectodermal lineages through the post-translational attenuation of Nanog expression levels60 (Table 1). More recently, the neuronal repressor element 1 (RE1)-silencing transcription factor (REST) has been reported to play a role in the maintenance of the self-renewal and pluripotency of mouse ES cells through, among other mechanisms, transcriptional suppression of miRNA-21 expression. miRNA-21 itself can suppress ES cell self-renewal by affecting the expression of the key self-renewal regulators Oct4, Sox2, Nanog, and c-myc.61 Bioinformatic approaches have also pointed to the presence of putative miRNA-21-binding sites in Sox2 and Nanog mRNAs61 (Table 1). Taken together, these observations from ES cells lend support to the functional role of miRNAs, their modulators and targets in early physiological networks regulating normal morphogenesis, tissue and organ development.

### 4.2 Skeletal and cardiac muscle

Myogenesis is characterized by the transcriptional activation of muscle-specific genes encoding contractile proteins, metabolic enzymes, ion channels, and neurotransmitter receptors. Transcription of muscle-specific genes is regulated by a set of basic helix-loop-helix muscle regulatory factors (bHLH-MRFs), which include Myf-5, MyoD, myogenin, and MRf4.62 Other more general factors, such as the myocyte enhancer factor 2 (MEF2), are essential for both skeletal muscle and cardiac development.63

Members of the miRNA-1 (miRNA-1-1, miRNA-1-2), miRNA-133 families (miRNA-133a-1, miRNA-133a-2, and miRNA-133b) and miRNA-206 have recently been described in key regulatory circuitries controlling: (i) skeletal muscle cell proliferation and differentiation; (ii) many aspects of cardiac biology, ranging from organogenesis in the embryo to hypertrophy in adulthood.64-67

miRNA-1 and miRNA-133 are specifically expressed in both skeletal muscle and heart, though their abundance differs in distinct stages of development.64-66 The vertebrate genome contains two distinct loci for the miRNA-1 and miRNA-133 bicistronic clusters that give rise to identical mature miRNA sequences. Although miRNA-133a is expressed in the same bicistronic unit as miRNA-1-1 or miRNA-1-2, miRNA-133b is

