Published in final edited form as:

Immunol Rev. 2013 May ; 253(1): 290–303. doi:10.1111/imr.12048.

Exploring the RNA World in Hematopoietic Cells Through the Lens of RNA-Binding Proteins

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Summary

The discovery of microRNAs has renewed interest in post-transcriptional modes of regulation, fueling an emerging view of a rich RNA world within our cells that deserves further exploration. Much work has gone into elucidating genetic regulatory networks that orchestrate gene expression programs and direct cell fate decisions in the hematopoietic system. However, the focus has been to elucidate signaling pathways and transcriptional programs. To bring us one step closer to reverse engineering the molecular logic of cellular differentiation, it will be necessary to map post-transcriptional circuits as well and integrate them in the context of existing network models. In this regard, RNA-binding proteins (RBPs) may rival transcription factors as important regulators of cell fates and represent a tractable opportunity to connect the RNA world to the proteome. ChIP-seq has greatly facilitated genome-wide localization of DNA-binding proteins, helping us to understand genomic regulation at a systems level. Similarly, technological advances such as CLIP-seq allow transcriptome-wide mapping of RBP binding sites, aiding us to unravel post-transcriptional networks. Here, we review RBP-mediated post-transcriptional regulation, paying special attention to findings relevant to the immune system. As a prime example, we highlight the RBP Lin28B, which acts as a heterochronic switch between fetal and adult lymphopoiesis.

Keywords

systems biology; genomics; post-transcriptional regulation; hematopoiesis; microRNA; RNA-Binding Proteins; Lin28; let-7

Introduction

The basis of cellular differentiation and function can be represented as integrated circuits that are genetically programmed. Identification of the master regulators within these complex circuits that can switch on or off a genetic program will enable us to reprogram cells to suit biomedical needs. A remarkable example was the discovery by Takahashi and Yamanaka (1) that somatic cells could be reprogrammed into induced pluripotent stem (iPS) cells via the ectopic expression of four key transcription factors. Interestingly, a specific set of microRNAs (miRNAs) could also mediate this reprogramming (2, 3), revealing a powerful layer of post-transcriptional regulation that is able to override a pre-existing transcriptional program (4). Similarly, miR-9 and miR-124 were sufficient to mediate transdifferentiation of human fibroblasts into neurons (5). Accordingly, we are enamored by the RNA world and pay special attention in our investigations to regulatory non-coding RNAs (ncRNAs), particularly miRNAs and long non-coding RNAs (lncRNAs) and how

they integrate with known genetic regulatory networks (*Fig. 1*). With the exception of certain ribozymes, regulatory RNAs generally do not work alone. Instead, they are physically organized as RNA-protein (RNP) complexes. Operationally, RNA-binding proteins (RBPs) and their interactome work in concert as post-transcriptional networks, or RNA regulons, in response to developmental and environmental cues (6). Inspired by this concept and other pioneering studies in the worm, we recently demonstrated that a single RBP Lin28 was sufficient to reprogram adult hematopoietic progenitors to adopt fetal-like properties (7). We discuss these and related findings, which begin to disentangle the complex functions of RBPs in the context of recent advances in post-transcriptional regulation, starting with the discovery of miRNAs.

The Lin28/let-7 circuit: from worm development to lymphopoiesis

Inspiration from the worm

Working in *C. elegans*, Ambros and Horvitz (8) identified a set of genes that control developmental timing, a category that they termed heterochronic genes. Heterochrony is a term coined by evolutionary biologists and popularized by the worm community to denote events that either positively or negatively regulate developmental timing in multicellular organisms. The discovery of two heterochronic genes, *lin-4* and *lin-28*, which encode a miRNA and RBP respectively, is particularly relevant to this review. The *lineage* (*lin*) mutants were previously identified and named because they displayed abnormalities in cell lineage differentiation. Furthermore, some of them were considered heterochronic, as adult mutants harbored immature characteristics (retarded phenotype) or, conversely, larval mutants displayed adult characteristics (precocious phenotype). It was not until 1993 that *lin-4* was characterized molecularly, because contrary to popular expectations, the gene did not encode a protein but instead a small RNA now appreciated as the first miRNA to be discovered (9). The *lin-4* miRNA acts in part by inhibiting the expression of the LIN-14 transcription factor through imperfect basepairing to sites in the 3' untranslated region (UTR) of *lin-14* mRNA (9, 10). However, it was not apparent initially whether *lin-4* or *lin-14* is evolutionarily conserved, potentially relegating these findings to be relevant only to the worm. Interestingly, *Lin28*, a gene conserved in mammals, was later identified to be a direct target of the *lin-4* miRNA (11). *Lin28* loss-of-function resulted in a precocious phenotype, whereas gain-of-function resulted in a retarded phenotype; thus, Lin28 acts as a heterochronic switch during *C. elegans* larval development (11).

