K.M.J. Menon
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Post-transcriptional Mechanisms in Endocrine Regulation

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Preface

The endocrine system is both diverse and complex, controlling a wide array of physiological processes and cellular responses. Hormones induce a variety of effects on cells, including regulation of metabolic processes, by increasing the levels or activity of enzymes and proteins in the target cells. Hormone responses can be amplified through cascades of signal transduction pathways leading to changes in gene expression including synthesis, processing, stability, and translation of mRNAs. Moreover, hormone responses can change enzyme activity through covalent modifications. While activation of gene expression and covalent modification of proteins have been the intense focus of studies for over the past several decades, recent advances in the RNA biology have provided new insights into the role of post-transcriptional mechanisms involved in cellular responses to hormones and other biomolecules.

Throughout biology, post-transcriptional regulatory mechanisms play an essential role in controlling dynamic gene expression. The outcome of this regulation includes control of the amount, timing, and location of protein expression. Regulation is mediated by cis-acting RNA sequences and structures and transacting RNA-binding proteins and noncoding RNAs, including microRNAs. Recent advances in the characterization of these regulatory factors have revealed enormous regulatory potential; thousands of new RNA-binding proteins and microRNAs, which control protein expression in fascinating ways, have been identified in mammals. The goal of this book is to highlight the advances made in the understanding of the regulatory mechanisms by which hormones control these processes.

The first chapter of this volume provides an overview of our current understanding of the various components of post-transcriptional mechanisms including regulatory factors, elements, and general mechanisms that control protein expression. The intent is to provide early stage investigators with an introduction, emphasizing key concepts underlying post-transcriptional regulatory mechanisms.

In subsequent chapters, specific system-based studies on post-transcriptional regulation are described. These chapters were composed by leading experts who summarize current state of knowledge, remaining key questions, and provide perspective for future research goals.

Chapter 2 describes the intricate regulation of expression of insulin and insulin-like growth factors (IGF). The mRNAs encoding these peptide hormones are subject to multiple regulatory mechanisms including alternative RNA processing, regulated mRNA stability, and modulation of translation efficiency. RNA-binding proteins, RNA degrading and modifying enzymes, and noncoding regulatory RNAs (i.e., microRNAs) control expression of insulin and IGF. This work has important relevance to the physiology of energy homeostasis, diabetes, and cancer.

In Chap. 3, mechanisms of regulation of cytokine mRNA during inflammatory response are presented. Emphasis is placed on the cis-acting elements of cytokine mRNAs including the now classic adenosine-uridine rich elements (AREs), which act as bifunctional switches to modulate cytokine mRNA stability. The authors introduce the concepts of combinatorial regulatory control and competitive binding, which form regulatory networks to control cytokine production and modulate innate immune responses. Defects in these control mechanisms contribute to inflammatory autoimmune diseases and cancer.

Chapters 4–7 deal with post-transcriptional regulation of target cell function by reproductive hormones. These processes have important roles in reproductive processes and dysfunction contributes to diseases such as infertility and cancer. In Chap. 4, a novel mechanism of regulation of luteinizing hormone receptor (LHR) mRNA expression by an mRNA-binding protein is presented. In response to physiological changes in the secretion of LH, the expression of LHR mRNA in the ovary undergoes rapid changes, the most striking change occurring after LH surge to induce ovulation. During this period the LH receptor mRNA expression is transiently downregulated by increasing LHR mRNA degradation that is mediated by an unanticipated LHR mRNA-binding protein—a steroid metabolism protein that “moonlights” as an RNA-binding regulator. This chapter highlights important concepts including regulation though mRNA localization, mRNA stability, and translational control. In Chap. 5, the regulation of estrogen receptor, a member of the nuclear receptor family, by its own ligand is described. Evidence is presented showing that estrogen stabilizes its receptor by increasing the binding of a specific protein to the receptor’s mRNA. Moreover, microRNAs contribute to translational control of estrogen receptor.

Chapter 6 focuses on the regulation of ovarian function by microRNAs. This chapter provides information on the synthesis and processing of microRNAs. The mechanisms of translational control and mRNA degradation caused by microRNAs are presented. The regulation of microRNAs by differential processing and competitive inhibition by endogenous competitor RNAs is also considered. The authors then describe the important roles of microRNAs and their processing enzymes in controlling ovarian gene expression during reproductive cycles.

Chapter 7 provides a detailed discussion of post-transcriptional control in the germline. Regulation of translation, RNA localization, and decay pervades the germline, gametes, and early embryos in part due to the quiescence of transcription during early developmental stages. Maternal mRNAs are deposited in developing oocytes, stored in a repressed state within RNA-protein granules, and subsequently activated to drive development in response to hormonal cues and fertilization.

Moreover, post-transcriptional control specifies body pattern formation and designates the primordial germline. This chapter highlights multiple key regulators that control these crucial events.

Chapter 8 focuses on VEGF-A, a hormone that controls angiogenesis during development and wound healing, among other processes. Proper control of VEGF-A is crucial and dysregulation contributes to cancer and other disorders. Control of VEGF-A is intricate, as the authors guide the reader through the diverse mechanisms that control VEGF-A expression and function including alternative mRNA processing, mechanisms of destabilization and stabilization of the mRNA, and translational control, mediated by cis-acting RNA elements, RNA-binding proteins, and microRNAs.

Prostaglandins are lipid hormones that control reproductive physiology and inflammation. Chapter 9 explores the pathway of prostanoid biosynthesis and the post-transcriptional mechanisms that control these enzymes. Multiple RNA-binding proteins and microRNAs control translation, stability, and localization of mRNAs encoding prostanoid biosynthesis enzymes. This regulation involves regulated and coordinated mRNA degradation pathways and enzymes. Additionally, mRNA localization to specialized intracellular granules contributes to the control of prostaglandin synthesis. These layers of regulation ensure proper control of prostaglandins, which is crucial since unregulated expression contributes to cancer and inflammatory diseases.

Chapter 10 explores post-transcriptional regulation of production of the secreted peptide hormone leptin. Leptin is produced by fat cells and contributes to the control of appetite and energy storage and homeostasis. This hormone has a major impact on obesity and dieting. Leptin production is controlled at the post-transcriptional level by insulin signaling through the important mammalian target of rapamycin pathway (mTOR), which controls translation efficiency of mRNAs.

Post-transcriptional mechanisms that control parathyroid hormone gene expression are explored in Chap. 11. Parathyroid hormone has crucial role in the control of mineral metabolism and bone strength. Regulation of this secreted peptide hormone is achieved by RNA-binding proteins that interact with the hormone's mRNA to stabilize or degrade the mRNA in response to serum calcium, phosphate, and vitamin D.

Chapter 12 illuminates the pathways of steroid hormone biosynthesis, which broadly influence metabolism, physiology, reproduction, and immune function. The authors explain the roles of post-transcriptional and post-translational mechanisms that operate to control steroid production, emphasizing the roles of microRNAs in translational control and mRNA degradation of the biosynthetic enzymes.

Glucocorticoids are steroid hormones that control immune function and stress response. Chapter 13 examines the post-transcriptional regulation by glucocorticoid including modulation of cytokine translation and mRNA stability. Multiple mechanisms appear to contribute to regulation. Glucocorticoid receptor, a ligand-activated transcription factor, regulates RNA synthesis to modulate signal transduction and the expression and activity of RNA-binding proteins and microRNAs. These effects drive changes in mRNA stability and translation. Surprisingly, recent evidence

indicates that glucocorticoid receptor can bind to mRNA directly to control degradation of specific mRNAs in the cytoplasm.

Chapter 14 surveys post-transcriptional regulation by brain-derived neurotrophic factor (BDNF) in the nervous system. Protein expression in neurons is controlled by intricate mechanisms including RNA localization to synapses and repression and activation of specific mRNAs in response to signals. These mechanisms contribute to the control of neuronal activity, synaptic plasticity, and longer-term memory formation. BDNF plays an important role in controlling activity-dependent gene expression in neurons by globally enhancing translation through mTOR pathway. Certain mRNAs are specifically affected by BDNF signaling, and this selectivity is determined by the activities of specific RNA-binding factors that control translation and mRNA stability.

We thank the authors for their time and effort to contribute to this book and for their cooperation for timely submission. We hope the information presented in this book will be a valuable source of current state of knowledge for experts as well as beginners who wish to pursue future research in this exciting area.

Finally we thank Springer for the opportunity to edit this volume dealing with post-transcriptional mechanism in endocrine regulation. Special thanks to former Editor, Meredith Clinton, of Endocrinology at Springer for her support in the initial stages and Kelly Wilson, Associate Editor, Endocrinology, Springer Science and Business Media, for her support in making our effort a reality. We appreciate the assistance of Kelly Studer, Administrative Assistant at the University of Michigan, who cheerfully provided us administrative support during the course of this project.

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Mechanisms of Post-transcriptional Gene Regulation

1

René Arvola, Elizabeth Abshire, Jennifer Bohn, and Aaron C. Goldstrohm

1 Introduction

Gene expression involves multiple sequential steps which are highly regulated and coordinated. Regulation of gene expression is crucial for proper function of the endocrine system. Over the past several decades, an abundance of data has accumulated which demonstrates the importance of post-transcriptional mechanisms in control of the endocrine function, which is the subject of this book. These mechanisms can control the amount, timing, and location of protein expression. Moreover, post-transcriptional mechanisms of RNA processing and editing can change the properties of expressed proteins. The purpose of this chapter is to provide an overview of post-transcriptional regulation, focusing on translation and mRNA degradation pathways. We also explore our current knowledge of the mechanisms that regulate protein synthesis and mRNA stability. We refer readers to recent comprehensive reviews on additional post-transcriptional mechanisms including RNA processing (Elkon et al. 2013; Fu and Ares 2014) and modifications (Wang and He 2014), nucleo-cytoplasmic transport (Wickramasinghe and Laskey 2015) and localization (Buxbaum et al. 2015).

Opportunities for post-transcriptional control are numerous. Messenger RNAs (mRNAs) contain extensive cis-acting sequences that can control RNA processing, translation efficiency, and mRNA stability. Furthermore, eukaryotic genomes encode a large repertoire of trans-acting regulators, including RNA binding proteins

Note: Due to the space limitations and the broad nature of this chapter, we were unable to cite many important contributions, thus we apologize to colleagues whose work could not be highlighted.

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and non-coding RNAs, with potential regulatory functions. Current estimates indicate that the human genome encodes some 1500 RNA binding proteins (Gerstberger et al. 2014) and thousands of non-coding RNAs, such as the small regulatory microRNAs (miRNAs) (Bartel 2004; Geisler and Coller 2013). Yet the function of the vast majority of this regulatory potential remains to be explored, especially in physiological systems. The chapters of this volume highlight elegant cases wherein intricate post-transcriptional regulatory mechanisms control responses to and responses from endocrine pathways.

2 Translation Regulation

Translation efficiency plays an important role in determining the level of protein expression. Globally, protein levels correlate poorly with mRNA levels, and translation efficiencies of mRNAs vary widely (Schwanhausser et al. 2011). Translation of mRNAs can be regulated on a global scale, for instance in response to stress or infection (Liu and Qian 2014). Moreover, translation of specific mRNAs or groups of mRNAs can be regulated to control specific pathways, environmental responses, and developmental programs (Curtis et al. 1995; Kong and Lasko 2012; Micklem 1995). In this section, we will provide an overview of the process of translation followed by an exploration of the major paradigms of translational control. For more detailed insight, we refer readers to several excellent reviews on this subject (Aitken and Lorsch 2012; Hinnebusch 2014; Jackson et al. 2010; Kong and Lasko 2012; Sonenberg and Hinnebusch 2009). To provide context for discussion of translational control and its role in endocrine function, we will review the mechanism of translation, and then present principles and examples of regulatory mechanisms that control translation.

2.1 Mechanism of Translation

The process of translation can be conceptualized by four steps: (1) mRNA activation, (2) initiation, (3) elongation, and (4) termination, each having unique mechanisms of control. (1) An mRNA is activated for translation when it is associated with the trans-acting factors necessary to begin recruitment of ribosomes. (2) Translation initiation involves assembly of the ribosome on the activated mRNA, which is facilitated by translation initiation factors (Aitken and Lorsch 2012). During initiation, the ribosome scans the mRNA to identify the translation start site (i.e. the initiation codon), which is recognized by base-pairs formed between the mRNA start codon and the initiator tRNA (tRNAi) anticodon (Hinnebusch 2014). As we will see in the following sections, initiation involves a series of carefully orchestrated events involving multiple translation factors, and therefore is a rate-determining step of protein synthesis that is subject to multiple mechanisms of regulation (Jackson et al. 2010; Sonenberg and Hinnebusch 2009). (3) Once the start site is identified, the ribosome catalyzes elongation of the nascent polypeptide chain, (4) which is terminated upon encountering a stop codon (Aitken and Lorsch 2012).

2.1.1 The Ribosome and Translation Factors

The process of translation is catalyzed by the ribosome, a large multisubunit ribonucleoprotein complex. Translation is facilitated by an assortment of eukaryotic initiation factors (eIFs), elongation factors (eEFs) and termination factors (Aitken and Lorsch 2012; Inge-Vechtomov et al. 2003; Riis et al. 1990).

The eukaryotic ribosome is comprised of two subunits: a large 60S subunit and a small 40S subunit comprised of 80 ribosomal proteins and 4 ribosomal RNAs (60S: 5S, 5.8S, and 25S rRNA; 40S: 18S rRNA). During the translation initiation step, the two subunits must join together on the mRNA to form the 80S ribosome, which is capable of catalyzing peptide bond formation. The mRNA is held at the interface of the two subunits, positioned to permit reading of the codons by incoming transfer RNA molecules (tRNAs) (Aitken and Lorsch 2012). For detailed information on the fascinating structure and function of the ribosome components, readers are directed to recent reviews (Korobeinikova et al. 2012; Wilson and Doudna Cate 2012; Yusupova and Yusupov 2014).

Transfer RNAs that are charged with amino acids are essential ingredients for translation. Each amino acid is covalently appended to the appropriate cognate tRNA by an amino-acyl tRNA synthetase. These charged tRNAs are then delivered to the ribosome as RNA-protein complexes with special translation factors. The tRNA involved in translation initiation, tRNAi, is charged with methionine (Met-tRNAi). Met-tRNAi forms a ternary complex with the GTP-bound form of translation initiation factor eIF2, and together they associate with the 40S ribosomal subunit to function during initiation of protein synthesis. During initiation, the Met-tRNAi is positioned in the Peptidyl-site (P-site) of the ribosome. The other charged tRNAs associate with the GTP-bound form of translation elongation factor eEF1A, which delivers them to the Amino-acyl site (A-site) of the ribosome as specified by the mRNA's codons (Dever and Green 2012; Ibba and Soll 2000).

2.1.2 Key mRNA Features Necessary for Translation

Messenger RNAs possess features that influence their translational efficiency, including two important non-templated modifications. The first is the 7-methyl-guanosine nucleotide cap at the 5′ end of the transcript, which is added during transcription in the nucleus. In the cytoplasm, this cap facilitates translation by interacting with the translation initiation factor, eIF4F, which is composed of subunits eIF4E, eIF4G, and eIF4A. All three subunits bind to the RNA, with eIF4E directly contacting the 5′ cap (Aitken and Lorsch 2012). The 3′ ends of all mRNAs (with the exception of replication-dependent histone mRNAs) are also enzymatically modified by addition of a polyadenosine tail. Addition of this poly(A) tail by poly(A) polymerase enzyme is coupled to 3′ end processing of the nascent transcript, which occurs through a sequence-specific endonucleolytic cleavage event (Elkon et al. 2013). The poly(A) tail is bound by poly(A) binding proteins (Kuhn and Wahle 2004). In the cytoplasm, the PABPC1 protein coats the poly(A) tail and enhances the efficiency of translation (Kuhn and Wahle 2004).

The protein coding capacity of the mRNA is specified by several features that determine where translation will begin and end, defining the open reading frame (ORF).

The translation initiation site is typically the first AUG codon, from the 5′ end of the mRNA, with the proper surrounding sequence context (Jackson et al. 2010). This context, originally characterized by Marilyn Kozak, surrounds the initiation site AUG (Kozak 1987). In vertebrates, the general "Kozak" consensus sequence is gccRccAUGG, where the underlined AUG initiation codon is flanked by uppercase nucleotides, denoting strong influence on initiation, and lowercase nucleotides denoting lesser importance for initiation. Note that the "R" indicates a purine nucleotide base. Using transcriptome-wide datasets, AUG codon contexts have now been defined for multiple species (Nakagawa et al. 2008). The 3′ end of the protein coding region is specified by an in-frame stop codon, either UAA, UGA, or UAG (Dever and Green 2012).

Messenger RNAs can contain other features that affect translation. Only a portion of the mRNA sequence encodes protein, while the remaining sequences are 5′ and 3′ Untranslated Regions (UTR). These UTRs can range from tens of nucleotides to thousands of nucleotides and—as we shall see throughout this chapter and book—play important regulatory roles to control and alter translation (Mignone et al. 2002).

2.1.3 Activated mRNAs and the Closed-Loop Conformation

Before engaging ribosomes, mRNAs must be activated. The 5′ and 3′ ends of the mRNA are brought together through interactions between cap-bound eIF4F and poly(A)-bound PABPC1. This "looping" is mediated by the eIF4G component of eIF4F, which bridges the cap binding protein eIF4E and PABPC1 via protein–protein interactions. Circular RNA-protein complexes have been observed by atomic force and electron microscopy (Afonina et al. 2014; Wells et al. 1998). Moreover, evidence in several systems demonstrates synergistic stimulation of translation mediated by the 5′ cap and poly(A) tail (Borman et al. 2000; Gallie 1991; Michel et al. 2000). Thus, mRNAs complexed with eIF4F and PABPC1 can be considered to be in an activated state that is potentiated for subsequent loading of ribosomes. It is interesting to note that replication-dependent histone mRNAs maintain a cap-to-tail closed loop, though they do not possess a poly(A) tail; instead, the histone mRNA closed loop is formed by specialized RNA binding proteins that recognize a unique 3′ end RNA structure, thereby promoting histone protein synthesis (Marzluff et al. 2008). Thus, closed loop formation is thought to be a generalized feature of activated mRNAs (Fig. 1.1).

2.1.4 Initiation: Assembly of the Pre-initiation Complex

Translation initiation requires at least 12 different initiation factors that act to bring together the ribosome subunits on the mRNA and, as the most complex stage of translation, this process is highly regulated. First, the small ribosomal subunit must locate the initiation codon, facilitated by base-pairing with the anticodon of the tRNAi. Initiation begins with the formation of a pre-initiation complex (Fig. 1.1a). First, the 40S small ribosomal subunit associates with initiation factors eIF1, eIF1A, eIF5, eIF3 and the eIF2 ternary complex (composed of GTP-bound eIF2 and met-tRNAi) to form the 43S pre-initiation complex (PIC). The 43S PIC is then joins an activated mRNA to form the 48S PIC, mediated by many protein and RNA contacts

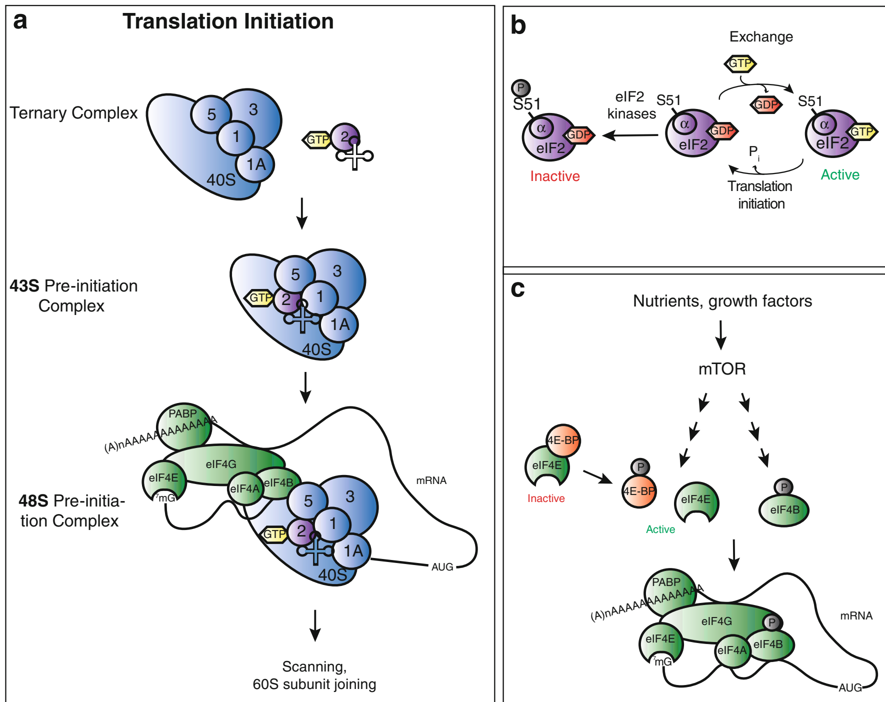

Fig. 1.1 Translation initiation and common mechanisms of regulation. The steps of translation initiation are outlined in (**a**). The ternary complex is comprised of eIFs 1, 1A, 3 and 5 joined to the 40S small ribosomal subunit. eIF2 bound to GTP and the initiator tRNA joins the ternary complex to form the 43S pre-initiation complex (43S PIC). The 43S PIC is then bound to an activated mRNA to form the 48S PIC. The 48S PIC performs scanning until the Kozak sequence is found. The 48S PIC is then joined by the 60S large ribosomal subunit, signaling the end of initiation and the start of elongation. (**b**) Demonstrates how translational efficiency is regulated negatively through phosphorylation of eIF2. eIF2 is bound to GTP, which is hydrolyzed during initiation. The eIF2α subunit of eIF2 can be phosphorylated at Serine 51 (S51) to negatively regulate its role in translation. Unphosphorylated eIF2 can initiate translation normally, whereas phosphorylated eIF2α prevents exchange of GDP for GTP, inhibiting initiation. (**c**) shows how members of the eIF4F complex are regulated by mTOR. Phosphorylation by various kinases of the mTOR signaling cascade enhances translational efficiency through inhibition of 4E-BP, as well as enhancing association of eIF4B with eIF4F

including those formed between eIF4F, PABP and mRNA with the multisubunit initiation factor, eIF3. Once these translation factors have assembled on the mRNA, the next step is to locate the proper initiation codon (Aitken and Lorsch 2012; Jackson et al. 2010).

2.1.5 Initiation: Scanning for the Initiation Codon

Once the 48S PIC is assembled, the ribosome must locate the translation start site (typically the first AUG codon) to initiate protein synthesis. In order for the 48S PIC to search for the AUG start codon, it must traverse the 5′ UTR in a process known

as ribosome scanning. 5′UTRs frequently contain RNA structures which can impede scanning and thus inhibit translation. In the event that the first AUG codon has a poor context, downstream AUG codons can be utilized to initiate translation, a process referred to as leaky scanning (Hinnebusch 2014).

Scanning through RNA structure by the 48S PIC is promoted by the eIF4A protein, which is an ATPase/helicase that can unwind secondary structure in the 5′UTR. Other helicases may also facilitate scanning. This process requires energy in the form of hydrolysis of adenosine triphosphate (ATP). eIF4B binds single stranded RNA (ssRNA) and also helps in unwinding. eIF4G is involved by facilitating the association of eIF4A (Hinnebusch 2014).

The factors eIF1, eIF1A, and eIF3 aid in scanning by stabilizing the open conformation of the mRNA entry channel of the small ribosome subunit, and also in start codon recognition. The 48S PIC slides along the mRNA, sampling the RNA until the first AUG codon in the proper sequence context is located. Once the AUG start codon successfully base pairs with its anticodon complement on the Met-tRNAi, eIF1 is released and the PIC adopts a more closed conformation. At this point, the Met-tRNAi is positioned in the P-site of the ribosome. The GTP bound to eIF2 is then hydrolyzed and eIF5 and GDP-bound eIF2 are released from the PIC. eIF1A is the only initiation factor from the PIC which remains bound throughout the entire process of initiation. The PIC is now more stably bound to the mRNA and tRNAi and poised for joining of the 60S subunit (Hinnebusch 2014).

2.1.6 Initiation: Formation of the 80S Ribosome

The next phase of initiation is assembly of the 80S ribosome through joining of the 60S subunit to the initiation codon associated 48S PIC. The 60S large ribosomal subunit first assembles with the GTPase protein eIF5B. Upon large and small ribosome subunit joining, eIF5B hydrolyzes its GTP. eIF5B and eIF1A are then released as the ribosome undergoes a conformational change. The resulting 80S ribosome is thereby primed to enter the elongation phase (Aitken and Lorsch 2012; Jackson et al. 2010).

2.1.7 Elongation

Once the 80S ribosome has assembled at the initiation site, protein synthesis can commence through ribosome-catalyzed peptide bond formation between the Met-tRNAi located in the P-site and the incoming amino-acylated tRNA in the A-site. The nascent polypeptide chain is extended through sequential rounds of peptide bond formation and translocation of the ribosome along the mRNA. Subsequent amino acid additions are specified through complementary base-pairing between tRNA anti-codons and the triplet codons of the mRNA. Elongation in eukaryotes is mediated by two elongation factor proteins: eEF1 and eEF2 (Dever and Green 2012). eEF1 is a multisubunit complex that delivers the amino-acylated tRNA to the ribosome (Sasikumar et al. 2012). Upon proper positioning of the tRNA in the A-site, the eEF1A subunit hydrolyzes GTP and eEF1 dissociates from the ribosome. The eEF2 factor facilitates the translocation of the ribosome and hydrolysis of GTP (Dever and Green 2012).

Typically, multiple ribosomes are sequentially assembled on and traverse the mRNA simultaneously. As the first ribosome elongates away from the initiation site,

new ribosomes can initiate and follow. The resulting mRNA with multiple associated ribosomes is referred to as a poly-ribosome or polysome (Slayter et al. 1963; Warner et al. 1963). The density of ribosomes on an mRNA is proportional to the length of the open reading frame and the rates of initiation and elongation (Ingolia 2014).

2.1.8 Termination

Translating ribosomes traverse the mRNA until they encounter a stop codon (UAA, UAG, and UGA) within the A-site, which signals the termination of polypeptide chain elongation. Since there is no tRNA anticodon complementary to the stop codon, no amino acid can be added to the end of the peptide chain. Instead, a release factor (eRF) binds the stop codon and triggers the release of the complete polypeptide from the ribosome. Eukaryotes have two release factors: eRF1, which is involved in stop codon recognition and hydrolysis of the nascent protein from the P-site bound tRNA, and eRF3, a GTPase which promotes polypeptide release. Upon termination of translation, the ribosome is disassembled into its large and small subunits, assisted by additional translation factors (Dever and Green 2012; Jackson et al. 2012).

2.2 Regulation of Translation

Translation can be regulated in multiple steps to control the amount, timing and location of protein synthesis. Cis-acting sequence elements and trans-acting factors can either activate or repress translation. Translation can be regulated globally, affecting protein synthesis from all mRNAs (as is the case with the mTOR pathway), or specifically from certain mRNAs (as with sequence-specific RNA-binding proteins and microRNAs). Groups of mRNAs can be translationally regulated in a coordinated fashion by common cis-elements and trans-factors (Abaza and Gebauer 2008; Jackson et al. 2010). One classic example of this type of post-transcriptional regulation is the 5′ terminal oligo-pyrimidine (TOP) mRNAs that encode multiple components of the translation apparatus and are coordinately regulated in response to stress (Meyuhas and Kahan 2015). Here, we will discuss control of translation and explore some of its general mechanisms (Fig. 1.2).

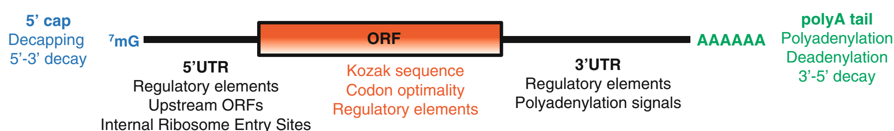

Fig. 1.2 mRNAs contain cis-acting regulatory information that controls translation efficiency and stability. Eukaryotic mRNAs have a 5′ 7-methylguanosine (7mG) cap that promotes translation and stability. The poly(A)denosine (poly(A)) tail is, recognized by poly(A) binding proteins PABP, promotes translation and stability. Removal of the cap and poly(A) tail result in subsequent mRNA degradation. Cis regulatory elements can be contained in the 5′ and 3′ untranslated regions (UTRs) of an mRNA. These can include binding sides of RNA binding proteins and non-coding RNAs that modulate stability and translation. The open reading frame (ORF) can also contribute to regulation through the Kozak sequence and codon optimality (how commonly the codons it contains are found in the cell)

2.2.1 Regulation of Translation Initiation Through the Closed Loop

The closed loop conformation of mRNAs promotes translation initiation through the cap-to-poly(A) tail interactions mediated by eIF4F and PABP. Formation of the closed loop represents an important regulatory stage. Trans-acting factors can disrupt the closed loop to inhibit translation by displacing eIF4F or PABP from the mRNA or by disrupting their protein–protein interactions (Kawahara et al. 2008; Weidmann et al. 2014; Zekri et al. 2013). Enzymatic removal of the 5′ cap structure or poly(A) tail can disrupt closed loop formation, thereby silencing translation and leaving the mRNA vulnerable to degradation, as described below. The length of the poly(A) tail can modulate translation like a rheostat, with longer poly(A) tails promoting protein expression whereas shortening of the tail reduces it. As such, factors that stabilize or shorten the tail can control translation efficiency (Goldstrohm and Wickens 2008).

Translation efficiency can be controlled by proteins that interact with PABP. PABP-interacting proteins (PAIP1 and PAIP2) bind to PABP to either stimulate or inhibit translation, respectively (Khaleghpour et al. 2001; Roy et al. 2002). PAIP1 shares homology with eIF4G and forms a complex with initiation factors eIF4A and eIF3 to enhance translation by bridging PABP's interaction with eIF4F and stabilizing the closed loop (Craig et al. 1998; Martineau et al. 2008). PAIP2 competes with eIF4G and PAIP1 to bind PABP, reducing its affinity for the poly(A) tail and reducing translation efficiency (Khaleghpour et al. 2001).

The 5′ cap and associated eIF4F complex are major targets for translational control mechanisms. Proteins that compete with eIF4F for binding to the 5′ cap can inhibit translation (Cho et al. 2005, 2006). A second, widely utilized control mechanism is mediated by proteins that directly bind to cap-binding eIF4F subunit, eIF4E. These eIF4E Binding Proteins (4E-BPs) competitively bind to the same region of eIF4E as the eIF4G subunit. In doing so, 4E-BPs disrupt eIF4F and the closed loop, thereby inhibiting formation of the PIC. In addition to repressing translation, several 4E-BPs have been shown to promote degradation of mRNAs (Andrei et al. 2005; Blewett and Goldstrohm 2012a, b; Igreja and Izaurralde 2011; Rendl et al. 2012).

Multiple signaling pathways intersect on 4E-BPs, providing a nexus for controlling translation. Unphosphorylated 4E-BPs have a high affinity for eIF4E whereas phosphorylation of 4E-BPs prevents their interaction with eIF4E (Fig. 1.1c). The Target of Rapamycin (TOR) pathway is a major regulator of translation that responds to the availability of nutrients and amino acids. TOR is an important regulator of cell growth and proliferation and is inhibited in response to stress conditions and starvation. TOR also integrates signals from hormones such as Insulin and Brain-Derived Neurotrophic Factor. In turn, TOR pathway regulates translation of peptide hormones such as Leptin. TOR promotes translation in several ways, including phosphorylation of 4E-BPs and activation of S6 kinase, which phosphorylates the small ribosomal subunit 6 and eIF4B, among other targets, to promote translation (Dennis et al. 2012; Ma and Blenis 2009; Tavares et al. 2015). This cascade of TOR signaling controls translation initiation on a broad level.

2.2.2 Regulation of Initiation Through Initiation Factor eIF2

Translation initiation depends on delivery of Met-tRNAi to the 40S subunit by GTP-bound eIF2 and subsequent PIC formation. Thus, eIF2 represents an important regulatory target. eIF2 is inhibited by phosphorylation at Serine 51 (S51) on the α subunit by various kinases in response to diverse signals (Baird and Wek 2012) (Fig. 1.1b). Kinases that phosphorylate eIF2 include: (1) PKR-like endoplasmic reticulum kinase (PERK), which is activated by the unfolded protein response; (2) General Control Nonderepressible 2 (GCN2), which is activated by diverse stressors, such glucose and amino acid starvation; (3) Protein Kinase R (PKR), which is activated by dsRNAs greater than 30 bp in length and plays an important role in anti-viral response; and (4) Heme-regulated inhibitor kinase (HRI) in erythroid cells, which is activated in response to heme deficiency (Baird and Wek 2012; Lemaire et al. 2008). Phosphorylation at S51 prevents exchange of GDP for GTP, thus the phosphorylated eIF2 cannot enter new rounds of translation. As a result, translation initiation is inhibited globally (Baird and Wek 2012).

2.2.3 RNA Binding Proteins Regulate Translation

The untranslated regions (UTRs) of many mRNAs can contain important regulatory information that controls translation (Mignone et al. 2002). RNA binding proteins (RBPs) often recognize these regions to regulate the translational efficiency and stability of target mRNAs. RBPs serve many important biological roles where gene expression needs to be quantitatively, temporally and/or spatially controlled, such as in response to hormone mediated signaling. RBPs can bind to specific RNA structures, (e.g. stem-loop structures), or they can bind to specific single-stranded sequence motifs. Upon binding to a transcript, RBPs can use diverse mechanisms to modulate translation by either repressing or activating protein synthesis (Abaza and Gebauer 2008). Here, we will explore some specific mechanisms of RBP translational repressors and activators.

RBP repressors can inhibit initiation by binding to the 5′ UTR of a target mRNA and blocking assembly of the PIC. A classic example of this mechanism is the Iron Response Protein (IRP), which, in response to low intracellular iron, binds to specific RNA stem-loop structure in the 5′UTR of ferritin mRNA, the Iron Response Element, to impede 43S joining and thus represses translation of ferritin (Muckenthaler et al. 1998).

RBP repressors can also bind to the 3′UTR of transcripts to control translation. One mechanism is to prevent assembly of the 80S ribosome. For instance, the RBPs hnRNP-K and hnRNP-E1 repress lipoxygenase mRNA by preventing 60S subunit joining to the 48S PIC (Ostareck et al. 2001). Other 3′UTR-bound RBPs can recruit 4E-BPs to a specific message to disrupt the closed loop and repress translation; examples of such interactions are numerous and include Bruno and Cup (Nakamura et al. 2004), Smaug and Cup (Nelson et al. 2004), and Puf5 and Eap1 (Blewett and Goldstrohm 2012a). This mechanism is illustrated by the RBP called Cytoplasmic Polyadenylation Element Binding Protein (CPEB), which binds to U-rich sequences known as Cytoplasmic Polyadenylation Elements (CPEs) in the 3′UTR of certain

mRNAs. One mechanism of CPEB repression is recruitment of the 4E-BP Maskin to inhibit translation of specific mRNAs, such as Cyclin B, during oogenesis (Groisman et al. 2000; Stebbins-Boaz et al. 1999).

Translation initiation can be inhibited by RBP-mediated recruitment of an eIF4E Homologous Protein (4EHP) that competes with eIF4E for binding to the mRNAs 5′ cap (Rom et al. 1998). However, unlike eIF4E, 4EHP does not interact with eIF4G and thus prevents translation initiation. The 3′UTR binding protein Bicoid recruits 4EHP to repress translation of specific mRNAs during Drosophila embryonic development (Cho et al. 2005).

RBPs can repress translation of specific mRNAs by causing shortening of the mRNAs poly(A) tail—a process referred to as deadenylation—thereby reducing or eliminating the occupancy of PABP to diminish translation initiation. One of the first examples of deadenylation mediated silencing was the finding that cytokine and growth factor mRNAs contained Adenine-Uridine Rich Elements (AREs) in their 3′UTRs which accelerated deadenylation and mRNA degradation, limiting protein expression (Wilson and Treisman 1988). These AREs can be bound by several RBPs, including the repressive Tristetraprolin (TTP) protein which binds and recruits a multisubunit complex of poly(A) degrading enzymes that shorten the poly(A) tail of TTP bound mRNAs (Lykke-Andersen and Wagner 2005). Likewise, members of the Pumilio and Fem3 Binding (PUF) family of sequence-specific RBPs bind to 3′UTRs and recruit specialized poly(A) degrading enzymes that remove the poly(A) tail to repress protein expression (Goldstrohm et al. 2006; Van Etten et al. 2012). CPEB, as mentioned earlier, also promotes deadenylation of the mRNAs to which it binds by recruiting the poly(A) specific ribonuclease (PARN), contributing to translational repression (Kim and Richter 2006).

3′UTR-bound RBPs can also repress translation by promoting removal of the message's 5′ cap structure. The TTP protein interacts with and recruits decapping enzymes to specific transcripts that contain ARE sequences in their 3′UTRs (Fenger-Gron et al. 2005; Lykke-Andersen and Wagner 2005). One PUF protein can promote decapping of mRNAs by using a 4E-BP to disrupt eIF4F and to recruit decapping factors to the message, resulting in translational repression and mRNA degradation (Blewett and Goldstrohm 2012a, b). Through these mechanisms, RBP mediated translational repression and mRNA degradation are directly interrelated, a subject that we shall revisit in subsequent discussion of mRNA decay pathways in post-transcriptional control.

2.2.4 RBP Activators

Translation can also be activated by cis- and trans-acting factors, which can boost the amount of protein produced by an mRNA. They can also reanimate mRNAs that have been stored in a quiescent status, a common event in developmental contexts (Gray and Wickens 1998; Ivshina et al. 2014). Just as the poly(A) tail is a target for repressive mechanisms, it can also be employed to activate mRNAs. Polyadenylation (that is, lengthening of the poly(A) tail) and the resulting increased recruitment of PABP can promote translational activation. Thus, dormant, deadenylated mRNAs can be activated by polyadenylation in the cytoplasm via recruitment of poly(A) polymerase enzymes, such as GLD2 (Ivshina et al. 2014).

Perhaps the best characterized example of polyadenylation mediated activation is the sequence-specific RBP CPEB (Charlesworth et al. 2013). As described earlier, CPEB represses mRNAs via deadenylation and Maskin-mediated inhibition of eIF4F. CPEB acts as a bifunctional regulator during oogenesis, switching from repression to activation in response to signal the steroid hormone progesterone (Groisman et al. 2002; Ivshina et al. 2014; Sarkissian et al. 2004). Aurora A kinase phosphorylates CPEB, thereby switching it to an activation mechanism wherein CPEB interacts with and recruits GLD2 poly(A) polymerase. CPEB-Gld2 mediated polyadenylation requires that the mRNA contain both a CPE and the polyadenylation element (AAUAAA) at the 3′ end of the mRNA. The polyadenylation element is recognized by a cytoplasmic version of the Cleavage and Polyadenylation Specificity Factor (CPSF). The CPEB-GLD2 complex extends the poly(A) tails of the target mRNAs and increases the occupancy of poly(A) binding proteins. Thus, CPEB activation includes derepression and polyadenylation resulting in increased efficiency of translation (Ivshina et al. 2014).

2.2.5 Regulation of Translation Elongation

The process of elongation is iterated with an average rate of 6 amino acid additions per second (Ingolia et al. 2011). Global analyses suggest that protein synthesis rates vary over a wide range (Schwanhausser et al. 2011). Various factors impinge on the elongating ribosome to influence its speed and the location and quality of protein expression. For instance, elongation rate can be influenced by synonymous codon usage and the availability of the necessary amino-acylated tRNAs (Pechmann and Frydman 2013; Presnyak et al. 2015; Quax et al. 2015; Tarrant and von der Haar 2014). For membrane bound and secreted proteins such as hormones, a signal peptide in the nascent polypeptide is recognized by the Signal Recognition Particle to direct the translating mRNA to the proper intracellular location. Chaperones (for example, heat shock proteins) associate with and fold the nascent peptide cotranslationally (Jha and Komar 2011). In several examples, signal transduction pathways have been shown to target elongation factors to influence the rate of protein synthesis (Dever and Green 2012; Sasikumar et al. 2012).

2.2.6 Alternative Mechanisms of Initiation: Cap-Independent Translation Initiation

Translation generally requires the 5′ cap; however, in specialized instances, translation can initiate in a cap-independent manner, mediated by internal ribosome entry sites (IRES). IRES are highly structured elements present in the 5′UTR of specific mRNAs which can allow translational initiation on that mRNA without the requirement of the 5′ cap and certain initiation factors through complex interactions with the ribosome, circumventing the process of scanning. IRES were first discovered in viral mRNAs, but examples of cellular IRES-containing mRNAs have emerged. These alternative initiation mechanisms permit translation of specific proteins when cap-dependent translation is turned off by the cell in response to viral infection, or other cellular stresses (Hellen and Sarnow 2001).

One well known example of a viral IRES-containing mRNA is that of the Hepatitis C Virus, which contains both 5′ and 3′ IRES elements (Fraser and Doudna 2007).

An extreme example of a viral 5′ IRES is that the Cricket Paralysis Virus, which is able to bypass the requirement for all translation initiation factors (including eIF2-tRNAi) by mimicking the initiator tRNA in the P site of the ribosome (Fernandez et al. 2014; Hellen and Sarnow 2001). A well-studied example of a cellular IRES-containing mRNA which will be discussed in Chap. 8 is Vascular Endothelial Growth Factor A (VEGF-A), a mitogen and important stimulator of angiogenesis (Akiri et al. 1998; Huez et al. 1998; Miller et al. 1998).

3 Regulation of Gene Expression by mRNA Degradation

All mRNAs undergo decay as a part of normal gene expression. Decay of individual mRNAs can be highly regulated to control proper levels of protein expression and to spatially and temporally restrict protein production. Further, intrinsic or extrinsic signals can alter the decay rates of specific transcripts, including endocrine signals that alter gene expression by effecting mRNA decay. In this section, we provide an overview of mRNA decay pathways in mammals. We then discuss mechanisms of post-transcriptional regulation through RNA decay, including important gaps in current knowledge.