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Localization</th>
<th>Biological function</th>
<th>Predicted/validated targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>Embryonic stem cells</td>
<td>Self-renewal suppression</td>
<td>Nanog, Sox261</td>
</tr>
<tr>
<td>miRNA-134</td>
<td></td>
<td>Ectodermal lineages differentiation</td>
<td>Nanog60</td>
</tr>
<tr>
<td>miRNA-1</td>
<td>Skeletal and cardiac muscle</td>
<td>Cardiogenesis, myogenesis, hypertrophy</td>
<td>Hand2, HDAC4, Delta64,65</td>
</tr>
<tr>
<td>miRNA-133</td>
<td></td>
<td>Myogenesis, inhibition of skeletal and cardiac hypertrophy</td>
<td>SRF65</td>
</tr>
<tr>
<td>miRNA-206</td>
<td></td>
<td>Myogenesis</td>
<td>B-ind1, Cx43, Mdm, Pola171</td>
</tr>
<tr>
<td>miRNA-208</td>
<td></td>
<td>Expression of β-Mhc, stress-dependent cardiac remodelling</td>
<td>THRAP175</td>
</tr>
<tr>
<td>miRNA-133b</td>
<td>Nervous tissue</td>
<td>Neuron maturation</td>
<td>Pitx378</td>
</tr>
<tr>
<td>miRNA-132</td>
<td></td>
<td>Neuronal morphogenesis</td>
<td>MeCP281</td>
</tr>
<tr>
<td>miRNA-124a</td>
<td></td>
<td>Neuronal identity</td>
<td>MeCP2, REST80</td>
</tr>
<tr>
<td>miRNA-223</td>
<td>Haematopoietic tissue</td>
<td>Granulopoiesis</td>
<td>NFI-A,90 Mef2c101</td>
</tr>
<tr>
<td>miRNA-424</td>
<td></td>
<td>Monocytopenosis</td>
<td>NFI-A94</td>
</tr>
<tr>
<td>miRNA-221/222</td>
<td></td>
<td>Erythropoiesis</td>
<td>klt88</td>
</tr>
<tr>
<td>miRNA-181</td>
<td></td>
<td>B-cell differentiation</td>
<td>Tc11102</td>
</tr>
<tr>
<td>miRNA-17-5p/20a/106a</td>
<td></td>
<td>Monocytopenosis</td>
<td>AML149</td>
</tr>
<tr>
<td>miRNA-17-5p/92</td>
<td>B-cell lymphomas</td>
<td>Cell proliferation</td>
<td>EZF95,99</td>
</tr>
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Abbreviations: Sox2, SRY-related HMG-box2; Hand2, heart and neural crest derivatives expressed 2; HDAC4, histone-deacetylase 4; Delta, notch ligand delta; SRF, serum response factor; B-ind1, butyrate-induced transcript 1; Cx43, connexin 43; Mdm, monocyte-macrophage differentiation-associated protein; Pola1, DNA polymerase α; largest subunit; THRAP1, thyroid-hormone-receptor-associated protein 1; Pitx3, paired-like homeodomain transcription factor; MeCP2, methyl CpG-binding protein 2; REST, RE1-silencing transcription factor; NFI-A, nuclear factor I-A; Mef2c, myocyte-specific enhancer factor 2C; kit, tyrosine kinase receptor; Tc11, T-cell leukaemia/lymphoma; AML1, acute myeloid leukaemia-1. Reference numbers are indicated.
expressed as a separate gene transcript. The molecular mechanisms regulating miRNA-1 and miRNA-133 processing have yet to be fully clarified. Interestingly, miRNA-1 and miRNA-133 share common cis-regulatory motifs and trans-regulation mechanisms, but have distinct biological functions. During skeletal myogenesis and in the developing heart, their tissue specific expression is largely controlled by myogenic transcriptional networks involving transcriptional regulators, such as MEF2, serum response factor (SRF), Myo-D, and myocardin. In skeletal myogenesis, miRNA-1 inhibits myoblast growth, thus favouring the differentiation pathway, whereas miRNA-133 promotes myoblast proliferation, thus suppressing differentiation. miRNA-1 and miRNA-133 expression levels can be regulated by cardiac and skeletal muscle-specific enhancers present upstream of their cluster gene. These enhancers are direct targets of Myo-D and MEF2 in skeletal muscle cells and of SRF in cardiac myocytes. In the developing heart, SRF binding to the pre-miRNA upstream promoter region up-regulates miRNA-1 expression.

In keeping with a cardiac requirement for SRF, mutation of the SRF site in the miRNA-1-2 regulatory region disrupts its cardiac expression. In cardiac myocytes, SRF induces the expression of miRNA-133, which in turn inhibits SRF itself in a feedback regulatory loop that affects differentiation and promotes progenitor proliferation. In skeletal muscle progenitors, miRNA-133 enhances myoblast proliferation by targeting SRF, whereas miRNA-1 promotes myogenesis by targeting the HDAC4. HDAC4 is a chromatin-remodelling enzyme that acts as a transcriptional repressor of MEF2, which is present in protein–protein complexes with Myo-D at muscle-specific and growth factor-inducible gene-promoter sites. Thus, miRNA-1 and miRNA-133, and their targets SRF and HDAC-4, participate in key negative feedback regulatory loops, thereby contributing to appropriate decisions regarding the proliferation and differentiation status of skeletal and cardiac muscle progenitors (Figure 2A). However, a variety of key regulators of cardiac and skeletal myogenesis, besides SRF and HDAC4, are targets of miRNA-1 and miRNA-133 activities, including Notch ligand Delta and Hand2 (a basic HLH transcription factor that promotes ventricular cardiomyocyte expansion).