The possibility that *lin-4* may be an oddity of the worm was dissolved with the discovery of the second miRNA, again in *C. elegans*, *let-7* (12). Unlike *lin-4*, the evolutionary conservation of *let-7* from sea urchin to human was quickly appreciated (13). Importantly, expression analysis showed that *let-7* expression is temporally regulated from molluscs to vertebrates in all three major clades of bilaterian animals, implying that its role as a developmental timekeeper is conserved (14). This established miRNAs as a field unto its own that has progressed rapidly with the identification of Drosha, Dgcr8, Dicer, and Argonaute (Ago) RBPs as core components of the miRNA pathway (15). Orthologs of *lin-4* were eventually found in mammals (*mir-125a*, *-b-1*, and *-b-2*) (16) along with hundreds of novel miRNAs from numerous organisms (17). We now recognize that miRNAs, in complex with the RBP Ago, frequently bind their cognate targets via imperfect complementarity to evolutionarily conserved sequences in 3' UTRs (18-20) and mediate post-transcriptional repression (21).

Lin28 and *let-7* in mammalian development

Two *Lin28* homologs exist in mammals (Lin28A and Lin28B) that share 77% sequence identity and contain common RNA-binding domains including an N-terminal cold shock

domain, and two CCHC-type zinc finger (ZnF) domains (11). Studies in mammalian cells led to the discovery that Lin28A and Lin28B (hereafter commonly referred to as Lin28) physically bind to unprocessed *let-7* RNA and inhibit biogenesis of mature *let-7* (22-25). Thus, the opposing functions and expression patterns of *let-7* and Lin28 suggest that this heterochronic regulatory axis may participate in the temporal regulation of mammalian development. Consistent with this notion, Lin28 gain-of-function mutations have been associated with increased body stature coupled with delayed onset of puberty in mice and humans (26-32). Furthermore, a gradual increase in *let-7* expression in neural stem cells from fetal to aging adult mice mediates repression of the self-renewal factor HMGA2 and results in declined stem cell function (33). Finally, mounting evidence indicate opposing roles for Lin28 and *let-7* in stem cell pluripotency and oncogenesis (34, 35). These findings are consistent with a role of the Lin28/*let-7* axis in regulating important heterochronic traits in mammals such as body height, longevity, and disease.

Heterochrony in immune development

In analogy to the principles of worm development, we have extended the term heterochrony to encompass the evolutionarily programmed changes in blood cell development that occur during ontogeny in vertebrates. Like any developmental process, timing is everything during hematopoietic ontogeny. In vertebrates, hematopoiesis takes place in anatomically distinct regions during embryogenesis. Primitive hematopoiesis in mice is first detected in the yolk sac around 7 days post coitus (dpc). In the embryo proper, the main site of hematopoiesis is sequentially localized in the aorta-gonad-mesonephros (AGM) region, then the fetal liver, and finally the bone marrow where hematopoietic stem cells reside throughout adult life (36). Pioneering chick/quail xenograft studies established that the thymus is seeded in temporally distinct waves during fetal and neonatal life (37, 38). With the invention of multicolor flow cytometry over 20 years ago, it was appreciated that fetal liver hematopoietic stem and progenitor cells (HSPCs) preferentially generated the innate-like B-1 B cells and $\gamma\beta$ T cells, while adult bone marrow almost exclusively generated conventional B-2 B cells and $\alpha\beta$ T cells (39, 40). These differences appeared to be intrinsically programmed in HSPCs and led to the important postulation by Leonore and Leonard Herzenberg (41, 42) that the mammalian immune system develops in a layered rather than a linear fashion, where ordered appearance of distinct hematopoietic stem cells (HSCs) gives rise to functionally distinct and increasingly evolutionarily complex lymphocyte lineages during ontogeny.

The layered immune system hypothesis potentially accounts for both the chronological sequence, in which our immune system evolved, and its breadth and complexity. The idea that the innate-like lymphocytes represent more primitive lymphocyte subsets is supported by their highly restricted antigen repertoire and by their disproportionate abundance in more primitive species such as birds and amphibians (43, 44). Also consistent with layered immune development, B-cell progenitors were recently found in the yolk sac of mouse embryos as early as in 9 dpc that strictly give rise to innate-like B lymphocytes (45). Evolutionary events leading to the acquisition of an adaptive immune system in higher organisms presumably took place in a way that preserved useful primitive functions resulting in a stratified immune system. Indeed, the ordered appearance of distinct lymphocyte subsets may confer an important advantage in protecting the vulnerable body surfaces of the neonate against common pathogens prior to the maturation of the adaptive immune system (46). Furthermore, a layered development of the immune system has been linked to the maintenance of fetal-maternal tolerance during the long gestational time of higher mammals (47, 48).

A switch in the fetal to adult type lymphopoiesis has been mapped to occur around 2-4 weeks after birth in mice (49-51). Thus, important clues into the evolution and development

of the adaptive immune system can be gained by interrogating this heterochronic change in HSPC developmental potential. Thus far, experimentation using mainly cellular immunological approaches has set the stage for elucidating the molecular basis underpinning the two distinct stem cell fates. To this end, the developmentally regulated expression of terminal deoxynucleotidyl transferase (TdT) was found to account for the lack of N-nucleotide additions during V(D)J rearrangement, contributing to reduced antigen receptor diversity in fetal and neonatal lymphocytes (52-54). In addition, fetal specific requirement of the transcription factor Sox17 distinguishes the transcriptional regulation of fetal HSCs from adult HSCs (55). However, a potential role of heterochronic genes in regulating the developmental switch during vertebrate hematopoietic ontogeny was not explored until recently.