RNA decay pathways are initiated from either the 3′ or 5′ ends of the transcript, or by endonucleolytic cleavage. Multiple pathways can overlap in order to efficiently degrade an mRNA. First, we consider a major pathway of transcript decay that proceeds through the processes of deadenylation, decapping and exonucleolytic decay (Fig. 1.3).

3.1 Deadenylases Remove the Poly(A) Tail

Decay of cytoplasmic mRNAs typically initiates by the progressive shortening of the 3′ poly(A) tail, a process referred to as deadenylation (Fig. 1.3). Deadenylation is frequently the rate-limiting step of mRNA decay and is an important node for control of translation and mRNA decay. Multiple enzymes catalyze deadenylation (Goldstrohm and Wickens 2008). These deadenylases are magnesium-dependent exoribonucleases that degrade mRNAs from the 3′ to 5′ end of the poly(A) tail, releasing 5′ AMP as a product. General mRNA deadenylation occurs in two phases. Once the poly(A) tail is shorted to a critical threshold, the mRNA is rapidly destroyed.

The first phase is comprised of an initial shortening of the poly(A) tail by the Pan2-Pan3 deadenylase complex (Boeck et al. 1996; Brown et al. 1996; Yamashita et al. 2005). The Pan2 subunit contains a DEDD type nuclease domain that catalyzes deadenylation. The activity of Pan2-Pan3 is stimulated by Poly(A) Binding Protein (PABP) (Uchida et al. 2004). This interaction occurs through a specific protein interaction motif located on Pan3 (Siddiqui et al. 2007). PABP therefore serves a dual role: during translation, PABP stimulates translation initiation, and it also participates in the first phase of mRNA decay. Pan2-Pan3 also has a WD40

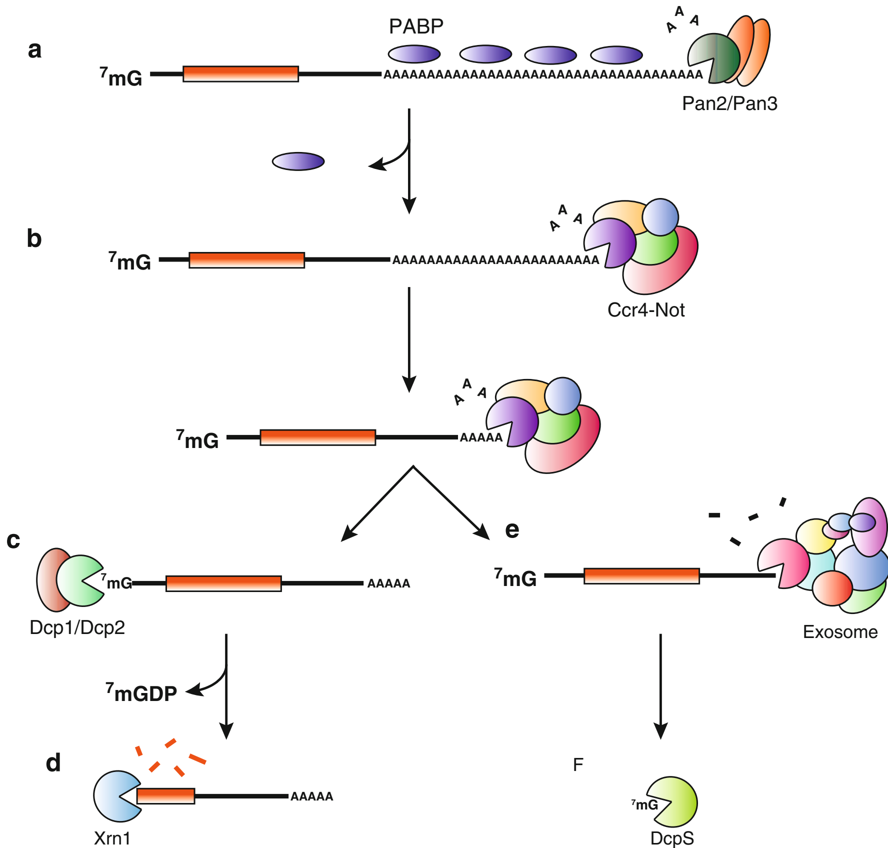

Fig. 1.3 Eukaryotic mRNA decay pathways. Decay of mRNA in eukaryotes begins with deadenylation by Pan2-Pan3 (**a**), which shortens the poly(A) tail and may dissociate PABP. The remainder of the poly(A) tail is degraded by the Ccr4-Not complex (**b**). Both deadenylase complexes release AMP molecules as the product of the deadenylation reaction. After deadenylation, mRNAs go through one of two pathways to decay the remainder of the mRNA. The 5′ to 3′ decay pathway starts with decapping by Dcp1/Dcp2 to remove the 7-methylguanosine cap (**c**) and is concluded by degradation of the remainder of the message by Xrn1 (**d**). The 3′ to 5′ decay pathway comprises of degradation of the RNA down to the cap by the Exosome (**e**) and subsequent scavenging of the cap by DcpS (**f**)

protein–protein interaction domain, suggesting that other protein partners may contact and regulate Pan2 activity. In support of this idea, Pan2 is known to associate with at least one RNA binding regulatory complex (Christie et al. 2013; Huntzinger et al. 2013; Kuzuoglu-Ozturk et al. 2012). Additionally, Pan2 has a catalytically dead ubiquitin hydrolase domain, though the function of this domain remains enigmatic. The Pan3 subunit contains a pseudokinase domain and zinc finger domain, both of which have been hypothesized to contribute to RNA binding (Jonas et al. 2014; Schafer et al. 2014; Wolf et al. 2014).

The second phase of deadenylation is catalyzed by the multisubunit CCR4-NOT deadenylase complex (CNOT) (Yamashita et al. 2005). Originally discovered in

yeast, the CNOT complex contains two active deadenylase subunits, orthologs of the Ccr4 and Pop/Caf1 proteins. In mammals, the Ccr4 orthologs include CNOT6 and CNOT6L proteins while the Pop2 orthologs include CNOT7 and CNOT8 proteins. The CNOT complex includes at least seven other components with various functions associated with regulation and coordination of deadenylase activity, in addition to functions beyond deadenylation (Collart 2003; Tucker et al. 2001). In contrast to Pan2-Pan3, which is stimulated by PABP, addition of PABP in in vitro experiments inhibits the activity of CNOT (Tucker et al. 2002). CNOT deadenylates the poly(A) tail down to a short oligo(A) that is incapable of binding PABP to facilitate translation initiation. Thus, PABP and CNOT have opposing stimulatory and inhibitory activities with respect to translation, placing poly(A) at the nexus of control of translation and mRNA decay.

The CNOT complex is regulated by protein interactions with multiple partners (Goldstrohm and Wickens 2008). For example, multiple members of the PUF family of sequence specific RNA binding proteins have been shown to bind the Pop2 subunit and recruit the CNOT complex to specific mRNAs (Goldstrohm et al. 2006, 2007; Weidmann et al. 2014). As a consequence, the poly(A) tails of the mRNAs are removed more quickly, the resulting deadenylated mRNA is translationally repressed, and its stability is decreased. A variety of RBPs have now been shown to recruit the CNOT complex to achieve repression, including the ARE binding protein TTP (Lykke-Andersen and Wagner 2005; Sandler et al. 2011) and the RBP Roquin (Leppek et al. 2013), both of which cause repression and degradation of cytokine mRNAs. RBPs can recruit the complex via interactions with specific subunits. The CNOT1 subunit is important for deadenylation, though it has no catalytic function itself. Instead, CNOT1 serves as a molecular scaffold and a target for RBP mediated recruitment to specific substrate mRNAs. For instance, TTP binds directly to CNOT1. Other CNOT complex subunits play additional functional roles, as recently reviewed by Shirai et al. (2014).

Other deadenylases have also been identified in mammalian systems. The presence of multiple deadenylases could lead to redundancy in their function, yet certain deadenylases have been shown to have specific biological functions such as control of development, anti-viral response, and regulation of metabolism (Goldstrohm and Wickens 2008). Also, some deadenylases have unique activities and features. The PARN deadenylase, expressed in many higher eukaryotes, is unique in that it binds to the 5′ cap of mRNAs, resulting in enhancement of PARN's ability to deadenylate the mRNAs 3′end (Gao et al. 2000; Martinez et al. 2000). This property indicates that PARN likely acts on translationally inactive mRNAs. PARN has been implicated in developmental processes in plants and *Xenopus laevis* and is also expressed in mammals. Of particular interest in the field of endocrinology, the deadenylase Nocturnin is controlled by the circadian clock (Green and Besharse 1996), and genetic analysis indicates that Nocturnin's main function is to regulate fat metabolism (Green et al. 2007). Still, much remains to be elucidated with regard to the functions of deadenylase family members and their roles in post-transcriptional regulation.

3.2 Decapping Enzymes Catalyze Removal of the 5′ Cap

In addition to its role in translation initiation, the 5′ 7-methylguanosine cap protects the 5′ end of the mRNA from exonucleases. These features put the 5′ cap at the nexus of regulation of translation and mRNA decay. The 5′ cap can be removed by hydrolysis of the 5′ to 5′ triphosphate linkage that covalently joins the 7-methyl guanosine to the first nucleotide of the mRNA. This process is referred to as decapping and is catalyzed by specialized decapping enzymes. Decapping typically follows deadenylation, coordinated by protein interactions between the deadenylase complex and the decapping machinery. However, deadenylation independent decapping has also been observed for some mRNAs (Badis et al. 2004; Fromont-Racine et al. 1993).

Multiple decapping enzymes have been identified (Li and Kiledjian 2010; Song et al. 2013). One of the best-characterized decapping enzymes is the highly conserved Dcp2 enzyme, which releases ^{7}mGDP from the mRNA 5′ end through activity of its Nudix domain (Wang and Kiledjian 2002). Dcp2 also possesses a Box A domain that functions in RNA binding.

Decapping enzymes are highly regulated by protein partners (Jonas and Izaurralde 2013). Dcp2 activity is stimulated by its protein partner Dcp1, which forms a stable complex with Dcp2 through the Dcp2 Box A domain (Piccirillo et al. 2003; She et al. 2006). Dcp2 is additionally activated by Dhh1, an RNA helicase, and Pat1, which recruits the Lsm complex. (Ling et al. 2011). The Lsm protein complex (Lsm 1-7) associates with the 3′ deadenylated end of mRNA and increases Dcp2 decapping efficiency (Tharun and Parker 2001). An additional decapping factor, Ge-1, is found in this complex in higher eukaryotes, further activating decapping activity (She et al. 2008; Yu et al. 2005).

RNA binding proteins greatly influence decapping rates of specific mRNAs, as explored below and in several recent reviews (Arribas-Layton et al. 2013; Li and Kiledjian 2010). For instance, the ARE-binding protein TTP recruits decapping factors to accelerate decapping of specific transcripts (Gao et al. 2001; Lykke-Andersen and Wagner 2005). Decapping of mRNAs is frequently the fate-determining step that targets mRNAs for destruction; however, recent evidence indicates that some transcripts may be recapped, suggesting that decapping and recapping could serve as a regulatory mechanism to repress and then activate mRNAs (Mukherjee et al. 2012).

3.3 Exoribonucleolytic Decay Can Initiate from the 5′ End

Following decapping, the exposed 5′ end of the mRNA can be attacked by exoribonucleases, degrading the mRNA in a 5′ to 3′ direction while releasing nucleotide monophosphate products. Several 5′ exoribonucleases have been identified in eukaryotes including XRN1 and XRN2 (Nagarajan et al. 2013). Both enzymes act on multiple types of substrate RNAs. Here we consider several important roles.

The exoribonuclease XRN1 is primarily responsible for 5′ mRNA decay in the cytoplasm. XRN1 has specificity for RNAs with a 5′ monophosphate, coinciding

with the product of DCP2-mediated decapping. This enzyme is fast and highly processive; decay intermediates are rarely detected (Nagarajan et al. 2013).

XRN2, a 5′ to 3′ exoribonuclease with homology to XRN1, is primarily located in the nucleus and is conserved across a range of species (Miki and Grosshans 2013). Functions of XRN2 include maturation of rRNA and snoRNAs, as well as transcription termination (Boisvert et al. 2007; Luo et al. 2006; Wang and Pestov 2011). XRN2 is also involved in RNA quality control pathways, decaying aberrant RNAs in the nucleus, as well as unspliced pre-mRNAs to control mRNA levels (Das et al. 2003).

Decapping and 5′ decay of mRNAs are coordinated via physical interaction of the XRN1 C-terminus with decapping factors (Fischer and Weis 2002; Sinturel et al. 2012). XRN1 is further regulated by interactions with RNA binding proteins such as the ARE-binding protein TTP.

3.4 Exoribonucleolytic Decay from the 3′ End

Messenger RNAs can also be degraded from the 3′ end by the exosome, a large multisubunit complex (10–12 subunits) which acts in a 3′ to 5′ direction. Two variations of the exosome complex have been characterized: one nuclear exosome and one cytoplasmic exosome. The exosome acts to process and/or degrade multiple types of RNA including mRNAs (Liu et al. 2006). The cytoplasmic exosome acts on mRNAs that have been deadenylated, degrading them from the 3′ end to produce nucleotide monophosphates. The Dis3 subunit is responsible for this activity (Reis et al. 2013). Once the mRNA is degraded to a capped m^{7}GpppN (where N is fewer than 10 nucleotides) product, a specialized scavenger decapping enzyme, DcpS, hydrolyzes the cap structure to produce 7-methylguanosine monophosphate and nucleotide diphosphate (Chen et al. 2005; Liu and Kiledjian 2005). DcpS action prevents the potentially toxic effects of accumulated capped mRNA fragments, which could competitively inhibit eIF4F function. Interestingly, defects in this final mRNA decay step cause a form of intellectual disability and neuromuscular disease (Ng et al. 2015).

As we have learned, mRNAs can be degraded from the 5′ end, the 3′ end, or both. The 5′ and 3′ decay pathways appear to compete to degrade certain mRNAs, whereas other mRNAs appear to be mainly degraded by one or the other pathway. The determinants and factors that affect these destructive choices remain incompletely understood.

3.5 Endonucleases Cut mRNAs to Initiate Decay

As an alternative pathway to exoribonucleolytic decay, select messages undergo endonucleolytic cleavage (Fig. 1.4). The resulting 5′ and 3′ fragments are subsequently degraded by exoribonucleases including XRN1 and the exosome. In fact, the Dis3 subunit of the exosome is also an endonuclease, in addition to its

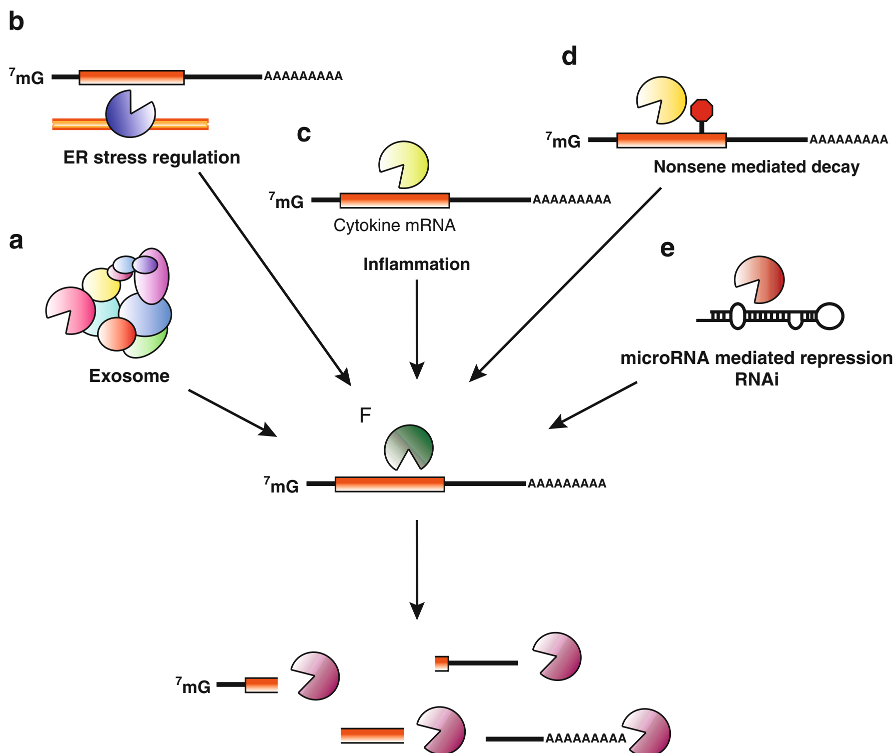

Fig. 1.4 Endonucleolytic decay pathways. Representative examples of eukaryotic endonucleolytic decay pathways are illustrated here. Components of the exosome, such as Dis3, have endonuclease activity (**a**). Under ER stress conditions, Ire-1 decays ER-associated mRNAs to reduce translation (**b**). Pro-inflammatory mRNAs are degraded by Regnase-1 to negatively regulate cytokine expression (**c**). In nonsense mediated decay, mRNAs with premature stop codons are cleaved by SMG6 before being degraded by exonucleases (**d**). In microRNA-mediated decay pathways and RNAi pathways, precursor miRNAs are processed by endonucleases such as Dicer and Drosha to make mature miRNAs (**e**). Endonucleolytic decay products typically serve as substrates for further decay by exonucleases (**f**)

exoribonuclease activity (Arraiano et al. 2010). Other examples of endonucleases highlight the diversity within this class of enzymes. One of the first characterized mammalian endoribonucleases was the PMR1 enzyme, which was found to be an estrogen induced factor that initiates mRNA decay (Pastori et al. 1991a, b). PMR1 associates with polysomes to efficiently degrade specific mRNAs. Recent evidence indicates that PMR1 has an important role in reducing parathyroid hormone mRNA levels, discussed further in the chapter by Naveh-Many (Nechama et al. 2009). The Regnase-1 endonuclease regulates immune function by degrading pro-inflammatory cytokine mRNAs (Matsushita et al. 2009) (Fig. 1.4). Regnase-1 is rapidly degraded upon stimulation of immune responses, and in mouse models, Regnase-1 is implicated in autoimmune disorders (Liang et al. 2010).

Endonucleolytic mRNA decay is also an important for stress response. The IRE1 protein is a transmembrane endoribonuclease located on the endoplasmic reticulum. During stress conditions, IRE1 decays mRNAs through the regulated IRE1 dependent decay (RIDD) pathway (Hollien et al. 2009; Maurel et al. 2014) (Fig. 1.4). IRE1 substrates are specifically associated with the ER and decay faster during stress conditions to reduce translation (Gaddam et al. 2013). Additionally, these substrate mRNAs are highly enriched for transcripts involved in secretory pathways, including the hormone insulin and multiple cell surface receptors (Han et al. 2009a).

Nonsense mediated decay (NMD) of mRNAs is a vital pathway for clearing mRNA transcripts with premature termination codons. These aberrant transcripts are targeted by NMD machinery for degradation, as reviewed by Popp and Maquat (2013). One NMD component, SMG6, is an endonuclease that cleaves the mRNA, resulting in subsequent exoribonucleolytic decay of the fragments (Franks et al. 2010; Schmid and Jensen 2008) (Fig. 1.4). Endonucleases function in a variety of other RNA decay and processing pathways in the cell, including the Argonaute, Dicer, and Drosha endonucleases that participate in RNA interference pathways, addressed in a later section of this chapter.

3.6 Regulation of mRNA Decay

Cis-acting RNA sequences, either linear motifs or secondary structures, can control decay of the transcript. These elements are recognized by sequence specific RNA binding regulatory factors. In turn, the regulators recruit the mRNA decay machinery to facilitate removal of the poly(A) tail, the 5′ cap, or to promote exonucleolytic or endonucleolytic decay. There are many hundreds of RNA binding proteins encoded in mammalian cells (Gerstberger et al. 2014). Moreover, mRNA decay can be controlled by small non-coding RNAs that form complexes with regulatory proteins (Jonas and Izaurralde 2015), as described in subsequent sections. Here, we a provide a few examples or regulators that control mRNA stability and refer readers to recent in-depth reviews (Garneau et al. 2007; Goldstrohm and Wickens 2008; Li and Kiledjian 2010; Schoenberg and Maquat 2012).

Two well-characterized instability elements are the Adenine and Uridine Rich Elements (AREs) and Guanine and Uridine Rich Elements (GREs), which are frequently found in 3′ UTR of mRNAs such as cytokine mRNAs (Bakheet et al. 2006; Vlasova-St Louis and Bohjanen 2011). Multiple RNA binding proteins recognize these sequences to control mRNA stability. Certain ARE binding proteins can recruit mRNA decay machinery to increase decay. For instance, TTP recruits the CNOT deadenylase complex, the DCP2 decapping complex, and exonucleases to mRNAs to promote their destruction (Chen et al. 2001; Fenger-Gron et al. 2005; Lykke-Andersen and Wagner 2005; Sandler et al. 2011).

AREs can act as a bifunctional switch, causing mRNA decay in one state while stabilizing mRNAs in other conditions. This is achieved by at least two mechanisms. First, stabilizing ARE binding proteins, such as HuR, can compete with

destabilizing factors such as TTP. Second, post-translational modifications can alter the activity of the ARE binding proteins (Garneau et al. 2007). Phosphorylation of TTP can alter its RNA binding, protein interactions, and protein stability (Brooks and Blackshear 2013). ARE mediated regulation will be further discussed in Chaps. 3, 5, 8, 9, 11, and 13.

GRE elements are bound by CUG-Binding protein, also referred to as CELF1 (Vlasova-St Louis and Bohjanen 2011) (see Chap. 3 of this volume). CELF1 plays multiple roles in mRNA processing, translation and stability. This evolutionarily conserved repressor represses protein expression by causing deadenylation and mRNA decay. The mechanism is incompletely understood, but evidence indicates that CELF1 interacts with the deadenylase PARN to promote deadenylation (Moraes et al. 2006).

3.7 Nonsense-Mediated, Non-stop, and No-Go mRNA Decay Pathways

Decay of mRNA is a vital point of normal gene regulation, but also plays an important role in quality control of gene expression (Ghosh and Jacobson 2010). Aberrant, defective mRNAs are degraded to ensure fidelity. These mRNAs can arise due to mutation, misprocessing, or breakdown in the complex processes necessary to decode them (i.e. translation). Nonsense mediated decay, discussed above, is responsible for decaying transcripts with premature termination codons, protecting cells from the potentially deleterious effects of producing truncated proteins with abnormal function (Popp and Maquat 2014). NMD destroys these aberrant transcripts via deadenylation dependent decay and endonucleolytic decay (Schoenberg and Maquat 2012). Messenger RNAs that lack a stop codon, as the result of mutation or misprocessing, are targeted by Non-stop decay pathway, wherein the exosome destroys the transcript. An additional quality control pathway, so-called No-Go Decay, clears faulty mRNAs stalled ribosomes stuck in the act of translation. This decay pathway releases the ribosome and then degrades the abnormal transcript via endonucleolytic cleavage. For more information on quality control mechanisms, we refer readers to recent reviews (Harigaya et al. 2010; Popp and Maquat 2013).

4 Post-transcriptional Regulation by Non-coding RNAs

The protein-coding sequence in the human genome only accounts for 1 % of the entire genome meaning that the majority of the genome is noncoding DNA (Mattick 2004). Much of this noncoding DNA is actually transcribed into noncoding RNAs (Bertone et al. 2004; Cheng et al. 2005; Kampa et al. 2004). This finding suggests that higher eukaryotes have evolved new and complex regulatory mechanisms, both structural and functional, that involve not just proteins but also noncoding RNAs. Many of these noncoding transcripts are synthesized similar to mRNAs including being capped at the 5′ end, often spliced, and polyadenylated at the 3′ end. Further

processing may take place once these RNAs are in processing complexes to generate functional noncoding RNAs. The regulatory potential of RNAs comes from the ability for it to interact with other nucleic acids and proteins allowing for intricate formation of regulatory RNA-protein (RNP) complexes. Such regulatory RNAs include small noncoding RNAs such as microRNAs (miRNAs) and short interfering RNAs (siRNAs) (Carthew and Sontheimer 2009). Long noncoding RNAs and circular RNAs are additional forms of noncoding RNA that have more recently been shown to contribute to gene regulation (Geisler and Coller 2013). These RNAs can serve many critical functional roles in the cell including control of transcription, post-transcriptional regulation of mRNA decay and translational control.

4.1 Small Non-coding RNAs

4.1.1 MicroRNAs

MicroRNAs are 22–25 nt small noncoding RNAs that participate in RNA-base pairing interactions with specific mRNAs in order to repress expression of target mRNAs (Bartel 2004). They do so by inhibiting translation and activating mRNA degradation (Jonas and Izaurralde 2015). Post-transcriptional gene regulation by microRNAs is widespread (Bartel 2004, 2009). Currently, over 1500 microRNAs have been identified in the human genome, each of which can regulate expression of multiple genes (Freedman and Tanriverdi 2013; Lewis et al. 2005). Some of these are conserved across mammals and even to lower eukaryotes, while others are endogenous to the human genome only. Many are expressed in a tissue specific manner, (Londin et al. 2015) while others are expressed in stage-specific manners during development (Krichevsky et al. 2003; Pasquinelli et al. 2000). Because of this, microRNAs are critical regulators of many tissue specific functions. For example: one pancreatic specific microRNA, miR-375, regulates several mRNA targets that are critical for the secretion of insulin from the islets of Langerhans (Poy et al. 2004). If miR-375 is lost, insulin secretion is upregulated and when miR-375 is abundant, insulin secretion is downregulated. Another microRNA, miR-143, was identified through a microarray analysis to play an important regulatory role in adipocyte differentiation (Esau et al. 2004). These are just a few examples of the numerous biological roles of microRNAs. This book will emphasize the regulatory roles of microRNAs in endocrine function, as discussed in Chaps. 2, 5, 6, 9, 12, 13 and 14.

Biogenesis of MicroRNAs

MicroRNAs are processed from precursor transcripts through several steps before they become functional. First, microRNAs are transcribed by RNA Polymerase II; these transcripts are usually capped and poly(A)denylated. Typically, these primary transcripts can encode for one or more microRNAs and can even code for clusters of microRNAs (Lagos-Quintana et al. 2001; Lau et al. 2001). Alternatively, one transcript can encode for both a microRNA and a protein simultaneously. In these cases, the microRNA sequence is many times encoded in the intronic sequence, so-called mirtrons (Berezikov et al. 2007; Ha and Kim 2014; Okamura et al. 2007).

The initial primary microRNA (pri-miRNA) has extra sequence extending past the microRNA itself (22–24 core nucleotides) both on the 5′- and 3′-ends of the transcript. Next, the pri-miRNA is folded into a stem-loop structure and is excised from the primary transcript while still in the cell's nucleus. A complex including an RNase III endonuclease, Drosha, carries out this cleavage step. Drosha is accompanied in this complex by DGCR8, a protein that serves to recognize the pri-miRNA. The Drosha-DGCR8 complex cleaves the pri-miRNA such that a 60–70 nucleotide stem-loop intermediate with a mature 5′-phosphate and a 3′-nucleotide overhang remains. This product of Drosha cleavage is referred to as a pre-miRNA and can be exported to the cytoplasm for further processing and maturation (Lee et al. 2003; Zeng and Cullen 2003).

In the cytoplasm, the pre-miRNA becomes a substrate for Dicer, an RNase III endonuclease which catalyzes a second cleavage event. Dicer specifically recognizes the 3′-nucleotide overhang that was previously generated during Drosha processing. This endonuclease event is responsible for removal of the terminal loop and consequently the release of a small double stranded RNA duplex. This Dicer cleavage event additionally dictates the length of the duplex RNA for the mature miRNA of 22–25 nucleotides. Once the mature miRNA is formed, it can enter into a functional regulatory complex referred to as the RNA-induced silencing complex (RISC) (Ha and Kim 2014; MacRae et al. 2007).

Regulation of MicroRNA Biogenesis

MicroRNA biogenesis can be regulated at each processing step, thereby altering the regulatory response controlled by the resulting microRNA (Ha and Kim 2014). Drosha processing activity can be modulated by DGCR8 autoregulation in the first step of microRNA processing (Han et al. 2009b). The DGCR8 mRNA contains conserved stem loop structures that closely resemble the structure formed by the pri-miRNA and therefore the Drosha/DGCR8 complex is able to cleave the DGCR8 mRNA, controlling the expression of DGCR8 at the post-transcriptional level. Furthermore, the DGCR8 component of the complex forms protein–protein interactions with Drosha in order to further stabilize Drosha creating a feedback mechanism to control the levels of this microprocessor complex (Han et al. 2009a, b). Importantly, this mechanism is highly conserved from humans to zebrafish to drosophila.

Another example of the regulation of microRNA biogenesis occurs at the stage of Dicer-catalyzed microRNA processing (Ha and Kim 2014). The best example is the let-7 microRNA and one of its canonical targets, Lin28. Lin28 is typically translationally repressed by the let-7 microRNA; however, when Lin28 protein is present, it binds to the terminal loop of the let-7 pre-miRNA leading. This interaction impedes the binding and cleavage by Dicer and thus the pre-let-7 miRNA maturation is inhibited (Carthew and Sontheimer 2009; Ha and Kim 2014). Instead, Lin28 recruits Terminal Uridyl Transferases (TUTs) to cause oligouridylation, or the addition of uridylate residues, to the 3′ end of the pre-let-7 miRNA and consequently degradation of the microRNA intermediate (Heo et al. 2012; Thornton et al. 2012). These types of mechanisms allow for tight control of microRNA production and processing and by controlling the levels of mature microRNAs, the cell is able to modulate regulation by microRNAs.

4.1.2 Short Interfering RNAs

Short interfering RNAs (siRNAs) are 21–23 nucleotide RNAs processed from exogenous double-stranded RNAs (dsRNAs). SiRNAs are fully complementary to their target mRNA sequences and work primarily by Watson-Crick base-pairing (Carthew and Sontheimer 2009). Targeting of an siRNA to an mRNA results in an endonucleolytic cleavage and subsequent exonucleolytic decay of the mRNA fragments. In research, siRNAs are often administered to cell culture for programmed knockdown of a particular gene via RNA interference (RNAi) or post-transcriptional gene silencing (PTGS). In cells, Dicer can also process double-stranded RNAs into functional siRNAs.

Another class of siRNAs, endo-siRNAs, are produced from endogenous cellular dsRNA precursors (Ghildiyal et al. 2008; Yang and Kazazian 2006). Endo-siRNAs differ from microRNAs in the ways in which they are generated and processed. These RNAs are synthesized and processed in a variety of different ways but typically require Dicer for cleavage of a double stranded RNA (dsRNA) precursor and/or RNA-dependent RNA polymerases in order to be synthesized and processed. RNA-dependent RNA polymerases can use mature mRNAs as templates for synthesis, and the products are cleaved by Dicer. The resulting endo-siRNAs can act as silencers of both transcription and translation (Ghildiyal and Zamore 2009). Endo-siRNAs produced from viral RNAs can protect the cell from viral infections (Li et al. 2013; Wang et al. 2006). Historically, there is strong evidence for this type of mechanism in plants and flies; however, more recently, these anti-viral RNAi response pathways have been observed in multiple mammalian cell lines, suggesting evolutionary conservation (Claycomb 2014; Li et al. 2013).

4.2 Assembly of the RNA Induced Silencing Complex (RISC)

To control gene expression, microRNAs and siRNAs must be loaded into protein complexes termed RNA induced silencing complexes (RISC) (Jonas and Izaurralde 2015) (Fig. 1.5). A core component of RISC is the Argonaute protein family (Meister 2013). When assembled, RISC contains one of several Argonaute (Ago) proteins that bind directly to the small RNAs. Eukaryotes often possess more than one Argonaute protein that share similar architecture including a domain with homology to endonucleases (Meister 2013). There are four human Argonautes (AGO1-4), each of which can bind to small RNAs; however, only AGO2, is enzymatically capable of endonucleolytic cleavage of bound mRNA targets.

Argonaute associates with one of the two strands of the non-coding RNA, referred to as the guide strand. First, the double stranded RNA duplex, produced by Dicer, is loaded onto Argonaute. Next, selection of the guide strand is determined by thermodynamics of base pairing at the 5′-ends of each of the two strands (Khvorova et al. 2003). The strand that is less tightly base paired will allow for easier loading and further strand separation (Khvorova et al. 2003). Removal of the RNA strand that is selected against is an intricate process that may involve an RNA helicase activity to aid in the unwinding of the small RNA duplex (Kawamata et al. 2009; Yoda et al.

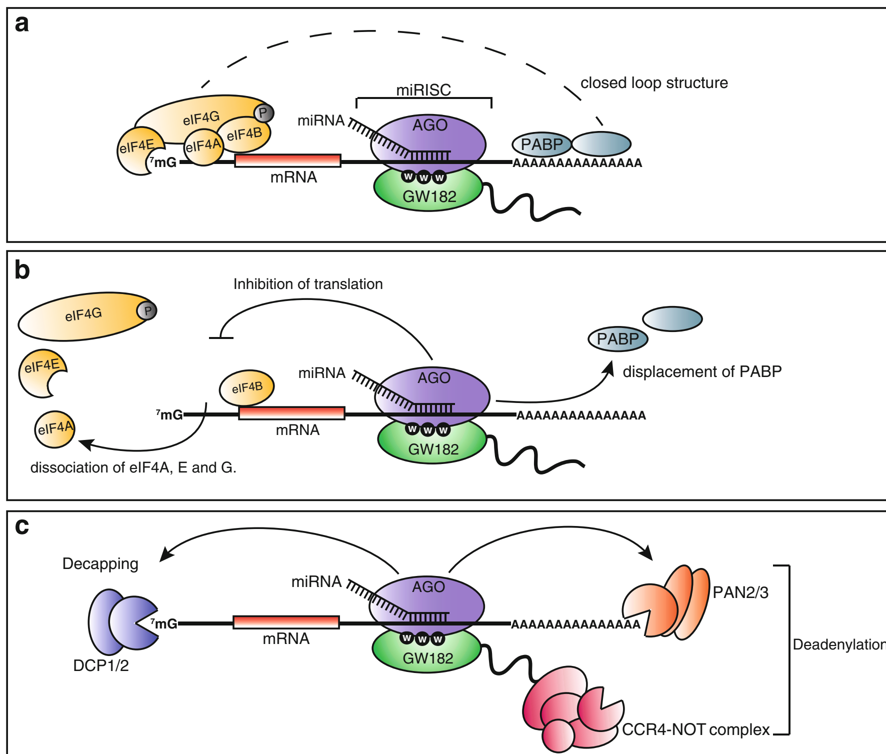

Fig. 1.5 MicroRNA mediated post-transcriptional regulation of translation and mRNA decay. (**a**) Assembly of the microRNA-induced silencing complex (miRISC) begins with the microRNA partially base-pairing with the 3′UTR of the mRNA. Argonaute proteins associate with the miRNA-mRNA duplex and recruit GW182, a glycine and tryptophan rich protein that mediates protein–protein interactions that underlie translational repression and mRNA decay. (**b**) Translational inhibition is mediated by displacement of PABP from the poly(A) tail, and disruption of the eIF4F complex bound to the cap. miRNA RISC thereby disrupts the translationally active closed loop structure of the mRNA to halt protein production. (**c**) MicroRNA RISC promotes deadenylation and decapping of the mRNA, resulting in mRNA destruction. As a consequence of PABP displacement, the poly(A) tail of the mRNA is left unprotected and susceptible to deadenylases such as PAN2/3 and the CCR4-NOT complex, both of which are recruited by the GW182 component of RISC. The miRISC also recruits the DCP1/2 decapping complex which triggers 5′→3′ decay of the mRNA as shown in Fig. 1.3

2010). The unloaded strand, or the passenger strand, dissociates and is enzymatically destroyed (Peters and Meister 2007). This preferential loading of the guide strand is the first step to RISC assembly and occurs similarly for RISC complexes containing either microRNAs or siRNAs. The Argonaute protein maintains tight interactions with the 3′-end of the guide strand allowing the 5′-end of the guide strand, including the seed site, to engage in Watson-crick base pairing for target scanning and recognition (Carthew and Sontheimer 2009; Ha and Kim 2014)

4.3 Targeting Mechanism of microRNAs

Both microRNAs and siRNAs function by base-pairing with target mRNAs but their degree of complementarity to target mRNAs influences the outcome of regulation. Complete complementarity of the small RNA to its target leads to endonucleolytic cleavage of the mRNA, catalyzed by the Argonaute protein. This mechanism predominates for silencing by siRNAs. Likewise, if a microRNA-mRNA interaction is fully base paired, mRNA cleavage can occur. However, most miRNAs are not fully complementary to their targets. Instead, miRNA recognition occurs by more limited Watson-Crick base pairing, with strong preference for pairing of the second through eighth nucleotide of the microRNA, referred to as the seed site. Functional microRNA targeting tends to exhibit perfect seed site complementarity and weaker, imperfect pairing for the remainder of the RNA. Functional miRNAs sites tend to be located in the 3′UTR of messages, though not exclusively. Rather than using endonuclease activity to cleave mRNA targets, microRNA RISC causes translational inhibition or canonical mRNA decay processes, as described in the following section.

4.4 Mechanism of microRNA Mediated Repression

MicroRNA-bound Argonaute associates with multiple protein partners to repress protein expression from target mRNAs (Fig. 1.5). One protein partner, GW182, is crucial for microRNA mediated repression. Argonaute proteins all have a conserved site on the surface of the protein that can tightly interact with conserved tryptophan residues that are characteristic of GW182 proteins (Jonas and Izaurralde 2015; Pfaff et al. 2013). GW182 functions as a critical adapter that mediates protein interactions necessary for downstream effects of microRNA regulation (Carthew and Sontheimer 2009; Jonas and Izaurralde 2015; Pfaff et al. 2013). This microRNA RISC complex regulates mRNA stability and translation by multiple mechanisms. RISC displaces PABP from the mRNA to disrupt closed loop conformation of translation (Zekri et al. 2013) (Fig. 1.5b). RISC also recruits both PAN2 and CCR4-NOT deadenylase complexes to accelerate removal of the mRNAs poly(A) tail. Additionally, RISC recruits DCP2 decapping complex to facilitate removal of the 5′ cap, thereby accelerating 5′ to 3′ mRNA degradation (Fig. 1.5c). Other mechanisms of microRNA mediated repression have been described including inhibition of translation initiation and elongation (Fukao et al. 2014; Fukaya et al. 2014; Zekri et al. 2013); this area of research remains highly active (Jonas and Izaurralde 2015). This combination of translational inhibition and mRNA degradation facilitate the effectiveness of microRNA repression. Moreover, repression also occurs in combination with other miRNAs and RBPs to ensure proper regulation of gene expression in a wide array of contexts, including response to endocrine signals.

4.5 Long Noncoding RNAs

Noncoding RNAs have long been known to play a variety of functions including catalysis of translation and splicing, scaffolding of protein complexes, chromatin/chromosome modifiers, and mediators of intracellular localization and sequence specific targeting (Cech and Steitz 2014; Geisler and Coller 2013). More recently, a large number of new long noncoding RNAs (lncRNAs) have been identified and the association of specific lncRNAs with disease states has primed interest in their molecular functions.

Long noncoding RNAs are generally classified as transcripts that are longer than 200 nucleotides that do not contain a high confidence open reading frame above a specific threshold length—typically 100 amino acids. The defining features of long noncoding RNAs are rapidly developing. The vast majority of newly reported long noncoding RNAs do not have a known function, but intense research efforts have revealed that some lncRNAs are key regulatory players in chromatin structure, transcriptional control or post-transcriptional control. Here, we focus on several examples of lncRNAs that act post-transcriptionally to control protein expression.

Many of these long noncoding RNAs look like typical mRNAs; they are transcribed by RNA Pol II and possess a 5′ cap and 3′ poly(A) tail. Long noncoding RNAs can originate from near classified genes (lncRNAs) or intergenic regions (lincRNAs). To date, thousands of these RNAs have been reported, facilitated by new detection methods such as next-generation sequencing. The abundance of lncRNAs varies widely, with some being among the most abundant in cells, whereas others are low abundance (Cabili et al. 2015). Furthermore, many lncRNAs have specific expression patterns related to developmental stage, tissue and cell type, hinting that these molecules have important yet undiscovered roles (Batista and Chang 2013). Evolutionary conservation of some lncRNAs also indicates that they have important biological functions (Ulitsky and Bartel 2013). Yet other lncRNAs appear to be recent additions to the genome. Given the propensity of RNA to adopt secondary and tertiary structures that are important for function, conservation of primary nucleotide sequence may be compensated by conserved structures of some parts of the RNA molecule (Diederichs 2014). Pseudogenes, gene duplicates that have lost the ability to produce functional protein, sometimes produce lncRNAs. These pseudogenes retain the ability to be transcribed, whether it is through its own promoter or from transcription read through at the neighboring gene locus.

4.5.1 LncRNAs Play a Role in mRNA Processing

Nascent transcripts are processed extensively by capping, splicing, editing and 3′ end cleavage and polyadenylation. Each of these steps can be regulated to control and diversify mRNAs. A prime example is splicing, which can be regulated to produce multiple mRNAs, and therefore multiple protein isoforms, from the same gene. Long noncoding RNAs are emerging as important regulators of alternative splicing events (Tripathi et al. 2010). For example, one class of lncRNAs, known as natural antisense transcripts (NATs), are antisense transcripts of protein-coding genes that hybridize to the mRNA and prevent alternative splicing. The highly abundant and stable lncRNA MALAT1 localizes to nuclear regions associated with

mRNA splicing factors, nuclear speckles, and evidence indicates that MALAT1 can modulate alternative splicing. Yet the molecular mechanism of how MALAT1 affects splicing remains unknown.

