



MicroRNAs and Long Non-coding RNAs in Genetic Diseases

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Abstract

Since the discovery and classification of non-coding RNAs, their roles have gained great attention. In this respect, microRNAs and long non-coding RNAs have been firmly demonstrated to be linked to regulation of gene expression and onset of human diseases, including rare genetic diseases; therefore they are suitable targets for therapeutic intervention. This issue, in the context of rare genetic diseases, is being considered by an increasing number of research groups and is of key interest to the health community. In the case of rare genetic diseases, the possibility of developing personalized therapy in precision medicine has attracted the attention of researchers and clinicians involved in developing “orphan medicinal products” and proposing these to the European Medicines Agency (EMA) and to the Food and Drug Administration (FDA) Office of Orphan Products Development (OOPD) in the United States. The major focuses of these activities are the evaluation and development of products (drugs, biologics, devices, or medical foods) considered to be promising for diagnosis and/or treatment of rare diseases or conditions, including rare genetic diseases. In an increasing number of rare genetic diseases, analysis of microRNAs and long non-coding RNAs has been proven a promising strategy. These diseases include, but are not limited to, Duchenne muscular dystrophy, cystic fibrosis, Rett syndrome, and β -thalassemia. In conclusion, a large number of approaches based on targeting microRNAs and long non-coding RNAs are expected in the field of molecular diagnosis and therapy, with a facilitated technological transfer in the case of rare genetic diseases, in virtue of the existing regulation concerning these diseases.

Key Points

Since non-coding RNAs' discovery and classification, their roles in genetic diseases have gained great attention.

Both microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) can be considered excellent markers for advanced diagnostic protocols.

Both miRNAs and lncRNAs are suitable targets for therapeutic intervention.

Diagnostics based on miRNA and lncRNA profiles might be a key factor for the development of personalized therapy in precision medicine of genetic diseases.

1 Key Roles of Non-coding RNAs in Gene Regulation

The expression of eukaryotic genes is a multistep and complex process, finely regulated by many different factors and levels of control [1], including (but not limited to) transcription factors (TFs), promoter sequences and enhancers [2–4], post-transcriptional regulation, and control of translation [5]. In this context, non-coding RNAs (ncRNAs) play a very important role [6–8]; they are composed of several classes, among which microRNAs (miRNAs) [9–11] and long non-coding RNAs (lncRNAs) [12–16] are of key interest.

MicroRNAs (19–25 nucleotides in length) are a family of small ncRNAs with the important function of inhibiting or reducing protein synthesis after formation of complexes with target messenger RNAs (mRNAs) through base pairing [9–11, 17, 18]. Target mRNAs can hybridize to complementary miRNAs at the 3'-untranslated region (3'-UTR), coding sequences (CDS) and 5'-untranslated region (5'-UTR) [17, 18]. When this biological function is considered in its deep complexity, it can be concluded that more than 60% of human mRNAs are regulated by miRNAs [17, 18]. The

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miRNA/mRNA interaction causes translational repression or mRNA degradation, depending on the degree of complementarity at the level of RNA-induced silencing complex (RISC) [19, 20]. At present, the number of human miRNAs identified and deposited in the miRBase databases (miRBase v.22, <http://www.mirbase.org>) is more than 2500 [21–23]. Several reports conclusively support the concept that miRNAs are involved in the onset and progression of rare genetic diseases [24–32].

As already mentioned, lncRNAs have been also deeply implicated in the control of gene expression [12–16]. lncRNAs are characterized by a length of more than 200 nucleotides, and their effects on gene expression in the development of diseases are based on a variety of mechanisms of action, including recruitment of chromatin modifiers, controlled recruitment of TFs (both activators and repressors of gene transcription), regulation of RNA splicing, regulation of chromosome looping, control of mRNA translation and decay, and miRNA sponging [17–21]. Figure 1 summarizes the interplay between miRNAs, TFs, and lncRNAs.

2 Non-coding RNAs as Possible Targets in Theranostics of Genetic Diseases

While miRNAs are firmly established as being involved in rare genetic diseases [24–32], examples of lncRNAs affecting gene expression in these diseases are fewer, but this research field is rapidly growing. Two major issues are related to the finding that ncRNAs are possible markers of rare genetic disease: (1) molecular diagnosis based on ncRNAs might open new avenues in the fields of diagnosis, prognosis, patient stratification, and response to therapy; (2) identification of molecular targets will help in developing

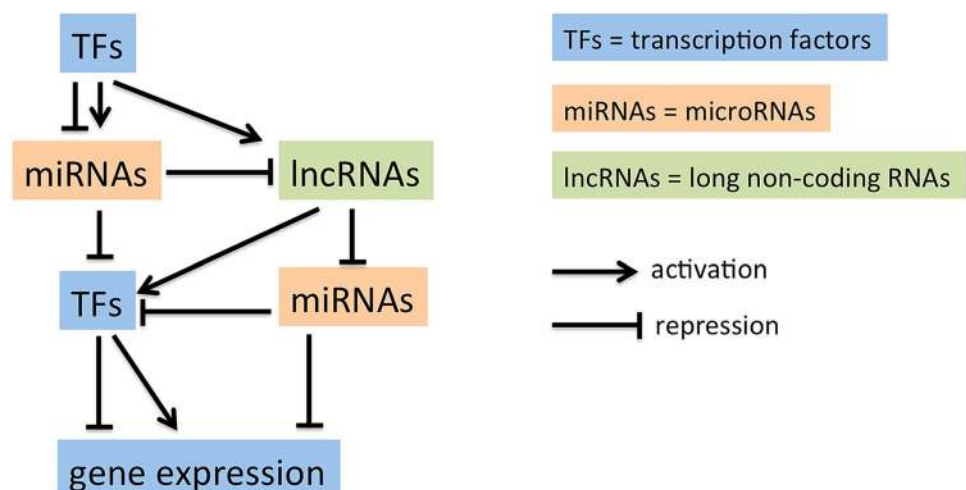
novel therapeutic approaches, possibly leading to novel strategies in precision medicine.

In the field of molecular diagnosis, in addition to well-established technologies, ncRNAs are very important markers for non-invasive diagnostic approaches, having implications in diagnosis and/or prognosis. Among these approaches, we would like to mention molecular analyses, which are an alternative to standard biopsies, known as “non-invasive liquid biopsies”, and aim to quantify and determine ncRNA patterns in body fluids (Fig. 2). For instance, the characterization of circulating cell-free miRNAs in serum helps in concluding diagnostic and prognostic assessments, based on the fact that disease-specific miRNAs can be identified in extracellular body fluids (such as serum, plasma, and saliva), supporting their potential as biomarkers [24–27, 30, 33–35]. The presence of miRNAs in body fluids is based on the fact that extracellular miRNAs are protected by exosome-like structures and small intraluminal vesicles released by a large variety of cells [36, 37].