The Lin28B/let-7 axis in lymphopoiesis

Our group looked to the miRNA world for clues on the developmental switch from fetal to adult HSPC fate and identified a global increase in the expression of let-7 family miRNAs in adult bone marrow HSPCs compared to fetal liver HSPCs in mice (7). There are twelve *let-7* paralogs encoded in the mouse and human genomes (Fig. 2). Collectively, the let-7 family represent one of the most abundantly and ubiquitously expressed miRNAs in the hematopoietic system (56). Thus, it is likely that, over evolutionary time, this highly conserved miRNA family may have become adopted to regulate the differentiation and function of the hematopoietic lineages. The peculiar expression pattern of let-7 miRNAs was explained when we discovered that *Lin28B* is specifically expressed in mouse and human fetal liver and fetal thymus and umbilical cord blood, while being strikingly absent in adult bone marrow or thymus (Fig. 3A). More importantly, we found that ectopic expression of either Lin28B or Lin28A could induce HSPCs from adult bone marrow to undergo multi-lineage reconstitution that resembles fetal/neonatal lymphopoiesis, including increased development of innate-like B-1a, marginal zone B, gamma/delta ($\gamma\delta$) T cells, and natural killer T (NKT) cells (Fig. 3B). The discovery that Lin28 can turn on the switch for fetal-like lymphopoiesis reveals a common post-transcriptional regulator linking the development of major innate-like lymphocyte subsets. In addition to lymphopoiesis, miRNA profiling of human reticulocytes from cord blood and adult blood revealed a developmentally controlled global increase in the expression of the let-7 family of miRNAs, curiously echoing the switch from fetal to adult hemoglobin expression (57). Taken together, these findings are consistent with a conserved heterochronic role of the Lin28B/let-7 axis in the hematopoietic system.

In addition to regulating the switch from fetal to adult type lymphopoiesis, aberrant expression of Lin28B has also been shown to promote T-cell activation, proliferation, and, over time, malignant transformation (58, 59). Derepression of *let-7* targets, including *Myc*, *Hmga2*, and *K-Ras*, likely contributes to the latter (58-60). An oncogenic feedback loop has been described in which *Myc* transactivates both the *Lin28A* (61) and *Lin28B* (62) loci; Lin28 in turn blocks the biogenesis of *let-7*, a repressor of both Lin28 (63, 64) and *Myc* (65) (Fig. 4). In T-cell leukemias, aberrant nuclear factor κ B (NF κ B) signaling caused by haploinsufficiency of the tumor suppressor ribosomal protein RPL22 was found to promote tumorigenesis through the induction of Lin28B (59). This report is consistent with *Lin28B* being under direct transcriptional control of NF κ B (66). Regarding the leukemogenicity of Lin28, it is likely context dependent, as we have never observed malignant or pre-malignant indications in aged bone marrow chimeric mice (> 1 year post adoptive transfer) reconstituted with a mixture of wildtype and Lin28 over-expressing adult HSPCs (data not shown). A negative regulator of Lin28 in HSPCs is miR-125, the mammalian ortholog of *lin4* known to enhance HSC and lymphoid progenitor expansion (67, 68). It remains to be clarified to what extent Lin28 repression is mediating miR-125 induced effects during

hematopoiesis. Nonetheless, this regulatory mechanism exemplifies another evolutionarily conserved miRNA:target relationship in bilaterian animals. Future efforts focused on genetic programs regulating fetal hematopoiesis will reveal whether any of these mechanisms contributes to the physiological pattern of Lin28B expression during hematopoietic ontogeny.

Emerging modes of Lin28 action

Recently, structural studies made clear that the zinc finger and cold shock domains of the Lin28A protein interact extensively with the conserved GGAG motif and a structure within the terminal loop of unprocessed let-7 RNA respectively (69, 70). To date, the selective inhibition of let-7 miRNA biogenesis is the best-understood mode of Lin28 action, and has been extensively reviewed elsewhere (34, 35, 71). However, it has become increasingly apparent that the expression of Lin28 and let-7 are not always coupled. For instance, overexpression of Lin28A in the mouse hypothalamic-pituitary-gonadal axis did not result in the expected decrease in mature let-7a or let-7g levels (26). Furthermore, let-7-independent effects of Lin28 have been observed during both myogenesis and gliogenesis (32, 72). These findings hint at the complexity and context dependent modes of Lin28 action.