Moreover, there is evidence supporting the regulation of other miRNAs by different myogenic regulators in the control of the muscle differentiation program, including: (i) the ability of myogenin and Myo-D to bind the genomic upstream sequences of different miRNA genes (e.g. miRNA-100, miRNA-191, miRNA-138-2, and miRNA-22); (ii) the induction of miRNA-206, a close homolog of miRNA-1 that is specifically expressed in skeletal muscle tissue and is measurable during myoblast–myotube transition of the mesenchymal C2C12 cell line. Interestingly, miRNA-206 is transcriptionally activated by Myo-D and affects myogenesis by reducing the levels of DNA polymerase, an important component of the differentiation program, and of Id1-3 and MyoR, two inhibitors of myogenic transcription factor activities.

Thus, miRNAs may be present in gradients of signalling and transcription factor networks that govern key aspects of skeletal and cardiac myogenesis and whose de-regulation may result in disease. Indeed, the knockdown of miRNA-133 expression causes cardiac hypertrophy in vivo, whereas miRNA-1 cardiac-specific over-expression in the embryonic heart inhibits cardiomyocyte proliferation and prevents expansion of the ventricular myocardium. Thus, miRNA-1, miRNA-133 as well as other miRNAs (e.g. miRNA-195 and miRNA-208, the latter encoded by an intron of the αMHC gene), are emerging as active players in cardiac disease and, therefore, as potential targets for therapeutic intervention.

4.3 Nervous tissue

Initial work in C. elegans highlighted the involvement of miRNAs in transcriptional regulatory circuits controlling nervous system patterning. Later studies revealed that neural progenitors sequentially express different miRNAs, resulting in the regulation of lineage-specific genes during mammalian neurogenesis. In mammalian adult brains, different miRNAs have displayed marked lineage specificity.

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**Examples of putative or established circuitry involving miRNAs and contributing to phenotypic cell determination**

**Figure 2** Transcriptional activation (curvy arrow), repression (curvy bold line), and post-transcriptional repression (curvy dotted line) of microRNAs (miRNAs) and/or transcription factors functioning in integrated regulatory loops. Reference numbers are indicated.
miRNA-124 and miRNA-128 are expressed at the highest levels in neurons; miRNA-26 and miRNA-29 are more strongly expressed in astrocytes than in neurons, while miRNA-23 expression is restricted to astrocytes. miRNA-133b is expressed in adult mammalian midbrain dopaminergic neurons. miRNA-133b regulates the maturation and function of midbrain dopaminergic neurons, being part of a negative feedback circuit that includes the paired-like homeodomain transcription factor Pitx3, a known regulator of neurons gene expression. Indeed, miRNA-133b is a direct transcriptional target of Pitx3 and its induction post-transcriptionally suppresses Pitx3, which results in a functional fine-tuning circuit for dopaminergic behaviours (e.g. locomotion) (Figure 2B). Worthy of note is the fact that miRNA-133b is deficient in midbrain tissue from patients with Parkinson’s disease.

miRNA-132 expression is strongly enhanced in neurons. miRNA-132 is transcribed from a conserved genomic region that contains, upstream of the pre-miRNA sequence, a cyclic AMP-response element recognized by the basic leucin zipper transcription factor CREB. CREB mediates the neurotrophin-dependent neuronal morphogenesis by binding to this consens sequence, which is present on its protein coding and non-coding target gene promoters. miRNA-132 expression is specifically increased by the brain-derived neurotrophic factor (BDNF) through the activation of CREB. However, BDNF gene is targeted by MeCP2, while MeCP2 translation is repressed by the brain-derived neurotrophic factor (BDNF) through the activation of CREB. Indeed, if MeCP2 levels increase, so do BDNF levels, the result being that miRNA-132 is induced and MeCP2 mRNA translation is repressed (Figure 2C). This negative feedback loop, involving the transcriptional regulation of miRNA-132, has been proposed as a regulatory mechanism for the homeostatic control of MeCP2 protein during neuronal morphogenesis. Interestingly, as a highly conserved CpG island is present on the entire miRNA-132 locus, both increases and decreases in MeCP2 levels can cause neurodevelopmental defects.