Also lncRNAs were reported to be of great interest for liquid biopsy, especially in the field of diagnostics of cancer [38–40]. While circulating lncRNAs have been proposed as useful markers in Crohn’s disease [41], gynecological diseases [42], coronary artery disease [43], Sjögren’s syndrome [44], and osteoporosis [45], their application to stratification of patients affected by rare genetic diseases is under investigation and limited to a few examples [46–49].

With respect to translational medicine, miRNAs are suitable targets for therapeutic intervention, as summarized in Fig. 3 [50–57]. A forced decrease of miRNA activity can be obtained using miRNA-inhibitor oligomers (such as in miRNA antisense therapy based on RNA, DNA, locked nucleic acid [LNA] and other DNA analogs) [58–63], miRNA sponges [64–66] and mowers [67], or through miRNA masking that interferes with miRNA function by masking the miRNAs binding site of target mRNAs through

Fig. 1 Interplay between miRNAs, TFs and lncRNAs. *miRNA* microRNA, *lncRNA* long non-coding RNA, *TF* transcription factor



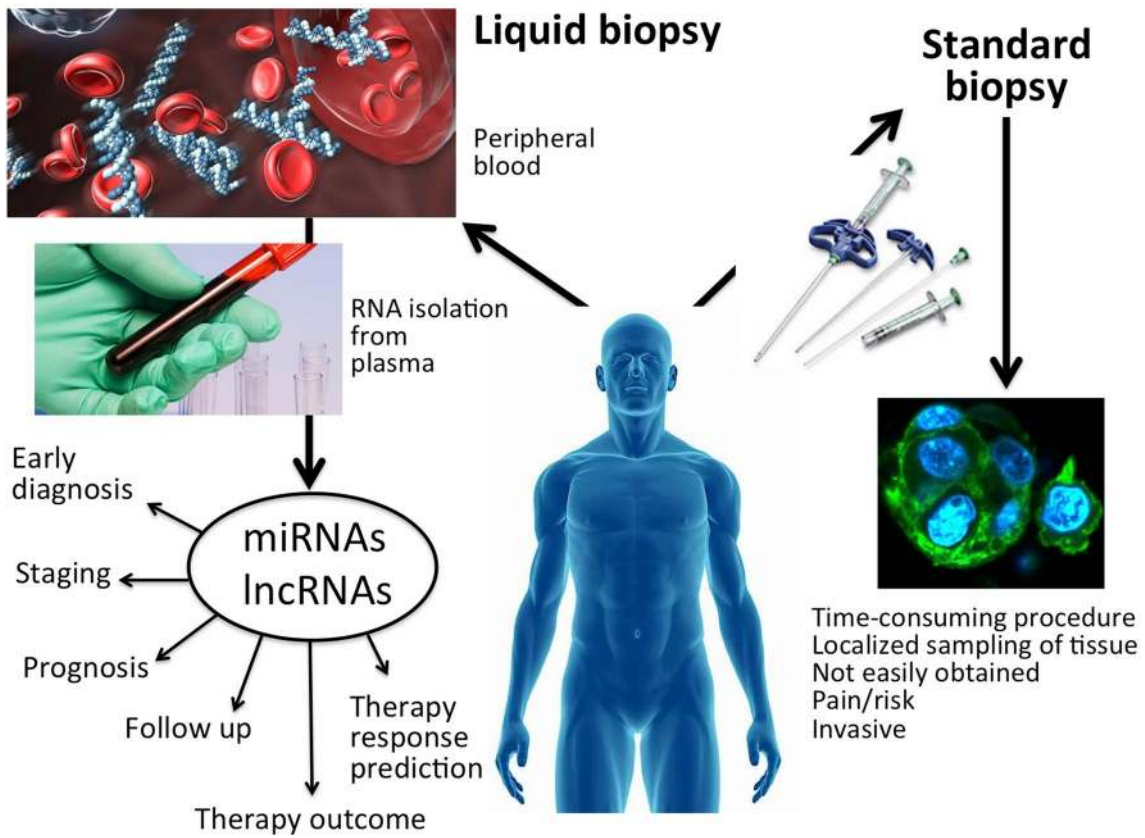
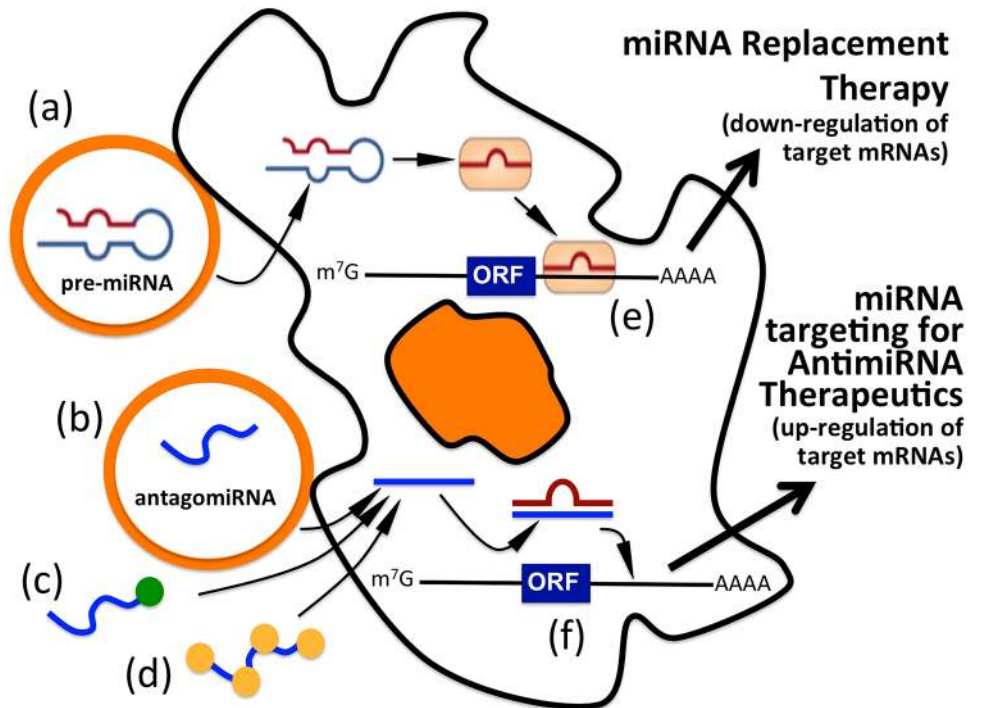


Fig. 2 Liquid biopsy versus standard biopsy in human pathologies. Analysis of non-coding circulating RNAs is helpful for patient stratification and to verify disease staging and response to treatment. The

liquid biopsy approach has been applied to some rare genetic diseases, such as DMD. *DMD* Duchenne muscular dystrophy

Fig. 3 Anti-miRNA therapy and miRNA replacement therapy. *miRNA* microRNA



hybridization with complementary molecules [68, 69]. In contrast, the use of modified miRNA mimetics, either synthetic or produced by plasmid or lentiviral vectors, might lead to potentiation of miRNA function (miRNA replacement therapy) [70–73].