Our own studies revealed one mechanism that uncouples Lin28 and let-7 expression. It is widely believed that the presence of Lin28 equals a coordinately regulated disappearance of let-7. However, miRNA profiling analysis from our laboratory demonstrated that mouse let-7c-2 (*mmu-let-7c-2*) is insensitive to ectopic Lin28 expression in mouse thymocytes (7) and NIH3T3 cells (R. Zahr and S.M., unpublished data). RNA immunoprecipitation studies in the latter revealed that *mmu-let-7c-2* fails to interact with Lin28 (L. Liu, J.Y., S.M., unpublished data). Mutation studies suggest that the distinct behavior of *mmu-let-7c-2* has evolved through the loss of a tetra-nucleotide GGAG or GAAG motif, conserved in the loop region of most let-7 primary or precursor (pri- or pre-) miRNAs (*Fig. 2A*, and L. Liu, J.Y., S.M., unpublished data). Furthermore, the G(G/A)AG motif is also absent among mammalian let-7c-2 orthologs (*Fig. 2B*), for example *hsa-let-7a-3* in humans. Our observations are supported by recent structural findings demonstrating a direct interaction of the GGAG motif to the zinc-finger motifs of Lin28 (69, 70). Consequently, *mmu-let-7c-2* is uniquely regulated among this family, and its ectopic expression can be used in experiments that take place in cell types expressing Lin28 because it is insensitive to Lin28-mediated inhibition. Analogous differences between paralogs within other miRNA families may be uncovered in the future and may provide one explanation as to why sometimes miRNA paralogs evolved. Specifically, differences in sequences within the terminal loop region of paralogous pri- or pre-miRNAs may afford differential post-transcriptional regulation of their expression as a result of divergent evolution. Other mechanisms are likely to exist that can compete with Lin28 binding to let-7 such as interception by other RBPs (73) and as yet unidentified RNAs.

The first evidence supporting a role for Lin28 in regulation of translation came from the observation that Lin28 co-sedimented in sucrose gradients with polysomes in undifferentiated P19 mouse teratoma cells (74) and differentiating myoblasts (75). Upon differentiation of C2C12 myoblasts, Lin28 expression is induced, followed by its increased association to polysomes and enhanced translation of IGF2 (75), a crucial growth factor during muscle development. More recently, conditional deletion of Lin28 in muscle was shown to disrupt glucose tolerance and insulin resistance, establishing a physiological requirement for Lin28 in the adult mouse (32). Although let-7 overexpressing mice display a similar phenotype of impaired glucose homeostasis (76), the unchanged let-7 expression in Lin28-deficient muscle tissue calls into question whether let-7 is the main physiological downstream mediator (32, 76). Two recent studies shed light on this conundrum by demonstrating that endogenous Lin28A is capable of directly binding thousands of protein

coding transcripts in ES cells in addition to the terminal loops of let-7 miRNAs (77, 78). Consistent with previous studies of Lin28 sequence recognition, Wilbert *et al.* (2012) demonstrated specific Lin28A binding to thousands of transcripts harboring the GGAGA motif. This interaction was found to be causative for the enhanced translation of several target transcripts. Notably, Lin28A was found to interact with its own mRNA consistent with a self-enforcing autoregulatory loop. Furthermore, the splicing factor TDP-43 protein expression was enhanced by Lin28, contributing to widespread downstream alternative splicing changes in a let-7-independent fashion (77). Thus, these recent studies suggest that direct mRNA targets also contribute to the Lin28 induced genetic program (*Fig. 4*). Consistent with this notion, HMGA1 is a direct mRNA target of Lin28 and a key regulator of glucose metabolism mutated in 5%–10% of type II diabetes patients (77, 79, 80) and may contribute to the let-7-independent metabolic effects induced by Lin28 in muscle cells (32). Recently, global polysome profiling studies (discussed below) indicates a negative regulatory effect of Lin28 binding on the translation of ER associated proteins (78). Thus, current understanding of Lin28-mediated translation indicates a context dependent mode of action.

A model is emerging in which Lin28 exerts its effects at multiple levels in addition to let-7 biogenesis (*Fig. 4*). The ability to orchestrate the fate of both coding and non-coding RNAs thereby remodeling the cellular protein landscape is consistent with its role as a master-regulator of fetal-like lymphopoiesis and its ability to facilitate iPS cell generation. Taken together, recent discoveries have significantly widened the scope of Lin28 action beyond let-7 inhibition and changed how we view this and other RBPs and their impact on development and disease.

RBPs as key regulators of the immune system

RBPs are multi-functional regulators

In addition to studying regulatory RNAs, a complementary approach to gain access into the RNA world of post-transcriptional gene regulation has been to perform loss- and gain- of-function studies aimed at elucidating the roles of RBPs. Within the immune system, characterization of the phenotypes caused by conditional deletion of known components of the miRNA biogenesis pathway provides an example of the value of interrogating RBP function. Tissue-specific inactivation of Dicer in the T-cell lineage resulted in impaired thymocyte survival, maintenance of peripheral CD8⁺ T cells, and dysregulated effector CD4⁺ T-helper cell differentiation (81, 82). In addition, impaired regulatory T (Treg) cell development and function in Dicer and Drosha-deficient mice results in loss of tolerance and spontaneous onset of inflammatory disease (83, 84). The lack of Dicer during B-cell development triggers a severe developmental block between the pro- and the pre-B-cell stage due to impaired survival and causes altered antigen receptor repertoire (85). Studies along these lines have proven to be invaluable approaches for the identification of junctures during immune development and function where miRNAs as a whole play a critical regulatory role.