Neuronal gene expression is transcriptionally silenced in non-neuronal cells by the REST factor. REST is a zinc-finger protein that mediates epigenetic silencing of target genes through its interaction with a conserved 23 nt RE1, where it recruits HDAC and MeCP2 activities. Among the transcriptional targets of REST, there are the upstream genomic regions of different pre-miRNAs, including that of the brain-specific miRNA-124a, which is highly expressed in the mature nervous system. In both neural progenitors and non-neuronal cells, REST inhibits miRNA-124a gene expression, which in turn results in the selective up-regulation of non-neuronal transcripts. During progenitor differentiation into mature neurons, REST leaves miRNA-124a gene loci, and non-neuronal transcripts are selectively degraded by the activation of this miRNA. Thus, the establishment and maintenance of neuronal identity appears to require both the transcriptional re-activation of REST-regulated genes and the post-transcriptional down-regulation of non-neuronal transcripts by miRNA-124a, whose expression levels are controlled by REST itself.

4.4 Haematopoiesis

Haematopoiesis is a life-long, highly regulated multistage process where a pluripotent self-renewing haematopoietic stem/progenitor cell (HSC) gives rise to all blood cell lineages. HSC self-renewal capacity as well as the growth and maturation of erythroid, granulocytic, and monocytic and megakaryocytic lineages are largely controlled by unique combinations of lineage-specific transcription factors, including AML1 (acute myeloid leukaemia-1), C/EBPα (CCAAT/enhancer-binding protein α), MAFB (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B) and PU.1, which cooperatively regulate promoters or enhancers present on their specific target genes. In mammalian cells, specific miRNAs whose expression varies during haematopoiesis have recently been identified. The relevance in haematopoietic lineage specification of some miRNAs has also been highlighted by the consequence of their silencing/ectopic expression in haematopoietic progenitor cells (Table 1). Down-regulation of miRNA-221 and miRNA-222 is required for erythropoietic differentiation, and that of the miRNA-17-5p-92 and 106a clusters for monocytogenesis. Ectopic expression of miRNA-181 increases the fraction of B-lymphoid cells, whereas increased miRNA-223 levels reprogram granulocytic differentiation. During granulocytic differentiation of human myeloid progenitors, miRNA-223 levels are modulated at the transcriptional level by the competitive binding of two transcription factors, the nuclear factor I (NFI-A) and the C/EBPα, for CAAT elements present on the pre-miRNA-223 sequence. NFI-A transcriptional activity maintains miRNA-223 at low levels, while its replacement by C/EBPα results in miRNA-223 up-regulation and granulocytic differentiation. It is noteworthy that C/EBPα is a transcription factor involved in myeloid differentiation, whereas NFI-A has been implicated in replication and in cell growth control. The relevance of NFI-A for myelopoiesis is supported by recent findings indicating NFI-A as a translational target of miRNA-424, a miRNA directly induced by the master regulatory factor PU.1 during human monocyte/macrophage differentiation (Figure 2E).