Targeting lncRNAs is a more complicated process and largely depends on the mechanism of action of the studied lncRNA. Inhibition of the recruitment of chromatin modifiers and/or TFs can be achieved with molecules altering the scaffold of lncRNAs; antisense molecules can be employed for alteration of the lncRNA-mediated regulation of RNA splicing, regulation of chromosome looping, and control of mRNA translation and decay; inhibition of miRNA sponging can be obtained either by knocking down lncRNAs with the use of antisense molecules activating RNase H or by competing with the lncRNA/miRNA interaction.

3 Examples of Non-coding RNAs in Rare Genetic Diseases

In this section, we will consider selected examples of miRNAs and lncRNAs that could be considered biomarkers of rare genetic diseases. The involvement of ncRNAs has

been proposed in studies based on a variety of biological samples, including tissue samples, primary cells (including stem cells) and body-derived fluids (including serum, plasma, and saliva). When available, examples of targeting of ncRNA for therapeutic protocols development will be considered. Table 1 [74–116] and Table 2 [46–48, 117–130] report a more extensive analysis of the available examples of miRNAs (Table 1) and lncRNAs (Table 2) in rare genetic diseases.

3.1 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a lethal X-linked neuromuscular disease caused by mutations in the dystrophin gene [131]. DMD is the most common muscular dystrophy affecting children and, characterized by a rapid progression of muscle degeneration, causing, in the absence of medical intervention, loss of ambulation and death within the second decade of life. Li et al. applied the “liquid biopsy strategy” to DMD and demonstrated that circulating miRNAs are biomarkers useful for the assessment of the clinical status of DMD patients [132]. This possibility is relevant for the development of non-invasive biomarkers in diagnostic/prognostic protocols and very useful for the replacement of

Table 1 Examples of miRNAs in rare genetic diseases

Genetic disease	OMIM	miRNAs	References
Duchenne muscular dystrophy	310200	miR-1, miR-21, miR-29, miR-30c, miR-31, miR-133, miR-181a, miR-206, miR-208a, miR-208b, miR-499	[24–27, 74–82]
Myotonic dystrophy (type 1)	160900	miR-1, miR-133a/b, miR-206	[83, 84]
Familial dysautonomia		miR-203a-3p	[85]
Amyotrophic lateral sclerosis	105400	miR-143-3p, miR-206, miR-208b, miR-374b-5p, miR-499	[24, 86]
Ullrich congenital muscular dystrophy	254090	miR-30c, miR-181a	[87]
Cystic fibrosis	219700	miR-9, miR-93, miR-145-5p, miR-181b, miR-454, miR-509-3p	[88–96]
Rett syndrome	312750	miR-29b, miR-92, miR-122a, miR-130, miR-146a, miR-146b, miR-199a, miR-199b, miR-221, miR-296, miR-329, miR-342, miR-382, miR-409	[24, 97]
Pulmonary arterial hypertension	178600	miR-9, miR-124, miR-130, miR-206	[98]
Facioscapulohumeral muscular dystrophy	158900	miR-411	[99]
Sézary syndrome	–	miR-18a, miR-21, miR-31, miR-199a2, miR-214, miR-233, miR-342, miR-486	[24, 31, 100–102]
Lesch–Nyhan disease	300322	miR-9, miR-181a, miR-187, miR-424	[103]
Multiple osteochondromas	133700	miR-21, miR-140, miR-145, miR-195, miR-214, miR-451, miR-483	[24, 104]
Hailey–Hailey disease	169600	miR-99a, miR-106, miR-125b, miR-181a	[105]
Li–Fraumeni syndrome	151623	miR-605	[106]
Hepatoblastoma	114550	miR-125a, miR-148a, miR-150, miR-214, miR-199a, miR-492	[24, 107]
MELAS syndrome	540000	miR-9	[108]
X-Chromosomal schizophrenia	181500	let-7f-2, miR-188, miR-325, miR-509-3, and miR-510, miR-660	[109]
β-Thalassemia	613985	miR-15a, miR-16-1, miR-26b, miR-96, miR-144, miR-155, miR-181a/c, miR-210, miR-320, miR-451, miR-486-3p, miR-503	[110–116]

MELAS mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, miRNA microRNA, OMIM Online Mendelian Inheritance in Man

Table 2 Examples of lncRNAs in rare genetic diseases

Genetic disease	OMIM	lncRNAs	References
Duchenne muscular dystrophy	310200	linc-31, linc-MD1	[46, 117, 118]
Myotonic dystrophy (type 1)	160900	MALAT1	[119]
Amyotrophic lateral sclerosis	105400	NEAT1_2	[47, 120]
Cystic fibrosis	219700	XIST, TLR8, HOTAIR, MALAT1, TLR8-AS1, BLACAT1, MEG9, BGas	[121–123]
Rett syndrome	312750	AK081227, AK087060	[124]
Pulmonary arterial hypertension	178600	MEG3, LncRPT	[125, 126]
Facioscapulohumeral muscular dystrophy	158900	DBE-T	[127]
MELAS syndrome	540000	LINC01405, SNHG12, RP11-403P17.4, CTC-260E6.6, RP11-357D18.1	[128]
β-Thalassemia	613985	DQ583499, XIST, lincRNA-TPM1, MRFS16P, lincRNA-RUNX2-2, HMI-LNCRNA, NR_001589, NR_120526, T315543	[48, 129, 130]

lncRNA long non-coding RNA, *MELAS* mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, *OMIM* Online Mendelian Inheritance in Man

invasive muscle biopsy in DMD patients. Serum levels of six muscle-specific miRNAs (miR-1, miR-206, miR-133, miR-499, miR-208a, and miR-208b, also known as myomiRs) were analyzed by Li et al. [132] and found to be all elevated in DMD patients. More recently, digital reverse transcription polymerase chain reaction (RT-PCR) was employed for quantification of miR-30c and miR-181a and validated for detection of these DMD serum biomarkers [34]. According to this and similar studies, a limited and validated list of “dystromirs” (miR-1, miR-133a, miR-133b, miR-206, and miR-31) have been proposed as serum biomarkers not only for monitoring the disease severity in DMD [27], but also to monitor progression of the disease and response to therapies [26]. Moreover, the study of the miRNA pattern allowed identification of strong differences between Becker’s muscular dystrophy and DMD patients [33]. In line with these findings, three serum biomarkers, miR-1, miR-133a, and miR-206, were characterized for limb-girdle muscular dystrophy, Becker’s muscular dystrophy and facioscapulohumeral muscular dystrophy [33].