Deficiency in Drosha, DGCR8, or Dicer did not always result in identical phenotypes (86–89). Consistent with this, miRNAs have been identified that are generated by Drosha-independent (88) or Dicer-independent mechanisms (90, 91). Furthermore, the functions Dicer and Drosha are not limited to miRNA biogenesis but have been found to be required for the processing of a diverse cohort of RNAs with secondary stem loop structures including precursors of endogenous siRNAs and Alu RNAs (87, 88, 92–100), as well as for normal centromere function (101–103). Therefore, considering that RNA-binding specificity is often determined by structural characteristics that can be shared by diverse RNA

molecules, it is important to consider potential miRNA-independent contributions by these RBPs – a lesson also evident from Lin28.

One diverse group of RBPs appreciated to be important in the immune system, even before the discovery of miRNAs, is distinguished by their ability to bind to AU-rich elements (AREs) often found in 3' UTRs of genes involved in inflammation, growth, and survival. Such RBPs are known as ARE-BPs and have been implicated in mRNA decay, alternative splicing, translation, as well as both alleviating and enhancing miRNA-mediated mRNA repression (104-107). Genetic inactivation of several ARE-BPs have been linked to aberrant cytokine expression due to impaired ARE-mediated decay (5, 108-111) (*Table 1*). In addition, deficiency of HuR and AUF1 has uncovered a pro-survival role for both in lymphocytes (112, 113), while ectopic expression of Tis11b (ZFP36L1) negatively regulates erythropoiesis by down-regulating Stat5b mRNA stability (114). The KH-type splicing regulatory protein (KSRP) originally identified as an alternative-splicing factor is a multi-functional RBP. It has been shown to associate with both Drosha and Dicer complexes to positively regulate the biogenesis of a subset of miRNAs including mir-155 and let-7 (73, 108, 115-120). In addition, KSRP, like many other ARE-BPs, mediate selective decay of mRNAs by recruitment of exosome complexes to mRNA targets (121) and constitutes a prime example of a multi-functional RBP.

Structural predictions combined with recent proteomic studies suggest upwards of 1000 RBPs in the cell (122-124). Thus, it is likely that further studies of RBPs will be in order. RBPs are involved in a plethora of biological processes and are not exhaustively reviewed here (*Table 1*). For example, the nonsense-mediated mRNA decay (NMD) pathway is involved in the detection and clearance of mRNA transcripts that contain premature termination codons. Recently, core RBPs in this pathway have been demonstrated to be required during thymocyte development for the clearing the large number of nonproductive rearrangements at the TCR β locus (125, 126); however, it remains possible that the NMD pathway has additional targets. Other RBPs of emerging importance include those that catalyze covalent modifications such RNA nucleotide deamination, adenylation, uridylation, and methylation. RNA editing is mediated by the ADAR enzymes (adenosine deaminases acting on RNA), which catalyze adenosine deamination and conversion to inosine. Advances in deep sequencing technology allowed for systematic comparison of genomic DNA and cDNA that revealed hundreds of RNA editing sites in seven human tissues within coding and ncRNAs (127). The TUTase family of terminal uridyl transferases has also emerged as important post-transcriptional RNA regulators and was found to target select mRNAs, miRNAs, mitochondrial pre-mRNAs, and small nuclear RNAs (snRNAs) to modulate their stability (128). Notably, recruitment of TUTase 4 (ZCCHC11), which then poly-uridylates pre-let-7, is one mechanism by which Lin28 inhibits let-7 biogenesis (129-131). Furthermore, two independent studies identified the methyltransferase BCDIN3 as responsible for the 5' terminal methylation of the lncRNA 7SK and miR-145 leading to increased half-life and impaired biogenesis respectively (132, 133). Targeted deletion of the genes encoding such RBPs will allow future assessment of their physiological function.

Organizing post-transcriptional 'regulons' of the immune system

A single RBP can link the fates of diverse RNA molecules through recognition of common secondary structures and consensus sequences. This aspect of RBP function has been found to orchestrate the splicing, export, stability, and translation of cohorts of functionally connected RNAs in a synchronized fashion, giving rise to the term post-transcriptional RNA regulon (6). Coordinated post-transcriptional regulation of mRNA fate by miRNAs and RBPs is of particular interest in the context of the immune system due to the need for precise temporal control of the order and duration of protein production in response to developmental and pro-inflammatory cues (134). The ability to rapidly modulate the

proteome makes RBPs suitable mediators of signaling pathways that require an immediate response. Indeed, a recent study indicates that mRNA decay contributes significantly to downregulation of trophic and survival factors following inhibition of phosphoinositide 3-kinase (PI3K) signaling (135). These changes of gene expression were largely dependent on Tis11b (ZFP36L1) and KSRP, consistent with both ARE-BPs being known targets of AKT (119, 136).