4.5.2 LncRNAs Affect Translation and mRNA Stability

LncRNAs can regulate the fate of mRNAs in the cytoplasm by affecting their translation efficiency and mRNA stability. Multiple mechanisms are emerging. For instance, lncRNAs can alter protein expression by competing with microRNAs for cis-acting sites on the mRNA, thereby reducing miRNA mediated repression (Ebert and Sharp 2010; Tay et al. 2014). Alternatively, lncRNAs can act as competing endogenous RNAs (ceRNAs) to sequester miRNAs away from their target mRNAs. This so called sponging or decoy action alleviates repression caused by specific microRNAs, thereby stabilizing the target mRNAs and increasing protein expression. An example of this competing endogenous RNA function is the PTENP1 pseudogene lncRNA (Poliseno and Pandolfi 2015; Poliseno et al. 2010). PTENP1 affects its protein-coding counterpart gene, PTEN, a well-documented tumor suppressor. PTEN is extensively regulated at the post-transcriptional level by microRNAs (He 2010). PTENP1 competitively binds and sequesters the microRNAs that repress PTEN, thus allowing for increased expression of PTEN at the protein level (Poliseno and Pandolfi 2015; Poliseno et al. 2010). Other lncRNAs may function in a similar manner to competitively disrupt regulatory switches, whether microRNAs or RNA binding proteins, that control expression of protein coding genes.

LncRNAs can anneal to specific mRNAs and mediate their degradation. Long noncoding RNAs with Alu repeats have been reported to form dsRNA duplexes with Alu-like elements in the 3′UTRs of targets, creating a high affinity binding site for the dsRNA binding protein, Staufen (Gong and Maquat 2011; Park and Maquat 2013). Following duplex formation and Staufen (STAU1) binding, the transcript is then degraded via Staufen mediated decay (SMD) (Park and Maquat 2013).

Translational control can also be affected by lncRNAs that interact with specific regions of mRNAs. Control of translation of the UCHL1 mRNA provides the primary example of this mechanism, whereby an antisense lncRNA promotes translation of UCHL1 protein in response to stress (Carrieri et al. 2012). This effect is driven by sequence complementarity between the lncRNA and a short interspersed repeat, SINEB2 elements in the transcript, which coincides with the site of translation initiation. Currently the mechanism of translational enhancement remains unknown.

4.5.3 Long Noncoding RNAs in the Endocrine System

LncRNAs are emerging as important regulators of endocrine function. Here, we discuss one example: lncRNA regulation of the glucocorticoid receptor (GR) protein (Knoll et al. 2015). GR controls gene expression during stress responses, causing growth arrest, dampening immune response, among other effects. The GR protein has a well-documented function as a transcriptional regulator. Glucocorticoid hormone binds to GR, triggering its binding to specific DNA sequence elements known as glucocorticoid response elements (GRE). More recently, GR was shown to also bind RNA and to regulate mRNA turnover in response to stress (Cho et al.

2015; Ishmael et al. 2011; Kino et al. 2010). Interestingly, GR activity is controlled by the lncRNA known as growth arrest specific 5 (Gas5). Gas5 inhibits the activity of GR by competitively binding to the protein, thereby preventing interaction with GRE containing DNA. Thus, Gas5 effectively dampens the cellular response to glucocorticoids and rather than arresting cell growth, these cells are shunted into pro-apoptotic pathways (Hudson et al. 2014; Kino et al. 2010). For more information on the regulation of glucocorticoid function, see Chap. 13 of this volume.

5 Conclusion

This chapter has provided the reader with fundamental knowledge of translation and mRNA degradation mechanisms. Importantly, key paradigms of post-transcriptional regulation were emphasized, supported by well documented examples. The cis and trans-acting factors that control translation and mRNA decay rates of specific mRNAs were discussed. As a result, the reader is now prepared to explore the roles of post-transcriptional regulation in the endocrine system, as illuminated by experts in the subsequent chapters.

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Post-transcriptional Regulation of Insulin and Insulin Like Growth Factors

2

Eun Kyung Lee and Wook Kim

1 Introduction

1.1 Insulin and Insulin-Like Growth Factors

Insulin and insulin-like growth factors (IGFs) are members of the same family of insulin-like peptides that share significant structural homology, and that engage common targets of downstream signaling pathways (Rajpathak et al. 2009; Cooke et al. 1991). There are, however, some major structural differences and tissue-specific activities attributed to the various family members. Both insulin and IGFs are composed of an alpha and a beta chain connected by disulfide bonds (Rinderknecht and Humbel 1978). Insulin is produced by proteolytic cleavage of a precursor protein, preproinsulin, solely in pancreatic β-cells; in contrast, IGFs are produced mainly by the liver and also in target tissues by a mechanism that does not require proteolytic cleavage of the c-peptide region. Similar to IGFs, insulin plays an important role in the regulation of cellular processes such as growth, proliferation, and survival in nearly all tissues. However, the major role of insulin is to tightly regulate glucose homeostasis in insulin-sensitive tissues, including liver, muscle, and adipose tissue. IGFs also have insulin-like activity as they can regulate glucose and lipid homeostasis by activating specific signal transduction pathways in peripheral tissues such as muscle (Clemmons 2006; Rajpathak et al. 2009). Both insulin and IGF synthesis are coordinately regulated in response to nutrients at the post-transcriptional level by

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trans-acting and mRNA-binding factors. Post-transcriptional mechanisms that regulate the stability and translation of mRNAs encoding insulin and IGFs include those that affect pre-mRNA splicing as well as mRNA stability and translation (Lee and Gorospe 2010; Panda et al. 2013); these regulatory mechanisms are particularly effective in eliciting acute changes in protein abundance in response to stimuli such as altered glucose levels.

In this chapter, we describe mRNA-binding factors involved in post-transcriptional regulation of mRNAs encoding insulin and IGFs and the influence of these factors on alternative splicing, mRNA stability, and translation.

1.2 RNA Binding Proteins and MicroRNAs

Tight regulation of gene expression is essential to maintain cellular homeostasis in response to various cellular signals. Besides transcriptional control, the production of insulin and IGFs are critically regulated at the post-transcriptional level. Post-transcriptional regulation at the RNA level in eukaryotic cells is broadly defined as the control of gene expression via the activity of pre-mRNA splicing factors, modulation of RNA maturation, and control of mRNA transport, storage, decay, and translation (Mitchell and Tollervey 2000; Orphanides and Reinberg 2002; Moore 2005; Lee and Gorospe 2010).

RNA binding proteins (RBPs) are varied in both structure and complexity and influence gene expression by affecting all aspects of RNA metabolism (Lunde et al. 2007). In particular, turnover- and translation-regulatory RBPs (TTR-RBPs) are involved in the regulation of mRNA stability and translation rates (Pullmann et al. 2007). RBPs dynamically interact with both coding and non-coding RNAs and form protein-RNA networks, which enables post-transcriptional regulation of gene expression (Lukong et al. 2008; Lunde et al. 2007; Moore 2005).

MicroRNAs (miRNAs) are a large group of small non-coding RNAs (ncRNAs) (19-23-nucletotide long) that negatively regulate gene expression by triggering mRNA decay and/or translational suppression (Bartel 2009; Mathonnet et al. 2007; Lim et al. 2005). miRNAs are assembled by the Argonaute (Ago) protein family into the miRNA-induced silencing complex and direct the RNA-induced silencing complex (RISC) to target mRNAs; this has a profound influence on various cellular processes, including proliferation, death, differentiation, and development (Kim et al. 2009; Chekulaeva et al. 2006). miRNAs are now recognized as pivotal post-transcriptional regulators of gene expression during physiologic and pathologic processes (Flynt and Lai 2008). Differential expression RBPs of miRNAs is implicated in the pathogenesis of human diseases such as cancer, neurodegenerative diseases and diabetes (Guay and Regazzi 2013; van Kouwenhove et al. 2011; Kim and Lee 2012; Lukong et al. 2008; Cooper et al. 2009).

Recently, the mechanisms by which several post-transcriptional regulators modulate the expression of *INS* and *IGF* mRNAs have been elucidated. In this chapter, the most recent findings regarding post-transcriptional regulation of insulin and IGFs by RBPs and miRNAs will be reviewed.

2 Post-transcriptional Regulation of Insulin

Insulin is synthesized in pancreatic β-cells in order to maintain a very narrow blood glucose range. Insulin biosynthesis is predominantly regulated in response to glucose through stimulation of *INS* transcription, as well as post-transcriptional regulation of *INS* mRNA (Leibiger et al. 1998; Welsh et al. 1985; Itoh and Okamoto 1980; Lee and Gorospe 2010). Post-transcriptional regulation of *INS* mRNA includes control of pre-mRNA splicing and maturation as well as the modulation of mRNA stability and translation (Mitchell and Tollervey 2000; Orphanides and Reinberg 2002; Moore 2005); all of these processes are predominantly governed by RBPs and miRNAs (Valencia-Sanchez et al. 2006; Keene 2007).

2.1 Alternative Splicing of *INS* mRNA

The *INS* gene contains three exons and two introns (Bell et al. 1980; Steiner et al. 1985). Alternative splicing generates variants of *INS* mRNA that are translated with different efficiencies (Shalev et al. 2002; Minn et al. 2005; Hernandez-Sanchez et al. 2003; Panda et al. 2010). The coding region of the human *INS* gene starts in exon 2, and therefore the 5′ UTR includes exon 1 and part of exon 2 (Bell et al. 1980; Steiner et al. 1985). Both introns are flanked by canonical splice sites, and a cryptic 5′ splice site exists in intron 1 (Fig. 2.1). Alternative splicing occurs at this splice site, thereby generating an *INS* mRNA splice variant retaining the first 26 bases of intron 1

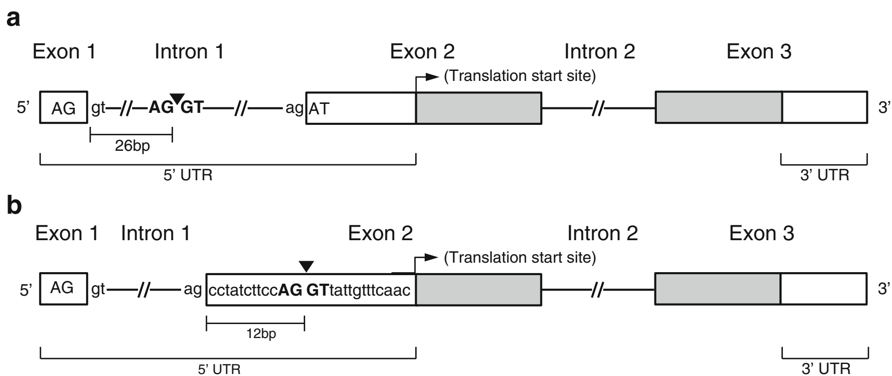

Fig. 2.1 Schematic representation of the human *insulin* (**a**) and mouse *insulin 2* (**b**) genes. The both genes consist of three exons (*boxes*), and two introns (*lines*). The coding region shown as *gray boxes* starts in exon 2 and the *arrows* indicate the translation start sites. Intron 1 in both the human *insulin* (**a**) and mouse *insulin 2* (**b**) genes is flanked by the canonical 5′-splice site sequence AG/GT and the 3′-splice site AG. The *arrowheads* indicate the 5′-alternative splice site within intron 1 in the human *insulin* gene (**a**) and 3′-alternative splice site within exon 2 in the mouse *insulin 2* gene (**b**). The 5′ UTR shown as *white boxes* includes exon 1 and part of exon 2. In humans, usage of the 5′-alternative splice site results in retention of the first 26 bp of intron 1 (**a**), while, in mouse, usage of the 3′-alternative splice site results in loss of the first 12 bp of exon 2 without altering the coding sequence (**b**) (Modified from Shalev et al. 2002 and Panda et al. 2010)

(Shalev et al. 2002). Through alteration of the 5′ UTR secondary structure, which may enhance ribosome binding, this engenders a higher translational efficiency of this splice variant in comparison to *INS* mRNA in vitro and in vivo. The expression of this splice variant is up-regulated in response to glucose and has been found to be significantly higher in diabetic/insulin-resistant mice than in wild-type littermates (Minn et al. 2005). The mouse *INS2* gene also undergoes alternative splicing to generate a variant that contains 5′ UTR lacking 12 bases; this does not alter the coding sequence (Panda et al. 2010) (Fig. 2.1). This *INS2* splice variant constitutes about 75 % of total *INS2* mRNA and is more efficiently translated in cells. Thus, alternative splicing of *INS* mRNA is also an important mechanism for post-transcriptional regulation of insulin biosynthesis. However, so far, the precise factors required for the regulation of *INS* mRNA alternative splicing remain to be identified.

2.2 Post-transcriptional Regulation of *INS* mRNA by miRNAs

The discovery of miRNAs has added a novel regulatory layer to post-transcriptional control of *INS* mRNA in pancreatic β-cells. The major miRNAs reported to play a direct role in this process in pancreatic β-cells are shown in Table 2.1 and have been discussed below.

2.2.1 miR-25 and 92a

In pancreatic β-cells, miRNAs regulate glucose-stimulated insulin secretion and β-cell survival as well as insulin biosynthesis (Kim and Lee 2012). Recently, the Jeyaseelan group reported two miRNAs (miR-25 and miR-92a) as direct regulators of insulin biosynthesis at the mRNA level (Setyowati Karolina et al. 2013). The 3′ UTR of *INS* mRNA contains binding sites for miR-25 and 92a that are conserved in the rat and mouse and are partially conserved in humans. Overexpression of miR-25 or miR-92a not only reduced *INS* mRNA but also its biosynthesis; conversely, introduction of anti-miR-25 or miR-92a increases insulin biosynthesis. Interestingly, significant up-regulation of miR-25 and miR92a is observed in the pancreas of diabetic rats, in which *INS* mRNA levels are decreased.

2.2.2 miR-196b

Although miRNAs typically target the 3′ UTR of the mRNA for functional inhibition(Pillai et al. 2007; Yekta et al. 2004), they can also target the 5′ UTR and coding regions of mRNA as well as increase translation of target mRNAs through the 3′ and 5′ UTR (Zhou et al. 2009; Orom et al. 2008; Vasudevan et al. 2007). Most recently, the Seshadri group has reported that miR-196b can directly increase *INS* mRNA translation by targeting the 5′ UTR (Panda et al. 2014). Mouse miR-196b specifically targets the 5′ UTR of *INS* mRNA and the binding site of miR-196b overlaps with that of HuD, a 5′ UTR-associated RBP that represses *INS* mRNA translation. Through this binding, miR-196b increases *INS* mRNA translation and this is likely Ago2-dependent process, because miR-196b-mediated activation of insulin expression is abolished when Ago2 levels are depleted (Panda et al. 2014). Interestingly, suppression of miR-196b expression causes increased association of

Table 2.1 Post-transcriptional regulators of insulin and insulin-like growth factors

Target mRNA	Regulators	INS/IGF levels	Function	Reference
INS	HuD	Down	Translational reppression of insulin mRNA	Lee and Kim (2012)
	PTB	Up	Glucose-induced stabilization of insulin mRNA	Tillmar L (2002)
	PDI and PABP	Up	Translational activation of insulin mRNA	Kulkarni SD (2011)
	miR-196b	Up	Translational activation of insulin mRNA	Panda AC (2014)
	miR-25 and -92	Down	Destabilization of insulin mRNA	Karolina DS (2013)
IGF-II	Zcchc11	Up	up-regulation of IGF-I by uridylation of IGF-I targeting miRNAs	Joens MR (2012)
	Nocturnin	Down	destabilizing of long form of IGF-I mRNA by deadenylation	Kawai M (2010)
	SF2/ASF		Exon 5 inclusion during myogenic differentiation, suclse hypertrophy, and myopathy	Smith PJ (2002)
	miR-1	Down	Translational inhibition of IGF-I in glucose-induced apoptosis	Yu X (2008)
	miR-1	Down	Translational inhibition of IGF-I in cartilage cell proliferation	Hu X (2013)
	miR-1	Down	Translational inhibition of IGF-I in muscle cell and inhibition of IGF signaling	Elia L (2009)
	miR-1/206	Down	Promotion of apoptosis after myocardiac infraction	Shan ZX (2009)
	miR-29	Down	promotion of myoblastic transition of HSC	Kwiecinski M (2012)
	miR-29	Down	inhibition of IGF-I signaling in aged brain	Fenn AM (2013)
IGF-II	IMPs	Down	translational reppression of IGF-II L3 mRNA during embryonic development	Nielsen J (1999)
	IMP3	Up	translational activation of IGF-II L3 mRNA in K562m glioblastoma	Liao B (2005, 2011)
	IMP2	Up	IRES translation activation of IGF-II L3 mRNA during embryogenesis	Dai N (2011)
	Lin-28	Up	Translational activation of IGF-II and promotion of myogenesis	Polesskaya A (2007)
	miR-125b	Down	Translational inhibition of IGF-II and inhibition of muscle differentiation	Ge Y (2011)
	miR-100	Down	Translational inhibition of IGF-II and inhibition of cell ;proliferation and survival signaing in breast cancer	Gebeshuber CA (2013)

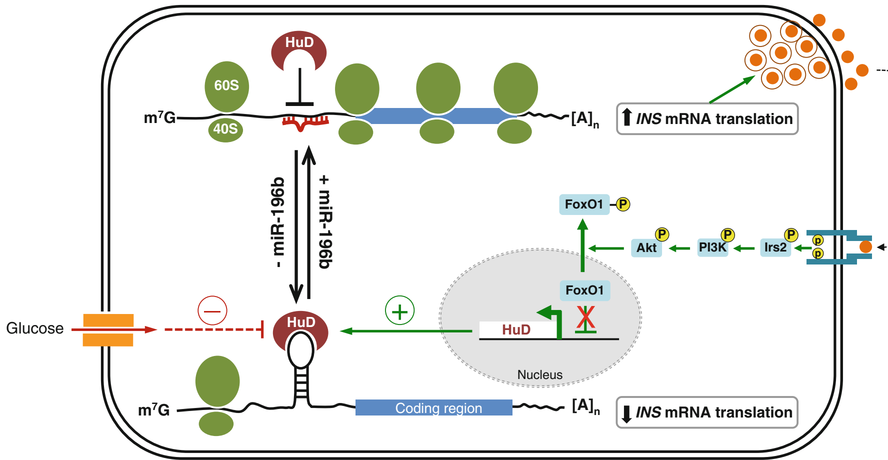

Fig. 2.2 A proposed model of HuD and miR-196b action on insulin biosynthesis

HuD with the 5′ UTR of *INS* mRNA and, conversely, this association is modestly decreased by overexpression of miR-196b, probably due to altered stem-loop structure of the 5′ UTR. HuD and miR-196b might compete for binding to the same site in the 5′ UTR of *INS* mRNA, providing an example of coordinated regulation of translation by miRNA and RBP (Fig. 2.2).

2.3 Post-transcriptional Regulation of *INS* mRNA by RBPs

Despite the short UTRs of *INS* mRNA, post-transcriptional regulation of *INS* mRNA is mediated through the cooperative action of a stem-loop in the 5′ UTR and the conserved UUGAA sequence in the 3′ UTR (Wicksteed et al. 2001). However, the identities of the specific factor(s) that associate with these elements had remained unclear until recently. Recently, several RBPs in pancreatic β-cells have been identified as pivotal post-transcriptional regulators of *INS* mRNA due to their effects on its stability and translation.

2.3.1 HuD

The Hu/ELAV (human antigen/embryonic lethal abnormal vision-like) family includes the ubiquitously expressed HuR (HuA) and the primarily neuronal HuB, HuC, and HuD. HuD/ELAVL4 is reported to specifically and directly affect post-transcriptional regulation of *INS* mRNA (Kim and Lee 2012). Like HuB and HuC, HuD expression was believed to be restricted primarily to neurons (Hinman and Lou 2008). However, recently it has been shown to be expressed in insulin-producing pancreatic β-cells (Lee et al. 2012; Poy et al. 2009), in which its levels are controlled by the insulin signaling pathway, sequentially implicating Irs2, PI3K, Akt, and FoxO1 (Fig. 2.2). Like other Hu/ELAV family proteins, HuD controls stability and translation of target mRNAs by binding to 5′ or 3′ UTR bearing

AU- and U-rich sequences through three highly conserved RNA recognition motifs (RRMs) (Hinman and Lou 2008; Pascale et al. 2008).

In pancreatic β-cells, HuD, but not HuR and other RBPs, including HuR, TIAR [T-cell-restricted intracellular antigen-1 (TIA-1)-related protein], heterogeneous nuclear ribonucleoprotein (hnRNP) K, and nuclear factor (NF) 90, as well as hnRNP C, Ago, and the fragile X syndrome protein (FMRP), associates with a 22-nucleotide segment in the 5′ UTR, but not with the coding region (CR) or 3′ UTR of mouse *INS2* mRNA (Lee et al. 2012). Binding of HuD to the *INS2* 5′ UTR represses insulin production by reducing the *INS2* mRNA translation, and not by altering *INS2* mRNA stability. This binding is repressed by glucose treatment, which induces a rapid and robust release of *INS2* mRNA from the HuD complex within 30 min of glucose stimulation thereby enables translation of *INS2* mRNA (Lee et al. 2012) (Fig. 2.2). Consistently, insulin levels are increased in pancreatic β-cells of *HuD*-null mice, and conversely, decreased in *HuD*-transgenic mice. This modulation of insulin by HuD in vivo likely contributes the homeostatic regulation of plasma glucose and insulin concentrations. In support of this is the finding that *HuD*-transgenic mice display impaired glucose clearance from the blood due to lower plasma insulin levels, thus defining HuD as a pivotal post-transcriptional regulator of insulin.

2.3.2 Polypyrimidine Tract Binding Protein

The polypyrimidine tract-binding protein (PTB), also known as hnRNP I, binds to pyrimidine-rich sequences of single stranded target mRNAs through four RRMs and plays an important role in several cellular processes such as alternative splicing (Garcia-Blanco et al. 1989; Valcarcel and Gebauer 1997; Wagner and Garcia-Blanco 2001), polyadenylation of pre-mRNAs (Lou et al. 1999; Moreira et al. 1998), cytoplasmic RNA localization (Cote et al. 1999), mRNA stability (Tillmar et al. 2002; Knoch et al. 2004) and translation initiation (Hellen et al. 1993). Glucose-induced stabilization of *INS* mRNA is a key event in the control of insulin biosynthesis and PTB plays an essential role in this process. In pancreatic β-cells, glucose induces the binding of PTB to the polypyrimidine-rich sequence located in the 3′ UTR of *INS* mRNA to stabilize *INS* mRNA, resulting in a glucose-dependent increase in *INS* mRNA (Tillmar et al. 2002; Tillmar and Welsh 2002). Glucose stimulation of β-cells enhances the cytoplasmic function of PTB by promoting its accumulation in the cytoplasm, upon which PTB binds and stabilizes *INS* mRNAs (Knoch et al. 2006). The cytoplasmic accumulation of PTB is regulated by cAMP and protein kinase A (PKA)-dependent phosphorylation (Xie et al. 2003; Knoch et al. 2006). Activation of PKA by elevation of cAMP levels causes the direct phosphorylation and nucleo-cytoplasmic transport of PTB (Fig. 2.3). Consistently, glucagon-like peptide 1 (GLP-1), which activates PKA and potentiates glucose-stimulated insulin gene expression and secretion by increasing cAMP levels in β-cells, promotes the phosphorylation of PTB, and conceivably, its cytoplasmic accumulation, which in turn enhances the levels of mRNAs containing PTB binding sites in their 3′ UTR (Knoch et al. 2006). However, GLP-1does not stabilize *INS* mRNA significantly in either rat insulinoma INS-1 cells or freshly isolated islets (Knoch et al. 2006).

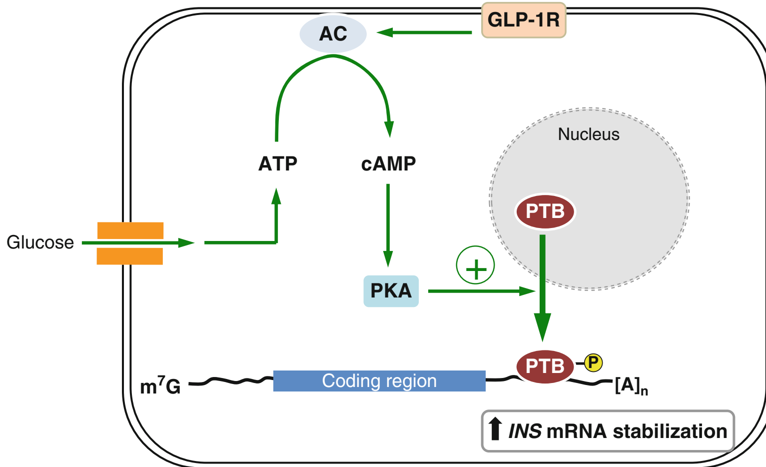

Fig. 2.3 A proposed model of PTB action on insulin biosynthesis

2.3.3 PDI and PABP

Poly(A)-binding protein (PABP) is a multifunctional RNA binding protein with an N-terminal RNA binding domain composed of four RRMs, and a C-terminal helical domain (Adam et al. 1986), which is important for its interaction with other proteins and for cooperative binding to poly(A) tails (Melo et al. 2003). Through poly(A) tail binding, PABP modulates the stability and translation of target mRNAs (Sonenberg and Dever 2003). Moreover, PABP can also bind to the A-rich sequence in the 5′ UTR to regulate key steps in mRNA translation (de Melo Neto et al. 1995; Oberer et al. 2005). In pancreatic β-cells, PABP binds specifically to the 5′ UTR of *INS* mRNA and increases the rate of ribosome recycling through its interaction with other translation factors; this leads to higher insulin translation during glucose stimulation (Kulkarni et al. 2011). The binding of PABP to the 5′ UTR of *INS* mRNA during glucose stimulation is regulated by protein disulfide isomerase (PDI), which was the first identified ER-resident catalyst of native disulfide bond formation (Anfinsen 1973). Glucose stimulation of β-cells induces PDI activation by promoting its phosphorylation. Activated PDI interacts with PABP and catalyzes the reduction of the PABP disulfide bond resulting in specific binding of PABP to the 5′ UTR of *INS* mRNA and increased insulin translation (Kulkarni et al. 2011).

3 Post-transcriptional Regulation of IGF

Insulin-like growth factor (IGF) is a protein ligand with high sequence similarity to insulin and functions as a key regulator of growth, survival, and differentiation in most cell types (Humbel 1990). There are various mechanisms by which IGF is itself induced and activated, and discrete pathways operate during proliferation,

apoptosis, differentiation, and metabolism (King et al. 1982; Panagakos 1993; Zheng et al. 2000). Production of IGF isoforms is tightly regulated at transcriptional, post-transcriptional, and translational levels (Chew et al. 1995; Adamo et al. 1991; Lowe et al. 1989; Hepler et al. 1990; Zhang et al. 1998; Foyt et al. 1991). Dysregulation of IGF levels is responsible for several pathological conditions such as diabetes, cancer, and aging (Cohen and LeRoith 2012). In this section, we review the post-transcriptional regulators of IGFs and their impact on IGF signaling.

3.1 Alternative Splicing of *IGF* mRNA

Although various splice variants of IGF-I and IGF-II have been characterized (Goldspink and Yang 2004; Velloso and Harridge 2010; Weller et al. 1993; Yang et al. 1995), the factors that govern the spicing factors are largely unknown. The serine-arginine protein splicing factor-1/alternative splicing factor (SF2/ASF) binds to a purine-rich sequence in exon 5 of *IGF-I* mRNA to promote its inclusion in the mature transcript (Smith et al. 2002). Exon 5 inclusion is responsible for myogenic differentiation, muscle hypertrophy, and myopathy (Barton et al. 2002; Matheny and Nindl 2011). The 5′ UTR composition of *IGF-II* mRNA varies throughout development due to alternative splicing (Ohlsson et al. 1994; Ekstrom et al. 1995; Monk et al. 2006), although the mechanisms and regulators required for this currently remain uncharacterized.

3.2 Post-transcriptional Regulation of *IGF* mRNA by miRNAs

3.2.1 Regulation of *IGF-I* mRNA by miR-1, miR-206, miR-29, and miR-320

IGF-I shows high sequence similarity with insulin and regulates various cellular processes such as cell growth, proliferation, survival, and insulin sensitivity in liver, muscle, and kidney (Lee and Gorospe 2010). Several miRNAs are known to modulate IGF-I expression in different model systems. Among them, miR-1 was shown to target the 3′ UTR of *IGF-I* mRNA and down-regulate its expression; in cardiomyocytes, miR-1 levels increased during glucose-induced apoptosis and relieved the anti-apoptotic actions of IGF-I (Yu et al. 2008). miR-1 also down-regulated IGF-1 expression in cardiac and skeletal muscle (Elia et al. 2009) and inhibited cell proliferation by directly targeting IGF-I in Chinese sika deer-derived cartilage cells (Hu et al. 2013). Furthermore, miR-1 and miR-206 levels were up-regulated after myocardial infraction, which resulted in a decrease in IGF-I (Shan et al. 2009). Down-regulation of IGF by miR-320 was also reported in myocardial microvascular endothelial cells isolated from type 2 diabetic Goto-Kakizaki rats (Wang et al. 2009). Recently, miR-29a and miR-29b were shown to increase during the myofibroblastic transition of hepatic stellate cells or in the aged brain, resulting in decreased IGF-1 levels (Kwiecinski et al. 2012; Fenn et al. 2013).

3.2.2 Regulation of IGF-II mRNA by miR-100 and miR-125b

IGF-II functions as an embryonic regulator of myogenesis by initiating myogenic differentiation; it is also essential for metastasis of breast cancers (McCann et al. 1996; Pravtcheva and Wise 1998; Florini et al. 1991). IGF-II expression is modulated by miR-125b and miR-100; miR-125b is involved in the down-regulation of IGF-II via direct targeting of 3′ UTR of *IGF-II* mRNA, and its expression decreases during myoblast differentiation (Ge et al. 2011). miR-125b negatively regulates myoblast differentiation by reducing the IGF-II level. miR-100 also down-regulates IGF-II expression, which leads to the inhibition of cell proliferation and reduces survival signaling in breast cancer cells; consistent with this, miR-100 expression has been found to decrease in human breast cancers (Gebeshuber and Martinez 2013).

3.3 Post-transcriptional Regulation of *IGF* mRNA by RBPs

3.3.1 Zcchc11

Zcchc11 (zinc finger CCHC domain-containing protein 11, terminal uridyltransferase 4, TUTase4) is a uridyltransferase that can suppress miRNA biogenesis by mediating terminal uridylation of miRNAs, including pre-let-7 and miR-26a (Hagan et al. 2009; Heo et al. 2009; Jones et al. 2009). Zcchc11 down-regulates let-7 in embryonic stem cells by uridylation of pre-let-7 via interaction with Lin28, which is essential for maintaining pluripontency (Hagan et al. 2009). Zcchc11 also catalyzes the 3′ UTR uridylation of miR-26a, thereby promoting expression of cytokines including interlukin-6 (IL-6) (Jones et al. 2009). In Zcchc11-deficient mice, the length and frequency of terminal uridines for diverse mature miRNAs, including miR-126b and miR-379 that target *IGF-1* mRNA, decreases (Jones et al. 2012). Uridylation of these miRNAs prevents them from suppressing IGF-I, thereby increasing its expression. Zcchc11 overexpression up-regulates IGF-I level by stabilizing *IGF-I* mRNA. This reduction in IGF-I level contributes to the increased mortality and reduced growth of Zcchc11-deficient mice because IGF-I is essential for post-natal growth and survival (Jones et al. 2012) (Fig. 2.4).

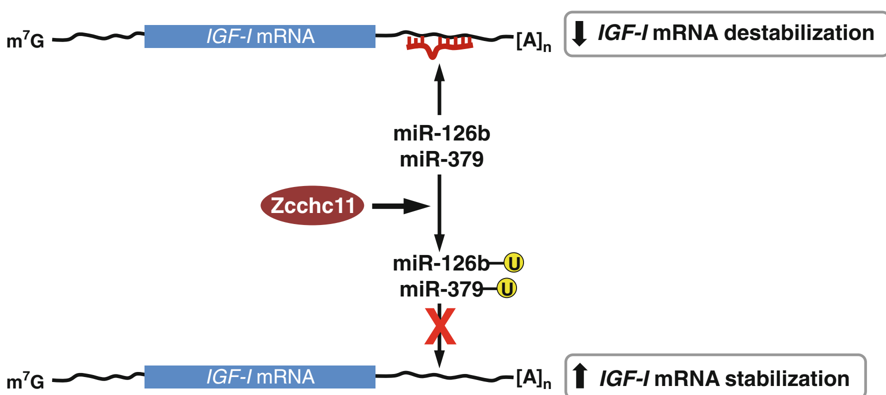

Fig. 2.4 A proposed model of Zcchc11 action on IGF-I biosynthesis

3.3.2 Nocturnin

Nocturnin (Noc, Ccrn4l) is a peripheral circadian-regulated gene product found in the liver, kidney, and testis where it functions as an mRNA deadenylase mediating mRNA degradation (Wang et al. 2001; Baggs and Green 2003; Douris and Green 2008; Kawai et al. 2010). Noc mediates post-transcriptional control of genes necessary for metabolic function including nutrient absorption, insulin sensitivity, lipid metabolism, adipogenesis, inflammation, and osteogenesis (Stubblefield et al. 2012; Green et al. 2007). The protein binds to the long-form 3′ UTR of *IGF-I* mRNA, which contains potential regulatory motifs involved in *IGF-I* mRNA degradation (Kawai et al. 2010). Noc overexpression reduces both mRNA and protein levels of IGF-I indicating that Nocturnin enhances *IGF-I* mRNA degradation, thereby reducing both its mRNA and protein levels. Interestingly, Noc interacts with *IGF-I* mRNA in a strain- and tissue-specific manner in mice (Kawai et al. 2010).

3.3.3 Lin-28

The small RNA-binding proteins Lin-28 was originally identified as a developmental regulator in *Caenorhabidits elegans* and is conserved in multiple species (Moss et al. 1997; Moss and Tang 2003; Thornton et al. 2012). Lin-28 suppresses the biogenesis of let-7 by binding to let-7 precursor and facilitating its terminal uridylation and degradation (Thornton et al. 2012; Hagan et al. 2009; Heo et al. 2009, 2012). Lin-28 is up-regulated during myogenesis and promotes myocyte differentiation. In mouse myoblast C2C12 cells, Lin-28 binds to *IGF-II* mRNA and increases its translation via enhancing its association with RNA-containing translation initiation complexes including eIF3β (Polesskaya et al. 2007).

3.3.4 IMPs

IGF-II mRNA binding proteins 1, 2, and 3 (IGF2BP1; IMP1, IGF2BP2; IMP2, IGFBP3; IMP3) belong to a conserved RNA binding protein family and function in various cellular processes, such as cell polarization, migration, proliferation, differentiation, embryogenesis and metabolism (Bell et al. 2013). IMPs bind to target mRNAs and regulate their expression post-transcriptionally via modulating mRNA stability, localization, and translation (Nielsen et al. 1999). IMPs associate at multiple sites with in the 5′ UTR of *IGF-II* leader-3 mRNA and repress translation during embryonic development (Nielsen et al. 1999). In contrast, IMP3 functions as a translational activator of *IGF-II* leader-3 mRNA, thereby promoting cell survival and tumor progression in various cell types, including K562 leukemia cells and glioblastoma cells (Liao et al. 2005, 2011; Suvasini et al. 2011). Together these data suggest that IMPs may influence IGF-II expression in a tissue- or cell-type specific manner. IMP2 binds to an internal ribosome entry site of *IGF-II* mRNA 5′ UTR and the binding of IMP2 is controlled by the mammalian target of rapamycin (mTOR) pathway (Dai et al. 2011). Phosphorylation of IMP2 on Ser162 and Ser164 residues by mTOR enhances the interaction between IMP2 and *IGF-II* mRNA, promoting translation of the latter (Fig. 2.5). IMP phosphorylation occurs in the mouse embryo as well as in adult tissues including islets of Langerhans, and seems to promote IGF2 expression, thereby regulating fetal growth and glucose homeostasis (Dai et al. 2011).

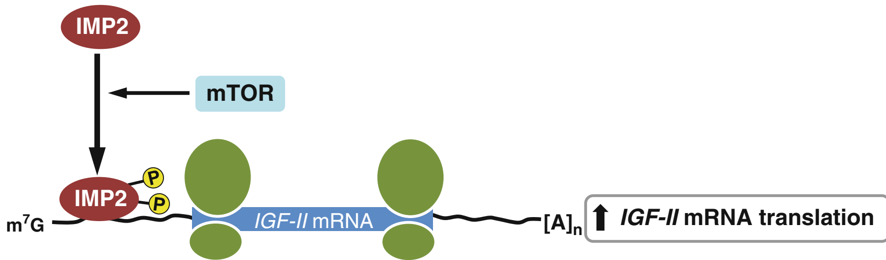

Fig. 2.5 A proposed model of IMP2 action on IGF-II biosynthesis

4 Conclusion

It is clear that the expression of insulin and IGFs is extensively regulated by post-transcriptional processes, including alternative splicing and mRNA stability and translation. Although the factors required for the regulation of *INS* and *IGF* mRNA alternative splicing are still largely unknown, the post-transcriptional regulation of *INS* and *IGF* mRNA is a complex process involving an array of different *trans*-acting factors such as RBPs and miRNAs. Here, we have described nearly all *trans*-acting factors that are known to directly bind to the UTRs of these mRNAs and modulate their expression. In addition, there are numerous factors, not described here, that indirectly regulate their expression at post-transcriptional levels that do not require direct binding to the UTRs. Through the cooperative regulation of both direct and indirect *trans*-acting factors, the expression of insulin and IGF expression is coordinately regulated. In response to acute changes in circulating glucose and other metabolic stimuli, the rate of insulin biosynthesis rises more dramatically within minutes by rapid increases in the stability and translation of the encoding mRNAs without de novo synthesis of *INS* mRNA (Brunstedt and Chan 1982; Itoh and Okamoto 1980). A long-term increase in insulin levels is dictated by both de novo synthesis and post-transcriptional regulation of *INS* mRNA. As described, although IGFs has insulin-like effects in glucose homeostasis, IGFs play a major role in the regulation of cellular processes such as growth, proliferation, and survival, which occur over longer periods. Thus, the IGF levels in these cases are likely changed by the long-term rather than the short-term regulation of the encoding mRNAs.

In addition to RBPs and miRNAs, long non-coding RNAs (lncRNAs), which are another type of post-transcriptional regulators and are non-protein coding transcripts longer than 200 nucleotides in their mature form, have also been linked to the insulin system. However, it is not known whether lncRNAs participate in the post-transcriptional modulation of *INS* and *IGF* mRNAs by direct binding to their UTRs. Further studies should therefore be performed to investigate whether and how lncRNAs, additional post-transcriptional modulators, or both increase or decrease insulin and IGF biosynthesis.

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Post-transcriptional Regulation of Cytokine Signaling During Inflammatory Responses

3

Irina Vlasova-St. Louis and Paul R. Bohjanen

1 Introduction

Cytokines are important mediators of cell to cell communication, controlling a variety of cellular activities such as inflammation, growth, differentiation, cell proliferation and apoptosis. In the immune system, cytokines play essential roles in native immunity, proper immune effector function and maintenance of peripheral tolerance. In addition, cytokines can act as hormones, regulating proper growth, cellular differentiation, and appropriate programmed cell death to help coordinate the gene expression pathways during development and maturation of multicellular organisms. Given the complexity and crosstalk between pathways involved in cytokine signaling, dysregulated cytokine networks are often associated with human diseases. For example, autoimmune diseases such as diabetes involve abnormal production of inflammatory cytokines, which lead to destruction of beta cells in the pancreas. Consequently, many of the medications used to treat autoimmune and inflammatory diseases target cytokines or kinase signaling downstream of cytokine

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receptors (reviewed in Beyaert et al. 2013; Kontzias et al. 2012; Nepom et al. 2013). Cytokine and growth factor dysregulation leading to uncontrolled cell growth is an important mechanism of cancer pathogenesis (Saharinen et al. 2011). Thus, it is crucial that cytokine signaling pathways are tightly regulated through multiple mechanisms, including transcriptional and post-transcriptional mechanisms (reviewed in Duan et al. 2013; Friedel et al. 2009; Ivanov and Anderson 2013; Seko et al. 2006).

Here, we review post-transcriptional regulation of cytokines and cytokine signaling, focusing on the role of AU-rich elements (AREs) and GU-rich elements (GREs) in coordinating signals transmitted by cytokines during inflammatory responses. AREs and GREs are conserved sequences in the 3′ untranslated region (UTR) of short-lived transcripts that encode a variety of proteins involved in cellular activation, growth and apoptosis, including components of cytokine signaling pathways. AREs function as post-transcriptional regulators of a variety of cytokines, and the mechanisms by which AREs mediate mRNA degradation have been studied extensively (reviewed in Anderson 2010). Current understanding of AREs has helped to explain the molecular mechanisms of post-transcriptional regulation of cytokine production (Anderson 2008) and has helped us to understand how networks of genes are coordinately regulated by conserved sequences in mRNA. In contrast, our knowledge about the role of GREs in coordinating gene expression is not as well understood. We discuss the role of GREs in regulating cytokine signaling networks, and suggest that AREs and GREs regulate distinct sets of transcripts but function in concert to coordinate cytokine expression with cytokine signaling.