With respect to possible applications in therapy, in vitro approaches have demonstrated that miRNA therapy can be successfully combined with other strategies. For instance Hildyard and Wells reported that miR-31 targeting might be of great interest [133], considering that translational suppression of dystrophin mRNA is caused by miR-31, which is elevated in the muscle of DMD patients. Therefore, they verified whether oligonucleotide-mediated exon skipping (leading to increase of dystrophin mRNA) could be conducted together with inhibition of miR-31 activity (leading to stabilization of the increased dystrophin mRNA levels), obtaining, at least in cell culture models, increased dystrophin production. Another very interesting approach involving miRNA targeting in DMD was recently reported by Mishra et al., and was based on the observation that the overexpression of utrophin, the autosomal homolog of

dystrophin, can to some extent compensate for the dystrophin absence [134]. Since several miRNAs (including let-7c) are able to inhibit utrophin mRNA, the hypothesis by Mishra et al. was that a forced inhibition of utrophin/let-7c interaction was potentially able to “repress the repression,” therefore increasing utrophin expression [134]. Following this hypothesis, an oligonucleotide was designed and validated for efficient annealing with the 3′-UTR of the utrophin mRNA. This was associated with inhibition of let-7c miRNA binding, upregulation of utrophin expression, and improvement of the dystrophic phenotype in vivo. A final remark is related to the opposing roles of different miRNAs on the progression of fibrosis in DMD. Zanotti et al. found that miR-21 and miR-29 were differentially regulated in muscle biopsies from DMD patients [135]. In Duchenne muscle biopsies, miR-21 expression was significantly increased, and correlated directly with transcript levels of the *COL1A1* and *COL6A1* genes. On the other hand, miR-29a and miR-29c were significantly reduced in Duchenne muscle and myoblasts, and miR-29 target transcripts, *COL3A1*, *FBN1* and *YY1*, significantly increased. Therefore miR-21 silencing on one hand and miR-29 mimicking on the other were able to reduce fibrosis in mdx mice, suggesting that pharmacological modulation of their expression has therapeutic potential for reducing fibrosis in this condition [135].

The involvement of lncRNAs in DMD was the subject of a limited number of studies, based on novel technologies such as transcriptome sequencing (RNA-seq), which highlight the possible importance of these novel RNAs [46]. For example, Ballarino et al. showed that the human lncRNA linc-MD1 is expressed during early stages of normal myoblast differentiation as well as in human primary myoblasts from DMD patients [117]. Twayana et al. clarified that its mechanism of action was based on “sponging” miR-133 and miR-135 [118]. Interestingly, linc-MD1 is also the host transcript of miR-133b, and their biogenesis

is mutually exclusive. Furthermore, Bovolenta et al. identified a variety of novel lncRNAs (both sense and antisense), whose expression mirrored that of the DMD gene [136]. These transcripts are intronic in origin, specifically localized within the nucleus, and are transcribed contextually with dystrophin isoforms or primed by MyoD-induced myogenic differentiation.

3.2 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motor neurons, denervation of target muscles, muscle atrophy, and paralysis [137]. Potential therapeutic targets for ALS were reported by Di Pietro et al., who found that changes in the expression of miR-206, miR-208b and miR-499 occur in the skeletal muscle of patients during progression of the ALS disease [29], and by Waller et al., who reported that serum miRNAs miR-206, 143-3p and 374b-5p are promising ALS biomarkers [30]. For the therapeutic point of view, it was reported that deficiency of miR-206 in ALS mouse models accelerates disease progression [86], supporting the concept that efficient regeneration of neuromuscular synapses after acute nerve injury requires miR-206. Therefore, miR-206 mimicking could be beneficial in ALS therapy.

A few reports are available on lncRNAs in ALS [47, 120, 138]. In this respect, Gagliardi et al. performed a whole transcriptome RNA-seq analysis to investigate the regulation of non-coding and coding RNAs in sporadic ALS (SALS) patients, mutated ALS patients and peripheral blood mononuclear cells (PBMC) isolated from matched controls [47]. A total of 293 differentially expressed lncRNAs were found in SALS patients, whereas a limited number of lncRNAs were deregulated in mutated patients [47]. These data highlighted the importance of extending the knowledge on the significance of regulatory lncRNA classes in the understanding of ALS disease. This study offers a starting point for new investigations into the pathogenic mechanisms involved in ALS disease.

3.3 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder affecting in addition to the lungs, other organs, such as the pancreas, liver, kidneys, and intestine [139]. As a result of frequent and heavy lung infection, CF patients exhibit important difficulty in breathing and frequent coughs with extensive mucus [140]. CF is caused by several classes of mutations of the gene for the CF transmembrane conductance regulator (CFTR) protein. With respect to the CF pathology, miRNA-based epigenetic regulation of expression of the *CFTR* gene and CFTR regulators has been recently proposed by different groups after extensive

studies performed using CF primary bronchial epithelial cells in vitro or from bronchial brushings ex vivo [88–96]. The general conclusion is that miRNAs are involved in CF. For instance, the expression of miR-145 and miR-494 was found anti-regulated with that of *CFTR* gene [88]. The interplay between miRNAs and TFs regulating the *CFTR* gene is supported by a study focusing on genome-wide expression of miRNAs in primary non-CF bronchial epithelial cells and demonstrating reduced expression of miR-138, a downregulator of SIN3A (a transcriptional repressor of *CFTR*) [90]. A direct effect of miRNAs on CFTR expression was on the other hand supported by the finding of a high expression in CF cells of miR-494 and miR-509-3p (able to interact with the 3'-UTR sequence of the CFTR mRNA) [91]. These in vitro findings were confirmed in ex vivo analyses demonstrating an increased expression of miR-494, miR-223 and miR-145 in CF brushings of airway cells [92]. Taken together, different miRNAs whose expression was found changed in CF primary bronchial epithelial cells can reduce *CFTR* expression, either by direct (miR-145, miR-223, miR-494, and miR-509-3p) or indirect (miR-138) effects. In this respect, it should be underlined that in addition to direct interaction with CFTR mRNA, miRNAs might regulate CFTR through binding to 3'-UTR sequences of mRNAs coding CFTR regulators, such as a large series of proteins known to interact with the CFTR protein in the quality-control mechanisms to regulate its folding and misfolding [141]. Among the so-called “CFTR interactome”, some proteins have been shown to interact with CFTR to stabilize its expression on the apical membrane of the epithelial cells [142, 143]. CFTR regulators include, but are not limited to, NHERF1, NHERF2, ezrin, PDZK1, PDZK2, and Shank2. The data presented by Fabbri et al. [93] showed that a peptide nucleic acid (PNA) directed against miR-145-5p causes miR-145-5p inhibition and increases the miR-145-5p regulated CFTR, after quantification of CFTR expression by quantitative reverse transcription PCR (RT-qPCR) and Western blotting. Therefore, miRNA targeting in CF might be considered as a useful strategy to increase CFTR content, supporting the possible application in the personalized therapy of cystic fibrosis. With respect to miR-145-5p involvement in CFTR expression, we have to underline that the promoter of this miRNA is under strong negative control, one of the involved TFs being Tcf4, as schematically represented in Fig. 4 [93, 144]. Interestingly, Tcf4 binds to an intronic enhancer present in the intron I of the *CFTR* gene [145]. Therefore, low expression of Tcf4 (as it occurs in CF) [146–148] might lead to low transcription of the *CFTR* gene, increased miR-145-5p transcription, and high miR-145-5p—dependent down modulation of CFTR mRNA. From the theoretical point of view, miR-145 might cause an increase of CFTR in consideration of the fact that miR-145-5p downregulates Sp1 [149], a TF modulating *CFTR*