Consistent with the notion that RNA regulons are important in processes that demand swift action, KSRP and HuR are both direct targets of the rapid phosphorylation-driven signaling triggered by DNA damage (117, 137, 138), trophic signaling (119, 135, 136, 139, 140), and pro-inflammatory cues (134). The clearest example of the action of a RNA regulon comes from the post-transcriptional regulation of genes during inflammation. While transcriptional events regulate the rate of mRNA production, post-transcriptional events are responsible for the rapid onset and timely resolution of immune effector functions through selective amplification of protein synthesis and mRNA decay. The half-lives of mRNAs encoding for immediate early genes such as cytokines and chemokines were found to be kinetically coordinated in a transcription-independent fashion (141-144). A study with important implications of our understanding of immunological tolerance demonstrated that self-reactive T cells harbor high levels of cytokine mRNA but do not generate the corresponding proteins due to a cytokine specific block in translation (145). This disconnect in mRNA and protein expression is at least in part mediated by an enrichment of conserved sequences within the 3' UTR of cytokine mRNAs including ARE sequences (145, 146). This finding is consistent with the aberrant cytokine expression observed upon perturbations of a growing group of RBPs (*Table 1*).

Technological advances

Recent technological advances have led to the development of several high-throughput assays useful in the mapping of RNA regulons. Many of these combine traditional biochemical methods with deep sequencing and sometimes proteomic methods (147). Analogous to ChIP-seq is CLIP-seq, also known as HITS-CLIP, which employs UV-crosslinking between RNA and protein followed by immunoprecipitation of the RBP, RNase treatment, deep sequencing, and bioinformatic mapping of protected fragments (148). This powerful technique allows high-resolution transcriptome-wide mapping of all RBP targets (148). For example, CLIP-seq of Ago has been successfully applied to catalog direct miRNA targets (149, 150). CLIP-seq of splicing factors promises unprecedented understanding of the regulation of alternative splicing. Accordingly, eIF4AIII CLIP-seq revealed transcriptome-wide mapping of the human exon junction complex (151). A variation of the method that aims to improve crosslinking of RNA to protein is photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP), which requires incorporation of a photoreactive ribonucleoside analog, such as 4-thiouridine (4-SU) or 6-thioguanosine (6-SG) into nascent RNA by cultured cells. UV cross-linking of 4-SU labeled transcripts to the RBP are subsequently revealed by thymidine to cytidine transitions upon cDNAs synthesis (152). Comparisons of native CLIP and PAR-CLIP suggest that detection of certain RBP-RNA interactions may be biased by one cross-linking chemistry over the other (122). More recently, these techniques have been adapted to allow transcriptome-wide profiling of RBP binding sites by enrichment of mRNA-protein complexes using oligo-(dT) beads instead of specific RBP antibodies (122, 123). This technology has been termed mRNA-protein interactome capture and in combination with mass spectrometry has identified the mRNA-binding proteome, identifying hundreds of novel RBPs (122, 123). RNase treatment of oligo-(dT) purified mRNAs identifies novel regulatory elements in the transcriptome and has confirmed the idea that 3' UTRs constitute important platforms of post-transcriptional regulation (123). Thus, it will be important to accurately annotate 3'

UTRs. To accomplish this task, a method known as 3P-seq has been developed to capture and sequence all 3' UTR termini (153). Other useful methods will surely be developed in time to address outstanding questions regarding post-transcriptional regulation.

Although RNA-seq in combination with bioinformatics analysis has identified many novel transcript isoforms and sites of RNA-editing, it fails to deliver quantitative measurements of many RBP mediated post-transcriptional functions on processes such as translation (77, 78, 127, 154). Ribosome or polysome profiling is an assay combining deep sequencing with RNase footprinting to reveal ribosome-protected mRNA fragments *en masse* (155, 156). This technique provides transcriptome-wide mapping of ribosome occupancy and thereby quantitative information about the position and rate of translation on a per transcript basis. Integrating data from ribosome profiling and CLIP-seq of specific RBPs have proven to be a particularly useful approach towards understanding RBP-induced changes in translation efficiency (78).

Recent technological advances have equipped us to begin systematic mapping of post-transcriptional regulatory networks. In addition to protein-coding transcripts, RBP interaction studies will provide much needed information on the regulation and function of lncRNAs. RNA-seq has uncovered over 9000 lncRNAs in humans (157, 158), and we predict that some of these will be involved in post-transcriptional regulation. Finally, reported dual RNA-DNA binding ability shown for proteins harboring domains such as ZnF, K homology (KH), SAF-A/B, Acinus, PIAS (SAP), and RNA recognition motifs (RRM) calls attention to the ability of some proteins to bind both DNA and RNA in a context-dependent fashion (122, 159-163). Parallel ChIP-seq and CLIP-seq experiments will be important to explore such possibilities. In summary, deep sequencing has enabled us to query gene regulation almost at will.

Conceptual advances

Recently, Pandolfi *et al.* (164) put forth the competing endogenous RNA (ceRNA) hypothesis that miRNAs and target transcripts thereof form a genetic regulatory network that facilitates extensive cross-talk. They and others have documented compelling evidence to support such a model (164-171). It has been reported that upon TCR activation, the transcriptome of CD4⁺ T cells undergoes widespread 3' UTR shortening (172). The authors further showed that this reprogramming of 3' UTR length is associated with avoidance of miRNA-mediated regulation. In light of the ceRNA hypothesis, miRNAs expressed in activated CD4⁺ T cells may be more potent, attributable to decreased competition for them. In addition to competition for miRNA targets, the RNA regulon hypothesis suggests that another layer of competition exists at the level of RBPs that may mediate cross-talk between transcripts as well as miRNAs. It will be important to consider this updated conceptual framework in building and testing models of genetic regulatory networks (*Fig. 1*).