2 Regulation by AREs

During inflammatory responses, cytokines and other early response gene proteins are induced transiently for a defined period of time through a preset program of gene expression events that include transcriptional induction, transcriptional repression, and regulated mRNA stability. For example, T cell receptor stimulation of human T lymphocytes induces ARE-containing cytokine gene expression, such as IL2, IFN-gamma, IL4, TNF-alpha, etc., through transcriptional pulses (Hao and Baltimore 2009; Raghavan et al. 2004). This transcriptional induction is followed by transcript degradation mediated by AREs found in the 3′UTRs of these transcripts (reviewed in Al-Haj and Khabar 2012; Khabar 2007; Schott and Stoecklin 2010). Figure 3.1 shows the intricate network of cytokines that are regulated by AREs in a variety of immune cell types. AREs function as instability elements that mediate cytokine transcript degradation by interacting with ARE-binding proteins that recruit the cellular mRNA degradation machinery to the transcript. Along with regulating mRNA decay, ARE-binding proteins control additional post-transcriptional events such as pre-mRNA processing, transport, intracellular localization, and translation (reviewed in Beisang and Bohjanen 2012; Raghavan and Bohjanen 2004; Stumpo et al. 2010).

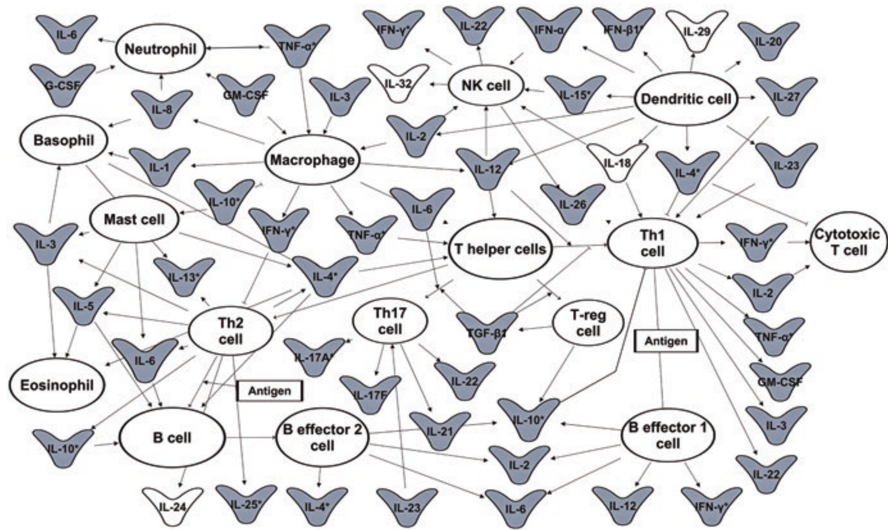

Fig. 3.1 The role of cytokines in mediating communication between immune cells. Transcripts in *grey* are cytokine transcripts that contain AREs in their 3′UTRs. Transcripts labeled with an *asterisk* (*) contain GREs in their 3′UTRs. *Arrows* indicate direct interactions and/or activations. *Blunt-ended lines* indicate inhibitory effects. Reproduced from Vlasova-St. Louis and Bohjanen (2014)

A variety of ARE-containing transcripts interact with many different ARE-binding proteins, resulting in complex patterns of post-transcriptional regulation. The destabilizing functions of different AREs, and their ability to interact with different ARE-binding proteins are not equivalent. The sequence characteristics and decay patterns of different AREs allowed them to be categorized into classes based on their sequence features and decay kinetics (Chen and Shyu 1995). A more extensive database of ARE-containing transcripts from mouse, rat and man has been generated using bioinformatics (Bakheet et al. 2003, 2006; Halees et al. 2008). This database groups AREs into five clusters depending on the number of overlapping AUUUA pentamers within the 3′UTR of a transcript with clusters 1–5 having five to one overlapping AUUUA pentamers, respectively. Cluster I AREs are enriched in secreted proteins, such as cytokines, and are involved in the growth of hematopoietic and immune cells. The other ARE clusters are found within a diverse set of transcripts. In total, ARE-containing transcripts compose approximately 5–8 % of the human transcriptome (Bakheet et al. 2001). AREs play decisive roles in regulating the effects of cytokines on inflammatory responses since mutation of the ARE in cytokine genes such as TNF-alpha or IFN-gamma resulted in profound autoimmune-like inflammatory syndrome (Hodge et al. 2014; Jacob et al. 1991).

AREs regulate mRNA decay by interacting with ARE-binding proteins that can either function to stabilize or destabilize the bound transcript. Numerous ARE-binding proteins involved in the regulation of mRNA turnover have been described in a variety of cell types (reviewed in Gratacos and Brewer 2010; Li et al. 2012; Von

Roretz et al. 2011). Typically, mRNA molecules move through different cellular compartments within messenger ribonucleoprotein (mRNP) complexes, dynamically associating with RNA-binding proteins (RBPs) that bind to conserved cis-elements found in subsets of transcripts (Turner and Hodson 2012a, b). The association of specific RBPs with subsets of transcripts containing conserved regulatory cis-elements coordinates the fate of these bound transcripts through post-transcriptional processes such as translation, intracellular localization, storage or mRNA decay (reviewed in Blackinton and Keene 2014; Keene 2007). Following cellular activation, the set of transcripts bound by a given RBP can be used to define post-transcriptional regulatory networks. RNA-immunoprecipitation (RNA-IP) experiments, in which RNA-binding proteins are immunoprecipitated from cellular lysates using an antibody against a given RBP, and co-purified mRNAs are identified, provided evidence that such networks of coordinately regulated RNAs exist. Target transcripts of several ARE-binding proteins, including HNRNPD (Wu et al. 2013), ZFP36 (Emmons et al. 2008; Stoecklin et al. 2008), ELAVL1 (Fan et al. 2011; Lopez De Silanes et al. 2004b; Mukherjee et al. 2009), KSRP (Winzen et al. 2007) and TIA-1 (Lopez De Silanes et al. 2005) were identified using RNA-IP techniques, and these targets represent distinct but overlapping sets of transcripts. ARE-binding protein targets include transcripts encoding cytokines and cytokine signaling components. Integration of the effects of multiple ARE-binding proteins likely decides the fate of ARE-containing transcripts (Mansfield and Keene 2009).

Several ARE-binding proteins, including ZFP36 (also known as TTP), BRF1, BRF2, KSRP, and HNRNPD (AUF1) destabilize transcripts by recruiting cellular deadenylases and enzymes involved in both $5' \rightarrow 3'$ and $3' \rightarrow 5'$ mRNA decay processes, whereas other ARE-binding proteins, such as ELAVL1 (also known as HuR) and ELAVL4, stabilize target transcripts by preventing deadenylation and decay (Bronicki and Jasmin 2013; Brooks and Blackshear 2013; Khabar 2010; Sarkar et al. 2011; White et al. 2013; Wu et al. 2013). ARE-containing mRNA can also be degraded by ribonucleases, such as ZC3H12A, which initiates decay by cleaving the stem loop mRNA structure in the near proximity of an ARE. Examples of ZC3H12A targets are TNF-alpha, IL1b, IL6 and IL12b, IL2, c-Rel and Ox40 transcripts (Matsushita et al. 2009; Uehata et al. 2013), which are important regulators of lymphocyte activation. These same ARE-containing transcripts are also targets of ELAVL1 (Fan et al. 2011; Lopez De Silanes et al. 2004b; Mukherjee et al. 2009). ELAVL1 has been reported to stabilize transcripts of multiple proinflammatory cytokines including, TNF-alpha, VEGF, IL13, IL17 COX2, GATA3 and a number of chemokines through AREs in their 3′UTRs, often leading to increases in both mRNA and protein levels (Stellato et al. 2011; Winzen et al. 2004). The stabilization of ARE-containing transcripts does not always lead to increased protein expression, however. For example when ELAVL1 is in a complex with TIA-1 protein, TNF-alpha and COX2 mRNAs were stabilized but their translation did not increase (Katsanou et al. 2005).

ARE-containing transcripts can interact with different ARE-binding proteins during the course of an inflammatory response through competitive binding. For example, the ARE-binding proteins ELAVL1 and ZFP36 compete with one another

for certain ARE-containing transcripts (Raghavan et al. 2001). More than 50 % of ZFP36 target sites in 3′UTRs also function as ELAVL1 target sites (Mukherjee et al. 2014). This potential for ZFP36 and ELAVL1 to compete for the same binding site on a single mRNA has implications for dynamic changes in binding during the course of adaptive immune responses. For example, shortly following T lymphocyte activation, cytoplasmic ELAVL1 levels increase transiently, stabilizing a network of ARE-containing transcripts and allowing their increased expression. Later in T cell activation, ZFP36 induction promotes the displacement of ELAVL1 from ARE-containing transcripts, allowing ZFP36 to mediate their rapid decay (Raghavan et al. 2001). This process allows the transient expression and subsequent degradation of ARE-containing transcripts during the course of immune responses. Cellular signals can also alter the functions of ARE-binding proteins over the course of innate immune responses. ZFP36 activity is regulated through its phosphorylation by p38 MAPK-activated protein kinase 2 (p38/MK2), following lipopolysaccharide (LPS) stimulation of macrophages. This phosphorylation of ZFP36 promotes its association with 14-3-3 protein and prevents ZFP36 from recruiting 3′ deadenylases to the bound transcript (Sun et al. 2007). Thus, through LPS-mediated phosphorylation of ZFP36, ARE-containing transcripts encoding inflammatory mediators such as CCL3, IL1, IL6, IL17, and COX2 are induced by virtue of transcript stabilization and thereby promote inflammation (Kang et al. 2011; Lee et al. 2012; Ronkina et al. 2010). As the immune response resolves, ZFP36 is dephosphorylated by the phosphatase PP2A, allowing ZFP36 to return to its baseline function, promoting the degradation of transcripts encoding these pro-inflammatory mediators (Frasca et al. 2010; Sun et al. 2007).

Understanding the role of AREs in balancing the expression of pro-inflammatory cytokines during inflammatory responses has led to the development of new anti-inflammatory therapies. For example, the novel drug, aganirsen, functions as an anti-inflammatory agent by impairing 14-3-3b-ZFP36 complex formation, preventing the stabilization and upregulation of ARE-containing cytokines, including IL8, TNF-alpha, IL21-beta, IL12, and IL22, during inflammatory responses (Colin et al. 2014). Thus, understanding ARE-mediated regulation of cytokine gene expression may lead to the further development of anti-inflammatory agents. In summary, the coordinate regulation of networks of ARE-containing transcripts, including cytokine networks, is a dynamic process following immune cell activation, which involves activation-induced expression of ARE-binding proteins, competition between ARE-binding proteins with opposing activities, and multiple phosphorylation events mediated through signaling pathways.

3 Regulation by GREs

Studies regarding AREs and ARE-binding proteins have led to identification of other cis-elements and proteins that coordinately regulate mRNA decay (Vlasova and Bohjanen 2008). For example, bioinformatic analysis of short-lived transcripts expressed in primary human T cells led to the identification of the sequence,

UGUUUGUUUGU (known as a GRE), to be highly enriched in the 3′UTR of transcripts that exhibited rapid degradation (Vlasova et al. 2008). Introduction of the GRE into the 3′UTR of a beta-globin reporter transcript induced rapid decay of the otherwise stable beta-globin transcript, demonstrating that the GRE functions as a mediator of mRNA degradation. The GRE binds to the protein CELF1, leading to the decay of GRE-containing transcripts. Knockdown of CELF1 in HeLa cells led to stabilization of GRE-containing reporter transcripts, further implicating CELF1 as a mediator of GRE-dependent mRNA degradation (Rattenbacher et al. 2010; Vlasova et al. 2008). A database of GRE-containing transcripts (Halees et al. 2011) was generated by categorizing GREs into five clusters based on the number of overlapping GUUUG pentamers found in the 3′UTR of a transcript, with clusters 1–5 having five to one overlapping GUUUG pentamers, respectively. These GRE-containing transcripts encode a variety of proteins with diverse biological functions including cellular signaling, growth, development and apoptosis regulation. Our recent analysis of CELF1 revealed that targets contained not only the GRE sequence UGUUUGUUUGU but also a GU-repeat sequence which could bind CELF1 and mediate mRNA decay. Based on these findings, the GRE sequence definition was revised to UGU[G/U]UGU[G/U]UGU (Rattenbacher et al. 2010).

CELF1 binds numerous target transcripts involved in cytokine signaling pathways (Vlasova-St. Louis and Bohjanen 2011, 2014; Vlasova-St. Louis et al. 2013). Genome-wide RNA-IP experiments in HeLa cells (Rattenbacher et al. 2010), primary human T cells (Beisang et al. 2012), and mouse myoblasts (Lee et al. 2010), identified hundreds of CELF1 target transcripts. Comparison of molecular functions of the CELF1 bound transcripts among the different cell types revealed enrichment of mRNAs encoding regulators of transcription, post-transcriptional control and cell cycle. In primary human resting T cells, CELF1 binds to and mediates the degradation of numerous transcripts involved in cellular activation and proliferation, presumably to maintain the cell in a quiescent state. Rapid changes in the expression of these GRE-containing transcripts occur immediately following activation of T lymphocytes. T cell receptor-mediated activation promotes the phosphorylation of CELF1, inhibiting its ability to bind to GRE-containing transcripts (Beisang et al. 2012). The lack of CELF1 binding to mRNA within first 24 h following T cell stimulation correlates with a transient increase in expression of GRE-containing transcripts involved in cellular activation and proliferation. Thus, CELF1 functions in resting T cells to mediate the degradation of GRE-containing transcripts that promote cell proliferation, and subsequent CELF1 phosphorylation following T cell activation prevents CELF1 binding to mRNA, leading to the stabilization and upregulation of GRE-containing transcripts (Vlasova-St. Louis and Bohjanen 2014).

Post-transcriptional networks are instrumental for cytokines to rapidly transmit intercellular signals to maintain homeostasis and direct the normal course of inflammatory responses. Post-transcriptional regulation through AREs impacts cytokine production by affecting the stability of cytokine transcripts. Numerous components of cytokine signaling, however, are regulated through GREs. Figures 3.2, 3.3, and 3.4 depict transcripts encoding signaling components downstream of various

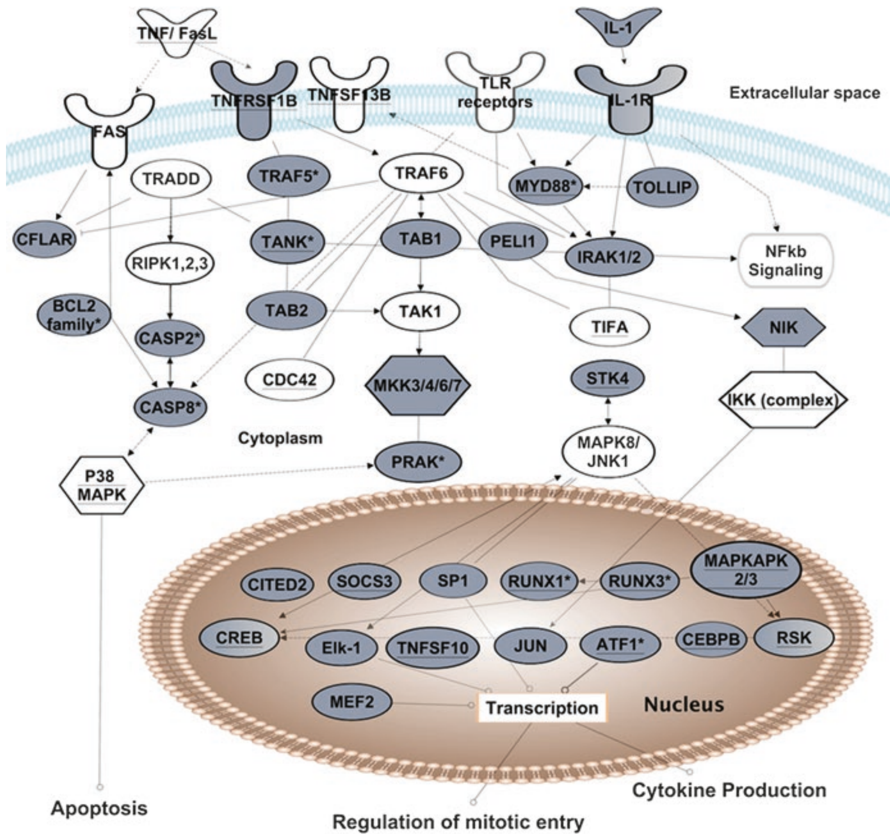

Fig. 3.2 The role of GREs and CELF1 in TNF, TLR or IL1 receptor signaling. Transcripts shown exhibited changes in steady-state levels following T cell receptor stimulation of primary human T cells (Raghavan et al. 2004). Transcripts *in grey* contain GREs. Transcripts with *underlined text* were identified as CELF1 target transcripts in human T cells by RNA-immunoprecipitation (Beisang et al. 2012). This figure is modified from (Vlasova-St Louis and Bohjanen 2014)

cytokine receptors whose steady state mRNA levels changed following immune activation. As can be seen, GRE-containing transcripts (grey transcripts) encode components of signaling pathways downstream from these receptors. Overall, these receptors transduce a broad range of intracellular signals that shift cell activation states, alter the rate of proliferation and production of other cytokines. These pathway analyses indicate that the GRE/CELF1 network plays important roles in regulating the dynamic expression of cytokine signaling components through mRNA degradation and thereby modulates the strength and duration of inflammatory responses.

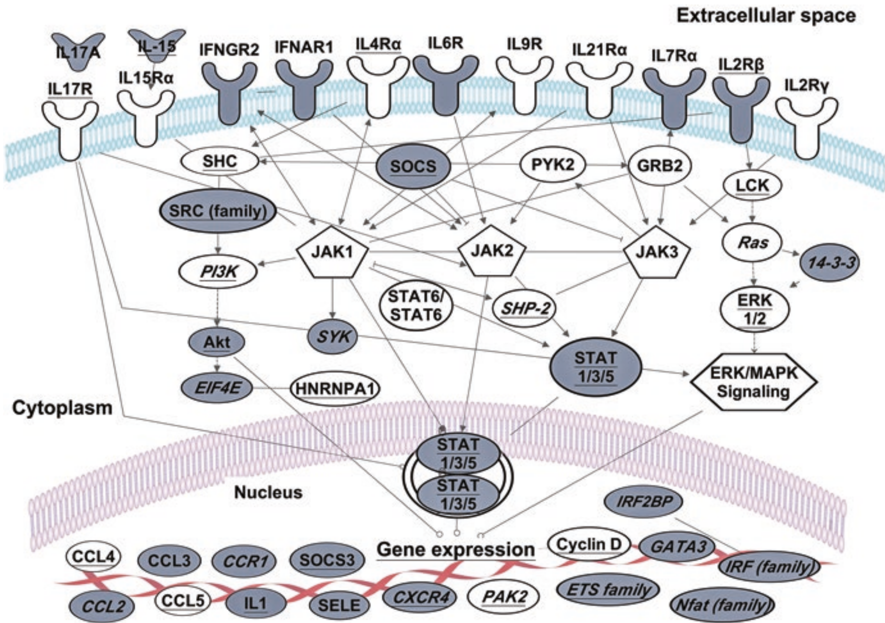

Fig. 3.3 The role of GREs and CELF1 in interferon and interleukin receptor signaling. Transcripts shown exhibited changes in steady-state levels following T cell receptor stimulation of primary human T cells (Raghavan et al. 2004). Transcripts in grey contain GREs. Transcripts with underlined text were identified as CELF1 target transcripts in human T cells by RNA-immunoprecipitation (Beisang et al. 2012). Transcripts marked with text in italics are also bound to ELAVL1 in activated cells (Mukherjee et al. 2009; Fan et al. 2011). This figure is modified from Vlasova-St. Louis and Bohjanen (2014)

4 Cross-Talk between Networks

The network of ARE-containing transcripts encodes various cytokines (Fig. 3.1) and cytokine signaling molecules that depends on circuits of GRE-containing transcripts to function. In particular, GRE-containing transcripts encode diverse components of cytokine receptor signaling (Figs. 3.2, 3.3, and 3.4). Moreover, numerous GRE-containing CELF1 target transcripts are also targets of the ARE-binding protein ELAVL1, indicating a functional interaction and potential competition between these RNA-binding proteins. RNA recognition sequences for CELF1 require precise GU repeats or GUUU repeats, whereas the recognition sequence for ELAVL1 is less precise, and ELAVL1 binds to a variety of U-rich sequences, including GU-rich sequences or a poly U sequence. Thus, CELF1 and ELAVL1 may compete for binding to a subset of target transcripts. In contrast to ELAVL1, other ARE-binding proteins that recognize precise AUUU repeats, such as ZFP36, do not bind to GRE sequences and do not compete for binding sites. The comparison of CELF1 targets that were immunoprecipitated from normal T cells (Beisang et al. 2012) with ELAVL1 targets from HeLa (Lopez De Silanes et al. 2004a) or Jurkat cells

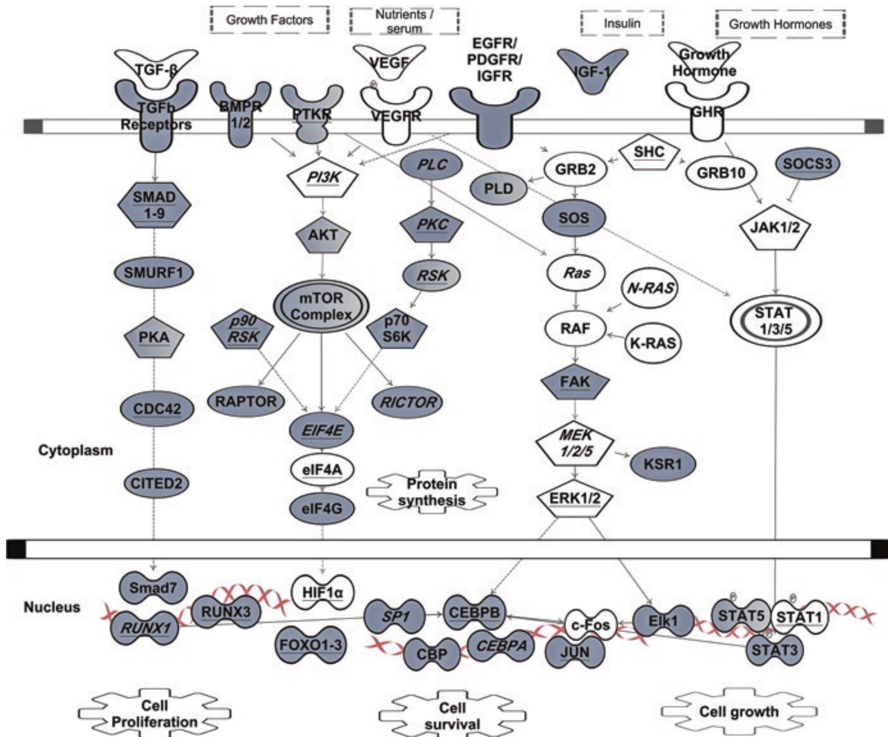

Fig. 3.4 The role of GREs in growth factor signaling. Signals transmitted through tumor growth factor receptors (TGFR), vascular endothelial, epidermal, platelet-derived or insulin growth factor receptors (VEGFR, EGFR, IGFR, PTDGFR) are directed through several major pathways, such as SMAD, mTOR, RAS, PLC, or JAK/STAT. Proper expressions of these transcripts cooperatively contribute to the overall cellular signaling outcome (such as cell proliferation, cell survival and cell growth). Transcripts in *grey* contain GREs. Transcripts with *underlined text* were identified as CELF1 target transcripts in resting human T cells by RNA-immunoprecipitation (Beisang et al. 2012). Transcripts marked with *text in italics* are also bind ELAVL1 in activated cells (Mukherjee et al. 2009; Fan et al. 2011). Arrows indicate direct interactions and/or activations. Blunt-ended lines indicate inhibitory effects. This network diagram was built using Ingenuity Pathway Assistant Software

(Mukherjee et al. 2009), showed a subset of transcripts involved in cytokine signaling that are targets of both proteins (Vlasova-St. Louis and Bohjanen 2014). Depending on which protein competitively binds to the GRE in the target transcript, dichotomous biochemical effects may result in either CELF1 mediating degradation or ELAVL1 mediating stabilization. We hypothesize that in resting cells these transcripts are bound to CELF1 and targeted for degradation, but in activated immune cells, CELF1 becomes phosphorylated and is rendered unable to bind to GRE sequences. ELAVL1 then binds to the GRE, displacing CELF1, stabilizing the transcripts, and allowing them to be translated. Later in the immune response, CELF1

becomes dephosphorylated and then displaces ELAVL1 to mediate transcript degradation. Although further evidence is needed, this model could explain the transient expression of certain GRE-containing transcripts following T cell activation.

The dynamic interplay of GRE and ARE networks appears to regulate nearly all known cytokine signaling pathways. For example, signals downstream of TNF-alpha receptors interact with signals downstream of FAS (also TNFRSF6) to regulate apoptosis. CELF1 regulates expression of a number of apoptotic proteins downstream of FAS receptor through destabilization of pro-apoptotic mRNAs, simultaneously affecting cell death rate and proliferation. CELF1 has been shown to co-immunoprecipitate with transcripts encoding TNF receptor super family members and other molecules downstream of TNF receptors (underlined transcripts in Fig. 3.2) (Beisang et al. 2012; Lee et al. 2010; Rattenbacher et al. 2010). Interestingly, CELF1 and ELAVL1 both bind to and affect the stability of TNF mRNA (Dean et al. 2001; Zhang et al. 2008) and other regulators including effector caspases and cytochrome C mRNA, which are activated downstream of TNF receptors involved in the regulation of apoptosis. Both CELF1 and ELAVL1 proteins regulate stability of transcripts encoding members of the BCL2 superfamily such as BCL2, BNIP3, BCL2L2, BAD and BAX, which modulate programmed cell death (Abdelmohsen et al. 2007; Beisang et al. 2012; Talwar et al. 2013). Thus, signaling through the TNF receptors is controlled by GRE-containing transcripts (also CELF1 targets), which cooperate with ELAVL1 target transcripts to regulate apoptosis.

As shown in Fig. 3.2, multiple GRE-containing transcripts encode signaling components downstream of the IL1 receptor. Proper signaling through this cytokine receptor requires coordination of ARE and GRE networks. Several IL1 receptor signaling components, including STK4, CDC42, MYD88, TIFA, MEF2, RSK, cyclin D have been shown to be ELAVL1 targets in RNA-IP experiments (Mukherjee et al. 2009). Many target transcripts, shared by CELF1 and ELAVL1, are transcription factors or early response genes such as RUNX1, JUN, myc or CREB that are transiently up-regulated following cellular activation and then down-regulated through rapid mRNA decay. Chronic imbalances in the turnover rates of these transcripts could promote onset and progression in diseases such as autoimmunity or cancer through aberrant immune cell activation, excess inflammation and inappropriate cell death (Candido and Hagemann 2013; Moudgil and Choubey 2011; Vlasova et al. 2005).

Many other interleukin and interferon receptors also utilize signaling proteins encoded by GRE-containing transcripts (Fig. 3.3), and rely on interplay between ARE and GRE networks to maintain homeostasis. Cytokines that bind to receptors containing the common gamma chain, such as IL2, IL4, IL7, IL9, IL15 and IL21, are pivotal for immune responses (Baker et al. 2007; Liao et al. 2011). These receptors activate Janus kinases (JAK1 and JAK2) to phosphorylate members of the STAT (Signal transducers and activators of transcription) family of transcription factors (Jenkins 2014). STAT proteins form hetero- or homodimers, and upon translocation into nucleus they induce the transcription of genes including cytokine, chemokine and adhesion molecule genes. One of the induced gene family, SOCS, plays a role in inhibiting JAK kinase activity and consequently, down-regulates immune

activation (Linossi et al. 2013). Interestingly, SOCS3 mRNA is also a CELF1 and ELAVL1 target, perhaps to allow its expression only at the appropriate time during the resolution phase of the immune response. Engagement of interferons by interferon receptors also activates JAK-STAT, PI3K and MAPK signaling pathways (Khabar and Young 2007). Interferon family members of type I, II, and III contain functional AREs that allow precise post-transcriptional control by ARE-binding proteins. Interferon receptor mRNAs contain GREs and likely function to maintain the balance in cytokine-receptor signaling and ultimately the course of immune responses (Fig. 3.3). Thus, the induction and control of interferon responses requires coordination of ARE and GRE networks.

Growth factor-induced cell proliferation, adhesion, and migration often depend on ARE and GRE post-transcriptional regulatory networks. Transforming growth factor-beta (TGF-beta) signals are transduced by trans-membrane type I and type II serine/threonine kinase receptors (TGFR1/2), which are encoded by GRE- and ARE-containing mRNAs (Fig. 3.4). TGF-beta maintains tissue homeostasis by regulating the cellular proliferation rate, differentiation and survival. The activated TGF receptor complex induces oligomerization of several downstream SMAD protein family members. Activated SMADs regulate transcription of a number of GRE-containing mRNAs (ex. CITED2, CXCL2, NKX, CDKN1A, VEGF, IL3RA, TGFBR1, etc.), which in turn, become a subject of post-transcriptional regulation by CELF1 (or ELAVL1), once they leave the nucleus. In pathological conditions, TGF-beta signaling, by activating RAS oncogenic pathway, upregulates a large set of ARE-containing genes and specifically VEGF mRNA (Kanies et al. 2008), perhaps due in part to dysfunctional mRNA decay pathways. The RAS–PI3K–Akt kinase–mTOR pathway is a well-established signaling axis that modulates the proliferation and survival of many cell types through growth factor receptor signaling (reviewed elsewhere Bitterman and Polunovsky 2012; Cao et al. 2008; Weichhart and Saemann 2008). Growth factors are beneficial for tissue growth and regeneration, but abnormal growth factor signaling can fuel inflammation and metastases (Carmeliet and Jain 2011). Since ARE and GRE-containing transcripts encode important regulators of apoptosis and cell cycle downstream of growth factor receptors, transformed cells may usurp these post-transcriptional networks to their advantage as they become malignant (Fuxe and Karlsson 2012).

Numerous studies describe an important role of ELAVL1 in the pathogenesis of inflammatory diseases that involve over-production of inflammatory cytokines or diseases characterized by cytokine deficiency. Most studies support a role for ELAVL1 malfunction in the promotion of inflammation and proliferation in human diseases such as autoimmune diseases and cancer (Khabar 2010; Srikantan and Gorospe 2012). Furthermore, genetic deletion of ELAVL1 in thymocytes (Papadaki et al. 2009) and myelocytes (Yiakouvaki et al. 2012) predisposed mice to exaggerated inflammatory responses and inflammation-driven oncogenesis. In contrast, CELF1 functions as an inhibitor of a network of transcripts that promote cellular activation and proliferation (Beisang and Bohjanen 2012). The opposing effects of CELF1 and ELAVL1 may have important implications for new therapies for proliferative diseases such as cancer or autoimmunity. Thus, understanding the crosstalk

of CELF1, ELAVL1 and other RBPs may uncover novel therapeutic strategies to fine-tune the balance in proliferative or proinflammatory pathways involved in human diseases. In the future, more work needs to be done to develop interventions targeting post-transcriptional mechanisms involved in inflammatory responses such as the severe reaction that occurs in sepsis, which involves self-amplifying networks of inflammatory mediators often referred to as 'cytokine storm'.

5 Summary and Perspective

Post-transcriptional networks defined by AREs and GREs regulate cytokine production and signaling. The ARE and GRE networks represent distinct subsets of transcripts that work in concert to coordinate the function of cytokines over the course of an immune response. AREs are critical regulators of cytokine production and play important roles in cytokine signaling, but a distinct subset of cytokine signaling components, regulated by GREs, is needed for precise physiological function of cytokines. Thus, an effective immune response requires crosstalk between ARE and GRE pathways to appropriately regulate cytokine responses over time. Perhaps, integrative studies of interactions between GREs, AREs and RBPs as modulators of coordinated gene expression could be translated to novel treatment strategies.

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Post-transcriptional Regulation of Luteinizing Hormone Receptor mRNA Expression in the Ovary

4

K.M.J. Menon, Bindu Menon, and Thippeswamy Gulappa

1 Introduction

In most mammals, the ovary undergoes continuous changes in structure and function during the reproductive period. In the human after menarche, the ovary secretes estrogens and progesterone in a cyclical manner to facilitate ovum production and preparing the endometrium for implantation of the blastocyst if fertilization has occurred. In the absence of blastocyst implantation, corpus luteum, the specialized structure that secretes progesterone, regresses and the cyclical pattern of hormone secretion repeats again. This process repeats continuously until menopause which occurs after the depletion of ovum resulting in the cessation of the cyclical changes in ovarian structure and function. In rodent and human, luteinizing hormone (LH) regulates androgen biosynthesis by the theca-interstitial cells during follicular development and these androgens are converted to estrogens by the granulosa cells (Bjersing 1968). The follicular growth is stimulated mainly by follicle stimulating hormone (FSH) which acts in concert with estradiol and insulin/IGF-1 system (Hirshfield 1991; Zeleznik 2004). More importantly, the growing follicles acquire LH receptors by the combined actions of FSH and estradiol (Zeleznik 2004; Menon et al. 2005). LH is also responsible for ovulation and for the biosynthesis of progesterone, a hormone required for preparing the uterus for the implantation of the blastocyst as well as maintaining the conceptus if implantation has occurred.

The action of LH is mediated by LH receptor which belongs to the glycoprotein subfamily of the large G protein-coupled receptor (GPCR) family with an unusually

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large extracellular domain, seven membrane spanning helices and an intracellular domain with two conserved palmitoylation sites (McFarland et al. 1989; Loosfelt et al. 1989). Its expression increases during follicle development and show a dramatic decline in response to a bolus of LH that occurs prior to ovulation when the oocyte reaches the metaphase of the second meiotic division (Baker 1972). During this period of transition, a refractory period of LH responsiveness occurs that is characterized by a transient loss of LH receptors and consequent loss of the LH responsiveness (Hunzicker-Dunn et al. 1979; Menon et al. 2004). The receptor levels then rises again reaching maximum levels by mid portion of the luteal phase to support progesterone production. The LH receptor expression then falls with the regression of the corpus luteum in the absence of pregnancy. The changes in LHR expression are exquisitely regulated to ensure successful reproduction. While FSH, along with other paracrine factors, is known to regulate the development of the primary follicles to preantral and antral stages, the LH receptor makes its appearance in significant amounts as a result of FSH stimulation (Zeleznik 2004; Channing et al. 1980), Once LHR expression is established in the growing follicle, subsequent changes in its expression during the ovarian cycle is caused by changes in the levels of its own ligand (Menon et al. 2004). We have examined the biochemical mechanism that is responsible for the changes in the levels of LHR expression using both rodent and human models. Since there is striking change in LHR following preovulatory LH surge under physiological conditions, we have used experimental conditions that mimic this situation to examine the biochemical mechanism that regulates LH receptor expression using a rodent model.

2 Post-transcriptional Regulation of LH Receptor mRNA Expression

Administration of hCG, a hormone with significant chemical identity to LH, produces a rapid decline in LHR mRNA and protein in the rat and human ovaries (Hoffman et al. 1991; Lu et al. 1993; LaPolt et al. 1990; Segaloff et al. 1990; Nair et al. 2002). Since G protein coupled receptors are internalized after ligand binding (Menon et al. 2004), the simplest explanation for the disappearance of LH receptor following exposure to high concentration of the ligand is that the ligand-receptor complex undergoes endocytosis and not replaced by newly synthesized receptor causing a reduction in cell surface expression. The steady state level of receptor expression is the balance between the rate at which the receptor is trafficked to the cell surface and the rate of its endocytosis. The loss of steady state levels of receptor expression could also be due to a temporary pause in synthesis of new receptor protein by decreased transcription, or an increase in degradation of mRNA. To examine these possibilities, we used ovaries from super-ovulated rats as a model system. Immature rats were treated with 50 IU PMSG followed by 25 IU hCG 56 h later to induce superovulation. On day 4 of superovulation, one set of animals was treated with 50 IU hCG, and a second control group received normal saline. Examination of LHR expression in the ovaries collected at different time intervals

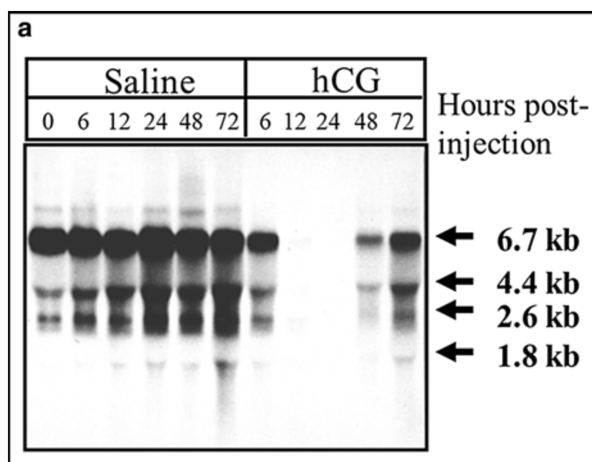

Fig. 4.1 LHR mRNA levels during hCG-induced downregulation. Northern blot analysis of steady state LHR mRNA levels in total RNA, isolated at the indicated times, from the ovaries of saline-injected (control) (*lanes 1–6*) or hCG-injected (downregulated) (*lanes 7–11*) rats. Blots were probed using a labeled cDNA encoding the LHR carboxyl terminus and a portion of the 3′-UTR (nucleotides 1936–2682). RNA was extracted from control and downregulated ovaries at 0, 6, 12, 24, 48, and 72 h after hCG injection. This research was originally published in J Biol Chem (Kash JC, Menon KMJ. *J Biol Chem.* 1998. 273:10658–64)

up to 72 h (Hoffman et al. 1991; Lu et al. 1993; Peegel et al. 1994) by Northern blot analysis, real time PCR and in situ hybridization analysis in tissue sections showed a steady decline in the expression levels of LH receptor mRNA starting 6 h following hCG treatment reaching undetectable levels by 24 h (Hoffman et al. 1991; Lu et al. 1993; Peegel et al. 1994). The recovery from downregulation was seen after 24 h and reached almost control levels by 72 h, as shown in Fig. 4.1. So, the loss of cell surface receptor via internalization of the ligand-bound receptor does not appear to be the sole mechanism responsible for the disappearance of the receptor during downregulation. The loss of mRNA was not expected, since the conventional thinking was that the rapid internalization of the ligand-receptor complex would be the major cause of the decrease of cell surface receptor expression. The in situ hybridization pattern of the receptor expression in the whole ovary was in full agreement with the Northern blot data which further suggested that the same corpora lutea that were depleted of the receptor mRNA regained the receptor (Peegel et al. 1994). It was interesting to note that the process of LH receptor mRNA loss was very specific since other mRNAs involved in ovarian function such as Cyp11A showed no decline. In fact, the expression of the steroidogenic enzyme mRNA expression showed an increase caused by the initial cellular response to hCG by increasing cyclic AMP production which has been known to activate the expression of key enzymes involved in ovarian steroidogenesis (Menon and Gunaga 1974; Marsh et al. 1966; Miller 2008). The loss of LHR in response to hCG treatment was mediated by increased production of cyclic AMP. This is further supported by the finding

that LHR mRNA downregulation can be achieved by chronic elevation of cyclic AMP levels by repeated administration of inhibitors of type 2 phosphodiesterase (Peegel et al. 2005). The evidence for the decrease in the steady state levels of LHR mRNA expression occurred through post-transcriptional mechanism came from the determination of transcription rates by performing nuclear run-on assays which showed no decrease in transcription rates during the time course of downregulation when compared to the control. In fact, the nuclei isolated from the hCG treated ovaries incorporated [^{3}H] uridine into total RNA at much higher levels compared to the control (Lu et al. 1993). These findings supported the view that the loss of LHR mRNA during ligand-induced downregulation was a result of increased mRNA degradation rather than a decrease in the transcription rate.

3 Accelerated LH Receptor mRNA Degradation as a Regulatory Mechanism

It is now well recognized that the expression of specific, highly regulated mRNAs is regulated, at least in part, at the level of mRNA degradation. There are several examples where the mRNA degradation rate controls the steady state levels of mRNA expression (Port et al. 1992; Bernstein et al. 1992; Sachs 1993; Shyu et al. 1989; Ross 1995; Moghul et al. 1994; Olivas and Parker 2000; Tharun et al. 2000; Pachter et al. 1987; Perez-Ortin et al. 2013; Garneau et al. 2007). In almost all instances where the mRNA expression is regulated by controlling its degradation, the changes in the stability of the mRNA have been shown to result from the binding of specific proteins to specific sequences and/or structures of the mRNA. The specific regions that the mRNA binding proteins interact with may be localized either on the 5′ untranslated region, the coding region or the 3′ untranslated region of the mRNA (Ross 1995). In general, the steady state levels of the mRNA expression are regulated by either increasing or decreasing the degradation rate resulting from the interaction with specific RNA binding proteins.

The possible existence of a binding protein that specifically recognizes LHR mRNA was examined by incubating [^{32}P] labeled LH receptor mRNA with a cytosolic fraction (100 × *g* supernatant) prepared from the ovary pretreated with hCG to downregulate LH receptor mRNA expression in the presence of ribonuclease inhibitors (Kash and Menon 1998, 1999). After treating the reaction mixture with ribonuclease T1 to degrade the unreacted RNA probe, and the ribonucleoprotein (RNP) complex was separated by electrophoresis on an 8 % native acrylamide gel and subjected to autoradiography. Two RNP complexes were identified, one prominent band corresponding to 50 kDa and a second less intense band of 45 kDa (Fig. 4.2). A threefold increase in the 50 kDa RNP complex was seen in samples derived from LHR downregulated ovaries but no significant change in the intensity of the 45 kDa band was found. Because of this reason, further studies focused on the larger 50 kDa ribonucleoprotein complex. The protein was named LH receptor mRNA binding protein and abbreviated as LRBP (Kash and Menon 1998, 1999).