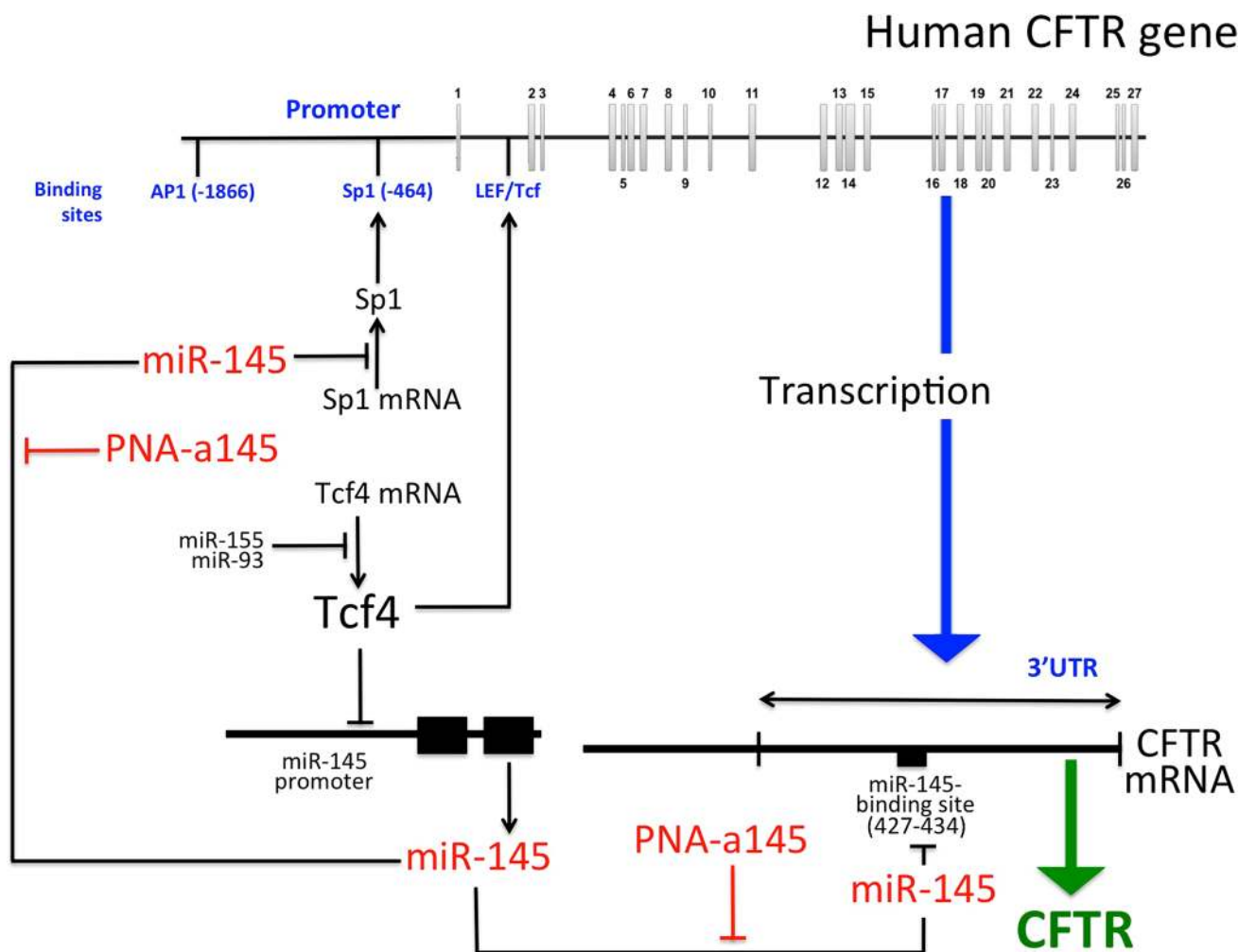


Fig. 4 The proposed interplay between transcription factors, CFTR regulation, miR-145-5p transcription and miR-145-5p target molecules [93]. This might have important implications for drug design,

suggesting development of approaches able to perform multi-targeting of microRNAs involved in complex activities. *CFTR* cystic fibrosis transmembrane conductance regulator

gene transcription (see Fig. 4). Therefore, PNAs targeting miR-145-5p might interfere with the direct binding of miR-145-5p to the 3'-UTR CFTR mRNA sequences; in addition, the same PNA might indirectly potentiate CFTR by interfering with the miR-145-5p/Sp1 mRNA interactions (Fig. 4). Furthermore, given the central role of Tcf4 in enhancing *CFTR* gene transcription and inhibiting miR-145-5p expression, a possible therapeutic strategy might consider enhancing Tcf4 [144, 145]. In this respect, considering that miR-155 is among the possible Tcf4 targeting miRNAs [150, 151], the development of anti-miR-155 PNAs might be of interest in the future [151].

In addition to miR-145-5p, several other miRNAs have been proposed to downregulate *CFTR* expression, such as miR-494, miR-509-3p, miR-101, and miR-433 [88–96, 152,

153]. Therefore, screening of PNAs targeting these miRNAs, identification of the most active, and combined treatments using the more efficient should be considered to reach a CFTR increase compatible with physiological effects.

Also lncRNAs are implicated in CF. In one study, McKiernan et al. demonstrated that lncRNAs are aberrantly expressed in vivo in the CF bronchial epithelium [121]. In a second study, the possible role of lncRNAs in CF was analyzed on CF cells infected by *Pseudomonas aeruginosa* [122], the leading cause of chronic lung infection in CF patients, associated with an exaggerated inflammatory response. Since lncRNAs are deeply involved in regulating innate immune response, Balloy et al. hypothesized that CF cells exposed to *P. aeruginosa* could express a specific lncRNA signature. This study identified temporal expression signatures of 26 lncRNA transcripts differentially expressed after 6 h of *P. aeruginosa* infection, which

differentiate CF from non-CF cells [122]. The differential expression of two lncRNAs (BLACAT1 and MEG9) was independently validated using real-time PCR.