Concluding remarks

Biological processes involved in the development and function of the immune system require programmed changes in protein production and constitute prime candidates for post-transcriptional regulation. While the ENCODE project initially aimed to identify all functional elements in the human DNA sequence, recent discoveries centered around miRNAs and multi-tasking RBPs, such as Lin28, have highlighted the need for a similar systematic effort in mapping post-transcriptional functional elements within the transcriptome. Integration of genomic, transcriptomic, and proteomic data remains a daunting but necessary task to achieve understanding of the full impact of genetic programs and the enigmatic roles of regulatory RNAs. Mastering the science of (re)programming cell fates promises to unleash the potential of stem cells for Regenerative Medicine.

Acknowledgments

We acknowledge Rami Zahr and Elizabeth Liu for contribution of unpublished results and Drs. Brenna Brady, Jeremy Daniel, and Chryssa Kanellopoulou for critical reading of the manuscript. The Integrative Immunobiology Unit is supported by the Intramural Research Program of the NIAID, NIH. The authors have no conflicts of interest to declare.

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Fig. 1. Updated model of gene regulation that integrates RBPs and ncRNAs

A cell's fate is determined by its transcriptome and proteome. Its transcriptome and translome is regulated by transcriptional and post-transcriptional networks. Here they are depicted as an integrated circuit that processes input (signal) to mediate an output, some form of cellular response (not depicted). For simplicity, post-translational and competing endogenous RNA networks are not depicted either. Chromatin regulators and transcription factors with the aid of lncRNAs control the accessibility and transcription rate of protein coding and non-coding genes while RBPs collaborate with ncRNAs to orchestrate the processing, transport, translation, and life-span of RNA transcripts. Since mRNA turnover can be slow, post-transcriptional regulation has evolved an important role in rapidly resetting the transcriptome in response to developmental and environmental cues that demand acute response not achievable by transcriptional regulation alone.

a			
UUAGGGUCACACCCACCACUG	GGAG AUAA	7a-1	MI0000556
UAGAGUUACAUCAAG	GGAG AUAA	7a-2	MI0000557
UCAGGGCAGUGAUGUUGCCCCUCC	GAAG AUAA	7b	MI0000558
UAGAGUUACACCCUG	GGAG UAA	7c-1	MI0000559
UUGGGCUCUGCCCCGCUC	UGCG GUAA	7c-2	MI0000560
UUAGGGCAGAGAUUUUGCCCCACA	GGAG UAA	7d	MI0000405
GAGGAAGACACCCGA	GGAG AUCA	7e	MI0000561
GUGGGGUAGUGAUUUUACCCUGUUUA	GGAG AUAA	7f-1	MI0000562
UUAGGGUCAUACCCCAUCU	GGAG AUAA	7f-2	MI0000563
UGAGGGUCUAUGAUACCACCCGGUACA	GGAG AUAA	7g	MI0000137
GGUCGGGUUGUGACAUUGCCCCGCUGU	GGAG AUAA	7i	MI0000138
GUGGGGUAGGGAUUUUAGGCCCCAGUAA	GAAG AUAA	Mir-98	MI0000586
b			
UUGGGCUCUGCCCCGCUC	UGCG GUAA	mmu-let-7c-2	MI0000560
UUGGGCUCUGCCCCGCUC	UGCG GUAA	rno-let-7c-2	MI0000831
UGGGGCUCUGCCCUGCUA	UGGG AUAA	hsa-let-7a-3	MI0000062
UGGGGCUCUGCCCUGCUA	UGGG GUAA	eca-let-7a-2	MI0012932
UGGGGCUCUGCCCUGCUC	UGGG UAAC	cfa-let-7a-1	MI0007989
UGGGGCUCUGCCCUGCUA	UGGG AUAA	ppy-let-7a-3	MI0014781
UGGGGCUCUGCCCUGCUA	UGGG AUAA	ptr-let-7a-3	MI0008399
UGGGGCUCUGCCCUGCUA	UGGG AUAA	mml-let-7a-3	MI0007572
UGGGGCUCUGCCCUGCUA	UGGG AUAA	bta-let-7a-3	MI0005452
UGGGGGUCGCUCCCUCUG	UCUG UGAG	mdu-let-7a-3	MI0005350
UUAGGGUUAUGCCCUGCC	UGUC AGAU	gga-let-7a-3	MI0001171
UGAGGGUUAACCCUUGC	UGUC AGAU	dre-let-7a-3	MI0001860
UUAGGGUCAUUCCCAUGC	UGUC AGAU	ola-let-7a-3	MI0019551

Fig. 2. Divergent evolution of a let-7 miRNA family member to evade Lin28 binding
 (A). Alignment of loop region sequences of all mouse pre-let-7 family members. (B).
 Alignment of loop region sequences of mouse pre-let-7c-2 orthologs in the indicated
 species. The box highlights the conserved absence of a consensus motif for Lin28 binding
 across vertebrates. *Red*: Canonical GGAG or GAAG motifs. *Blue*: motifs predicted not to
 bind Lin28. All sequences for alignments were obtained from miRBase (www.mirbase.org)
 (17).