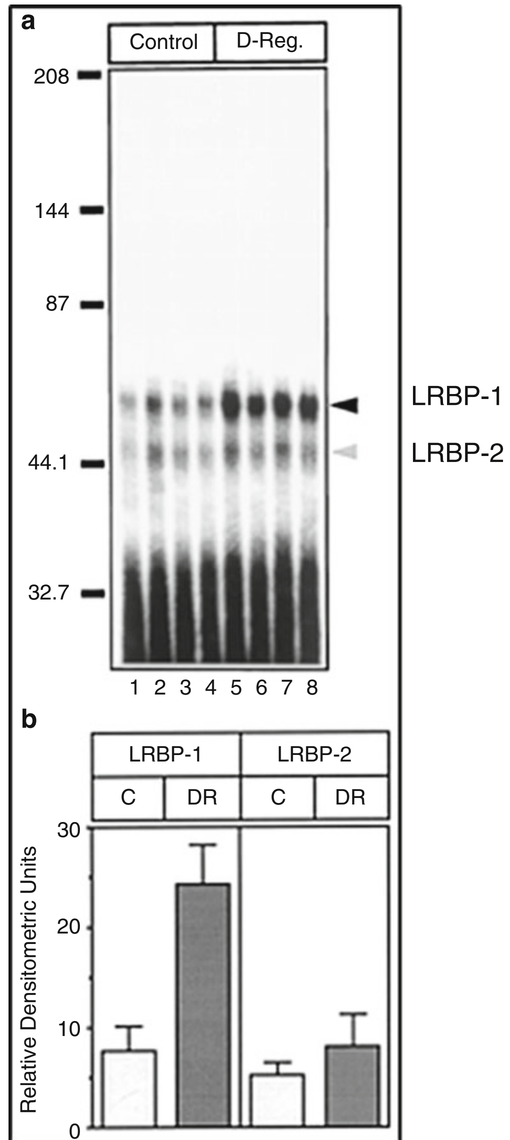

Fig. 4.2 Binding of LHR mRNA to LRBP-1 and LRBP-2 during hCG-induced downregulation. Ovaries were collected from saline-injected (*Control*) and hCG (12 h) downregulated (*D-Reg.*) rats. S100s from the control (*lanes 1–4*) and hCG downregulated (*lanes 5–8*) ovaries were subjected to RNA gel mobility shift analysis by incubation with radiolabeled (1×10^5 cpm) LHR RNA followed by 8 % native polyacrylamide gel electrophoresis. (**a**) Autoradiogram of gel shift analysis. *Arrows* indicate LRBP-1 and LRBP-2. (**b**) *Bar graph* representing the mean densitometric scans ± S.D. of the LRBP-1 and LRBP-2 complexes shown in (**a**) (Modified from Kash, J.C. and Menon, K.M.J., 1998, Fig. 6 with permission from the Journal of Biological Chemistry)

The binding of LHR mRNA to LRBP showed that the recognition of the mRNA resided in the amino terminal portion of the LH receptor corresponding to nucleotides 102–282. The RNP complex formation was always higher in the ovarian tissue derived from LH receptor downregulated ovaries with no change in the non-target tissues (Kash and Menon 1998). Further truncation of this region showed that the contact site resided between nucleotides 188–228. RNA hydroxy-radical footprinting revealed a bipartite polypyrimidine rich sequence, (UCUCX7-UCUCCCU)

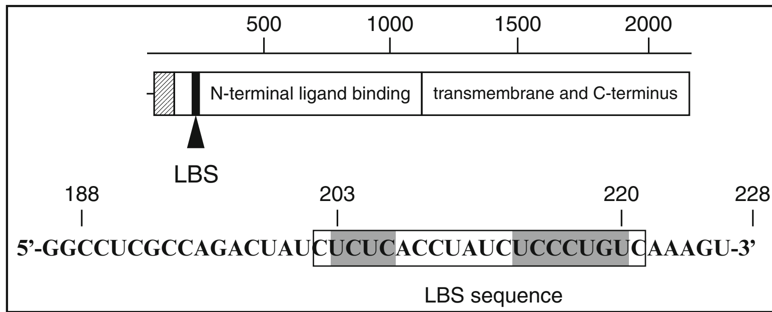

Fig. 4.3 Diagram of the LRBP RNA binding site. LH/hCG receptor mRNA open reading frame showing the position of the LRBP binding site (LBS). The sequence of the LHR:188–228 is also shown (Modified from Kash, J.C. and Menon, K.M.J., 1999, Fig. 8 with permission from Biochemistry)

corresponding to nucleotides 203–220 of LHR mRNA that specifically interacted with LRBP (Fig. 4.3). Mutation of the C residues within the bipartite sequence revealed that all C residues participated in binding to LRBP (Kash and Menon 1999). The contact site was further confirmed by performing RNA electrophoretic mobility shift assays. The binding was specific, since the RNP complex formation using [^{32}P] labeled LH receptor mRNA probe (nucleotides 203–220) was not displaced by probes corresponding to other regions of LH receptor mRNA. The interaction of LHR mRNA with LRBP exhibited high affinity with equilibrium dissociation constant (Kd) in the range of 4×10^{-9} M.

After establishing that the ovarian LRBP recognizes LH receptor mRNA with high affinity and specificity, the changes in LRBP activity in relation to tissue levels of LH receptor mRNA were examined. It has been established that LHR mRNA expression increases during maturation of the ovarian follicles in response to FSH. The levels then fall immediately after the preovulatory LH surge and the levels rise again with the growth and development of the corpus luteum. The relationship between LHR mRNA expression and LRBP activity was examined in ovarian tissues after treating immature rats with pregnant mare serum gonadotropin to induce follicle growth and to increase LHR mRNA expression levels. At this time point, the LRBP activity in the S100 fractions prepared from the ovaries showed low levels of activity. Thus a negative correlation between tissue levels of LHR mRNA expression and LRBP activity was observed. However, when LHR expression was suppressed during ligand-induced downregulation by treatment with hCG, the LRBP activity was increased several fold in a time dependent manner. Thus, when the LH receptor mRNA expression level was high, the RNA binding protein activity was at the lowest level. Conversely, the LH receptor mRNA binding activity was high when the mRNA levels began to fall. This inverse relationship suggested that LRBP is an endogenous regulator of LH receptor mRNA expression.

4 Signaling Pathways Involved in Ligand-Mediated Downregulation of LH Receptor mRNA

Since the immediate response of the ovarian tissue to hCG treatment is to increase the production of cyclic AMP (Menon and Gunaga 1974; Marsh et al. 1966), the involvement of cyclic AMP in LHR mRNA downregulation was presumed. In fact, this assumption turned out to be true by the demonstration that chronic elevation in cyclic AMP production in ovaries of superovulated rats with multiple injections of rolipram, an inhibitor of type 2 phosphodiesterase (Peegel et al. 2005), showed downregulation of LH receptor mRNA similar to that produced by the treatment with hCG. The ovaries were then processed for REMSA in order to determine whether there was increased LRBP activity when LH receptor mRNA was downregulated. A substantial increase in the RNA binding activity was seen in ovarian S100 fractions at time intervals when the LH receptor mRNA expression was downregulated. These findings suggested that the downregulation of LH receptor mRNA in response to hCG treatment was caused by an increase in cyclic AMP production. In addition, we were able to demonstrate that cyclic AMP-mediated activation of PKA and ERK1/2 signaling pathways is involved in ligand-mediated LH receptor downregulation in human granulosa cells (Menon et al. 2011). The participation of PKA and ERK1/2 signaling pathways was demonstrated with the use of H89 and U0126, respectively. Furthermore, transfecting cultured human granulosa cells with ERK1/2-specific small interfering RNA abrogated LH receptor downregulation in response to hCG treatment thereby conclusively demonstrating the participation of ERK1/2 signaling pathways in inducing downregulation (Menon et al. 2011).

5 In Vitro mRNA Degradation by the LH Receptor mRNA Binding Protein

After establishing a relationship between the expression of LH receptor mRNA and the RNA binding protein under in vivo conditions, the direct role of the RNA binding protein in LH receptor mRNA degradation was demonstrated by employing a cell-free mRNA decay system developed by Ross and colleagues (Ross 1993) under in vitro conditions. The assay essentially determines the degradation rate of a specific exogenous RNA in the presence and absence of the RNA binding protein under controlled conditions. Although all mRNAs are prone to degradation, the rate of degradation varies depending on the cellular environment. Some mRNAs are more labile than others, a property conferred by the presence of appropriate *trans*-acting factors that interact with specific structures or sequences present in the mRNA. Initially, we showed that the rate of decay of LH receptor mRNA was very rapid in ribosomes isolated from the ovaries of rats treated with hCG, compared to the degradation of LH receptor mRNA by ribosomes isolated from ovaries of saline treated control animals (Nair et al. 2002), but the rate of decay of exogenously added ovarian RNA by ribosomes isolated from saline treated rats was accelerated by the addition of a partially purified ovarian LH receptor mRNA binding protein

(Nair et al. 2002). The rate of LH receptor mRNA decay was not affected by unrelated proteins added to the reaction mixture. These experiments demonstrated that the LHR mRNA binding protein plays a role in LH receptor mRNA degradation.

6 Characterization of LH Receptor mRNA Binding Protein

Purification of RNA binding proteins from tissue samples presents many challenges including the relatively time consuming assays to follow the RNA binding activity using RNA electrophoretic gel mobility shift assay and its low abundance in the cell. Covalently linked RNA binding sequence to sepharose followed by column chromatography showed that the binding protein was retained on the columns, but the attempts to elute the binding protein retained on the matrix were not successful. Therefore, conventional techniques were used to purify the LH receptor mRNA binding protein from the $100 \times g$ supernatant fraction of the ovarian homogenates. The ovaries were initially downregulated by treatment with the ligand in order to increase the yield of the binding protein. The supernatants were subjected to chromatography on a strong cation exchange resin (Macro-Prep high S support) and eluted with 150 mM KCl. The eluates were concentrated and subjected to SDS-PAGE to separate the proteins. The [^{32}P] LHR mRNA binding activity associated with the protein band on the gel was identified by an overlay assay (Northwestern blot) using [^{32}P] labeled LH receptor mRNA fragment (203–220) as the probe. After extensive standardization of the assay, the protein band corresponding to the band that showed the RNA binding activity was cut, eluted and renatured. The eluted protein was electrophoresed again to determine the purity of the preparation. The electrophoretically homogeneous protein band was then subjected to amino terminal analysis as well as MS-MALDI analysis to establish its identity. Both analyses revealed the purified protein to be mevalonate kinase (Nair and Menon 2004). The gene encoding rat mevalonate kinase was then cloned and overexpressed in 293 T cells (Nair and Menon 2004). The recombinant protein exhibited a concentration dependent increase in binding LHR mRNA probe (Nair and Menon 2004). The binding exhibited all the characteristics of the expected LHR mRNA binding protein with respect to specificity for binding to the previously identified contact site (nucleotides 203–220), competition by unlabeled LHR mRNA fragment (203–220), dependence on C residues in the ligand binding site and immunoreactivity of the recombinant protein similar to that seen for the electrophoretically purified rat mevalonate kinase by Western blot analysis. Furthermore, since mevalonate kinase is known to have two binding sites, one for ATP and a second site for mevalonate, the involvement of these sites in LH receptor mRNA binding activity was then determined. It was seen that the binding of the [^{32}P] labeled LH receptor mRNA binding sequence to mevalonate kinase was inhibited by ATP and mevalonate. The inhibitory effect was even more pronounced in the presence of both ATP and mevalonate. These results clearly confirm the identity of the RNA binding protein as mevalonate kinase. Mevalonate kinase is a metabolic enzyme involved in cholesterol biosynthesis which catalyzes the conversion of mevalonate to phosphomevalonate, the precursor

of cholesterol and many natural products. In fact, we have shown that mevalonate kinase expression is regulated by LH in the corpus luteum both at the mRNA level as well as at the protein level (Wang and Menon 2005). During LH/hCG-induced downregulation, mevalonate kinase expression was induced in the corpus luteum. The induction of expression of other SRE containing genes such as HMG CoA reductase, farnesyl pyrophosphate synthase and LDL receptor were also seen during this phase (Wang and Menon 2005). These results suggest that mevalonate kinase expression plays a regulatory role in LH receptor mRNA expression in the corpus luteum.

Depletion of mevalonate kinase expression by 25 hydroxycholesterol resulted in the abrogation of LH receptor mRNA downregulation in cultured human granulosa cells (Menon et al. 2011; Wang and Menon 2005). The inhibitory effect of mevalonate kinase on LH receptor expression was documented by Ikeda et al. 2008 who showed that overexpression of mevalonate kinase can abrogate FSH-induced increase in LH receptor mRNA expression in rat ovarian follicles.

The notion that mevalonate kinase, a metabolic enzyme, acts as an mRNA binding protein is consistent with a similar role of several other metabolic enzymes that have been recently described (Menon et al. 2005; Hentze 1994). The iron response element binding protein, which plays a role in iron homeostasis in cells, is an enzyme in the citric acid cycle. Similarly, thymidylate synthase, dihydrofolate reductase, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase and lactate dehydrogenase all have been shown to serve as RNA binding proteins that regulate different aspects of RNA metabolism. In the case of mevalonate kinase, structural studies have shown that it belongs to a family of ATP binding proteins containing a conserved glycine motif. Members of this group include galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase. These proteins share a common fold, a left handed β-α-β loop known as the ribosomal protein S5 domain 2 like fold. This fold is similar to that found in proteins that interact with nucleic acids including elongation factor G and ribonuclease P (Zhou et al. 2000). Thus from a structural point, it is conceivable that mevalonate kinase can serve as an LH receptor mRNA binding protein to regulate cellular expression of LH receptor under select conditions.

7 Mechanism of LH Receptor mRNA Decay by LRBP

The endogenous association of LRBP with LH receptor mRNA was seen during downregulation supporting the view that LRBP is likely responsible for rapid degradation of LHR (Nair et al. 2002). The evidence comes from studies using ovarian extracts from the downregulated ovaries. The RNP complexes isolated from these ovaries were immunoprecipitated with anti LRBP antibodies and RNA extracted from the immunoprecipitates. RNAs were reverse transcribed and then subjected to PCR amplification using specific probes for LH receptor. Significant amplification of LHR was seen in the downregulated ovarian extracts suggesting that the association of LRBP with LHR mRNA occurs during downregulation (Nair et al. 2002).

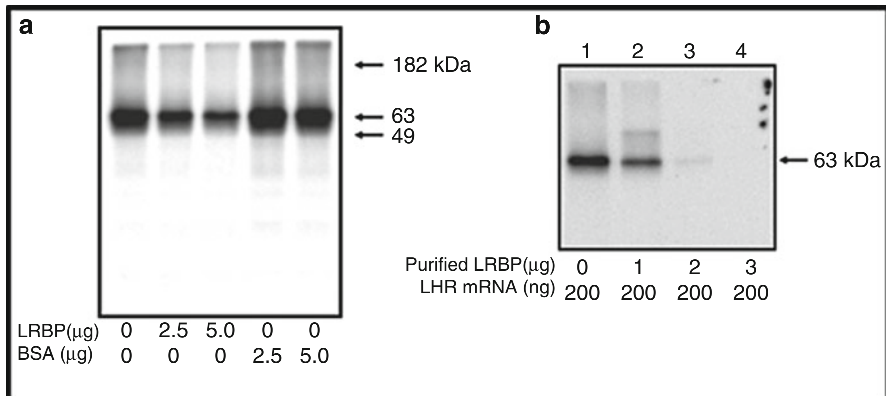

Fig. 4.4 Translational suppression of LHR mRNA by LRBP in vitro. (**a**) FLAG-tagged rat LHR mRNA (200 ng) was in vitro translated using a Flexi rabbit reticulocyte lysate system in the presence of [^{35}S] methionine and partially purified rat LRBP from the ovary or fatty acid-free bovine serum albumin (*BSA*) at different concentrations (2.5 and 5.0 µg). The translated LHR protein was immunoprecipitated with anti-FLAG antibody, separated on 10 % SDS-PAGE, and processed for autoradiography. (**b**) In vitro translation of FLAG-tagged rat LHR mRNA using [^{35}S]methionine in the presence of increasing concentrations (1, 2, and 3 µg) of purified rat LRBP, followed by immunoprecipitation, SDS-PAGE and autoradiogram. This research was originally published in J Biol Chem. (Nair AK et al. *J Biol Chem.* 2005, 280, 42809–42816)

Since the coding region of LHR mRNA is recognized by LRBP, it is quite likely that LRBP might act as a translational suppressor of LHR mRNA. This was indeed the case. We developed a cell free protein synthesizing system in which FLAG tagged LHR mRNAs were efficiently translated using an in vitro rabbit reticulocyte lysate system to yield 63 kDa protein species corresponding to non-glycosylated LHR. Addition of purified LRBP blocked the translation of LH receptor mRNA while addition of a region of corresponding to LRBP binding sequence (LBS) in which all the cytidine residues were mutated to uridine residues were not affected (Fig. 4.4). The wild type LBS effectively inhibited the translation of LH receptor mRNA. This clearly showed that LRBP acts as a translational suppressor of LH receptor mRNA and the loss of LH receptor mRNA during downregulation suggested that untranslatable LH receptor mRNA might be targeted for degradation. The translation of eukaryotic mRNAs is a complex process. In the cytoplasm, the fully processed mRNA can exist either in a polysome-associated, translationally active form or in an inactive messenger ribonucleoprotein (RNP) complex (Xu et al. 2000; Xu and Grabowski 1999; Mathews et al. 2000; Hershey 1991). The exchange between these two compartments modulates the fraction of mRNA available for translation (Kozak 1989). A clear link between translation and decay are intimately connected since the factors and processes required for mRNA translation and decay are closely connected (Ross 1995; Mathews et al. 2000; Jacobson and Peltz 1996) and translational arrest, in general, alters the degradation rate of most eukaryotic mRNAs (Ross 1995; Mathews et al. 2000).

Since LRBP is a cytosolic protein, we further examined its translocation from cytoplasm to ribosomes during downregulation to cause translational suppression. We were able to show that Western blot analysis of ribosome rich fractions from FPLC-assisted gel filtration of post-mitochondrial fractions showed the presence of LRBP in translating ribosomes from Downregulated ovaries , but not from the control ovaries. Increased association of LH receptor mRNA with LRBP was seen in the polysomes isolated from the downregulated ovaries when compared to the control group. The increased association of LH receptor mRNA with LRBP in polysomes from downregulated ovaries was associated with decreased translational effectiveness of these polysomes again showing the translational suppression by LRBP (Menon et al. 2009). Thus, during downregulation, an increase in the translocation of LRBP to ribosome and its increased association with LHR mRNA leads to its increased degradation.

Increased translocation of LRBP to polysome and increased association in translating ribosomes during downregulation suggested that these processes precede a decrease in LH receptor mRNA levels. The effect of these early events during downregulation showed that the association of LRBP with ribosomes and translational suppression led to its rapid degradation by facilitating decapping. Immunoprecipitation of the complex with 5′ cap structure antibody followed by real time PCR analysis using specific LH receptor mRNA probes revealed progressive loss of capped LH receptor mRNA during downregulation (Menon et al. 2013a). RNA immunoprecipitation confirmed the dissociation of eukaryotic initiation factor 4E from the cap structure which is required for decapping. Furthermore, RNA immunoprecipitation with antibody against the P body marker protein, DCP1A revealed that LH receptor mRNA was associated with the P bodies, the cytoplasmic foci that contain RNA degradative enzymes and decapping complex (Menon et al. 2013a). Interestingly, immunohistochemical studies using antibodies against LRBP and DCP1A followed by confocal analysis showed co-localization of LRBP with DCP1A during LHR downregulation. The association of LRBP and LH receptor mRNA in the P bodies during downregulation was also confirmed by examining the association of a second P body marker rck/p54. These results are supportive of the notion that during downregulation, LRBP forms an untranslatable ribonucleoprotein complex which is then translocated to P bodies leading to decapping and degradation by exonucleases (Fig. 4.5).

8 Interaction of LRBP with Other Trans Factors during Downregulation

Since LRBP is devoid of any nuclease activity capable of degrading LH receptor mRNA, the possibility of other proteins that selectively interact with LRBP and if such proteins exist, their functional roles in directing LH receptor mRNA to decay pathways was examined. We used a yeast two hybrid system to screen cDNA library which was constructed from LHR downregulated ovaries (Wang et al. 2010). Using this strategy we identified five potential candidate proteins that interacted with

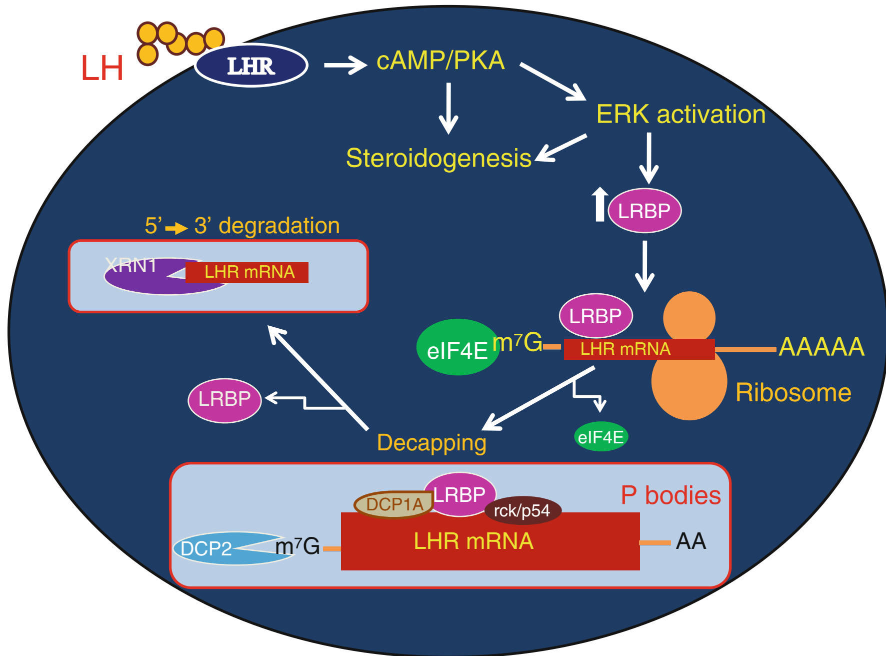

Fig. 4.5 Proposed mechanism of LRBP mediated degradation of LHR mRNA in the P bodies. LH signaling through its receptor activates cAMP/PKA/ERK pathways, leading to increased levels of LRBP. LRBP binds to the coding region of LHR mRNA in the ribosomes and an untranslatable ribonucleoprotein complex is formed. LRBP also associates with the P body components such as DCP1A and rck/p54 resulting in the P body localization of this mRNP complex. This is followed by the dissociation of eIF4E from the cap structure and the decapping of LHR mRNA in the P bodies, initiated by DCP2 enzyme. Final step is the 5′-3′ exonuclease (XRN1) mediated degradation of LHR mRNA

LRBP during downregulation. Among these, UBCE2i which is now recognized as the sumoylating enzyme has been implicated in nuclear-cytoplasmic transport in addition to other cellular functions. SUMOplotT revealed that LRBP has two potential sumoylation sites at K 345 and K 256. Overexpressed LRBP in 293 T cells was assayed for sumoylation (Wang et al. 2010) and revealed that both lysine 256 and 345 were sumoylated whereas mutation of the lysine residues to arginine blocked sumoylation. Although the actual functional significance of sumoylation of LRBP is not understood, it is likely that sumoylation might play a role in the translocation of LRBP-bound LH receptor mRNA to P bodies for degradation.

A second LRBP interacting protein identified was eukaryotic initiation factor 5A (eIF5A). Its functional role in RNA metabolism has been a topic of investigation in several laboratories since it has been shown that it participates in many aspects of RNA metabolism including RNA decay, transport and most recently, translation elongation (Rossi et al. 2014; Zanelli and Valentini 2007; Ruhl et al. 1993; Zuk and Jacobson 1998; Gutierrez et al. 2013; Dever and Green 2012; Saini et al. 2009; Schrader et al. 2006). EIF5A has been shown to undergo hypusination, a unique

post-translational modification which is essential for its function (Park et al. 1993, 2010) including RNA binding. Since eIF5A was found to interact with LRBP in the LHR mRNA-downregulated state, we examined whether eIF5A plays a role in LH receptor downregulation. First we showed that during downregulation, eIF5A undergoes hypusination by transferring 4-aminobutyl group from the polyamine, spermidine to the ∈ amino group of lysine by following the incorporation of [^{3}H] from [^{3}H] labeled spermidine into eIF5A. After electrophoretic separation by SDS-PAGE, [^{3}H] labeled eIF5A (hypusinated) was detected by fluorography. A time dependent increase in the incorporation of [^{3}H] into eIF5A was detected as early as 4 h after instituting ligand treatment to induce downregulation. The appearance of [^{3}H] labeled eIF5A was blocked by GC7, an inhibitor of hypusination. Pretreatment of rats with GC7 2 h prior to treatment with hCG, showed a marked decrease in the association of eIF5A with LRBP. Even more interesting was the finding that GC7 pretreatment reversed the ligand-induced downregulation of LH receptor mRNA as well as the appearance of functional receptors on the ovarian cell surface. Analysis of the equilibrium dissociation constant for the ligand-receptor interaction showed comparable Kd values for the control group and GC7 treated group, but the number of the binding sites in the GC7 treated group was significantly higher than the group without GC7 pretreatment (Menon et al. 2014a). Recent studies have shown that hypusination of eIF5A also participates in the formation of P bodies, the presumed site of mRNA decay (Gregio et al. 2009) and in the nucleocytoplasmic translocation of HIV-1Rev and human T cell leukemia virus type 1Rex RNA export factors (Elfgang et al. 1999). We have also shown that LH receptor mRNA-LRBP complex is shuttled from ribosomes to P bodies for degradation (Menon et al. 2009). However, since there was no discernible increase in the association of eIF5A with DCP1A, during downregulation the role of hypusinated eIF5A in shuttling the mRNP complex to the degradation machinery was ruled out. It is likely that eIF5A might act by augmenting translational suppression of LH receptor mRNA-LRBP complex which is then targeted for degradation, as summarized in Fig. 4.6.

9 Role of microRNA-122 (miR-122) in LH Receptor Expression

Since LRBP plays a crucial role in LH receptor expression during ligand-induced downregulation, the regulatory factors controlling LRBP expression would be expected to have a major influence on LH receptor mRNA expression. Mevalonate kinase (LRBP) is a member of the family of enzymes encoded by genes containing oxysterol responsive element. Oxysterols act by suppressing the proteolytic cleavage of the precursor of sterol response element binding protein (SREBP) to the active form. The cleavage product, the active form of SREBP binds to sterol response element (SRE) which causes transcriptional activation. Conversely, when cellular concentrations of oxysterols are low, the cleavage of the precursor of SREBP is enhanced to produce active form of SREBP which then accelerates transcription of genes containing SRE elements in the promoter region. Mevalonate

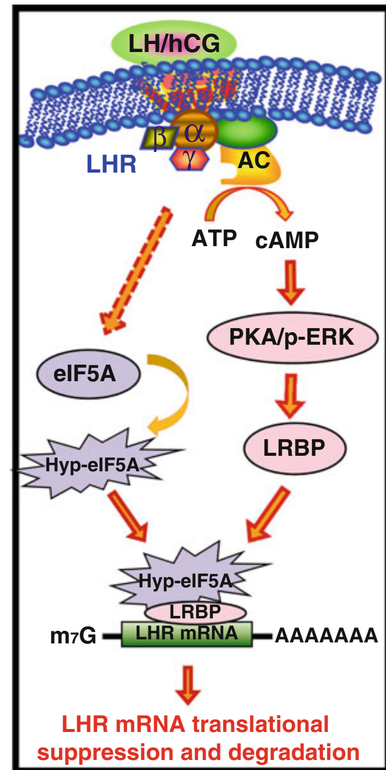

Fig. 4.6 Role of hypusinated eIF5A in LRBP-mediated LHR mRNA downregulation. Binding of LH/hCG to LH receptor stimulates the expression and hypusination of eIF5A, which then forms a complex with LRBP-LHR mRNA leading to the translational suppression and degradation of LHR mRNA

kinase, phosphomevalonate kinase, hydroxymethyl glutaryl CoA reductase are representative of enzymes encoded by SRE containing genes. Our studies have shown that addition of 25 hydroxycholesterol to cultured human granulosa cells suppressed LRBP expression as well as ligand induced downregulation of LH receptor mRNA expression. Krutzfeldt et al. (2005 and Schrader et al. (2006) have identified miR-122 as a positive regulator of MVK expression in the liver. It is therefore possible that miR-122 might regulate LHR mRNA expression during ligand-induced downregulation by regulating LRBP levels. We examined the expression of miR-122 in rat and human ovary by fluorescent in situ hybridization using 5′-fluorescein isothiocyanate labeled miR-122 locked nucleic acid probe, and by real time PCR (Menon et al. 2013b). Our results showed that miR-122 is expressed in the ovary and that the expression levels are increased by treatment with hCG. This suggested that miR-122 might play a regulatory role in ligand-induced downregulation of LH receptor mRNA expression by controlling the expression of LRBP since ovarian tissue endogenously expresses this micro RNA. The expression of miR-122 showed a time dependent increase at earlier time periods after inducing LHR mRNA downregulation and preceded the increases in LRBP mRNA and protein expression. These increases were followed by the downregulation of LHR mRNA expression. Inhibition of protein kinase A and ERK1/2 signaling pathways by H-89 and U0126, respectively blunted ligand-induced increases in miR-122 expression suggesting

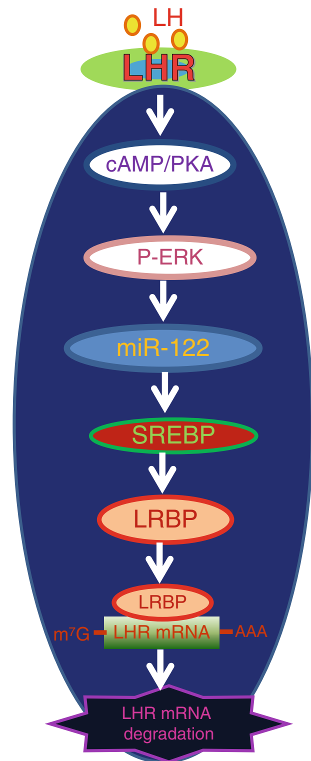

Fig. 4.7 Schematic model depicting the proposed signaling pathway in LH/hCG-induced miR-122-mediated LHR mRNA downregulation. Binding of ligand to LH receptor induces activation of cAMP/PKA signaling followed by ERK1/2. This leads to an increase in the expression of miR-122 which causes the activation of SREBPs. Activated SREBPs increase the expression of LRBP. LRBP binds to LHR mRNA, ultimately leading to its degradation

that ligand-mediated activation of miR-122 was mediated by cyclic/AMP/ERK1/2 signaling pathway. The involvement of miR-122 was further confirmed by inhibition of LRBP levels by treatment with locked nucleic acid-conjugated antagomir of miR-122 during ligand-induced downregulation. Furthermore, the levels of active forms of SREBP-1a and SREBP-2 were increased during downregulation. The role of miR-122 as an upstream activator of SREBP comes from studies where we showed that SREBP activation was blocked by injecting the miR-122 antagomir into the bursa of the ovary prior to treatment with hCG. Additional support for the role SREBP in LHR mRNA downregulation was obtained by blocking LHR downregulation by pre-treatment with fatostatin, an inhibitor of SREBP processing in the endoplasmic reticulum, prior to treatment with hCG (Menon et al. 2014b). Thus ligand-induced downregulation of LHR mRNA expression is mediated by an increase in miR-122 through cyclic AMP/PKA/ERK signaling pathway leading to increased levels of active forms of SREBPs which, in turn regulate, LRBP expression (Fig. 4.7).

10 Conclusion

The regulation of LHR mRNA expression at the post-transcriptional level in a constantly changing hormonal environment is advantageous to the cell. It provides a means to regulate LHR mRNA levels and consequently the receptor density on the

cell surface by a relatively simple mechanism by changing LHR mRNA stability without having to reprogram the more complex transcriptional machinery. By maintaining appropriate levels of the receptor to meet a number of specific physiological needs of the ovarian cell such as stimulation of the biosynthesis of different classes of steroids with unique functions at different phases of the ovarian cycle, ovulation, differentiation of granulosa cells to luteal cells, stimulation of VEGF production to support angiogenesis and regression of the corpus luteum to end of each cycle. These processes are essential for successful reproduction. The regulatory mechanisms to maintain the appropriate levels of LHR at different phases of the ovarian cycle appear to be programmed exquisitely to meet these challenges. Thus, the post-transcriptional mechanism that we have described here is an efficient means to fine tune the expression levels of LHR mRNA and its translation product, LHR in the ovary to transduce appropriate signals that are necessary to carry out its vital functions crucial for successful reproduction.

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Post-transcriptional Regulation of Steroid Hormone Receptors

5

Nancy H. Ing

1 Introduction

Genome studies of diverse species indicate that mammalian physiology is unique not because of greater numbers of genes, but rather because of greater complexity of gene regulation (Mattick 2001; Maniatis and Reed 2002). Post-transcriptional regulation of gene expression (including mRNA stability and translation) is one of the ways that organisms control and modify the flow of genetic information into the proteome (Keene and Tenenbaum 2002). Within the continuum of gene expression from transcription to protein degradation, regulated mRNA stability is increasingly being recognized as a major effector of gene regulation.

The first discoveries of steroid hormone effects on the stabilities of specific mRNAs were made in highly responsive animal tissues that were initiating large-scale production of new proteins. Early studies determined that estrogen regulated stabilities of mRNAs in the livers of egg-laying animals at the initiation of oogenesis (McKnight and Palmiter 1979; Dodson and Shapiro 2002). In one example, estrogen stabilized the vitellogenin mRNA in frog liver by 40-fold, increasing the half-life of the mRNA from 16 to 600 h. While estrogen stabilized mRNAs encoding egg proteins, it also destabilized mRNAs encoding serum proteins in the frog liver.

The rate of mRNA degradation has been established as equally important as the rate of synthesis in regulating the steady state concentration of the mRNA (Hargrove et al. 1991). The average mRNA half-life in mammalian cells is 24 h, with short-lived messages such as c-fos (*FOS*) mRNA having 20 min long half-lives and long-lived RNAs, such as *28S* and *18S* rRNAs, having 4 day long half-lives (Hargrove and Schmidt 1989). The half-lives of mRNAs are important in determining how long each mRNA is present to act as a template for translation of proteins.

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In addition, the half-life of the mRNA also controls how rapidly its steady state concentrations can be altered. Expression of genes with short half-lived mRNAs are rapidly regulated by changes in transcription and degradation rates, while long-lived mRNAs may take days to achieve a new steady state level in response to a cell signal such as a steroid hormone. When comparing transcriptional and post-transcriptional up-regulation of gene expression, mRNA stabilization may be advantageous to organisms because it lacks the lag phase and energetic costs that occur for responses that increase the rates of gene transcription.

Current reports elucidate post-transcriptional regulation that finely tunes the expression of steroid hormone receptor genes in animal tissues that are healthy or changing with the progression of a disease (Klinge 2012). This review focuses on post-transcriptional regulation of mRNAs encoding the nuclear steroid hormone receptors.

2 RNA Sequences and Binding Factors that Regulate mRNA Stability

Messenger RNAs are composed of three, functionally diverse regions: the 5′ untranslated region (UTR), the coding sequence (cds) and the 3′UTR (Fig. 5.1). The 5′UTR is usually short (100–300 bases long). The function of the 5′UTR is to be scanned by ribosomes, which identify an optimal Kozak sequence for the initiation of translation at the AUG start codon. The length of the cds relates to the length of the protein encoded by the mRNA. Ribosomes initiate translation at the AUG start codon and proceed to translate the cds of the mRNA until they reach a stop codon. The 3′UTRs are highly variable in length between different mRNAs. For example, the length of the 3′UTR of the *ESR2* "variant a" mRNA is 108 bases while the 3′UTR of the *PGR* mRNA is 9492 bases long (Fig. 5.1). The function of the 3′UTR is regulation of mRNA stability. The 3′UTRs of mRNAs are unique in that they maintain stable structures with RNA folding and binding proteins that are not disrupted by the movement of ribosomes.

The 3′UTR sequences of mRNAs are predicted to form stable structures with algorithms such as MFOLD (Zuker 2003). For example, the folded structure of the 4307 base long 3′UTR of *ESR1* mRNA (depicted in Fig. 5.2) has numerous single stranded regions that are susceptible to cleavage by common ribonucleases such as RNase A. Cleavage at any position in the 3′UTR separates the 5′UTR and cds from the polyA tail ("A_n" in Figs. 5.1 and 5.2) and results in rapid degradation of the mRNA (Bevilacqua et al. 2003). In the example of estrogen up-regulation of vitellogenin gene expression in frog liver, estrogen treatment lowers the rate of the cleavage of vitellogenin mRNA within its 3′UTR (Dodson and Shapiro 2002). The 3′UTR sequences of an mRNA are fairly well-conserved across related species, although insertions and deletions of sequence are well tolerated (Mitchell and Ing 2003; Manjithaya and Dighe 2004). The 3′UTR sequences, structures, and binding molecules (proteins and microRNAs) confer its function: regulating the lifespan of the mRNA.

As in the case of mRNA synthesis (transcription) in the nucleus, rates of mRNA degradation in the cytoplasm are regulated by finite sequence elements in the 3′UTR and the proteins that bind them (Guhaniyogi and Brewer 2001). The most well characterized mRNA elements are AU-Rich Elements (AREs). These were first identified as instability elements in the 3′UTRs of oncogene and cytokine mRNAs, e.g. those encoding c-fos (*FOS*) and tumor necrosis factor (*TNF*; Zhang et al. 2002; Bevilacqua et al. 2003). There are four distinct classes of AREs (Stellato 2004). The simplest is individual AUUUA elements scattered in 3′UTR sequences, as in follicle-stimulating hormone β-subunit (*FSHB*) and epidermal growth factor receptor (*EGFR*) mRNAs (Balmer et al. 2001; Shim and Karin 2002; Manjithaya and Dighe 2004). More complex AREs have arrays of several AUUUA elements in nearby regions of the 3′UTR, such as those in many oncogene mRNAs. Other sequence elements involved in stability have been identified, such as C-rich elements on the 3′UTR of the *LHCGR* mRNA encoding the luteinizing hormone receptor (Menon et al. 2004; Ing 2005).

There are multiple families of proteins that bind AREs, including AU-binding factors (e.g. *HNRPD* gene products, also known as AUF1), zinc-finger proteins (e.g. tristetraprolin, the *ZFP36* gene product) and relatives of the Drosophila RNA-binding ELAV protein (e.g. ELAV1 which is also known as HuR; Staton et al. 2000;

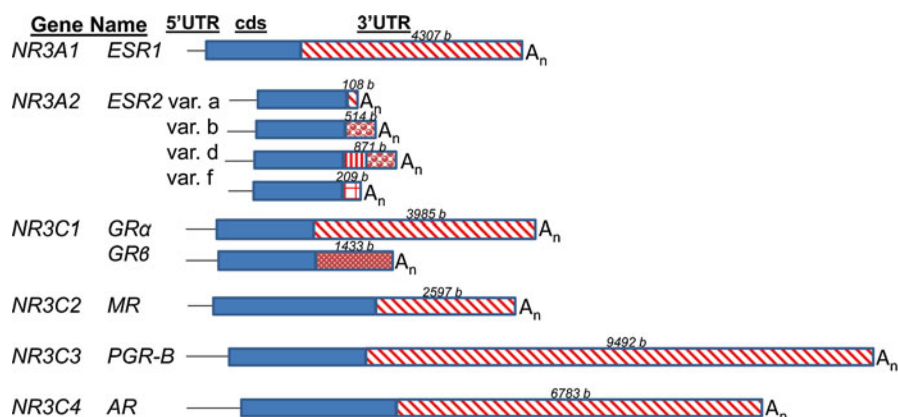

Fig. 5.1 A diagram of the human mRNAs encoding steroid hormone receptors. The mRNAs encoding estrogen receptor α (*NR3A1* or *ESR1*), estrogen receptor β (*NR3A2* or *ESR2*), glucocorticoid receptors α and β (*NR3C1* or *GR*), mineralocorticoid receptor (*NR3C2* or *MR*), progesterone receptor (*NR3C3* or *PGR*) and androgen receptor (*NR3C4* or *AR*) are diagrammed. The 5′ untranslated regions ("5′UTR") are shown as *narrow black lines*, the coding sequences ("cds") are *solid blue boxes*, the 3′untranslated regions ("3′UTR") are *red patterned boxes*, and the polyA tail is indicated by "A_n". For the different 3′UTRs in the variant ("var.") mRNAs of *ESR2* and *GR*, the different patterns in the 3′UTR boxes indicate unique sequences. The lengths of the 3′UTRs is indicated in bases ("b") above the 3′UTR box. The information is from the reference mRNA sequences from GenBank: NM_000125.3 (*ESR1*); NM_001437.2, NM_001040275.1, NM_001214902.1, NM_001271876.1 (*ESR2*); NM_000176.2, NM_001020825.1 (*GR*); NM_000901 (*MR*); NM_000926.4 (*PGR*); NM_000044.3 (*AR*)

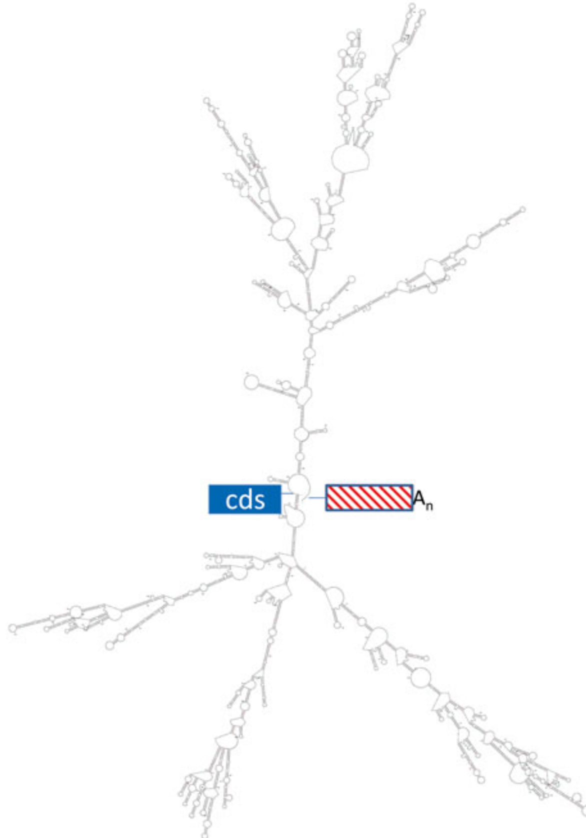

Fig. 5.2 Predicted structure in the 3′UTR of *ESR1* mRNA. The MFOLD algorithm predicted the folded structure shown for the 5′ end of the 3′UTR (1330 bases) of the human *ESR1* mRNA. The end at *left* joins the coding sequence ("cds") while the end at *right* joins the rest of the 3′UTR and the polyA tail ("An"). The free energy of the predicted structure is −474 kcal/mol

Shim and Karin 2002). While some ARE-binding proteins (such as tristetraprolin) destabilize the ARE-bearing mRNA, others (such as HuR) have stabilizing effects. The *HNRPD* gene produces four AUF1 proteins AUF1p37, AUF1p40, AUF1p42 and AUF1p45 by alternative splicing of the mRNA. All of the proteins bind to AREs. However, AUF1p37 and AUF1p42 destabilize mRNAs while AUF1p40 and AUF1p45 stabilize the mRNAs they bind (Wagner et al. 1998; Loflin et al. 1999; Tolnay et al. 1999; Chen et al. 2001; Mukherjee et al. 2002; Sarkar et al. 2003; Lal et al. 2006). Most RNA binding proteins are ubiquitously expressed although there is some degree of tissue-specificity in the distribution of some of them (Brewer 2001; Hollams et al. 2002; Zhang et al. 2002; Lu and Schneider 2004). Interestingly, the expression of some genes encoding ARE-binding proteins are regulated by steroid hormones via their receptor proteins (Cuadrado et al. 2003; Arao et al. 2002, 2004; Ing 2010).