3.4 Rett Syndrome

Rett syndrome (RTT) is a complex X-linked neurological disorder associated with mutations in the gene coding for the methyl CpG binding protein 2 (*Mecp2* gene) [154], causing a severe phenotype. The high frequency (one out of every 10,000–15,000 live births) makes the RTT disorder the second most frequent cause of mental retardation in females. The miRNA miR-199a was found by Tsujimura et al. to link MeCP2 with mammalian target of rapamycin (mTOR) signaling, and its dysregulation leads to RTT phenotypes. In RTT, miRNAs dysregulation caused by *Mecp2* absence has been reported. In particular, it was demonstrated that the most commonly disrupted miRNAs were miR-146a, miR-146b, miR-130, miR-122a, miR-342, and miR-409 (downregulated) and miR-29b, miR-329, miR-199b, miR-382, miR-296, miR-221 and miR-92 (upregulated) [155]. As far as lncRNAs are concerned, the study by Petazzi et al. focused on the impact of *Mecp2* impairment on lncRNAs pattern using an RTT mouse model [124]. Using a microarray platform, an aberrant lncRNA transcriptome was identified in the brain of RTT mice. In particular, the transcripts of the *AK081227* and *AK087060* genes were found to be upregulated in *Mecp2*-null mice brains. Furthermore, this study highlighted that the overexpression of *AK081227* mediated by the *Mecp2* loss was associated with the downregulation of its host coding protein gene, the gamma-aminobutyric acid receptor subunit Rho 2 (*Gabbr2*) [124]. These observations support the concept that dysregulation of lncRNAs transcription upon *Mecp2* loss might contribute to the neurological RTT phenotype.

3.5 Sézary Syndrome

This is a rare and aggressive leukemic variant of cutaneous T-cell lymphoma (CTCL). This disease is characterized by the presence of neoplastic lymphocytes named Sézary cells in the skin, lymph nodes, and peripheral blood [156, 157]. Studies based on deep-sequencing demonstrated that the miR-199a2/214 cluster represents the vast majority of aberrantly expressed miRNAs in Sézary syndrome (SS) [31]. On the other hand, Ballabio and colleagues reported that a high proportion of SS-associated miRNAs were downregulated, consistent with previously reported abnormalities of the genomic copy number [100]. Altered miR-223 levels were found to discriminate SS samples from healthy controls; inhibition of apoptosis was found to be associated in SS samples with downregulation of miR-342 and was hypothesized to be involved in the pathogenesis of this disease [100]. The

involvement of miRNAs in SS was also strongly supported by another study in which a miRNA signature was identified that was potentially able to discriminate patients with unfavorable and favorable outcomes [101]. Among the miRNAs identified, miR-21 appears to be important. In line with this hypothesis, van der Fits and colleagues demonstrated that miR-21 expression is increased in SS cells when compared with cells from healthy subjects. Functional studies focusing on miR-21 in SS were able to demonstrate that silencing of miR-21 in SS cells is associated with increased apoptosis, suggesting a role for miR-21 in the leukomogenic process. These studies propose miR-21 as a possible molecular target in protocols finalized for the therapeutic treatment of SS [102].

As far as lncRNAs are concerned, among the few studies available, one reported by Lee et al. was based on bioinformatics, which allowed the identification of SS-associated lncRNAs [158]. To our knowledge, this was, among the studies on SS, the only one that, starting from the characterization of the SS transcriptome, supports an implication of lncRNA dysregulation in SS.

3.6 Multiple Osteochondromas

Multiple Osteochondromas (MOs) are characterized by a high number of mutations affecting the *EXT1/EXT2* genes [104]. At the same time, a significant population of MO patients does not exhibit alterations in the *EXT* genes. Intra- and interfamilial variability of the MO phenotype is present, for which the molecular basis is largely unknown. For this reason, appropriate diagnostic/prognostic markers and personalized clinical management for both benign and malignant lesions are not available so far [104]. In this respect, a miRNA signature could help in patient stratification. Zuntini et al. reported the miRNA pattern of MO, differentiating disease-specific from normal cartilage miRNA signatures [104]. In particular, a signature of eight miRNAs (miR-21, miR-140, miR-145, miR-214, miR-195, miR-199a, miR-451, and miR-483) was associated with MO and was useful to distinguish MO patients from unaffected subjects. The obtained data indicate that differentially expressed miRNAs may contribute to the molecular signaling responsible for normal proliferation and differentiation of chondrocytes, playing a role in both pathogenesis and clinical outcome [104]. To the best of our knowledge, no examples of lncRNAs associated with MOs are so far available.

3.7 β -Thalassemia

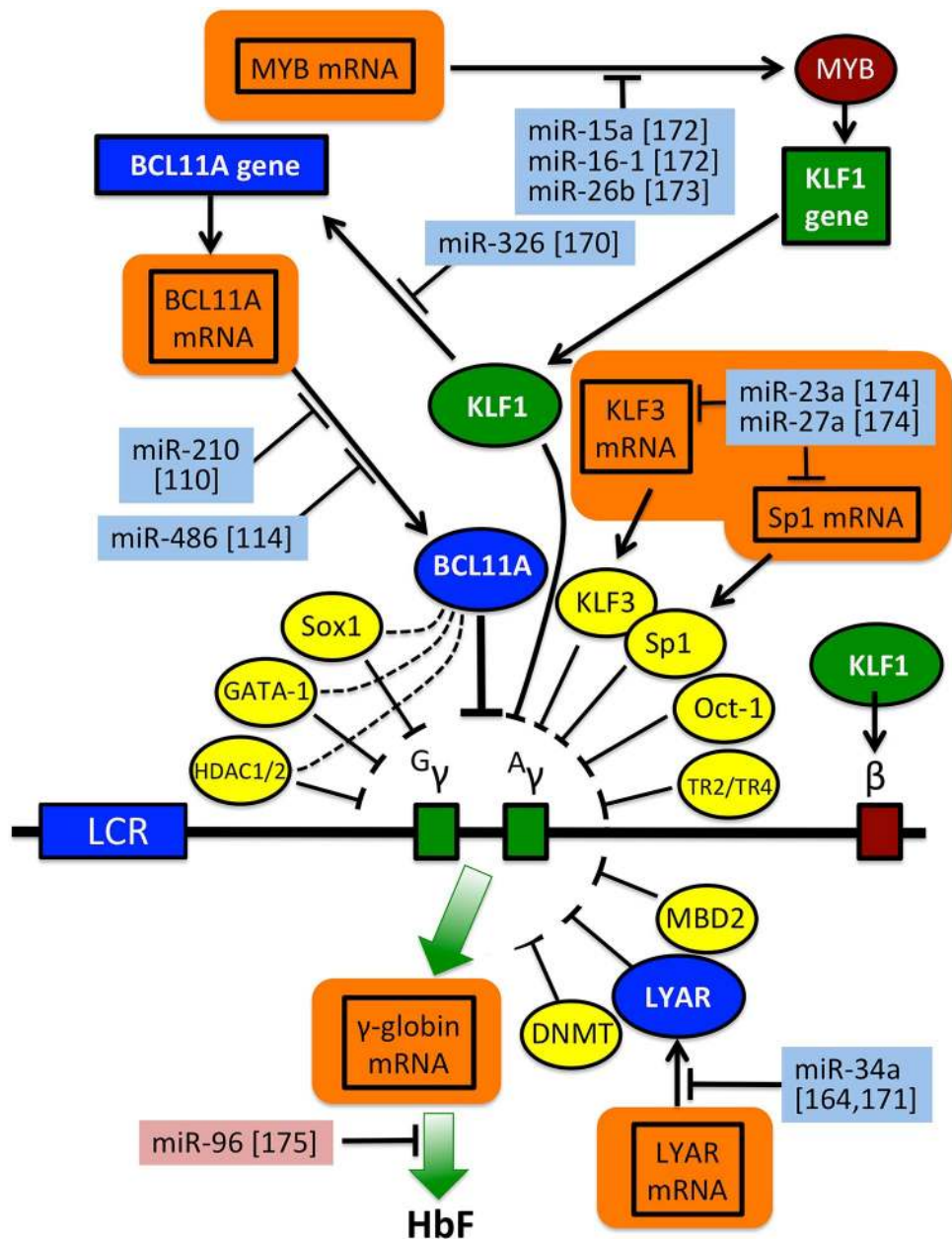
β -Thalassemia is a recessive genetic disease caused by more than 300 mutations affecting the β -globin gene [159]. This leads to absence or lower production of the adult β -globin and, consequently, hemoglobin. The management