Fig. 3. *Lin28b* promotes fetal-like lymphopoiesis

(A). Model depicting how *Lin28b* and *let-7* expression shifts between fetal and adult hematopoiesis. The immune system develops in waves during ontogeny, being initially populated by cells generated from fetal HSCs and later by cells derived from adult HSCs. *Lin28b* is highly expressed in fetal hematopoietic stem/progenitor cells (HSPCs) present in the fetal liver and thymus in humans and mice but is down-regulated in the neonate and undetectable in adult HSPCs. The expression of *Lin28b* correlates with the potential of fetal HSPCs for development of innate-like lymphocytes and inversely correlates with expression of mature *let-7* family members. (B). Ectopic expression of *Lin28* reprograms hematopoietic HSPCs from adult bone marrow, endowing them with the ability to mediate multi-lineage reconstitution that resembles fetal lymphopoiesis.

Fig. 4. Lin28 controls multiple cellular processes post-transcriptionally via distinct mechanisms Lin28 is a multi-functional RBP regulating growth and differentiation through inhibition of let-7 biogenesis as well as selective regulation of mRNA translation. Since let-7 is predicted to directly repress hundreds of target genes including Myc, Igf2bp2, Hmga2, IL-6, and K-ras, loss of mature let-7 expression could result in a dramatic effect on a cell's gene expression program. CLIP-seq has identified additional direct targets of Lin28 including its own mRNA, splicing factors and a collection of transcripts destined for translation in the ER. Knowing that Lin28 recognizes a consensus sequence and structure shared by many RNA molecules, we speculate that it could interact with lncRNAs as well to control many cellular processes. Dashed lines indicate indirect interactions, and dotted lines indicate hypothetical interactions that may be in effect depending on cellular context.

Table 1

A selection of RBPs with known function in the hematopoietic system

RBP	RNA-binding domain	Implication in hematopoietic system	Known target RNAs	References
Ago2 (eIF2C2)	Piwi (RNaseH-like), PAZ	B cell development and erythropoiesis	Mature miRNA guide strand, miRNA targets, a subset of pre-miRNAs (eg. miR-451)	(90, 91, 173)
Dicer	RNaseIII, PAZ, dsRBD, helicase	Lymphocyte development, survival and effector functions	Most pre-miRNAs, endo-siRNA precursors	(81, 82, 84, 85, 174)
Drosha	RNaseIII, dsRBD		Most pri-miRNAs, Dgcr8, Neurogenin2	(83, 97-99)
Dgcr8	dsRBD			
MCPIP1 (Zc3h12a)	PIN-like RNase, CCHC-type ZnF	Inflammation	Cytokines, miR-155	(175)
Lin28A	CCHC-type ZnF, Cold Shock	Mediator of miR-125 induced leukemogenesis	pri- or pre-let-7, Hmga1, Lin28a, TDP-43	(68)
Lin28B		Fetal lymphopoiesis, leukemia		(7, 58, 59)
TUTase4 (Zcchc1)	CCHC-type ZnF, PAP associated and nucleotidyl transferase domains	Lin28 dependent let-7 poly-uridylation, directs cytokine production through miR-26	pre-let-7, miR-26	(128-130)
Roquin (Rc3h1)	CCHC-type ZnF	Repressor of ICOS, T cell activation, immune homeostasis	Icos	(176-179)
Tis11 (TTP, ZFP36)		Repressor of NFkB activity, MAPK target, mRNA decay		(140, 180-184)
Tis11b (ZFP36L1)		Pro-apoptotic tumor suppressor, thymocyte development, hematopoiesis		(114, 185-187)
Tis11d (ZFP36L2)				(185, 188)
AUF1 (Hnripd)	RNA-recognition motif (RRM)	B cell maintenance and function, anti-apoptotic, inflammation, aging, cell cycle, component of LR1 transcription complex	A U-rich elements in 3' UTRs	(106, 113, 189-193)
Nucleolin (Ncl)		Hematopoiesis, cell survival, DNA damage response		(111, 194-196)
HuR(Elav1)		HSC maintenance, B cell and megakaryocyte development		(105, 112, 137, 192, 197-201)
RMB15		Anti-inflammatory, alternative splicing		(161, 202, 203)
Tia1	K homology (KH)	Anti-inflammatory, DNA damage response, selective regulation of miRNA biogenesis		(204-206)
KSRP		NFkB complex subunit, lymphocyte activation, DNA repair		(107, 108, 115, 117, 119, 136)
Rps3		tumor suppressor, upstream repressor of Lin28b		(159, 207-209)
RPL22	L22e	Hematopoiesis, thymocyte development, NMD	40S rRNA, Rps3	(59, 210)
UPF1	RNA helicase UPF1, 2 interacting domain		60S rRNA, EBER1	(126)

RBP	RNA-binding domain	Implication in hematopoietic system	Known target RNAs	References
UPF2				(125)
RIG-I	DEXD/H helicase	Innate immune receptor, PAMP recognition	Viral dsRNA	(211)
TLR3	Leucine-rich repeats (LRR)			(212)
TLR7,8			Viral ssRNA	(213-215)