While some post-transcriptional regulation of mRNA stability is the result of RNA binding proteins (either stabilizing or destabilizing), other post-transcriptional regulation is performed by non-coding regulatory RNAs such as microRNAs. MicroRNAs are short (about 22 bases) single-stranded, non-coding RNAs that regulate expression of protein encoding genes, usually by destabilizing the mRNA or repressing translation. The microRNAs that effect the changes in gene expression of the greatest magnitude usually do both (Baek et al. 2008). MicroRNAs exist in large complexes with proteins, called RNA-Induced Silencing Complexes or RISC. Within the RISC, microRNAs are very stable even in bodily fluids, such as serum, that contain high levels of RNAses. MicroRNAs act by binding their 5′ seed sequence to a complementary sequence within the 3′UTR of the mRNA, which directs the RISC complex. Subsequently, the mRNA is degraded in the exosome. While there are only approximately a thousand microRNA genes in the human genome, each individual microRNA can regulate the expression of hundreds of protein-encoding genes. It is estimated that microRNAs regulate more than a third of human genes (Ing et al. 2009). Therefore, it is not surprising to note that microRNAs regulate the expression of genes encoding the steroid hormone receptors. For discussion of microRNAs regulating steroid hormone action by their effects on nuclear receptor cofactors and kinase signaling cascades, as well as regulation of the expression of microRNA genes by the steroid hormones via the steroid hormone receptor proteins, see current reviews (Ing 2009; Pandey and Picard 2010; Klinge 2012; Manavathi et al. 2013).

3 Autoregulation of the Stabilities of Messenger RNAs Encoding Steroid Hormone Receptors

Estrogen is a steroid hormone that autoregulates the stability of *ESR1* mRNA, which encodes its dominant, active receptor, estrogen receptor α (ERα), in most tissues of the body. Estrogens stabilize *ESR1* mRNA in fish liver during the initiation of oogenesis and in mammalian endometrium during the preovulatory surge of estrogen (Flouriot et al. 1996; Mitchell and Ing 2003; Boyce-Derricott et al. 2010). In both cases, the stabilization and resultant up-regulation of *ESR1* mRNA are dependent upon ERα protein, because ERα antagonists block the effect. The conservation of this estrogen action across these diverse species implies that it is an important mechanism for augmenting further estrogen responses. In sheep endometrium, a single physiological dose of estradiol up-regulates *ESR1* mRNA concentrations fivefold in 24 h, during which time there is no increase in the transcription rate of the *ESR1* gene (Ing et al. 1996). The primary mechanism of *ESR1* mRNA up-regulation, by increasing *ESR1* mRNA stability, was directly demonstrated in vivo with pulse-chase labeling and ex vivo using explants cultured with a transcription inhibitor (Ing and Ott 1999).

The molecular mechanism by which estrogen stabilizes *ESR1* mRNA was discovered using the sheep uterus as a model system. Human and sheep *ESR1* mRNAs carry 14 and 10 ARE elements in their respective 3′UTRs. (There are no ARE elements in the 5′UTRs or cds regions of human or sheep *ESR1* mRNAs.) The AREs are probably responsible for the inherent instability of the *ESR1* mRNA

and of the heterologous mRNAs that have the *ESR1* 3′UTR sequence transferred to them (Mitchell and Ing 2003; Kenealy et al. 2000). Using an in vitro stability assay developed with cytoplasmic extracts from endometrial samples from control (ovariectomized) and ovariectomized, estradiol-treated ewes, two discrete (82 bases long) Minimal Estradiol-Modulated Stability Sequences (MEMSSs) were identified in the vast (4366 bases long) 3′UTR of the sheep *ESR1* mRNA (Mitchell and Ing 2003). These MEMSSs conferred estradiol-enhanced stability to heterologous mRNAs when they were transferred to them. UV-crosslinking identified several endometrial proteins that were induced to bind the MEMSS by estradiol treatment of ewes. One predominant MEMMS binding protein is AUF1p45, one of four protein products of the *HRNPD* gene (Ing et al. 2008). The three other estradiol-induced MEMSS binding proteins of 27, 34 and 70 kDa sizes remain to be identified. When recombinant AUF1p45 fusion protein was produced and purified from bacteria, it stabilized *ESR1* mRNA in vitro in the presence of endometrial extracts from ovariectomized ewes. Similar analyses with recombinant HuR, a 34 kDa RNA stabilizing protein that binds AREs, did not stabilize *ESR1* mRNA in vitro (Ing et al. 2008). Intriguingly, estradiol treatment of ewes increased concentrations of *AUF1p45* mRNA and protein apparently by stabilizing the *AUF1p45* mRNA (Ing 2010). These data lead to the model of the molecular mechanism of estrogen up-regulation of *ESR1* gene expression in Fig. 5.3.

In contrast, estradiol treatment has also been reported to destabilize *ESR1* mRNA in the MCF7 breast cancer cell line (Saceda et al. 1998). The explanation of these contrasting effects of estrogens may become clear when the molecular mechanism(s) that regulate *ESR1* mRNA stability are further investigated.

Estrogens also regulate gene expression by binding estrogen receptor β, which is encoded by the distinct *ESR2* gene. There are numerous variant *ESR2* mRNAs. These

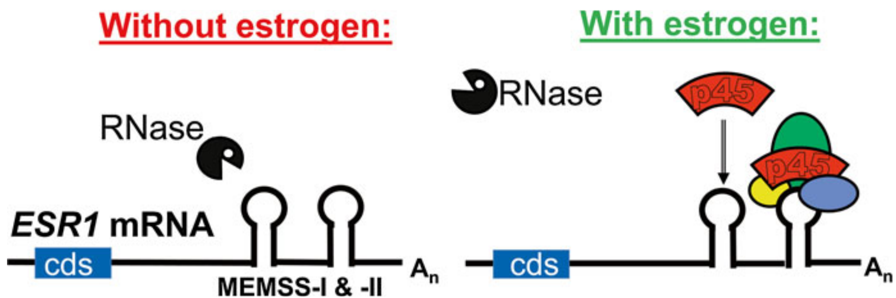

Fig. 5.3 A molecular model for estrogen induction of a stabilizing ribonucleoprotein complex on the 3′UTR of *ESR1* mRNA in the sheep uterus. The cartoon of sheep *ESR1* mRNA shows the cds as a *blue box* that is 5′ to the extensive 3′UTR (4366 bases long). Within the 3′UTR are two 82 base long Minimal Estrogen Modulated Stability Sequences ("MEMSSs"), shown as the hairpin structures predicted by MFOLD. Each carries a common 10 base long U-Rich Element ("URE") on the loop of the hairpin. At *left*, in the absence of estrogen, the long 3′UTR is susceptible to cleavage by ribonuclease ("RNase"). However, estrogen treatment induces the formation of a stabilizing protein complex that includes AUF1p45 ("p45") on the MEMSS, which protects the 3′UTR of *ESR1* mRNA from cleavage by RNase (*right panel*)

result from usage of three promoters on the *ESR2* gene, alternative splicing in 5′ and 3′ UTRs and alternative polyA signal usage. Three protein isoforms (estrogen receptor β-1, -2, and -5) are produced. They vary mainly at a small region of the C-terminus. Representative *ESR2* mRNAs with 3′ UTR differences are shown in Fig. 5.1 (from human reference sequences in GenBank). In sharp contrast to the *ESR1* mRNA, the 3′ UTRs of *ESR2* mRNAs are very short. It is interesting to note that splicing within the 3′UTR near the stop codon can trigger nonsense mediated mRNA decay (Ni et al. 2007; McGlincy and Smith 2008; Saltzman et al. 2008). Studies of *ESR2* gene expression in normal breast and prostate and cancers derived from those tissues have focused on different 5′ UTR sequences and their effects on translational efficiencies (Smith et al. 2010; Lee et al. 2013). However, the dissimilar 3′UTRs interact with 5′UTR sequences to alter translational efficiencies. Estradiol treatment appears to inhibit translational efficiencies of two of 15 human *ESR2* mRNA variants (Smith et al. 2010). However, estradiol treatment of rainbow trout does not alter the stabilities of *ESR2* mRNAs in liver (Boyce-Derricott et al. 2010).

The glucocorticoid receptor proteins GRα and GRβ are encoded by the same *NR3C1* gene. The mRNAs that encode GRα and GRβ differ by alternative splicing of the last exon, exon 9 (Fig. 5.1). The GRα protein has 50 amino acids encoded by exon 9a prior to the stop codon, while GRβ has only 15 amino acids encoded in exon 9b (Schaaf and Cidlowski 2002). The shorter C-terminus of GRβ does not allow glucocorticoid binding, but GRβ regulates transcription of distinct genes from GRα. GRβ also dimerizes with GRα to act as a dominant negative factor (Vandevyver et al. 2014). The transcripts that encode GRα and GRβ have unique 3′UTRs that carry 10 and 4 type I AREs (AUUUA sequence elements), respectively (Schaaf and Cidlowski 2002; Stellato 2004). Mutation of all ten AUUUA elements to GUUUA in the mRNA encoding GRα stabilizes the mRNA and increases GRα protein levels in transfected COS-1 cells (Schaaf and Cidlowski 2002). Similar effects occur with mutation of just one AUUUA of the four in the 3′UTR of the mRNA encoding GRβ. It is fascinating to note that the mutation occurs naturally in humans, is associated with rheumatoid arthritis, and likely contributes to glucocorticoid resistance (Derijk et al. 2001). While glucocorticoids transcriptionally repress the *NR3C1* gene, they also rapidly destabilize *NR3C1* mRNA in many cell types (Schaaf and Cidlowski 2003; Ramamoorthy and Cidlowski 2013). Ligand activated GRs are known to act via AREs and kinase pathways to down-regulate a number of genes involved in inflammation (Stellato 2004). There is at least one report that implies that dexamethasone treatment of rats decreases *GR* mRNA in muscle concurrent with increasing *AUF1* mRNA (Sato et al. 2011). However, in that study, *GRα* and *GRβ* and the four *AUF1* transcripts (two encode proteins that destabilize mRNAs while the other two encode mRNA stabilizing proteins) were not distinguished from each other.

The *NR3C2* gene encodes the mineralocorticoid receptor (MR). Although the *MR* mRNA has a long 3′UTR (2597 bases long, Fig. 5.1) that carries eight AREs (AUUUA elements determined using the AREsite 1.0 algorithm; Gruber et al. 2011), very little has been published about its regulated stability. There is an indication that restraint stress of rats down-regulates *MR* heteronuclear RNA in nuclei of the hippocampus within 2 h (Herman and Watson 1995). In that study, decreased levels of

MR mRNA in the cytoplasm were not detected. However, the technique used was in situ hybridization, which is considered to be semiquantitative. In addition, the in situ hybridization probes could bind partially degraded *MR* mRNA as well as the full length mRNA, so perhaps degradation was not complete within the 2 h time frame.

The *NR3C3* gene encodes progesterone receptors A and B (PGR-A and PGR-B). Their mRNAs are similar to *ESR1* mRNA in that they carry a very long 3′UTR (9492 bases, Fig. 5.1). The 3′UTR of *PGR* mRNA bears numerous AREs: 28 AUUUA elements, with all but three conserved across mammalian species (AREsite 1.0 algorithm). The mRNAs that encode PGR-A and PGR-B differ in 5′ sequence because the transcription of *PGR-B* mRNA initiates at a site upstream of the *PGR-A* mRNA transcription initiation site. The PGR-B protein is longer and has a more efficient transcriptional activation domain than PGR-A. In contrast to the bidirectional autoregulation of *ESR1* mRNA by estrogen, progestins have only been reported to stabilize *PGR* mRNA (Tseng and Zhu 1997). The progestin medroxyprogesterone acetate increased the *PGR* mRNA half-life from 6 to 12 h in primary cultures of stromal cells from human endometrium (Saceda et al. 1998).

The *NR3C4* gene has a single transcript that encodes androgen receptor (AR). Androgens autoregulate the stability of *AR* mRNA in both positive and negative directions (Yeap et al. 1999, 2004). The direction (up or down) of regulation depends upon dose of androgen and the tissue or cell line examined. For instance, androgens stabilize *AR* mRNA in a prostate cancer cell line but destabilize it in the MDA-453 breast cancer cell line. The extensive 3′UTR of *AR* mRNA (6783 bases long, Fig. 5.1) does not carry many AREs (only five AUUUAs, AREsite1.0 algorithm). However, the 3′UTR of *AR* mRNA bears a UC-rich element that is conserved across species. This *AR* mRNA element binds a poly(C)-binding protein as well as HuR (Yeap et al. 1999, 2004). These two proteins cooperate in a cell-type-specific manner to determine whether androgen stimulation stabilizes or destabilizes the *AR* mRNA (Yeap et al. 2004). In prostate cancer cell lines, the ErbB3 binding protein 1 (EBP1) destabilized *AR* mRNA by binding the same UC-rich element in the 3′UTR (Zhou et al. 2010).

4 microRNA Regulation of Messenger RNAs Encoding Steroid Hormone Receptors

The best studied gene encoding a steroid hormone receptor that is regulated by microRNAs is the *ESR1* gene (Ing 2009; Pandey and Picard 2010). One reason for this is that ERα is critical to *PGR* gene expression: so microRNAs that down-regulate the expression of the *ESR1* gene will also down-regulate the *PGR* gene. Other reasons are that *ESR1* gene expression is critical to many aspects of mammalian health and may be dysregulated in disease (Klinge 2012). And finally, the 3′UTR of *ESR1* mRNA is long and is replete with 65 putative binding sites for microRNAs predicted by algorithms such as TargetScan (Pandey and Picard 2010). There are 13 different microRNA binding sites on the 3′UTR of *ESR1* mRNA that have been validated in various model systems (Table 5.1; Pandey and Picard 2010; Manavathi et al. 2013; Klinge 2012; and references therein).

Table 5.1 Validated miRNA target sites on 3′UTRs of mRNAs encoding human steroid hormone receptors

Gene name	3′UTR	miR sites	References
NR3A1/ESR1	4307 b	Let-7	Manavathi et al. (2013)
		miR-137	Zhao et al. (2008)
		miR-145	Zhao et al. (2008)
		miR-17-5p	Zhao et al. (2008)
		miR-18a/19b/20b	Manavathi et al. (2013)
		miR-181	Pandey and Picard (2010)
		miR-19	Pandey and Picard (2010)
		miR-193b	Klinge (2012)
		miR-206	Klinge (2012); Manavathi et al. (2013)
		miR-221/222	Pandey and Picard (2010); Klinge (2012); Manavathi et al. (2013)
		miR-22	Pandey and Picard (2010); Klinge (2012); Manavathi et al. (2013)
		miR 26ab/1297	Pandey and Picard (2010)
		miR-302	Klinge (2012)
NR3A2/ESR2	871 b	miR-92	Al-Nakhle et al. (2010)
NR3C1/GRα	3985 b	miR-101	Riester et al. (2012)
		miR-130b	Tessel et al. (2011)
		miR-124/506	Vreugdenhil et al. (2009); Ledderose et al. (2012)
		miR-142-3p	Riester et al. (2012); Lv et al. (2012)
		miR-18a	Uchida et al. (2008); Vreugdenhil et al. (2009)
		miR-433	Riester et al. (2012)
		miR-96	Riester et al. (2012)
NR3C2/MR	2597 b	miR-124/506	Sõber et al. (2010)
		miR-135a	Sõber et al. (2010)
NR3C3/PGR	9492 b	miR-126-3p	Cui et al. (2011)
		miR-181	Maillot et al. (2009)
		miR-200a	Haraguchi et al. (2014)
		miR-26ab/197	Maillot et al. (2009)
NR3C4/AR	6783 b	miR-135b	Östling et al. (2011)
		miR-185	Östling et al. (2011)
		miR-297	Östling et al. (2011)
		miR-299-3p	Östling et al. (2011)
		miR-34 ac	Östling et al. (2011)
		miR-371	Östling et al. (2011)
		miR-421	Östling et al. (2011)
		miR-449ab	Östling et al. (2011)

Length of the 3′UTR in bases (“b”) is indicated for the mRNAs of the named genes. All sites are conserved across species except none of the 3′UTRs of the four variant mRNAs encoding ESR2 have any conserved sites (Pandey and Picard 2010; TargetScanHuman v6.2)

Several studies demonstrated that microRNAs regulate expression of the *ESR1* gene in breast cancer. One study began with interest in microRNAs that had increased concentrations in ERα-negative breast tumors compared to ERα-positive breast tumors (Iorio et al. 2005). Among those microRNAs, the investigators focused on the few microRNAs that also had predicted binding sites within the 3′UTR of *ESR1* mRNA (Adams et al. 2007). This lead to the discovery that experimentally decreasing the concentrations of miR-206 increased *ESR1* gene expression in MCF7 cells. The mechanism by which miR-206 decreased *ESR1* gene expression was by destabilization of *ESR1* mRNA. Further studies demonstrated that estradiol treatment of breast cancer cells decreased concentrations of miR-206, leading to increased *ESR1* mRNA stability and ERα protein concentrations. This describes a second mechanism by which estrogens post-transcriptionally up-regulate *ESR1* gene expression, in addition to that described in the sheep uterus (Fig. 5.3). Another group searching for microRNAs that regulate *ESR1* gene expression identified 12 microRNAs that were more highly expressed in ERα-negative breast cancer cell lines compared to ERα-positive lines (Zhao et al. 2008). These investigators predicted that miR-221/222 would target the 3′UTR of *ESR1* mRNA. By altering levels of the microRNA in the MCF7 and T47D breast cancer cell lines, they demonstrated that miR-221/222 decreased ERα protein concentrations but not *ESR1* mRNA concentrations, indicating that this microRNA inhibits translation of *ESR1* mRNA. In breast cancer patients who have received chemotherapy, high levels of miR-221 in plasma is predictive of chemoresistance (Zhao et al. 2008). Some have suggested that down-regulation of miR-221 with antagomiR oligonucleotide drugs may be a useful approach to treating breast cancer (Piva et al. 2013).

Studies of a human pancreatic cancer cell line lead to the discovery that treatment with curcumin (a naturally occurring flavonoid that inhibits cancer growth) increased miR-22 and decreased miR-199a-3p concentrations (Sun et al. 2008). Experimental up-regulation of miR-22 suppressed ERα protein concentrations along with those of Sp1, which is a transcription factor that cooperates with ERα to activate transcription on many gene promoters that lack conventional estrogen-responsive elements. Thus, miR-22 appears to inhibit ERα and ERα/Sp1 responsive gene promoters by reducing the concentrations of those transcription factors. These examples show that several different microRNAs regulate the expression of the *ESR1* gene in a variety of tumor cells.

As explained in the preceding section, the *ESR2* gene is distinct from that of *ESR1* and its variant mRNAs have short 3′UTR sequences. Therefore, it is not surprising that only one predicted microRNA site, miR-92, is reported in the literature for "*ESR2* variant a" mRNA (Table 5.1; Al-Nakhle et al. 2010). *ESR2* gene expression is down-regulated in many breast cancer tumors while miR-92 concentrations increased. In the MCF7 breast cancer cell line, anti-miR-92 down-regulated miR-92 while *ESR2* gene expression was upregulated (Al-Nakhle et al. 2010). Similar results occurred when the 3′UTR of *ESR2* mRNA was cloned 3′ to a reporter gene.

Within the 3′UTR of the mRNA encoding GRα (3985 bases long; Fig. 5.1), TargetscanHuman v6.2 identified two miR-124/506 binding sites that were conserved across species. Several studies have implicated these and other microRNAs

in down-regulating *GRα* gene expression (Table 5.1). In the adrenal gland, adrenocorticotrophic hormone increased concentrations of miR-101a, miR-142-3p, miR-433, and miR-96 while *GRα* gene expression decreased (Riester et al. 2012). Similarly in T-cells from acute lymphoblastic leukemia patients, antagomirs to miR-142-3p up-regulated *GRα* gene expression (Lv et al. 2012). In T cells from patients with sepsis, glucocorticoid resistance was associated with decreased *GRα* gene expression and increased levels of miR-124 (Ledderose et al. 2012). In cell lines derived from multiple myelomas, miR-130b down-regulated *GRα* gene expression (Tessel et al. 2011). In rat brain, increased levels of miR-124/506 and miR-18a were associated with down-regulation of *GRα* gene expression (Uchida et al. 2008; Vreugdenhil et al. 2009). Although TargetScanHuman v6.2 identified two sites each for miR-181a and miR-4262 within the distinct 3′UTR of the mRNA encoding GRβ (1433 bases long; Fig. 5.1), to my knowledge no sites have been validated.

The *MR* mRNA has a 2597 base long 3′UTR that bears several predicted microRNA binding sites that are conserved across vertebrate species (Söber et al. 2010). In HeLa cells transfected with a luciferase reporter carrying the *MR* mRNA 3′UTR, transfection with miR-124 and miR-135a expression plasmids down-regulated luciferase activity (Table 5.1). Intriguingly, the miR-124 site is homologous to that in the 3′UTR of the related *GRα* mRNA. The repression of luciferase activity by miR-124 and miR-135a appeared to be due to a repression of translation, as the luciferase mRNA level was not lowered similarly. However, transfection with the miR-135a expression vector had a slight trend toward down-regulating the concentrations of endogenous *MR* mRNA.

The 3′UTR of the mRNA encoding PGR-B is the longest of any of the mRNAs encoding steroid hormone receptors (9492 bases; Fig. 5.1). However, there are just four reports of regulation by microRNAs (Cochrane et al. 2012). TargetScanHuman v6.2 predicted only six conserved microRNA binding sites within the 3′UTR of *PGR* mRNA. Two of those, miR-181a and miR-26a, are down-regulated by estradiol treatment in breast cancer cell lines with simultaneous stimulation of proliferation (Table 5.1). When either miR-181a or miR-26a was transfected into MCF7 breast cancer cells, *PGR* gene expression was down-regulated at the levels of mRNA and protein concentrations with a concurrent decrease in proliferation (Maillot et al. 2009). In mice, it was noted that when proliferation of cervical epithelium was low, Pgr protein levels were low and miR-200a levels were high (Haraguchi et al. 2014). The 3′UTR of mouse *Pgr* mRNA is much shorter (3473 bases) than the human's. In the same report, the authors cloned the 3′UTR of the mRNA encoding human *PGR* (with and without the miR-200a binding site mutated) 3′ to a luciferase reporter gene. When transfected into COS cells, derived from monkey kidney cells, miR-200a down-regulated reporter gene activity (Haraguchi et al. 2014). In epithelial cells from mouse mammary gland, miR-126-3p binds the 3′UTR of mouse *Pgr* mRNA and down-regulates both *Pgr* gene expression and cell proliferation (Cui et al. 2011). These studies pave the way for future research relating microRNAs to *PGR* gene expression.

Post-transcriptional regulation of the expression of the *AR* gene was investigated because of its role in prostate cancer (Zhu et al. 2013). The mRNA encoding AR

carries an extensive (6783 bases long) 3′UTR (Table 5.1). Screening of 1179 microRNAs identified 13 microRNAs that down-regulated AR protein levels in a prostate cancer cell line (Östling et al. 2011). All of them bound the 3′UTR of *AR* mRNA. Intriguingly, transfection of expression vectors for each of these 13 microRNAs also down-regulated the androgen-dependent proliferation of the prostate cancer cell lines.

5 Conclusions and Future Directions

The post-transcriptional regulation of mRNAs that encode steroid hormone receptors is a molecular mechanism with extensive effects on the expression levels of many genes in diverse tissues. The bidirectional control of hormone receptor mRNA stabilities allows positive or negative gene regulation that finely tunes hormone responsiveness. Interactions between the RNA sequence elements and the proteins and microRNAs that bind them are an intriguing area of research (Jing et al. 2005). Mechanistic information about how steroid hormones regulate mRNA stability may provide new targets for therapeutic control of expression of specific genes. Some investigators are already attempting to interrupt crucial mRNA-protein and mRNA-microRNA interactions for therapeutic interventions (Coulis et al. 2000; DeJong et al. 2002). Alternatively, expression or function of the trans-acting factor, such as a steroid hormone-induced RNA-binding protein, could be individually selected for inhibition or augmentation. To target the proteins that regulate mRNA stability by binding directly to cis-elements on the mRNA, oligonucleotide mimics of the cis-element (also called “RNA decoys”) could be used to bind and sequester those RNA-binding proteins and interrupt the proteins’ effect on mRNA stability and translation (Makeyev et al. 2002). For example, if uteri were treated with the *ESR1* mRNA regions responsible for its stabilization (such as MEMSS), the AUF1p45 and other binding proteins could be sequestered and unable to stabilize *ESR1* mRNA. This would block the up-regulation of the expression of the ER gene, as well as the genes it subsequently up-regulates. This approach is unique because it could spare the beneficial influences of basal levels of estrogens on bone, brain and circulatory system health.

Elucidation of the molecular mechanisms by which microRNAs regulate the stability and translation of target mRNAs opens new therapeutic avenues for altering both normal and abnormal physiology of animal tissues. Recent advances in oligonucleotide therapeutics make it feasible to either up- or down-regulate key microRNAs in cell culture and, subsequently, in vivo (Esau and Monia 2007; Ford and Cheng 2008; Zhao et al. 2008). For example, Krutzfeldt et al. (2005) used antisense microRNAs “antagomirs” successfully in mice to down-regulate an individual microRNA. A particular microRNA might be targeted by an antisense RNA to reverse the actions of the microRNA on expression of critical genes. Increased knowledge about microRNA actions may lead to new diagnostics and therapies that utilize microRNAs to fight diseases such as cancer. In another example, plasma levels of miR-221 increase with breast cancer (Piva et al. 2013). An antagonist to

miR-221 lowers miR-221 and proliferation of MCF7 breast cancer cells in routine culture conditions as well as in tumors created in nude mice. Other anti-miR reagents discussed above may themselves be drugs for treating hormone-dependent cancers. Alternatively, therapies might increase concentration of microRNAs that act as tumor suppressors, such as miR-17-5p in breast cancer (Hossain et al. 2006).

As our knowledge of the post-transcriptional regulation of mRNAs encoding steroid hormone receptors grows, so will our ability to alter tissue physiology in order to enhance human and animal health.

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6 MicroRNA Regulation of Endocrine Functions in the Ovary

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1 Introduction

The ovaries have two main roles in maintaining normal reproductive functions: an *exocrine* role of producing the oocyte, and an *endocrine* role of producing sex hormones. The endocrine properties of the ovaries appear in a cyclical manner starting at puberty and continue throughout a female's reproductive lifespan, with each cycle resulting in the maturation of an ovarian follicle or atresia of non-dominant follicles. For successful reproduction to occur, the ovary must complete follicular development, ovulation, formation of a corpus luteum, and luteolysis. Successful networking and cooperation between the somatic cells (granulosa, theca, luteal, endothelial, immune, and epithelial cells) and the germ line (oocyte) is necessary to establish this pattern of regular ovulatory cycles. The gene regulatory networks are temporally and spatially regulated through intricate transcriptional and post-transcriptional regulatory mechanisms. Transcriptional regulation has been well studied and reviewed in detail (Lavoie and King 2009; Richards 1994; Espey and Richards 2002), but much less is known about post-transcriptional regulation in reproductive organs.

Post-transcriptional regulation can involve a multitude of events downstream of transcription, including mRNA splicing, RNA editing, RNA transportation, RNA storage, all the way through translation. This form of regulation is important because it allows for fine-tuning of gene expression, in a tissue specific manner independent of transcription (Carletti and Christenson 2009). These post-transcriptional regulatory mechanisms, however are difficult to systematically study, since they are often gene specific and the cell physiologic context can have dramatic influences on the regulatory mechanisms.

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Thus, much of the evidence for post-transcriptional regulation in ovarian endocrine function has arisen on a gene-by-gene basis typically when differences in mRNA and protein expression are observed to be out of synch with each other. An exception to this limitation is in the study of microRNA (miRNA, miR) mediated post-transcriptional regulation. This is largely due to the use of targeted gene deletion of several key enzymatic steps in the miRNA biosynthetic pathway. MicroRNA signaling is crucial for control of ovarian function by regulating aspects of folliculogenesis, oocyte maturation, ovulation and luteal function. In this review we will focus on miRNA's role in regulation of ovarian endocrine function. Important aspects of miRNA regulation not directly related to ovarian endocrine function have been previously reviewed (Christenson 2010; Imbar and Eisenberg 2014; Donadeu et al. 2012; Hossain et al. 2012; Nothnick 2012; McGinnis et al. 2015), so they will not be repeated in-depth in the present review.

2 MicroRNA Biogenesis and Mechanisms of Action

MicroRNA are a class of small, non-coding RNA that are comprised of 21–24 single-stranded nucleotides. These regulatory RNA have key roles in a variety of cellular processes by negatively regulating target messenger RNA (mRNA) through sequence-specific binding that results in inhibition of translation and decreased mRNA stability (Bartel 2009). A few studies have indicated the ability for positive regulation by miRNA, however, this method of miRNA action has been observed only experimentally and more studies are needed to verify this mechanism of action (Place et al. 2008; Orom et al. 2008). MicroRNA are predominately transcribed by RNA polymerase II into primary microRNA (pri-miR) and get processed into shorter (70–100 base pair) hairpin structures called precursor microRNA (pre-miR) by the microprocessor complex, comprised of Drosha and its binding partner, DiGeorge Syndrome Critical Region Gene 8 (DGCR8; Fig. 6.1). In turn, the pre-miR short hairpin structures associate with the nuclear export receptor, exportin-5 (XPO5), to get translocated into the cytoplasm (Bohnsack et al. 2004). Exportin-5 cooperates with the small GTPase Ran, to mediate the directional transport to the cytoplasm. Following export to the cytoplasm, GTP is hydrolyzed and the pre-miRNA is released into the cytoplasm where it is further processed by the RNAse III enzyme, Dicer, to create a short double stranded miRNA duplex. One of the strands will associate with the RNA induced silencing complex (RISC) and is then referred to as the guide strand or mature miRNA. The opposite strand referred to as the passenger strand then undergoes degradation.

In addition to this canonical pathway of miRNA biogenesis, several non-canonical pathways have been identified. A subset of miRNA called "mirtrons", do not depend on Drosha for their initial processing (Fig. 6.1). These miRNAs are processed from introns of transcripts after splicing (Ruby et al. 2007). The lariat-debranching enzyme involved in splicing resolves the RNA branch site and results in a pre-miRNA hairpin that gets exported to the cytoplasm like other pre-miRNA to be further processed by Dicer (Berezikov et al. 2007). Yet another alternative miRNA biogenesis mechanism can be seen in miR-451, which is processed in a Dicer independent fashion. In the case of miR-451, pre-miR-451 gets loaded into argonaute-2 (Ago2)

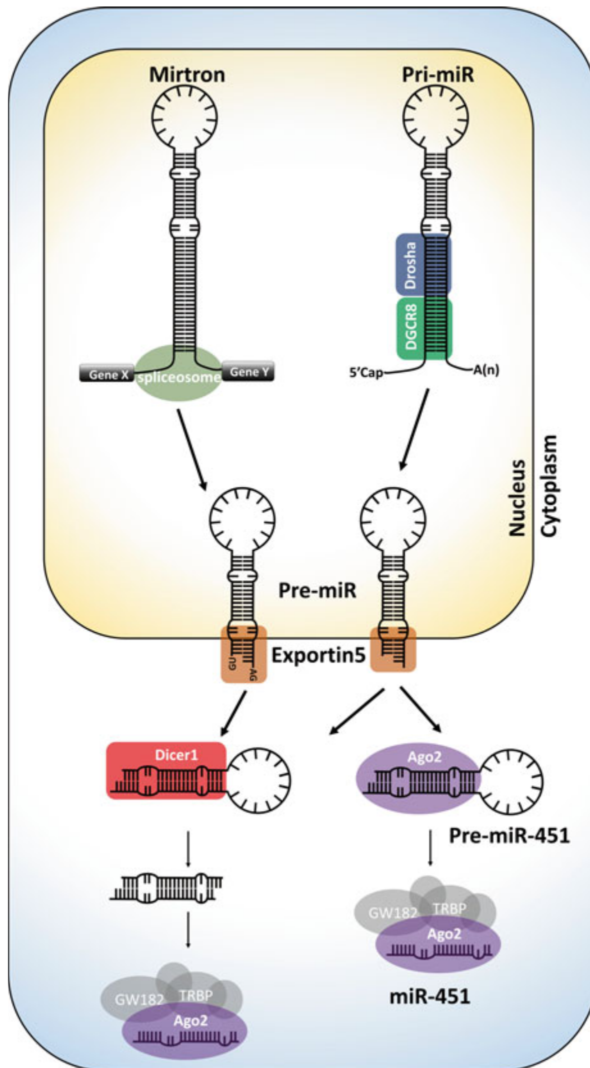

Fig. 6.1 Canonical and alternative miRNA biogenesis pathways. Most miRNA are transcribed by RNA Polymerase II to form long primary transcripts (pri-miRNA) which form complex structures that are then recognized and processed into precursor miRNA (pre-miRNA) by the microprocessor complex that includes the DGCR8 and RNAse III enzyme Drosha. These pre-miRNA hairpins are transported into the cytoplasm by exportin-5 and a Ran GTPase. In the cytoplasm, pre-miRNA are further processed into ~22 base pair duplexes by another RNAse III enzyme, Dicer, in association with GW182 and TAR RNA binding protein (TRBP). The mature miRNA then gets incorporated into the RISC complex, where it can then bind to the 3′-untranslated region (UTR) of target mRNA to block translation or degrade the transcript, or both. Also illustrated is the biogenesis of miRNA through the non-canonical pathways, including an intronic splicing mechanism. In these cases, the pre-miRNA is generated directly bypassing the need for Drosha and DGCR8, afterwards the remaining steps of processing are similar to the canonical miRNA maturation pathway. Another alternative process occurs specifically with miR-451, this miRNA utilizes the beginning steps of the canonical pathway however, instead of Dicer the pre-miR is processed by Ago2 in the cytosol

instead of Dicer, and is cleaved by the Ago2 catalytic center where it is then trimmed into a mature miRNA (Cheloufi et al. 2010).

Regardless of the mechanism of biogenesis, the mature miRNA are bound to the RISC to affect their biologic action within the cell. The RISC complex contains numerous proteins, but the primary effector of miRNA-mediated repression reside within a family of proteins known as the argonaute proteins (Gregory et al. 2005). The human genome contains eight argonaute-family proteins, but Ago2 is the only one with RNA cleavage activity and is thought to play the lead in miRNA-mediated silencing. Proteomics approaches have identified dozens of proteins that associate with Ago and RISC, and the combination of protein partners in this complex controls the regulation of RISC activity and the degree of miRNA-mediated repression (Hock et al. 2007). Association of the mature miRNA with RISC and the cognate mRNA at the 3′-UTR can result in repression of translation, or destabilization of the mRNA through decapping and degradation. The mechanistic details about these two methods of post-transcriptional repression remain an area of intense investigation (Wilczynska and Bushell 2015).

Mature miRNA can themselves be subjects to regulation in several ways. The mature miRNA transcript can be bound by other RNA transcripts called competing endogenous RNA (ceRNA) (Tay et al. 2014). Another class of RNA that can affect levels of mature miRNA are called circular RNA (circRNA), which are formed by a covalent link between the 5′ and 3′ ends of an exon by the spliceosome (Hentze and Preiss 2013). Both the ceRNA and circRNA can function as sponges and titrate mature RNA from the mRNA targets they may bind to. The function of these RNA families in the ovary has not yet been examined, but could provide useful information for understanding the biology of the endocrine functions of the ovaries.

3 Consequences of Global miRNA Depletion in Ovarian Cells

Targeted gene deletion studies in mice suggest a critical role for miRNA regulation throughout the body, including the ovary. Initial studies showed that deletion of the Dicer eliminated almost all miRNA from the developing embryo and caused embryonic lethality at E6.5 due to a loss of pluripotent stem cells and impaired angiogenesis (Bernstein et al. 2003; Yang et al. 2005). Similarly, loss of Ago2 or Drosha was also embryonic lethal by E6.5–E7.5 (Kaneda et al. 2009; Yuan et al. 2014). To overcome these embryonic lethal phenotypes, Dicer conditional knockout (cKO) mouse models were developed and the essential roles of Dicer were demonstrated in a number of critically important tissues and cell types (Luense et al. 2009). In the reproductive organs, Dicer has been deleted using the oocyte-specific zona pellucida-3 (Zp3) promoter linked to Cre recombinase (Murchison et al. 2007), another oocyte-specific knockout using the DEAD (Asp-Glu-Ala-Asp) box polypeptide-4 (DDX4 or vasa) Cre and the anti-Mullerian hormone receptor-2 (*Amhr2*) Cre (Hong et al. 2008; Lei et al. 2010; Nagaraja et al. 2008; Gonzalez and Behringer 2009; Yuan et al. 2014), which is expressed in ovarian granulosa cells and derivatives of the Mullerian duct (oviduct, uterus, and cervix) and pituitary.

The targeted deletion of Dicer in the oocyte using Zp3-Cre resulted in infertility due to disorganized spindles, chromosome alignment defects, and oocyte arrest at metaphase-I of meiosis (Murchison et al. 2007; Tang et al. 2007). The Zp3-cKO of Ago2 resulted in similar chromosome abnormalities although these oocytes are able to mature to metaphase-II (Kaneda et al. 2009). Deleting Dicer at an even earlier stage of oocyte development (E15) caused female infertility due to failure of follicle growth (Yuan et al. 2014). Thus, Dicer, and Ago2 are necessary for normal oocyte meiosis, early follicle development and female fertility. This was originally interpreted to indicate that miRNA had minimal roles in oocyte development or in the crosstalk of the oocyte with the cumulus cells of growing follicles but that miRNA were critical in the oocyte at later stages for the completion of meiotic maturation prior to ovulation. However, more recent studies have discovered that Dicer and Ago2 are also involved in the biogenesis of endogenous small interfering RNA (siRNA) as well as miRNA (Flemr et al. 2013; Yuan et al. 2014). Two studies have helped to differentiate between the siRNA and miRNA pathways in the oocyte (Suh et al. 2010; Yuan et al. 2014). DGCR8 and Drosha are essential for miRNA but not siRNA biogenesis pathways. Therefore, DGCR8 was conditionally deleted from oocytes using the Zp3-Cre and the DDX4-cre, similar to the studies with Dicer and Ago2 (Suh et al. 2010; Yuan et al. 2014). Interestingly, deletion of DGCR8 or Drosha resulted in normal follicular development and oocytes that matured, fertilized and developed to healthy live born offspring indicating a requirement for siRNA but not miRNA in mouse oocyte maturation (Suh et al. 2010; Yuan et al. 2014). Another recent study specific to rats and mice discovered that the Dicer protein in the oocytes ($Dicer^{O}$) of these species is different from the Dicer expressed in their somatic cells (Flemr et al. 2013). Expression of the $Dicer^{O}$ transcript is driven by an alternative promoter that originates from a retrotransposon. As a result, $Dicer^{O}$ has a higher affinity for double stranded RNA that have perfect complementarity, thus $Dicer^{O}$ generates siRNA instead of miRNA (Flemr et al. 2013). These findings explain the severe phenotypes seen in the oocyte-specific knock-outs of Dicer and Ago2, but that are not found in the cKO of DGCR8 and Drosha. Because this oocyte-specific isoform of Dicer is not present in humans or other mammals, the implications only affect studies performed in rodents. It is clear that the function of small RNAs is necessary for correct development and function of the oocyte, however, much remains to be discovered in this young field. The mouse may not be an adequate model for the study of miRNA in the oocyte, but it may be suitable for studies in somatic cells because these cells have the full length Dicer that is required for miRNA processing.