of β -thalassemia patients is mostly based on blood transfusion, chelation therapy and, alternatively, on bone marrow transplantation [160]. Recently, novel therapeutic options are explored, such as gene therapy and fetal hemoglobin (HbF) induction [161]. Despite the fact that these approaches are promising, these therapeutic strategies are at present still under deep experimental development, involving a limited number of clinical trials. Furthermore, it should be considered that new HbF inducers and/or new approaches for reactivation of γ -globin genes are required, since important limitations are exhibited by hydroxyurea (the most used HbF inducer in β -thalassemia), including (1) toxicity; (2) lack of response by about 50% of β -thalassemia patients; and (3)

development of drug resistance with long-term treatments [161].

In this respect, of great interest is the demonstration of the fact that the transcriptional regulation of the human γ -globin genes is under the control of several transcriptional repressors, including MYB, BCL11A, KLF-1, KLF-2, Sp1, and LYAR [162–164]. This has greatly facilitated the identification of specific targets for the development of possible pre-clinical approaches to reactivate γ -globin gene expression (and consequently HbF production) by targeting these TFs. Among possible additional strategies for HbF potentiation is the use of miRNA-based approaches. In fact, miRNAs involved in the control of γ -globin gene repressors have been

Fig. 5 The network composed of transcription factors and regulatory miRNAs. These interactions contribute to the level of fetal hemoglobin production by erythroid cells from β -thalassemia patients. Proposed miRNA targets are indicated [110, 114, 164, 170–175]. miRNA microRNA



identified and validated. Examples are miR-486-3p (targeting BCL11A mRNA), miR-23a (targeting KLF-2) miR-15a and miR-16-1 (targeting MYB mRNA), and miR-27a (targeting Sp1) [110] (Fig. 5).

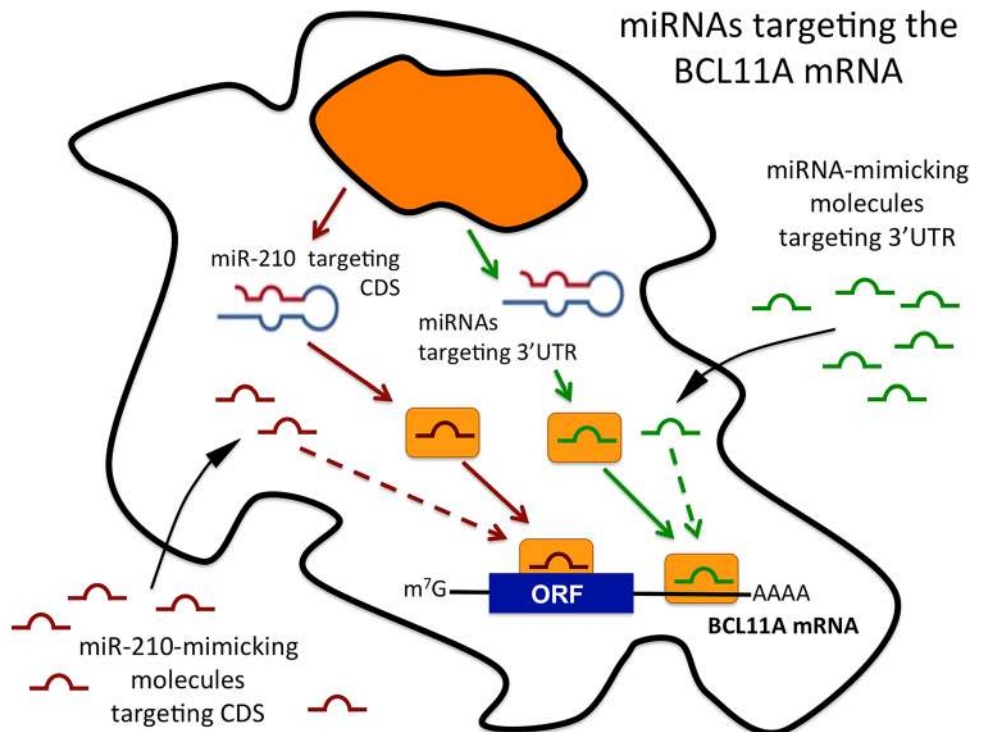
While with respect to other HbF-associated miRNAs, information is still lacking, miR-210 has been suggested by different groups to be somehow involved in HbF production. It has been reported that miR-210 is involved in erythroid differentiation and, possibly, in γ -globin gene upregulation [165–169]. For instance, a recent paper by Bavelloni et al. [168] confirmed the data published by Bianchi et al. [166], demonstrating an increase in miR-210 in K562 cells treated with mithramycin, one of the most powerful HbF inducers in erythroid cells. Sarakul et al. demonstrated upregulation of miR-210 under hypoxic conditions in K562 cells and in β -thalassemia/HbE (hemoglobin E) progenitor cell cultures [167]. Inhibition of miR-210 expression leads to reduction in γ -globin gene expression, indicating that miR-210 contributes to hypoxia-induced erythroid differentiation. The possible role of miR-210 in HbF production was also proposed in the communication by Sawant et al. that suggested that HbF induction by hydroxycarbamide works through miR-210 in sickle cell anemia patients [169].