Another transcriptional regulatory loop that is relevant to monocytic-macrophage differentiation and maturation of human haematopoietic progenitors involves the transcriptional regulation of the miRNA 17-5p-92 and 106a-92 cluster promoters by AML1. AML1 is a transcription factor that is widely expressed in multiple haematopoietic lineages and regulates the expression of a variety of haematopoietic genes. AML1 is also a translational target of miRNA 17-5p/20a/106a activation. In early haematopoietic progenitors, miRNA 17-5p/20a/106a repress AML1 translation, thus maintaining their undifferentiated status. In contrast, during the monocytic-macrophage maturation of haematopoietic precursors, miRNA 17-5p/20a/106a is down-regulated, thereby unblocking AML1 translation. Increased AML1 levels induce the transcriptional silencing of the miRNA 17-5p-92 and 106a-92 clusters through the binding of AML1 to its site in promoter regions. This leads to a negative transcriptional regulatory loop, which further increases AML1 translation (Figure 2F). Thus, during lineage specification of haematopoietic multipotent progenitors, feedback transcriptional regulatory pathways may allow fine titration of the levels of both transcriptional regulatory factors and miRNA, whose key role in normal haematopoiesis is only now emerging. Therefore, the transcriptional or epigenetic de-regulation of pathways involving miRNA genes may be relevant for the
pathogenesis of haematological diseases. In keeping with this hypothesis, the myelopoesis-regulator miRNA-223 has been reported to be a key transcription target of the AML1/ETO oncoprotein, the fusion product of the t(8;21), which is the most frequent chromosomal translocation associated with acute myeloid leukaemia.\textsuperscript{50} AML1/ETO triggers the heterochromatic silencing of the miRNA-223 gene through its interaction with an AML1 binding site present upstream of the pre-miRNA-223. Here, AML1/ETO recruits HDAC and DNMT activities that de-acetylate histone proteins and methylate sparse CpGs, all assembled in the upstream, body and downstream regions of the pre-miRNA-223 sequence. Newly methylated CpGs act as docking sites for the DNA-methyl CpG binding protein MeCP2. Through the silencing of the miRNA-223 gene, the AML1/ETO associated complex contributes to the differentiation block of myeloid progenitors, thus linking the epigenetic silencing of a miRNA locus to leukaemogenesis.

Various bodies of evidence point to a functional pathogenic role of de-regulated transcriptional pathways involving miRNA genes in haematopoietic cells. Among these, the enforced expression of the miRNA 17-5p-92 cluster in conjunction with its transcriptional regulator c-myc resulted in mouse B-cell lymphomas which were characterized by the absence of the high apoptosis levels normally associated with c-myc-induced lymphomas.\textsuperscript{94} Interestingly, both c-myc and miRNA-17-5p-92 influence cell proliferation and apoptosis and possess oncogenic functions. The activation of the oncogenic transcription factor c-myc induces the expression of miRNAs within the 17-5p-92 cluster through the direct binding to its recognition sites (E-boxes), present in conserved upstream regulatory regions.\textsuperscript{95,96} Worthy of note is the fact that two of the miRNAs included in the miRNA-17-5p-92 cluster, miRNA-17-5p, and miRNA-20a, act as negative regulators of the expression of the transcription factor E2F1. E2F1 is one of the main drivers of G1/S transition during the cell cycle. In addition, c-Myc and E2F1 can activate each other’s transcription, thereby establishing a network in which c-Myc activates the transcription of E2F1, which simultaneously inhibits its translation.\textsuperscript{97,98} Thus, upon c-myc activation, miRNA-17-5p-92 may act as a brake on the c-myc/E2F1 positive feedback loop to ensure tightly controlled expression of these two proteins.\textsuperscript{98} Moreover, two consensus sequences for E2F binding present on the miRNA-17-5p-92 promoter recruit all three E2F family members for transcriptional activation,\textsuperscript{99,100} thus rendering the regulatory networks involved in the control of proliferation, apoptosis, and tumorigenesis even more complex (Figure 2G).

5. Concluding remarks

Evidence is emerging that transcription factors, chromatin remodelling activities and miRNAs ultimately determine the correct organization of cell-type specific gene arrays, which control developmental timing, tissue differentiation and maintenance of tissue identity. The fact that miRNAs repress the expression of multiple target genes suggests that their biogenesis, processing and activity is crucially controlled and integrated into the molecular pathways governing these physiological processes. Indeed, miRNAs participate in complex epigenetic regulatory circuitries, involving cis-acting elements for different transcription factors in their putative promoter regions. Moreover, putative binding sites for distinct miRNAs are present in the 3' UTR of transcription factor miRNAs. The transcriptional/epigenetic regulation of miRNA genes and the factors responsible for their tissue-specific expression have only recently started emerging as a unitary mechanistic model for cell fate decision. Thus, one challenge for the future will be to unravel the contribution of individual signal cascades in the complex and highly controlled regulatory networks that are responsible for the differential expression of genes and dictate cell phenotypic changes throughout development and during adult life, as the de-regulation of such networks might explain the pathogenesis of a number of diseases.

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MicroRNA and regulatory networks for cell fate determination


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