Targeted knockout of Dicer has also been conducted using the *Amhr2*-Cre which deletes Dicer from the mesenchyme of the developing Mullerian ducts and postnatally in ovarian granulosa cells and mesenchyme-derived cells of the female reproductive tract (Gonzalez and Behringer 2009; Hong et al. 2008; Nagaraja et al. 2008; Pastorelli et al. 2009). Female mice with *Amhr2*-cKO of Dicer suffer from complete infertility due to abnormalities in all of these tissues. Progesterone (P4) and estrogen levels tested normal in these mice (Hong et al. 2008). However uteri were short and immature. Oviduct development was severely affected, especially at the isthmus, which showed a lack of smooth muscle leading to bulbous distended

sac-like structures and a general disorganization of the oviductal epithelial layer (Nagaraja et al. 2008; Hong et al. 2008; Pastorelli et al. 2009). While the primary cause of infertility in the *Amhr2*-cKO Dicer mice was due to the trapping of oocytes and embryos within the enlarged sacs of the oviduct, these mice also exhibited reduced ovary weight and lower ovulation rates demonstrating a need for Dicer in ovary and follicular development (Hong et al. 2008). In addition, the cKO of Dicer in follicular cells led to luteal insufficiency and failure to maintain pregnancy (Otsuka et al. 2008). Studies such as these alter miRNA in a global manner, and consequently disrupt the more than 2500 miRNAs that have been described (see http://microRNA.sanger.ac.uk). Further research is necessary in order to understand the effect of specific miRNA and their targets on endocrine regulation within the ovary.

4 Ovarian miRNA Expression Profiling

Another mechanism used by researchers to identify whether miRNA might be involved in ovarian function has been through the profiling of miRNA expression in ovarian tissues. Initial studies focused on miRNA gene expression in whole ovarian tissues comparing different ages of animal (i.e., fetal/newborn to adults) (Ahn et al. 2010; Ro et al. 2007; Mishima et al. 2008; Tripurani et al. 2010; Huang et al. 2011) and different physiological states (follicular versus luteal phase) (Hossain et al. 2009). Mouse and cow have been the models most commonly used to identify miRNA expression patterns in the ovaries of adult and fetal animals, but studies on, pigs, goats, and sheep have also been performed (Li et al. 2011; Ling et al. 2014; McBride et al. 2012). While these studies identified ovarian specific miRNA, the advent of more robust techniques such as small RNA sequencing and deeper analysis of more tissues, has left very few miRNA for consideration as ovarian specific. Another major drawback of these studies is that the ovary is comprised of a very heterogeneous population of different cell types and that even within a cell type, the function and therefore gene expression can vary wildly dependent on the stage of the estrous or menstrual cycle at which the cells are evaluated.

Several studies have used more defined populations of ovarian somatic cells or have compared the changes in miRNA expression in extracellular vesicles (i.e., exosomes and microvesicles) isolated from follicular fluid collected at discrete periods of follicle development. Briefly, comparisons of miRNA expression of granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle using RNAseq identified significant differential miRNA expression (Salilew-Wondim et al. 2014). In granulosa cells isolated from mice following a superovulation protocol, a small number of miRNA in granulosa cells were found to change temporally with respect to administration of an ovulatory dose of the LH mimic, human chorionic gonadotropin (hCG) (Fiedler et al. 2008). Within the highly steroidogenic corpus luteum, small RNAseq was used to identify 15 miRNA that were differentially expressed in pregnant versus non-pregnant cows and these miRNA were predicted to target genes involved in apoptosis and immune signaling pathways (Maalouf et al. 2014). Lastly, several studies have characterized

the miRNA associated with extracellular vesicles derived from follicular fluid (Diez-Fraile et al. 2014; Roth et al. 2014; Sohel et al. 2013; Sang et al. 2013; da Silveira et al. 2012). In mares and women, age was shown to influence miRNA expression in extracellular vesicles (da Silveira et al. 2012; Diez-Fraile et al. 2014). Altered miRNA abundance was also seen in the follicular fluid of women with polycystic ovarian syndrome compared to normal controls (Roth et al. 2014; Sang et al. 2013). In another study, differences in miRNA abundance in extracellular vesicles compared to miRNA in vesicle-free fractions of follicular fluid was observed (Sohel et al. 2013). MicroRNA were found in both vesicle and vesicle-free fractions of bovine follicular fluid, and the miRNA showed differential abundance in these two fractions. While this finding is intriguing, the biological function of miRNA within the extracellular vesicles and extracellular vesicle-free miRNA remains to be determined. Furthermore, most studies assessing changes of miRNA abundance in follicular fluid lack experimental controls necessary to determine the parental cell of origin that generated the miRNA. Unfortunately, those studies of the corpus luteum showing differential miRNA expression still suffer from being derived from a very heterogeneous population of cell types (i.e., immune, endothelial, luteal). These studies highlight our current lack of understanding of miRNA expression within ovarian tissues and the need to perform more robust studies aimed at understanding the functional role these small molecules have on ovarian function.

In conclusion, the genetic depletion of critical regulators of miRNA biogenesis and function, and the evidence of differential expression of miRNA within tissues and cells collected at discrete functionally relevant time points, does support a role for these molecules on normal ovarian function.

5 MicroRNA Regulation Within the Antral Follicle

As a major functional unit within the ovary, follicles undergo a progressive transformational process as they grow from primordial to ovulatory follicles. During this developmental process the antral follicle becomes the first tissue to produce significant levels of the major endocrine hormone, estradiol (E2). This estrogen dominates during the follicular phase and is critical for regulation of the hypothalamic/pituitary/gonadal axis, secondary sex characteristics, reproductive tract function, and reproductive behavior. Copious numbers of studies have examined the pathway of E2 production and the role gonadotropins play in support of the endocrine functions of the antral follicle (Knobil and Neill 2014). Indeed, the well-known ‘two cell two-gonadotropin theory’ describes how compartmentalization of the steroidogenic enzymes and gonadotropin receptors within the two different follicular cells, facilitate thecal cell production of androgens and subsequent granulosa cell aromatization of those androgens to estrogen (Knobil and Neill 2014) (Fig. 6.2). Furthermore, estrogen has negative and positive endocrine effects at the pituitary that are essential for regulation of the LH and FSH secretion that drives folliculogenesis and ultimately the surge of LH that signals the end of

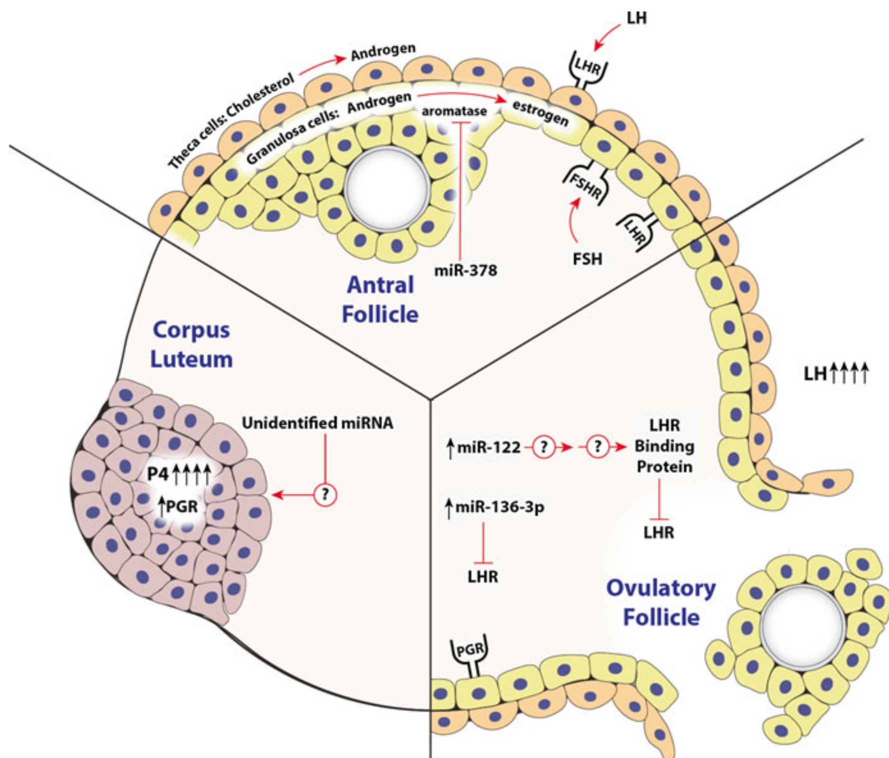

Fig. 6.2 MicroRNA regulation of ovarian endocrine system. Ovarian endocrine regulation is divided into three main temporal periods: the antral follicle, ovulation, and corpus luteum. The cell types involved (theca, granulosa and luteal), the primary endocrine hormones (FSH and LH) and their respective receptors (FSHr and LHr), and the primary steroids (progesterone, P4; androgens, A4; and estrogen, E2) produced by these cells are illustrated. Those miRNA that have been implicated in the direct regulation of these hormone secretion pathways or the regulation of the receptors are shown. Details including the references related to these findings are described in the appropriate sections within this chapter

follicular development and the ovulation of the oocyte. While a substantial number ~26 papers have examined the putative functions of miRNA in mural granulosa cells isolated from antral follicles, most of these have focused on other critical cellular elements involved in folliculogenesis such as proliferation and apoptosis (see Reviews McGinnis et al. 2015; Donadeu et al. 2012). A very limited number of studies have examined the role miRNA play specifically in the regulation of steroidogenesis within the antral follicle, those papers and supporting papers from other areas of biology are described below.

Ovarian endocrine function is dependent on androgen signaling through interactions from the theca cells and granulosa cells (Hu et al. 2004). Androgens can affect ovarian steroidogenesis directly through androgen receptors (AR), or indirectly by the process of aromatization to estrogen. Androgens play a critical role

in early follicle development, as demonstrated by mice that lack a functional AR. These mice are less fertile and have a shorter reproductive window than controls (Hu et al. 2004; Lyon and Glenister 1980). Androgen receptors are highly expressed in granulosa cells and theca cells of early stage follicles (Gervasio et al. 2014). AR can enhance the expression of miR-125b, which has anti-apoptotic effects and may regulate follicular atresia by promoting survival of granulosa cells (Sen et al. 2014). In primary mouse granulosa cells, AR binds to the promoter of the miR-125b primary transcript at three androgen response elements, in an androgen dependent fashion, indicating a transcriptional regulation of miR-125b. Interestingly, the transcriptional regulation of miR-125b was dependent upon nuclear and extranuclear actions of androgen, because the transcriptional upregulation of miR-125b was dependent on interactions of AR and paxillin, a scaffolding mediator of kinase cascades. The *in vivo* effect of miR-125b on ovarian function was demonstrated using bursal injections of miR-125b inhibitors, which significantly increased the expression of pro-apoptotic proteins BAK, BAX, BMF, and p53 in granulosa cells and induced apoptosis. This study demonstrated the *in vivo* effect of a miRNA on follicular development, in an androgen-dependent manner. MicroRNAs targeting the AR have also been identified systematically in prostate cancer cell lines (Ostling et al. 2011). A gain-of-function screen of 1129 miRNA in a panel of human prostate cancer cell lines was used to quantify changes in AR protein. Identification of 13 miRNA were validated using a luciferase reporter assay containing the AR 3′UTR: (miR-135b, miR-185, miR-297, miR-299-3p, miR-34a, miR-34c, miR-371-3p, miR-421, miR-449a, miR-449b, miR-634, miR-654-5p, and miR-9). Briefly, these assays work by linking the 3′-UTR of the gene of interest (i.e., AR, PGR) to the luciferase gene, followed by transfection of this reporter into cells where the investigator either blocks or enhances the expression of a specific miRNA. The contribution of these AR-modulating miRNA within the ovary remains to be determined.

During folliculogenesis and steroidogenesis, E2 acts together with FSH and is critical in enabling pituitary neuron cells to exhibit fluctuating endocrine patterns allowing for the preovulatory LH surge (Xu et al. 2011). Because of the importance of E2, the regulation of aromatase (CYP19A1) has been extensively studied (reviewed Stocco 2008). In porcine granulosa cells, miR-378 down regulates the expression of CYP19A1 (and therefore estrogen) (Xu et al. 2011). MicroRNA-378 targeted both CYP19A1 and progesterone receptor (PGR), and the down regulation of this miRNA appeared to be necessary for granulosa cell differentiation to the preovulatory stage. These studies were both performed *in vitro*, and it remains unclear how the miRNA is regulated *in vivo*.

Several additional miRNA can target genes involved in E2 production by mouse granulosa cells. MicroRNA-224 can affect E2 production by targeting Smad4, a transcription factor involved in the transforming growth factor-beta1 (TGF-β1) pathway that can activate expression of a variety of proliferation genes (Yao et al. 2010a). These studies, however, are difficult to interpret because TGF-β did not induce miR-224 expression consistently. The same group identified miR-383 as being down regulated by TGF-β1. MicroRNA-383 can target RNA binding motif, single stranded interacting protein 1 (RBMS1), thus increasing E2 release (Yin

et al. 2012). Another miRNA associated with E2 synthesis is miR-133b, which directly targets the transcription factor Forkhead L2 (Foxl2), which inhibits transcription of steroidogenic acute regulatory protein (StAR) and CYP19A1, thus leading to an increase in E2 production after FSH stimulation (Dai et al. 2013). Both isoforms of the estrogen receptor (ERα and ERβ) are important in fertility, as demonstrated in KO mouse models. Null ERα (αERKO) mice are infertile, while null ERβ (βERKO) mice are subfertile (Couse et al. 2003). The αERKO mice contained hemorrhagic and cystic ovaries, and displayed abnormally high levels of serum LH, which resulted in elevation of steroidogenic genes (Cyp17a1, Cyp19, and HSD17b3). ERβ is expressed in granulosa cells, and the βERKO mice had a predominant phenotype in follicles, with evidence of unruptured follicles after superovulation as well as decreased numbers of recovered oocytes (Krege et al. 1998). While the two estrogen receptors are essential to fertility, the contribution of miRNA to their regulation is poorly understood in the ovary. Several studies have identified miRNA that target ERα and ERβ in cancer cell lines. In hepatocellular carcinoma cell lines ERα was shown to be directly targeted by miR-18a and miR-22 (Liu et al. 2009), while in breast cancer cell lines miR-206 was shown to affect ER directly (Pandey and Picard 2009; Adams et al. 2007). Fewer studies have analyzed ERβ as a target of miRNA, limited to miR-92 in MCF-7 breast cancer cells (Al-Nakhle et al. 2010).

Additionally, endocrine hormones such as FSH that affect antral follicle development and function can also alter the expression of miRNA (Yao et al. 2010b). Specifically, 12 h post-FSH treatment in primary rat granulosa cells, miR-29a and miR-30d were significantly down regulated, but increased in expression after 48 h. However, the specific targets of these miRNA were not identified, and the biologic consequences of the biphasic response at 12 and 48 h were not explored. Importantly, many of the identified miRNA involved in steroid production pathways have been identified through *in vitro* experiments. Few studies have confirmed miRNA effects on steroidogenesis *in vivo*.

6 MicroRNA Regulation Within the Ovulatory Follicle

At the completion of follicular growth, increasing follicular estrogen signals to the hypothalamus and pituitary to initiate a surge of LH secretion that facilitates the release of the oocyte and the conversion or luteinization of the granulosa and thecal cells. During this periovulatory period, P4 becomes highly concentrated in follicular fluid (Emori and Drapkin 2014), and it is critical for the final stages of folliculogenesis and for ovulation (Duffy et al. 2010). Simultaneously, the LH surge induces the transient expression of progesterone receptor (PGR) in the luteinizing granulosa cells of rodents, and in mammals with a true luteal phase, the LH surge induces long-term expression of PGR (Gougeon 2010; Hatzirodos et al. 2014; Iwai et al. 1990; Revelli et al. 1996). In mice the function of the two different isoforms of PGR (A and B) have been evaluated and loss of PGR-A was shown to cause a 70 % decline in the number of oocytes ovulated while in contrast, loss of PGR-B had no effect detectable on ovulation (Conneely et al. 2003; Shao et al. 2003). The transcriptional expression of PGR is clearly upregulated in granulosa cells following

hormonal gonadotropin signaling through FSH and LH (Shimada and Terada 2002; Clemens et al. 1998), but post-transcriptional regulation by miRNA has also been shown to be involved (Toms et al. 2014). In the pig, PGR was inversely correlated with expression of miR-378-3p in developing follicles and *in vitro* studies using isolated granulosa cells showed that miR-378-3p could directly target PGR mRNA as evidenced by 3′-UTR luciferase reporter assays (Toms et al. 2014). The expression of miR-378-3p is lower in porcine large follicles, suggesting that by repressing this miRNA during late folliculogenesis, the repression of genes essential for granulosa cell differentiation (CYP19A1 and PGR) is alleviated. Additionally, in three breast cancer cell lines, miR-513a-5p decreased the luciferase activity in a PGR luciferase reporter assay, indicating that this miRNA also directly targets PGR (Cochrane et al. 2012). Supporting miR-513a-5p as a direct regulator of PGR, this miRNA was also induced by P4 (Cochrane et al. 2012), whether this miRNA is expressed in the ovary and if it may function there has not been assessed.

In another approach to examine whether miRNA can elicit changes in steroidogenesis *in vitro*, primary granulosa cells were harvested from normally cycling women after ovariectomy due to non-metastatic cervical cancer and were transfected with 80 different pre-miRNA constructs (Sirotkin et al. 2009). Markers of proliferation, apoptosis, and secretory activity of P4 were assessed to identify miRNA involved in ovarian function (Sirotkin et al. 2010). Pre-miR-15a treatment enhanced the expression of proliferative and apoptotic signals, and promoted the release of P4 and testosterone, but not E2 (Sirotkin et al. 2014).

In vivo experiments performed in mice identified several miRNA in granulosa cells including miR-21, miR-132 and miR-212 that changed significantly following the LH surge (Fiedler et al. 2008). Inhibition of miR-21 by injection of inhibitors beneath the bursa of mice blocked ovulation (Carletti et al. 2010). Conversely, none of these three miRNA have shown effects on ovarian steroidogenesis or luteinization *in vitro or in vivo* (Carletti et al. 2010; Fiedler et al. 2008; McGinnis et al. 2015). Lastly, the LH receptor (LHr) itself has been shown to be under regulation by miRNA. Studies performed in whole ovary digestion experiments in rats identified miR-122 as positively correlated with an increase of LHr mRNA binding protein (LRBP) after treatment with hCG, through an indirect and unknown mechanism (Menon et al. 2013). The authors postulate that this mechanism may be through the regulation of sterol regulatory element binding proteins-1 and -2 and the temporal patterns of expression does support this claim, yet direct evidence for this remains to be shown. Another study performed using whole rat ovary homogenates identified miRNA-136-3p as being overexpressed 6 h after hCG treatment and bioinformatic analyses indicated that 3′-UTR of the rat LHR has a miR-136-3p site within it. The biologic activity of miR-136 was then tested in primary rat granulosa cell culture and was shown to directly target the rat LHr (Kitahara et al. 2013). It should be noted that bioinformatic (TargetScan) analysis of the human LHr 3′-UTR does not detect a miR-136-3p site within it. This does not preclude the possibility that a miR-136-3p site is present, but considering that the 3′-UTR of genes have the greatest sequence variability, this would need to be tested empirically. It is thus critical that we do not generalize results across species and that until confirmatory results are produced *in vivo* the functional roles of miRNA can at best be speculative.

7 MicroRNA Regulation Within the Corpus Luteum

The granulosa and thecal cells following exposure to the LH surge, differentiate (i.e., luteinize) to form the corpus luteum (CL). This luteinization process involves the rapid hypertrophy of the granulosa and thecal steroidogenic cells and rapid proliferation of endothelial cells that allows invasion of capillaries around the previous avascular granulosa cells. These profound cellular and tissue remodeling events enable this newly formed tissue to produce exceptionally high P4 levels that are critical for normal cycles and for establishment and maintenance of pregnancy. To date, the female infertility observed in a mouse Dicer hypomorph provides the best evidence that miRNA play a role in luteal function (Otsuka et al. 2008).

The Dicer hypomorphic female mice were infertile due to CL insufficiency resulting, in part, from impaired growth of new capillaries in the ovary (Otsuka et al. 2008). This phenotype was partially due to the lack of miR-17-5p and let7b, two miRNA that function in angiogenesis by down regulating anti-angiogenic tissue inhibitor of metalloproteinase (TIMP1). The angiogenesis defect was rescued by re-administering the miRNA-17-5p and let7b, however full-term pregnancy was not maintained indicating that the miRNA are not the only factors controlling these complex processes. Recently, global expression analysis of miRNA from CL of sheep, pigs and cows have provided additional evidence for miRNA involvement in luteal function.

In sheep, miRNA involved in angiogenesis and cell survival pathways were highlighted in the transition from pre-ovulatory follicle to CL (McBride et al. 2012). In this study, cells were isolated from pre-ovulatory follicles, early CL, late CL and corpus albicans and subjected to RNAseq PCR then confirmed by Northern blot analysis and qRT-PCR (McBride et al. 2012). Overall, the most abundantly expressed miRNA included miR-21, miR-125b, let-7a and let-7b contributing 40 % of all miRNA in the ovarian tissues examined. Some miRNA were expressed at higher levels in the follicles (miR-199a-3p, miR-125b, miR-145 and miR-31) while others were much higher in the CL (miR-503, miR-21 and miR-142-3p) (McBride et al. 2012). Potential gene targets were determined from miRTarBase and it was found that the majority of these miRNA were involved in the regulation of cell cycle, survival, differentiation and angiogenesis. Increases in these pathways makes sense since the CL consists of multiple cell types that must differentiate, multiply and survive long-term in the case of pregnancy.

Global expression analysis of miRNA from CL of pregnant (day 17) versus non pregnant cows identified 15 miRNA that were differentially expressed (Maalouf et al. 2014). Interestingly, similar to studies in sheep (McBride et al. 2012) miR-21, let-7, and miR-199 were among the highest miRNA expressed in the cow ovaries and confirmed an earlier report from the cow (Hossain et al. 2009). Pathway analysis implied miRNA are especially involved in the regulation of cell survival and apoptosis in the bovine CL, a similar theme to that identified in the sheep (McBride et al. 2012). In the pig, miR-378 expression increased during CL development and decreased during luteolysis indicating a possible function in CL formation and maintenance (Ma et al. 2011). Since miR-378 promotes cell survival and

angiogenesis in other cell types (Lee et al. 2007) these known functions support the proposal that miR-378 may play similar roles in the CL (Ma et al. 2011). However, in a more recent study miR-378-3p was found to be inhibitory to PGR expression in pig granulosa cells (Toms et al. 2015). Since the PGR activity is critical to maintenance of the CL (Maybin and Duncan 2004), further studies are needed to understand these contradictory, possibly species-specific roles for miR-378.

These few studies indicate essential roles for miRNAs in luteal function. However, more studies are needed to identify and fully define these pathways. There is also a critical need for *in vivo* based studies to determine the true effects of miRNA on the physiology of cells and tissues.

8 MicroRNA in Reproductive Diseases

With the importance of miRNA in ovarian physiology described above, differences in miRNA expression might be expected in ovarian diseases. Surprisingly, few studies of miRNA function have been conducted on human patients with ovarian diseases and of those few studies that have been reported, only one disease polycystic ovary syndrome (PCOS) is specifically related to endocrine regulation within the ovary. As one of the most common endocrine-metabolic disorders in women of reproductive age (Imbar and Eisenberg 2014; Norman et al. 2007), PCOS results in menstrual disorders and infertility. Since miRNA were first discovered in follicular fluid (da Silveira et al. 2012), additional studies have compared miRNA contents between PCOS and healthy women (Roth et al. 2014; Sang et al. 2013). Combined genome-wide deep sequencing and TaqMan miRNA arrays then followed-up with cell culture experiments were used to identify miRNA involved in follicle steroidogenesis in PCOS patients (Sang et al. 2013). From their microarray results, they selected miRNA that were postulated to function in steroidogenesis in other tissues. MicroRNA-mimics or inhibitors were generated and transfected into a steroidogenic human granulosa-like tumor cell line (KGN) and cultured to determine the intracellular effects of these miRNA. The results identified five miRNA that regulated E2 in KGN cells: miR-24 decreased E2 secretion while miR-132, miR-320, miR-520c-3p and miR-222 all increased E2 secretion. Three miRNA were found to regulate P4 secretion (miR-24, miR-193b and miR-483-5p) (Sang et al. 2013). Follow-up testing of follicular fluid from PCOS and non-PCOS patients confirmed that there were decreased levels of miR-132 and miR-320 in the PCOS patients (Sang et al. 2013). In spite of the decline in miR-132 and miR-320 and that these two miRNA caused an increase in E2 secretion from KGN cells *in vitro*, the levels of E2 and P4 were not altered in the follicular fluid of PCOS patients. Therefore, the *in vivo* effects of these miRNA could not be confirmed *in vitro* and further studies are needed to determine if these miRNA serve a critical function within the follicle.

In another study of PCOS patients, 325 miRNA were identified in human follicular fluid and 29 of them showed expression differences between PCOS and control women (Roth et al. 2014). MiR-32, miR-34c, miR-135a, miR18b and miR-9 were more abundant in PCOS patients and pathway analysis correlated them to insulin

regulation and inflammation. Using a bioinformatics analysis, they identified interleukin 8 (IL8), synaptogamin 1 (SYT1) and insulin receptor substrate 2 (IRS2) as potential targets of these miRNA and as expected for targets of miRNA, all three were decreased in the follicular fluid of PCOS patients (Roth et al. 2014). However, in these two studies, there were no overlapping miRNA that were significantly different between the two groups. This may be because one study used Taqman based miRNA arrays (Roth et al. 2014), whereas the other study performed deep RNAseq (Sang et al. 2013) or it may be due to a different population of controls used in each study. These few studies indicate a potential role for miRNA regulation and their perturbation in PCOS, a disease which profoundly affects ovarian endocrine function. The drive for discovery of miRNA therapeutics for the treatment of human disease (Broderick and Zamore 2011), will hopefully initiate more research into the roles of miRNA in ovarian functions such as folliculogenesis, steroidogenesis, corpora lutea and diseases of the ovary and reproduction in general.

9 Conclusions

The studies highlighted in this review indicate an essential role for miRNA in the regulation of endocrine functions of the ovary. However, many studies assess miRNA expression in ovarian tissue composed of multiple cell types and make it difficult to interpret the results. Controlled experiments that demonstrate a functional and physiologic role of miRNA within the tissues of the ovary need to be performed in order to understand the role in each ovarian cell type (i.e., theca, granulosa and luteal cells). Lastly, advances in the field of exosomes and microvesicles have identified novel considerations for the study of miRNA functions, in physiologic and pathophysiologic conditions. This avenue will also surely bring new insight into the role of miRNA in ovarian endocrine functions.

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7 Translational Control in the Germ Line

Marco Conti, João Pedro Sousa Martins, Seung Jin Han, and Federica Franciosi

1 Introduction

Given their critical function in transmitting the genetic information across generations, germ cells are unique cells of the body and the only ones with the capacity to generate a complete organism. They use a specialized meiotic cell cycle to generate haploid cells, and, although differentiating into one of the most specialized cell of the body, they maintain some of the traits specific to pluripotent cells. To accomplish this, they often use unusual molecular strategies to control gene expression during their life cycle. Among these unique strategies is the extensive use of post-transcriptional control of mRNA to regulate important transitions throughout lineage specification, lineage expansion, and differentiation. In model organisms, maternal mRNAs accumulated during growth drive development up to the midblastula transition prior to gastrulation and with few exceptions in the absence of significant transcription (Lee et al. 2014). Moreover, the polarity of the female gamete is defined by mRNA deposited at one pole of the oocyte marking the region of the cytoplasm that will be inherited by and will define the germ lineage. The presence of the so called "germ granules" in the cytosol of most germ cells is the physical manifestation of mRNA storage/accumulation found in one form or another in most organisms. Although mammalian embryos have adopted a different strategy and become independent of maternal input earlier during embryo development (Li et al.

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2010), regulation of translation, in the absence of transcription, is the only form of gene expression that drives oocyte maturation and early embryo development up to the activation of the zygote genome (ZGA) (Clarke 2012). Similarly, translational regulations control critical spermatogenesis steps at the time of transition from mitosis to meiosis, during meiosis itself, as well as during the differentiation of the haploid gamete into a spermatozoon.

The teleological explanation for the extensive use of translational regulations on a background of reduced or absent transcription is matter of debate. It may include the need to rapidly reprogram the genome to totipotency in a short period of time, hence the need to transfer regulation of gene expression to the cytoplasm; it may be due to the need to generate polarity in a large cell such as the oocyte, or to induce rapid changes in protein expression necessary for progression through the meiotic cell cycle. Regardless of the actual reason, translational controls are intimately interwoven with the generation of a totipotent cell and the shift in gene expression control from maternal to embryonic.

Translation of a mRNA is initiated by the assembly of a pre-initiation complex that interacts with the 5′ 7-methyl-guanosine cap structure of the mRNA, and involves the eukaryotic initiation factor 4E (eIF4E) complex and the 40S subunit of the ribosome. This is followed by scanning of the 5′UTR, recognition of the translation initiation site, recruitment of the large 60S subunit of the ribosome, and elongation. For a subset of translational regulations, the cap complex interacts with additional complexes assembled at the other end of the mRNA, the 3′UTR. *Cis*-acting elements and cognate RNA binding proteins (RBPs) are responsible for the organization of these complexes. The complex assembled on the 3′UTR of a mRNA often controls the length of the poly(A) of a mRNA or polyadenylation, one of the most common mechanism involved in promoting translation. Indeed, changes in poly(A) tail length mediated by the cytoplasmic polyadenylation element binding protein, CPEB, was one of the first to be discovered.

An in depth analysis of the mechanisms of translational initiation are outside the scope of this review and the reader is directed to comprehensive recent review of the field. Due to space limitation, we will also not review the role of non-coding RNAs in translational regulation in germ cells. Here, after a brief summary of the major regulatory circuits controlling translation in germ cells in model organisms and conserved during evolution, we will concentrate on the most relevant mechanisms involved in the development of the male and female gamete in mammals and their function up to the formation of the zygote. The theme that will emerge from this survey is that a set of translational regulators are used over and over again through the life cycle of germ cells from worms to humans. This survey is not meant to be comprehensive but rather provide some basic concepts on the mechanisms controlling translation during germ cell development and the most important *trans*-acting proteins involved.

2 Control of Translation during Gametogenesis in Model Organisms

2.1 Translational Control of the Mitosis-to-Meiosis Transition in *Caenorhabditis elegans*

The gonad of the XX hermaphroditic nematode produces both sperm and oocytes whereas the XO male nematode contains only the spermatogenic lineage (Kimble and Crittenden 2007). The mechanisms of gamete production in the two states are similar and have been a powerful model to discover regulatory circuits involving RBPs. A cluster of Germ Stem Cells (GSCs) at the distal tips of the gonad interacts with somatic cells of the niche required for maintenance of the pluripotency state and to control self-renewal of these adult stem cells (Kimble and Crittenden 2007). Once they move away from this niche, GSCs continue with mitotic divisions until they reach a transition point where they enter into meiosis. Completion of meiosis is followed by differentiation into the haploid gamete. Several critical decisions are made during the germ cells differentiation, including the decision to maintain the proliferative state and the pluripotency state, the decision to transition from the mitotic cell cycle to a meiotic cell cycle, and the commitment to final differentiation to a haploid cell. These transitions depend on translational regulations and the function of a number of RBPs or proteins involved in RNA metabolism. Here we will highlight some of the major concepts on the organization of these circuits, underscoring how the same regulatory cassettes may be utilized during mammalian gametogenesis.

Self-renewal and the mitotic cell cycle in *Caernorhabditis elegans* GSCs are maintained through Notch signals originating from the somatic niche (Kimble and Simpson 1997); this controls expression of several key regulators including two RBPs with critical role for the GSC fate. Interestingly, Notch signaling is also involved in mammalian gametogenesis, being essential for formation of the ovarian follicle (Vanorny et al. 2014) and contributing to the testis stem cell niche (Garcia et al. 2014). The PUF proteins, Fbf-1 and Fbf-2 (collectively known as Pumilio and FBF proteins or Pumilios in mammals), function as hubs of complex networks within germ cells controlling both self-renewal and the mitosis/meiosis decision (Kimble 2011). These proteins act predominantly as translational repressors of a large number of mRNAs. Among the critical targets are mRNAs coding for components of the meiotic cell cycle machinery, for transcription factors involved in differentiation, as well as RBPs or mRNA modifiers required for entry into meiosis (Kimble 2011). Meiotic entry occurs prematurely in worms double mutant for *fbf-1* and *fbf-2*. The gonad of these mutants produces only sperm indicating an additional role for these RBPs at later stages during gametogenesis. Puf proteins repress the translation of several genes that are required to enter into meiosis including *gld-1*, *gld-2*, and *gld-3*. Gld1 is a member of the STAR/GSG/quaking-type RBP family and functions as a translational repressor of cyclin E (Marin and Evans 2003), a cyclin required for maintaining the mitotic cell cycle in germ cells. Gld-2 and Gld-3 form a heterodimer with the function of a cytoplasmic poly(A) polymerase and

translational activators. By repressing the expression of these components necessary at different stages of the germ cell differentiation, the Puf protein maintains the germ cell mitotic state. Mechanistically, the Puf proteins repress translation through several mechanisms that include recruitment of the Ccr4-Pop2-NOT deadenylase complex (Goldstrohm et al. 2006), interaction with Ago to stall translation (Friend et al. 2012) or by interacting with other deadenylases (Quenault et al. 2011).

Nos-3 is one of the three *C. elegans* homologs of Drosophila Nanos (Kraemer et al. 1999). In the sperm/oocyte switch of the hermaphrodite germline, Fbf together with Nos-3 represses *gld-1* and *fem-3* mRNAs and promotes oogenesis (Kraemer et al. 1999; Crittenden et al. 2002).

Fog-1 (feminization of the germ line 1) is a *C. elegans* homolog of the cytoplasmic polyadenylation element binding protein (CPEB) which is required for sperm development. Mutations affecting the *fog-1* locus cause a phenotype of sexual reversal with cells that would produce sperm instead differentiate into oocytes (Barton and Kimble 1990). There are additional members of the family of CPEBs with critical function at different stages of gamete development in the nematode. *Cpb-1* mutants do not produce sperm but this RBP functions at a later stage of spermatogenesis compared to Fog-1 (Luitjens et al. 2000). Cpb-3 has been implicated in meiotic progression (Luitjens et al. 2000) functioning in concert with the interacting partner Daz-1. Worms double mutants for *cpb-3* and *daz-1* are sterile (Maruyama et al. 2005). Both functional and physical interactions between members of the Cpb and Fbf proteins have been described. For instance, Fbf-2 and Cpb-1 form a complex that represses translation (Campbell et al. 2012).

Thus, numerous protein/protein interactions and feedbacks contribute to the stability of the control system and its robustness as well as to enforcing irreversibility of the decision switch. Many of these RBPs and some of the feedback arrangements are maintained in the mammalian germ line (see below) (Table 7.1).

Table 7.1 Orthologs of the most relevant RBPs involved in the control of translation in the germline of invertebrate and vertebrate animal models, and in mammals

Family of RBPs	*C. elegans*	*D. melanogaster*	*X. laevis*	*M. musculus*	*H. sapiens*
PUF	Fbf-1	Pum	Pum1	PUM1	PUM1
	Fbf-2		Pum2	PUM2	PUM2
	Puf-8				
Nanos	Nos-3	Nos	Nanos1		
			Nanos2	NANOS2	NANOS2
			Nanos3	NANOS3	NANOS3
CPEB	Fog-1	Orb	Cpeb1	CPEB1	CPEB1
	Cpb-1	Orb2	Cpeb3	CPEB3	CPEB3
	Cpb-3		Cpeb4	CPEB4	CPEB4
DAZ	Daz-1	Bol		BOLL	BOLL
			Dazl	DAZL	DAZ1 DAZLA
DAZAP		Hrb27C	Dazap1	DAZAP1	DAZAP1
			Dazap2	DAZAP2	DAZAP2

2.2 Translational Control of the Mitosis to Meiosis Transition in Drosophila

At the tip of the ovariole in the Drosophila germarium, two germ line stem cells (GSC) divide asymmetrically into a daughter cell which is committed to differentiate and one that replenishes the pool of stems cells (Lin and Spradling 1993; Xie and Spradling 2000). The committed cell, termed cystoblast (CS), undergoes four round of mitosis with incomplete cytokinesis as these cells are connected by cytoplasmic bridges, the ring canals. Two cells of the 16 cell cyst are connected by four cytoplasmic bridges whereas the other cells are connected by three or fewer bridges. One of the cells connected with four bridges will become an oocyte and the other cells will differentiate into nurse cells. Once the oocyte is designated, it receives material from the nurse cells via these ring canals.

Like in nematode gametogenesis, the GSC/CS and mitotic/meiotic transition in Drosophila is regulated by mechanism involving predominantly translational control. Like in worms, Pumilio (Pum) and Nanos (Nos) play a central function in repression of transcripts required for differentiation of the gametes (Miller and Olivas 2011).

The bag-of-marbles (Bam) and benign gonial cell neoplasm (Bgcn) proteins function in both males and females to promote gamete differentiation. Bam is expressed at a critical time when the cystoblast replicates and becomes committed to meiosis entry (Chen et al. 2011b; Slaidina and Lehmann 2014). Bam and Bgcn form a complex that binds and antagonizes both Pumilio and eIF4A proteins. An additional protein included in the complex required for inhibition of GSC factors is Mel-P26, a member of the tripartite motif containing TRIM family of proteins (Li et al. 2013). They also regulate the mRNA coding for another RBP, Nanos, via its 3′UTR (Li et al. 2009; Shen et al. 2009; Kim et al. 2010). Therefore, Bam and Bgcn are part of a network of RBPs that control differentiation of GSCs. A mammalian homolog of Bam was identified and termed GM114 (Tang et al. 2008). However, disruption of this locus has no effect on spermatogenesis (Tang et al. 2008). Since GM114 is remotedly related to Bam, it is still possible that another Bam-ortholog is present in the mammalian genome and involved in translational control during gametogenesis.

Similar players are also involved in differentiation of GSC into a gonioblast of the Drosophila testis (Gonczy et al. 1997; Fuller 1998). However, *bam* may also be required for entry into meiosis.

2.3 Translational Control of Egg and Embryo Polarity in Drosophila

A fascinating concept based on observation in model organism is that patterning during development is generated by mRNA localization and restricted translation in distinct subcellular domains of a cell. The *Drosophila* oocyte and embryo is one of the best understood experimental models in terms of development of polarity

generated by mRNA localization/translation. In Drosophila the specification of the anterior fate of the embryo is dependent on the localization of maternal mRNA bicoid (*bcd*) whereas the development of the posterior end depends on the proper localization of *osk* and *nanos* (Kugler and Lasko 2009). The localization and translation of these mRNAs generate the anterior/posterior axis. Here we will highlight some very basic concept regarding these mechanisms whereas exhaustive reviews of this process are available (Kugler and Lasko 2009; Ghosh et al. 2012).

Oskar mRNA localization coupled to its translational control is essential for normal development. *Osk* mRNA is produced by nurse cells and transferred to the oocyte by a complex of proteins including components of the exon junction complex, Staub, and the eIF4E interacting protein Cup and Bruno. Association of the complex with dynein and with microtubules is necessary for the transport. Once in the oocyte, *osk* localizes in a discrete cytoplasmic structure called the Balbiani body. The site of Oskar protein synthesis determines where germ plasm is assembled. Repression of Osk mRNA translation during this journey is dependent on the eIF4E interacting protein Cup in complex with the RBP Bruno. A Cup ortholog is expressed in mouse oocytes (Clast4) but its function in this species is unknown (Villaescusa et al. 2006). Recently, Clast4 was identified in a screen for genes important for oocyte meiotic reentry although its function was not explored further (Pfender et al, 2015).

Gurken protein is a member of the transforming growth factor-a (TGF-A) family, which functions as ligand for the Drosophila EGF receptor (DER or torpedo). Local mRNA accumulation, local secretion of Gurken and localized occupancy of the EGFR on follicular cells is essential for establishing the antero/posterior axis of the oocyte and the embryo. EGF-like growth factors also play a critical role in oocyte maturation and ovulation in mammals (Conti et al. 2006).

There are two CPEB-like proteins expressed in Drosophila termed Orb (ool8 RNA binding) and Orb2; both of them are required for germ cell development. *Orb* mutant flies are sterile (Lantz et al. 1994). Null mutant for *orb* prevents the last replication, and failure to produce oocytes or nurse cells. Later on during differentiation, the Orb protein is localized in the differentiating oocyte indicating an important function in this cell.

Orb undergoes well defined patterns of localization during oocyte development accumulating first in the anterior pole and becoming gradually enriched in the posterior pole of the oocyte. During early embryogenesis, this cytoplasm region will be inherited by primordial germ cells (PGCs). This localization of Orb is necessary for the localization and translation of the *osk* and Gurken mRNAs. The 3′UTR of *osk* mRNA plays a critical role in both localization and translation. While *osk* mRNA is localized to the posterior pole, Gurken mRNA is first deposited to the posterior region of the oocytes and then is translocated together with the nucleus to the anterior dorsal region (Cheung et al. 2011). *Orb* mutations interfere with both localization processes. Gurken acts both early and late in the oocyte assembly process (Cheung et al. 2011). Consequently, since Orb is involved in both phases of Gurken mRNA localization, Orb also acts both early and late in oocyte assembly (Christerson 1994). From the mechanistic standpoint, it has been shown that Orb and

poly-adenosine polymerase (Pap) control the polyadenylation and translation of oskar mRNA confirming their function as translational activators.