In a recent study, we analyzed a coding sequence of BCL11A mRNA as a possible target of miR-210 [110]. This miR-210 site of BCL11A mRNA is conserved through molecular evolution, possibly indicating that this sequence plays a key biological function. Interactions between miR-210 and the miR-210 BCL11A mRNA site were

demonstrated by surface plasmon resonance (SPR)-based biomolecular interaction analysis (BIA), and forced expression of miR-210 leads to a decrease in BCL11A mRNA and an increase in γ -globin mRNA content in erythroid cells, including erythroid precursors isolated from β -thalassemia patients. While most validated miRNA/mRNA interactions involve the 3'-UTR of the target mRNAs [170–178], functional interactions between miRNAs and coding sequences of target mRNAs have been reported in several studies [179–181]. Our study suggests that, in addition to the already reported binding of miR-486-3p to the 3'-UTR sequence of the BCL11A mRNA, also the coding mRNA sequence of BCL11A can be targeted by miRNAs (such as miR-210). Therefore, upregulation of γ -globin gene expression can be achieved by different miRNA-based approaches, including the use of pre-miRNA targeting the 3'-UTR region of the BCL11A mRNA, or the use of pre-miR-210, targeting the coding region of the mRNA. Both approaches (that at least in theory can be combined) might lead to BCL11A downregulation and induction of γ -globin gene expression (Fig. 6), in consideration of the fact that BCL11A is a transcriptional repressor of γ -globin genes. This is of interest, since inhibition of BCL11A is a recognized strategy for HbF induction for treatment of β -thalassemia, as suggested by several papers and patent applications [110, 173].

One of very few examples of validated lncRNAs in β -thalassemia [48, 129, 130] was described by Morrison et al., who characterized a novel 1283 bp transcript (HMI-LNCRNA) that was transcribed from the enhancer region

Fig. 6 Scheme outlining the possible miRNA mimicking approach for targeting BCL11A mRNA. Downregulation of *BCL11A* gene expression will lead to upregulation of γ -globin mRNA [110]. mRNA messenger RNA, miRNA microRNA



of the *MYB* gene coding for one of the major transcriptional repressor of β -globin genes [129]. Within erythroid cells, HMI-LNCRNA was almost exclusively present in the nucleus, and was significantly higher in erythroblasts derived from cultured adult peripheral blood CD34⁺ cells, which expressed adult hemoglobin (HbA), compared to erythroblasts from cultured cord blood CD34⁺ cells, which expressed much more HbF. Downregulation of HMI-LNCRNA in HUDEP-2 cells significantly upregulated HbF expression both at the mRNA (200-fold) and protein levels. Therefore, HMI-LNCRNA might be a potential therapeutic target for HbF induction treatment in sickle cell disease and β -thalassemia.

4 Concluding Remarks

Since ncRNAs' discovery and classification, their roles have gained great attention for several reasons, including their possible employment as novel disease markers as well as novel therapeutic targets. In this respect, miRNA and lncRNAs are key players (see Fig. 1), as they have been firmly linked to regulation of gene expression and to the onset of human diseases. While the majority of examples available in the literature are focused on cancer, examples of the involvement of ncRNAs in genetic diseases (including rare genetic diseases) are growing in number. Most of the examples available are based on the analysis of miRNAs, and only a limited (but growing) number have been reported for lncRNAs.

As far as applications in diagnostic and therapeutic protocols are concerned, both miRNAs and lncRNAs can be considered excellent markers for advanced diagnostic protocols, including liquid biopsy (see Fig. 2). In addition, progress has been made with respect to genetic mutations of miRNAs as well as of miRNA-binding sites affecting the 3'-UTR sequences of miRNA-regulated genes. Since these genetic alterations can be detected in rare cellular subpopulations, taking advantage of the knowledge driven by liquid biopsy approaches, we would expect future applications also in non-invasive prenatal diagnosis, which is particularly relevant in the case of genetic diseases and is based on the detection of genetic mutations in fetal cells circulating within the mother's peripheral blood.

With respect to translational medicine, miRNAs and lncRNAs are suitable targets for therapeutic intervention [51–57]. Inhibition of miRNA activity has been achieved by different approaches [58–69]. Increase of miRNA function (miRNA replacement therapy) was also considered [70–73]. Also in the case of therapeutic interventions (as it occurs for diagnostic protocols), the examples of targeting lncRNAs for the development of therapeutic approaches are fewer compared with miRNAs. This is probably related

to the fact that targeting in this case largely depends on the mechanism of action of the studied lncRNA. For instance, lncRNAs could act by sponging miRNAs, recruitment of chromatin modifiers, controlled recruitment of TFs (both activators and repressors of gene transcription), regulation of RNA splicing, regulation of chromosome looping, and control of mRNA translation and decay [12–16]. Very different approaches are therefore necessary depending on the lncRNA mechanism of action in the target cells.

Considering the key role of miRNAs and lncRNAs also in rare genetic diseases, a final comment should be focused on the issue of development of protocols based on these molecules. This issue is considered of key interest by an increasing number of research groups and patient associations. In the case of rare genetic diseases (unlike drugs and protocols developed for cancer, infectious diseases, and cardiovascular pathologies), it should be considered that even if excellent and innovative therapeutic strategies are available exhibiting high activity levels and promising specificity in pre-clinical tests, they are not expected to attract the interest of large pharmaceutical companies in consideration of the limited number of patients to be treated [182, 183]. The importance of taking this issue into consideration is also strongly related to the possibility of developing personalized therapy in precision medicine of rare genetic diseases [184]. In this context, the possibility to develop and propose “orphan medicinal products” [185] might be a key action, considering the deep advantages associated with these designations [186–188]. In Europe, the European Medicines Agency (EMA) continuously evaluates the applications for Orphan Medicinal Product designation on the basis of reports obtained from the Committee of the Orphan Medicinal Products (COMP). A similar activity is coordinated in United States by the Food and Drug Administration (FDA) Office of Orphan Products Development (OOPD) [204–206]. The major focuses of these activities are the evaluation and development of products (drugs, biologics, devices, or medical foods) considered to be promising for diagnosis and/or treatment of rare diseases or conditions, including rare genetic diseases. In conclusion, a large number of approaches are expected in the field of molecular diagnosis and therapy based on targeting miRNAs and lncRNAs, with a facilitated technological transfer in the case of rare genetic diseases.

In our opinion, the analysis of miRNAs and lncRNAs will be a key diagnostic and prognostic tool in the near future, helping clinicians to choose the therapeutic strategy to be activated in patients stratified with respect to the production of these ncRNA species. The protocols that will be developed are expected to significantly impact in the field of personalized therapy and precision medicine. In this respect, optimization of delivery systems, combined treatment targeting multiple miRNAs, and/or the

development of protocols involving miRNA replacement (affecting pathology-linked downregulated miRNAs) associated with antagomiRNA therapy (affecting pathology-linked upregulated miRNAs) are expected to significantly contribute to the set-up of advanced therapeutic protocols in the future.

Compliance with Ethical Standards

Conflict of interest All the authors (AF, EF, IL, JG, MB and RG) declare they have no conflicts of interest that are directly relevant to the content of this review.

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