2.4 Translational Control of the Meiotic Cell Cycle in Danio Rerio Oocytes

Oocyte maturation in zebrafish is triggered by MIH (17a,20b-Dihydroxy-4-pregnen-3-one) and governed by activation of pre-MPF, which consists of cyclin B and inactive Cdk1 (Brownlie et al. 2003). As with Drosophila, some mRNAs are deposited in zebrafish oocytes in a polarized fashion. Cyclin B mRNA is concentrated in RNA granules along the cytoplasm at the animal pole through an interaction with the RNA binding protein Pumilio1. Formation of RNA granules depends on actin filaments since cytochalasin treatment leads to cyclin B mRNA release from the granules. After stimulation by MIH, the cyclin B mRNA disperses into the cytoplasm, facilitating its translational activation (Kotani et al. 2013). Cyclin B mRNA localization within the oocyte is dependent on sequence elements within the open reading frame (ORF) as well as the 3′UTR (Yasuda et al. 2010).

As in other species, zebrafish cyclin B mRNA is regulated by RBPs. Recently Pumilo and Igf2bp3 (insulin-like growth factor 2 mRNA-binding protein 3) were reported to repress translation by binding the 3′UTR of cyclin B (Takahashi et al. 2014; Kotani et al. 2013). Compared to other model organisms CPEB dependent translation of cyclin B mRNA is considerably less efficient, probably due to different positioning and sequence of the CPEs (Zhang and Sheets 2009), indicating that the optimized level of cyclin B for the oocyte maturation is species specific. Zebrafish oocytes express two classes of CPEBs, the oocyte specific Zorba together with the embryonic type ElrA (O'Connell et al. 2014). Also Zorba mRNA localizes at the animal pole at stage II of oogenesis (GV stage). This localization seems to be independent of microtubules and microfilaments (Bally-Cuif et al. 1998).

2.5 Translational Control of the Meiotic Cell Cycle in *Xenopus laevis* Oocytes

Frog oocytes have been a model used for extensive studies on the role of translational control of germ cells. Cpeb was first described in frog oocytes and much of what is known about the function of this protein comes from studies in Xenopus. In frogs like in most species studied, maternal mRNAs accumulate during oocyte growth but they are not translated into proteins (Clarke 2012). The polyadenylation state of these mRNAs is thought to be central to the repressed state and for translational activation (Richter 2007; Charlesworth et al. 2013). In frog oocytes, a large macromolecular complex is formed involving RBPs (Cpeb1, Cpsf, ePab), an adenylase (Gld2), a deadenylase (Parn), and the scaffold/adaptors Symplekin and Maskin, as well as components of the cap complex (eIF4E, eIF4E-T). This complex is thought to prevent translation by maintaining a short poly(A) tail

(Fernandez-Miranda and Mendez 2012; Ivshina et al. 2014). Other complexes have been described as important for repression, including Pumilio proteins (Kim and Richter 2007) or Cup/Orb in Drosophila (Wong and Schedl 2011). Further function of these complexes is discussed below in the context of mammalian oocyte.

3 RBPs and Translational Control in Mammalian Primordial Germ Cells (PGCs)

In the mouse fetal gonad, male PGCs exit mitotic cycle and enter a period of quiescence, whereas female PGCs become committed to enter meiosis. The signal for this decision is extrinsic and derives from somatic cells. It is commonly accepted that retinoic acid (RA) induces female germ cells to enter meiosis and that this signal is suppressed in the male through rapid metabolism of RA (Bowles et al. 2006). A key regulator of meiotic cell cycle entry is stimulated by retinoic acid 8 (Stra8) with probable function of transcription regulator. However, RBPs play a key role in the control of Stra8 expression. Nanos 2 and 3 proteins are essential for PGC development in a dymorphic fashion with Nanos 2 expressed only in male germ cells. Nanos 2 function is to promote the male fate but repress the female fate in germ cells (Saga 2010). Nanos acts as a translational repressor that prevents meiotic entry by suppressing translation of several mRNAs including *Stra8* itself.

The RBP DAZL (deleted in Azoospermia-like) is also considered an important component required for entry into meiosis. It has been proposed that DAZL has a permissive function as a "licensing" factor, priming germ cells to respond to molecular cues and engage germ cells in sex differentiation (Gill et al. 2011) but the molecular mechanisms involved are unclear. Traditionally DAZL is viewed as an activator of translation but it has been recently proposed that it may also function as repressor. Indeed DAZL interacts with Pumilio proteins which are usually repressor of translation (Urano et al. 2005). CPEB1 also plays a role because CPEB1 knockout prevents PGC entry into meiosis and SYCP3 expression (Tay and Richter 2001). CPEB is necessary for DAZL protein accumulation and it is likely that CPEB1 and DAZL function sequentially and synergistically during female PGC commitment to enter meiosis.

4 Translation Control during Adult Mammalian Spermatogenesis

In the adult mammalian male gonad, the production of gametes is divided into three stages: a proliferation stage, where spermatogonia undergo five rounds of mitotic division (Oatley and Brinster 2008; Hermo et al. 2009), a maturation phase, where diploid spermatocytes undergo two meiotic divisions to yield haploid spermatids (Zickler and Kleckner 1999) and spermiogenesis where haploid spermatids differentiate into spermatozoa (Braun 2001). The progression through each of these

events relies on a tightly controlled network of specific genes regulated at the transcription and post-transcriptional level.

The temporal regulation of the protamine 1 (*Prm1*) mRNA translation during spermiogenesis is a classic example of translational regulation and uncoupling of transcription and translation. Transcription of this mRNA initiates during the round spermatid stage (Braun et al. 1989) but the mRNA is not translated immediately. The translation of the *Prm1* mRNA is dependent on a highly conserved sequence, the translational repression being mediated by a 17-nucleotide translational control element (TCE) located in the 3′UTR of this mRNA. Mutation of this TCE *cis*-acting element causes premature synthesis of protamine protein and sterility. The *Prm1* mRNA is stored as a cytoplasmic ribonucleoprotein (mRNP) particle in spermatids that are also required for maintaining this repressed state. Translational activation of stored *Prm1* mRNA in elongated spermatids requires the trans-activation responsive RNA-binding protein 2 (TARBP2). Mice mutant for *Tarbp2* are defective in proper translational activation of the *Prm1* and *Prm2* mRNAs and are sterile (Zhong et al., 1999). *Tarbp2* is expressed at high levels in post-meiotic spermatids. The cold shock domain Mouse Y-box protein 2 (MSY2) is one of the proteins responsible for repression of *Prm1*. This is believed to prime transcripts (with a Y-box region within their promoter) to cytoplasmic repression and RNA stability (Yang et al. 2005). In MSY2 knockout models, no spermatozoa are found in the seminiferous tubules, in part due to reduced chromatin condensation (Yang et al. 2005)

Additional RBPs have been described both in the nuclear and cytoplasmic components of cells, some of which shuttle between the nucleus and cytoplasm. Once in the cytoplasm, transcripts are encapsulated in RNPs stabilized by RBP protein–protein and protein–RNA interactions. The effect on mRNA processing by RBPs is further augmented by post-transcriptional modifications of the RBPs (methylation, ubiquitination, phosphorylation, acetylation) that remodel the structure of RNPs and alter their activity (Bettegowda and Wilkinson 2010; Idler and Yan 2012). For example, knockdown of the KH domain-containing protein SAM68 results in infertility associated with germ cell apoptosis and aberrant elongating spermatids. This protein binds polyadenylated transcripts with the AUUAAA motif and upon phosphorylation translocates from the nucleus to the cytoplasm promoting function of signal transduction, alternative splicing and translation upregulation (Lin et al. 1997; Najib et al. 2005; Paronetto et al. 2009; Paronetto and Sette 2010). Here we will focus on the properties and function of four classes of RBPs that are critical for spermatogenesis progression (Fig. 7.1).

4.1 Deleted in Azoospermia Family of RBP

The germ cell specific Deleted in Azoospermia (DAZ) family of proteins is composed of three members; the primate and catarrhine lineage specific protein DAZ, located within the long arm of the Y chromosome (Reijo et al. 1995); the autosomal and family ancestor Boule, with homologs described from flies and worms to mice and humans (Karashima et al. 2000); and the vertebrate specific DAZ-like (DAZL)

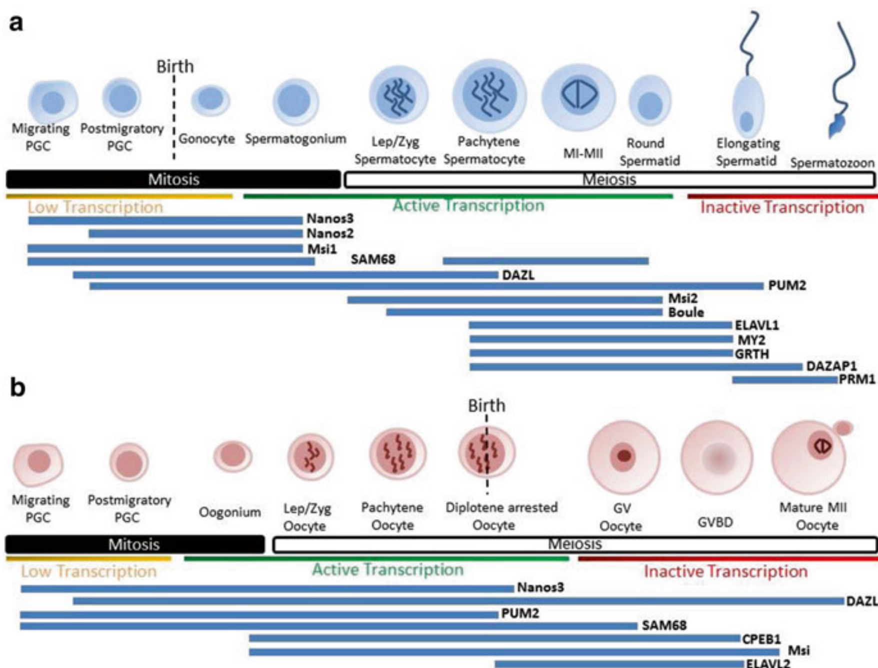

Fig. 7.1 Expression profile of RBPs throughout mouse spermatogenesis (**a**) and oogenesis (**b**). Progression through gametogenesis is highly regulated at the translational level, both in the mitotic and meiotic phases. Developmental progression is regulated by complex network of RBPs which control, translation activation, repression and RNA stability. The following references contain details on the expression of genes expressed during spermatogenesis and ovogenesis: Pum2 (Xu et al. 2007), Nanos 2 (Barrios et al. 2010), Nanos 3 (Lolicato et al. 2008), Dazl (Ruggiu et al. 1997; Saunders et al. 2003; Chen et al. 2011a), Boule (Xu et al. 2001). Sam68 (Paronetto et al. 2009), Elavl2 (Chalupnikova et al. 2014), CPEB1 (Tay and Richter 2001; Chen et al. 2011a), Musashi (Gunter and McLaughlin 2011)

(Bielawski and Yang 2001). This family of proteins owes its name to the phenotype of patients were it was first identified. In a genetic screen of azoospermic men, 14 % of cases had deletions within the area containing the DAZ coding region (Reijo et al. 1995). The other members of the DAZ family have also been associated with male infertility as reported in several SNPs studies (Chen et al. 2010).

All members of the DAZ family are required during spermatogenesis. DAZ and Boule are necessary for spermatid development, with Boule-null mice showing a developmental arrest at step 6 of spermatids (VanGompel and Xu 2010). DAZL phenotype can be detected much earlier compared to its orthologues, with a spermatogenic arrest at prophase of meiosis I (Ruggiu et al. 1997; Saunders et al. 2003). This phenotype becomes more penetrant in a pure BL6/C57 background, with apoptosis observed at embryonic day 15.5 of mouse development (Lin and Page 2005). Genetic studies have associated this phenotype with an impairment of a canonical PGC feature, sex differentiation.

The nuclear and cytoplasmic localization of DAZL (Xu et al. 2001), the ability to bind mRNA (Tsui et al. 2000b; Venables et al. 2001), the association with polyribosomes (Tsui et al. 2000b) and the interaction with several known RBPs (Brook et al. 2009) suggest a role in mRNA processing. Several molecular functions have been associated with DAZL, ranging from mRNA repression (Urano et al. 2005; Padmanabhan and Richter 2006) mRNA transport (Lee et al. 2006; Kim et al. 2012), miRNA-mediated repression protection (Takeda et al. 2009), but to date the best characterized role for DAZL is in translation activation (Collier et al. 2005; Reynolds et al. 2005, 2007; Chen et al. 2011a). In vitro studies have demonstrated that DAZ family members all share a common function as translation activators. Tethering assays, where DAZL-RNA binding activity is circumvented, showed that this family upregulates translation of artificially bound luciferase reporters (Collier et al. 2005). In vivo experiments have since demonstrated translation of endogenous transcripts (Reynolds et al. 2005, 2007; Chen et al. 2011a). Insight into the mechanism of DAZ-family translation activation demonstrates that these proteins act at the step of translation initiation. A direct interaction with PABP1 (but none of the other canonical translation initiation factors) was shown to be necessary to promote translation in a reconstitution system. The observation that DAZL was able to stimulate translation of non-adenylated reporter mRNAs indicated that this can work distinctly from the canonical CPEB translation activator (Pique et al. 2008) by promoting PABP recruitment in a poly(A) independent manner (Collier et al. 2005).

DAZL interacts with transcripts with a U-rich 3′UTR (Tsui et al. 2000b). A variety of assays identified the trinucleotide GUU has the minimal binding motif of DAZL (Tsui et al. 2000b; Ruggiu and Cooke 2000; Venables et al. 2001). The crystal structure of DAZL RNA recognition motif (RRM) demonstrated that this presents the highest affinity towards GUU[U/C] sequences (Jenkins et al. 2011). The relatively short sequence of this element greatly challenges the identification of DAZL targets. In vitro evidence for the requirement of multiple DAZL binding sites for a maximal translation activation can in the future help to overcome this challenge (Collier et al. 2005; Chen et al. 2011a). Several studies have identified DAZL targets (Brook et al. 2009). In spermatogenesis, pull down experiments have demonstrated an interaction with messages involved in sperm development (Tpx1, Grsf-1, Trf-2, TssK proteins, etc.) (Jiao et al. 2002; Zeng et al. 2008). Nonetheless, to date the only two bona fide spermatogenic targets for DAZL are Sycp3 and Mvh (Reynolds et al. 2005, 2007).

4.2 Pumilio Family of RBPs

Whereas the role of Puf proteins in model organisms have been extensively investigated, comparable little information is available on the role of Pumilio proteins, Pum1 and Pum2, in spermatogenesis and oogenesis in mammals. A gene trap disruption of *Pum2* was reported to produce a decrease in testis weight and some disruption in spermatogenesis but the exact steps affected were not investigated (Xu et al. 2007). Pum1 knockout mice have decreased fertility and decreased sperm

count and 25–30 % reduction in testis weight. It has been proposed that this is due to deregulation of the apoptotic pathway in the Pum1$^{-/-}$ mice during spermatogenesis (Chen et al. 2012). Pum1-dependent repression of Map3k1 is required to maintain P53 inactive. Since Pum1 targets a large number of mRNAs in cell lines and in the testis it is most likely that Pum1 functions go well beyond P53 regulation. In model organism, Pumilios play a critical role in maintaining the germ cell fate and prevent germ cell differentiation. It may be possible that accelerated differentiation of spermatogenic cells in the Pum1 knockout model leads to unbalanced ratio/germ cell supporting cells in the testis, a condition known to trigger apoptosis and spermatogenic failure.

4.3 Deleted in Azoospermia Associated Protein 1 (DAZAP1)

DAZAP1 is a member of the heterogeneous nuclear ribonuclear protein family (hnRNP). This protein was initially identified in a study looking at DAZL interacting proteins (Tsui et al. 2000a). But despite this interaction, both proteins differ in their protein expression pattern and were described to be associated with different subsets of target mRNAs, indicating that both proteins can have different roles (Kurihara et al. 2004).

DAZAP1 is ubiquitously expressed but highly enriched in testes (Dai et al. 2001). Whereas it is predominantly found in the nucleus of somatic cells (Lin and Yen 2006), in testes, DAZAP1 shows a dynamic distribution. In germ cells DAZAP1 is found in the nucleus from mid-pachytene spermatocytes to round spermatids, and relocates to the cytoplasm in elongating spermatids (Vera et al. 2002).

As with other hnRNPs, DAZAP1 binds newly synthesized transcripts and, through processes of splicing, export and translation, controls expression of specific messages (Martinez-Contreras et al. 2007). Recent evidence further elucidated the mechanism employed by DAZAP1 in splicing control based on extracellular cues. Through interaction with hnRNPs governed by 3′UTR *cis*-elements of target transcripts, DAZAP1 promotes splicing of weak exons. Phosphorylation of the C-terminal proline rich domain of DAZAP1 by MEK/Erk pathway alters the ability of DAZAP1 to maintain protein interactions with splicing factors and induces cytoplasmic translocation of DAZAP1 (Choudhury et al. 2014).

The molecular mechanisms of translational modulation of DAZAP1 are still not fully understood. It binds RNA at a consensus sequence AAAUAG and $GU_{[1-3]}AG$ (Yang et al. 2009), and the function is dependent on the protein partners to which it binds. Protein–protein interactions have been described with DAZL, KH-type splicing regulatory protein (KHSRP), hnRNPs, DEAD box polypeptide 20 (DDX20), let 7 and many other RBPs (Yang et al. 2009). The function as a translation activator has been studied in vitro models where DAZAP1 proteins were tethered to luciferase reporters. These show that both mouse and human DAZAP, as observed for *X. laevis*, stimulates translation in a cap-independent manner and it shows preferential translation towards nonadenylated messages (Smith et al. 2011).

Interestingly, DAZAP1 is itself a target for translation control. Although initially present at mid-pachytene spermatocytes, in situ analysis of DAZAP1 detects high levels of this transcript in spermatogonia and early spermatocytes (Vera et al. 2002). Through alternative usage of cleavage and polyadenylation sites, translation of each isoform is regulated by different mechanisms. At day 12 post-partum association with polyribosomes shows that both isoforms are actively translated. Upon reaching puberty the isoform with the longest 3′UTR relocates to the translational inactive mRNP fraction with a concomitant shortening of the poly(A) tail. The smaller isoform is also recruited to the mRNP fraction but this shows no changes in poly(A) length. The germ cell specific DAZL was shown to preferentially bind in vivo to the shorter isoform of DAZAP1, and in vitro assays also described a preferential DAZL promoted translation of a reporter with the 3′UTR of the shorter isoform (Yang and Yen 2013).

4.4 Gonadotropin-Regulated Testicular RNA Helicase (GRTH)

Ddx25 gained its common name, GRTH, from the fact that translation of this protein is dependent on hormonal cues. Three consensus Kozak sequences can be identified in frame with the coding region of GRTH. Whilst the second AUG is used by Leydig cells, germ cells preferentially use the first and third start codons. Although, treatment of rats with hCG, resulted in a shift towards the second AUG by round spermatids, but not spermatocytes (Sheng et al. 2003). This translational control is believed to result from induced paracrine factors that regulate the internal ribosomal entry site mechanisms.

GRTH promotes survival of spermatocytes by regulating apoptotic pathways. In *Grth*-null models, spermatogenesis arrests at step 8 spermatids due to increased apoptosis in spermatocytes at stage XII (Tsai-Morris et al. 2008). Despite not totally understood how GRTH regulates translation, an initial mechanism was directly linked to the function of RNA helicases that control RNA unwinding and nuclear transport (Tsai-Morris et al. 2010). An association with chromatoid bodies and polyribosomes further supported a role of this protein in translation (Tang et al. 1999; Sheng et al. 2006; Tsai-Morris et al. 2004b). Animal models provided the direct link to translation control by providing evidence that decrease levels of GRTH in germ cells resulted in decreased levels of specific proteins (TNPs and tACE) with no differences observed in mRNA levels (Tsai-Morris et al. 2004a). GRTH is present in the nucleus and cytoplasm, and is coupled with nuclear export, dependent on its phosphorylation status. Depletion of GRTH resulting in smaller chromatoid bodies and a deficit in the cytoplasmic ratios of proteins associated with nuclear histone-protamine (Tsai-Morris et al. 2004b; Sheng et al. 2006).

In humans, a mutation on GRTH is associated with non-obstructive azoospermia. This mutation results in a nonphosphorilated form of GRTH, and was proposed to impair RNA-binding and protein–protein interactions due to the location of the substituted residue in a hydrophobic pocket (Tsai-Morris et al. 2007).

5 Regulation of Translation in Mammalian Oocytes

The post-natal ovary is endowed with a pool of quiescent primordial follicles. Upon primordial follicle activation, the oocyte enters a growth phase that is characterized by intense transcriptional activity (Pan et al. 2005). However not all the transcribed mRNAs are immediately translated; a fraction is stored in a translationally-repressed form. As the oocyte approaches its fully grown size, the transcriptional activity ceases (Bouniol-Baly et al. 1999; Liu and Aoki 2002) and the final stages of differentiation and maturation, as well as fertilization and early embryo development occur in absence of transcription. Thus, the meiotic cell cycle progression and genome reprogramming rely on unmasking and translation of stored maternal mRNAs. This dependence continues until the embryo genome becomes activated, an event that occurs with a timing that is species specific (2-cell stage for major embryonic genome activation (EGA) in mice Latham 1999). As mentioned, this unique control of gene expression at the oocyte-to-embryo transition likely confers developmental plasticity by regulating transcript recruitment and translation in the cytoplasm, while the chromatin is free to undergo the reprogramming required for establishing totipotency and supporting embryogenesis.

Here we will present the most relevant mechanisms involved in the regulation of maternal mRNA translation, organizing the discussion into mechanisms controlling the activation, degradation and repression during oocyte development.

5.1 Mechanisms Controlling Repression of Translation and mRNA Degradation

Our current understanding of translational regulation during oocyte growth and maturation is mainly based on findings in the *X. laevis* oocyte model. In the frog system, it has been proposed that a combinatorial code of *cis*-acting elements in the 3′UTR of the messages regulates protein synthesis according to the temporal requirement of meiosis arrest and progression, as well as early embryo development.

One of the best characterized mechanisms of translation regulation in frog oocyte requires the *cis*-acting element CPE and the cognate binding proteins CPEB. By controlling the length of the cytoplasmic poly(A) tail, Cpeb1 represses translation in immature oocytes and, when phosphorylated upon cell cycle reentry, activates translation (McGrew et al. 1989). A mammalian isoform of CPEB, with 80 % identity with the frog Cpeb, was first described in mouse oocytes as a protein bound to the *c-Mos* mRNA (Gebauer and Richter 1996). Early studies of the tissue-type plasminogen activator (tPA) mRNA provided initial evidence that maternal mRNA repression and translation in mammals are regulated similarly to frogs (Huarte et al. 1992; Stutz et al. 1997). For instance, tPA is stored in a silenced form with a short poly(A) tail (30–40 nt) in GV stage oocytes. Upon meiotic re-entry, the poly(A) tail is elongated (to about 200 nt) and tPA becomes translated (Huarte et al. 1992). The role of a CPE *cis*-acting element was soon recognized as an important player in this

regulation. By inserting a CPE or deleting an existing one, the translation is respectively repressed or activated, together with shortening or lengthening of the poly(A) tail (Huarte et al. 1992; Gebauer and Richter 1996).

How CPEB functions in repressing poly(A) elongation and translation of maternal transcripts in mammals is still not fully understood. At least three different models have been proposed in frog oocytes, depending on the composition of the repressor complex, as reviewed in (Villalba et al. 2011). One possibility is that Cpeb simultaneously recruits the poly(A)-ribonuclease Parn and the poly(A)-polymerase Gld-2. The competition between the two enzymatic activities maintains a short poly(A) tail, hence promoting repression (Kim and Richter 2006). Alternatively a repressor protein, identified as Maskin, may bind to Cpeb and eIF4E, blocking the formation of the cap-binding complex on the mRNA, resulting in translational inhibition (Stebbins-Boaz et al. 1999). Similarly, a third model predicts that Cpeb bind to Clast 4 (also known as 4E-transporter (4E-T)), which in turn recruits isoforms of eIF4E, like eIF4E1b, that have low affinity for the cap structure (Minshall et al. 2007).

Rather than being mutually exclusive, these models may be integrated in the control of translational repression of different mRNA species or during different steps of oogenesis. Moreover extra layers of complexity are achieved through multiple regulatory *cis* and *trans*-acting factors. For instance additional regulation can be mediated by Pumilio binding elements (PBEs) that are frequently present in mRNAs that possess CPEs (Ota et al. 2011). As previously discussed in model organisms, Pumilio proteins (PUM1, PUM2) are mainly repressor proteins, but they can also stabilize CPEB binding and promote poly(A) tail elongation in some context (Padmanabhan and Richter 2006; Pique et al. 2008). However their role in mammalian oogenesis has not been elucidated yet. Interestingly, it has been reported that Pum1 protein in zebrafish oocytes is required to localize cyclin B1 to ribonuclear particles and this co-localization was confirmed in mouse oocytes (Kotani et al. 2013).

Another *cis*-acting element known to repress translation in immature oocytes is the translational control sequence (TCS). The corresponding *trans*-acting factor has been recently discovered in *X. leavis* and it is represented by zygote arrest 1 (Zar1) and Zar2. Zar1 and Zar2 bind to *Mos* and *Wee1* mRNA 3′UTRs via a zinc finger. The TCS-mediated repression of translation is exerted during oocyte growth and initial phases of meiotic resumption until the metaphase I (MI) stage (Charlesworth et al. 2012; Yamamoto et al. 2013). It is not clear if the translational activation of Zar2 target mRNAs occurs following partial degradation of Zar2 and release of the repressive state, or if the control requires more than one *trans*-acting factor, as proposed for CPE. Based on the observation that mice embryos that are null for *Zar1* arrest at the 1-cell stage due to failure in EGA, mammalian ZAR family proteins were initially proposed to be transcriptional regulators (Wu et al. 2003). However, a role for ZAR proteins in translational control in mammalian oocytes cannot be excluded. In this view, protein product(s) of ZAR-target mRNAs would be responsible of EGA, and the absence of ZAR would prevent their timely translation with subsequent failure in embryonic transcription and development.

In *X. laevis* oocytes CPE-induced translation is responsible for activating synthesis of RBPs, like the zinc finger protein C3H-4, that are required in the later stages of maturation for deadenylation of transcripts carrying A-U rich elements (AREs) (Belloc and Mendez 2008). In mammals, the embryonic lethal abnormal vision like 2 (ELAVL2) protein regulates ARE-mediated mRNA repression during oocyte growth (Chalupnikova et al. 2014). ELAVL2 decreased in abundance as the oocyte reaches a fully grown, meiotically competent stage of development, which is characterized by the transition from the non-surrounded nucleolus (NSN) chromatin configuration into the transcriptionally quiescent surrounded nucleolus (SN) configuration. ELAVL2 is absent in MII oocytes and zygotes. Mammalian proteins that function analogous to the frog C3H-4 protein to mediate ARE dependent deadenylation and repression during oocyte maturation have not been identified.

A regulatory feedback involving CPE-mediated translation to recruit *decapping mRNA1* and *2* (*Dcp1a* and *Dpc2*) has been recently described in mice (Ma et al. 2013). Decapping, i.e. the removal of the 5′ 7-methyl-guanosine cap, exposes mRNAs to exonucleases and represents a critical step in the 5′→3′ transcript degradation. Both the regulatory and catalytic subunits, DCP1A and DCP2, are synthesized between MI and MII and the inhibition of their accumulation interferes with the proper EGA execution. It is not clear at this point whether and how the DCP1A/DCP2-mediated degradation could be selective. One of the possibilities is that their involvement is the downstream event in a multi-step process that first requires mRNAs to be destabilized and tagged for degradation by sequence specific regulatory factors.

Destabilization can occur through deadenylation (Wu and Brewer 2012) and CDC2A-mediated phosphorylation of the Y box binding protein 2 (YBX2, also known as MSY2), an RBP required for oocyte mRNA stability (Medvedev et al. 2008, 2011). Furthermore, deadenylation does not necessarily target the mRNA for immediate degradation, but it can simply induce a translationally-repressed state. In support of this hypothesis in *X. laevis* oocytes deadenylation can be dissociated from decapping (Gillian-Daniel et al. 1998). In mice oocytes, a subset of messages that are not recruited to the polysome in metaphase II (MII) are degraded, whereas another subset exits the polysome fraction while remaining stable (Chen et al. 2011a). It would be interesting to investigate whether these transcripts are decapped, have a shorter poly(A) tail and/or a YBX2 motif.

Transcript profiling of oocytes and embryos represents one of the most informative approaches to monitor transcript stabilization or degradation. Using a system for oocyte transcript amplification not biased toward polyadenylated mRNA species and microarray approach, Su and co-Authors characterized the extent of transcript degradation during mice oocyte maturation by comparing fully grown—germinal vesicle (GV) stage oocytes and MII stage oocytes (Su et al. 2007). Selective degradation was found during oocyte maturation, targeting transcripts involved in oxidative phosphorylation, pyruvate and citrate metabolism and macromolecule synthesis, while transcripts involved in signal transduction were amongst the more stable. Degradation of the transcripts coding for ribosomal proteins was also a major

component in the culling of maternal mRNAs. However, a limitation to this approach is that it cannot provide information on protein synthesis.

5.2 Mechanisms Controlling Activation of Translation during Oocyte Maturation

A survey of the genome-wide pattern of translation was conducted by profiling maternal mRNAs recruited to the polysomes after centrifugation on sucrose cushions or density gradients. The isolation of the polysome fraction allowed for a direct analysis of mRNAs undergoing translation at precise time points during oocyte maturation (Chen et al. 2011a). Using this polysome array approach, stereotypic pattern of maternal mRNA association with the polysome was identified during progression through the meiotic cell cycle. A similar approach has been applied to study the changes in translation at the oocyte to embryo transition, revealing that around 2000 transcripts are differentially translated between MII stage oocytes and 1-cell embryos (Potireddy et al. 2006). Maternal mRNAs fall into three distinct patterns of polysome recruitment during oocyte maturation: Class I, the majority, constitutively recruited to the polysome independently of the meiotic stage; Class II, approximately 20 % of the transcripts, associated to the polysome only at the GV stage and dissociating thereafter; Class III, another 20 %, recruited for translation during the transition to MII oocytes. In addition to cell cycle components, this last class of transcripts was mainly represented by transcription and chromatin remodeling related transcripts, indicating that the machinery used for nuclear reprogramming and transcriptional activation in the zygote is assembled earlier on. This finding explains the observation that somatic nuclear reprogramming is more efficiently supported by the cytoplasm of MII oocytes rather than GV oocytes, even though critical factors removed with the GV of the oocyte may be an additional possibility. Specific motifs for RBPs were also identified by bioinformatics analysis in the 3′UTRs of Class III transcripts, providing mechanistic insights on the regulation of translation at defined times during oocytes maturation (Chen et al. 2011a). In both these genome-wide studies the most abundant motif identified in the 3′UTRs of transcripts activated in MII stage oocytes was the CPE (Potireddy et al. 2006; Chen et al. 2011a).

As described above, CPE plays a major role in driving the activation of translation by promoting the elongation of the poly(A) tail. The CPE consensus sequence (A)UUUUA(A)U is usually located within 100 nt upstream the nuclear polyadenylation signal AAUAAA (Hex) (Pique et al. 2008) which binds the cleavage and polyadenylation specificity factor (CPSF) (Sheets et al. 1994). In *X. laevis* Cpeb is phosphorylated by the kinase Aurora A in response to progesterone (Mendez et al. 2000a). This post-translational modification increases Cpeb affinity for Cpsf (Mendez et al. 2000b) and Gld-2 (Barnard et al. 2004), while decreasing the affinity for Parn (Kim and Richter 2006). Other events required for Cpeb-promoted polyadenylation and activation of translation are the displacement of Maskin (Stebbins-Boaz et al. 1999) and/or 4E-T (Minshall et al. 2007), to allow the recruitment to the

40S ribosomal subunit. Also in mouse oocytes CPEB1 phosphorylation seems to be a crucial player in controlling maternal transcript translation upon meiosis resumption (Chen et al. 2011a) but the exact sequence of events has not been further elucidated and some differences with the frog should be pointed out: (1) *de novo* protein synthesis is essential in frog for meiosis re-entry, while in mice oocytes it is only required for the progression from MI to MII (Hashimoto and Kishimoto 1988); (2) polyadenylation and activation of translation occur at MI in mice, whereas they precede the GV breakdown (GVBD) in frog (McGrew et al. 1989); (3) the kinase responsible for CPEB phosphorylation has not been identified in mammals and is likely not Aurora A (Andresson and Ruderman 1998; Mendez et al. 2000a, b); (4) the homologue of frog Maskin, transforming acidic coiled coil containing protein (TACC3), does not have a domain for interaction with CPEB (Barnard et al. 2005). Given these possible species differences, further studies are needed to elucidate CPEB function in mouse oocytes maturation.

In *Cpeb* null mice, the development of normal adult gametes and gonads are precluded, likely due to a defect in the translation of synaptonemal complex protein mRNAs that prevents the transition from the Pachytene to the Diplotene stage (Tay and Richter 2001; Tay et al. 2003). Consequently loss of function models cannot be used to elucidate the role of CPEB during oocyte maturation. Loss of function experiments have been conducted by morpholino oligonucleotides injection, showing that CPEB1 depletion, but not CPEB3 and 4, decreases the efficiency of progression through meiosis (Chen et al. 2011a). However, the extent of depletion of these proteins from the oocyte after morpholino microinjection could not be determined.

After phosphorylation, CPEB1 is targeted for degradation. The degradation of CPEB1 at the time of metaphase I (MI) and the identification of several other RNA motifs that correlate with recruitment to polysomes indicates that CPEB independent mechanisms also contribute to generate temporal pattern of translation throughout maturation. In frog oocytes, it has been proposed that Cpeb1 induces the translation of Cpeb4, a related RBP but with slightly different properties (Igea and Mendez 2010). The accumulation of this RBP which binds to a CPE element stabilizes CPE-dependent polyadenylation and translation. It is most likely that a similar mechanism operates in mammalian oocytes. As reported above, a first attempt to define the role of CPEB3 and CPEB4 using morpholino oligonucleotide knockdown and extrusion of the polar body as readout produced minimal disruption. However, other RBPs may be involved in this kind of sequential regulation. One of the motifs identified in the 3′UTRs of transcripts actively translated during oocyte maturation is represented by the consensus sequences for the DAZ family of proteins (Daz, Dazl, and Boule). Through its CPE, *Dazl* transcript is recruited to polysomes and DAZL protein increases fivefold, reaching a maximum in MII. In addition to the CPE, *Dazl* also contains DAZL consensus sequences. The newly synthesized DAZL therefore increases translation of its own mRNA, establishing a self-reinforcing, positive feedback loop (Chen et al. 2011a). It is most likely that DAZL promotes the translation of several transcripts coding for cell cycle

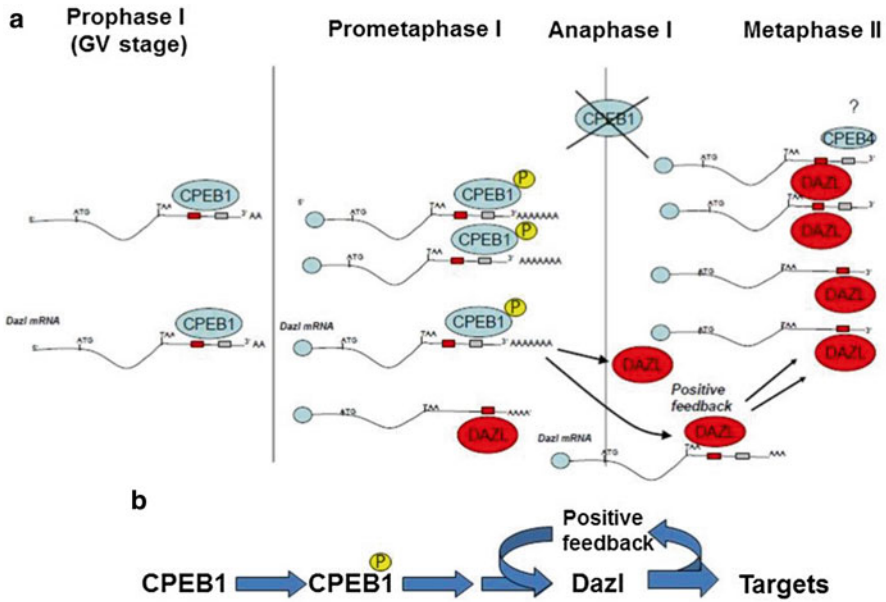

Fig. 7.2 Proposed model for the regulation of translation during mouse oocyte maturation. (**a**) Class III mRNAs including *Dazl* mRNA are represented in a repressed translational state at Prophase I of meiosis (GV stage) with CPEB bound to them and a short poly(A) tail. Upon progression through Prometaphase I and CPEB1 phosphorylation, Class III mRNAs, including *Dazl mRNA,* undergo poly(A) elongation and become actively translated. The increase in DAZL protein promotes a positive feedback auto-regulatory loop which amplifies *Dazl* mRNA translation. This positive feedback plays a role in prolonging an activated state of translation once CPEB1 is degraded at the Anaphase I. The CPEB1-dependent activation of Dazl translation may be complementary to other similar regulations, as a CPEB1-dependent translation of CPEB4 in the late stages of oocyte maturation has been reported in frog oocytes (Igea and Mendez 2010). (**b**) CPEB1 phosphorylation triggers DAZL translation and installs the positive feedback loop that promotes translation of DAZL targets (adapted from Chen et al. 2011)

regulators and chromatin remodelers and its depletion causes disruption of the spindle assembly (Fig. 7.2).

Because Pumilio Binding Elements have been identified between the motifs enriched in transcripts recruited to the polysomes in MII (Chen et al. 2011a), the RBPs Pumilio 1 and 2 may participate to translational activation, possibly by stabilizing the CPEB-mediated polyadenylation, as described in *X. laevis* oocytes (Padmanabhan and Richter 2006).

Early polyadenylation events and activation of translation have been described in *X. laevis* oocytes in a CPE-independent manner. This is the case for instance of the *Mos* mRNA, which is controlled by binding of Musashi to the polyadenylation response element (PRE) on the 3′UTR (Charlesworth et al. 2002). Synthesis of Mos is necessary to induce the activation of mitogen-activated protein kinase (MAPK) that occurs upstream (and is needed for) CPE-directed translation (Charlesworth et al. 2006). The Musashi-CPEB progressive activation is another example of how

the temporal control is exerted on translation to secure the proper progression through meiosis. Musashi is expressed in mammalian cells and, while it is usually associated with translational repression, it acts as an activator of translation in some conditions (MacNicol et al. 2011). Whether Musashi may have a similar function in mammalian oocyte is an open question.

Cell cycle progression and genome reprogramming strictly rely on the coordinated translation of stored maternal mRNAs at the oocyte to zygote transition. Consequently the proper execution of the translational program is a key factor for a successful embryo development. Rather than being completely cell autonomous, meiotic progression and translational activation are modulated by environmental inputs acting through the activation of the follicular EGF network (Chen et al. 2013). These somatic signals activate the PI(3)K-AKT-mTOR pathway in the oocyte soon after GVBD and promote translation of a subset of maternal mRNAs critical for the onset of the oocyte developmental competence (Fig. 7.3).

In this chapter, we have explored the extensive post-transcriptional regulatory factors and mechanisms that control development of the germline, integration of somatic signals, differentiation of gametes, and the earliest stages of embryonic development. Clearly there is a great deal yet to be learned, especially in mammalian reproductive systems.

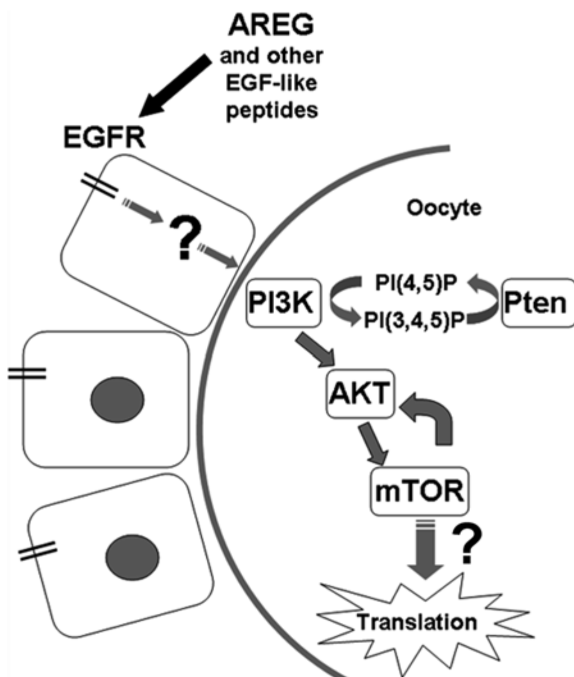

Fig. 7.3 Proposed model of the signaling pathway involved in somatic cell control of translation in the oocyte. The scheme represents an oocyte surrounded by its cumulus cells. Amphiregulin and other EGF-like growth factors, released in response to the LH surge, activate EGF receptors expressed on cumulus cells. Through the activation on undefined signaling pathway(s) across the cumulus and oocyte plasma membranes, PI(3)K is activated in the oocyte. The resulting phosphorylation of AKT and mTOR activation leads to an increase in translation of a subset of maternal mRNAs

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Post-transcriptional Regulation of VEGF-A

8

Hervé Prats and Christian Touriol

1 Introduction

Vascular Endothelial Growth Factor A (VEGF-A) is a protein of critical importance in embryonic development and during adulthood. Even a slight variation in its normal level can have serious physiological consequences and as a result its expression is stringently regulated at every conceivable stage. Thus, the expression of VEGF-A represents a useful model that demonstrates gene regulation at its most sophisticated.

VEGF-A acts on endothelial cells where it is required for cell survival, growth and migration. It is the main protein involved in the induction of angiogenesis, the formation of new blood vessels, and is therefore essential for adult organ growth and repair. VEGF-A plays an important role in physiological situations that affect blood vessels, such as the menstrual cycle, wound repair, adaptation to hypoxia and, importantly, embryonic development (Carmeliet 2005a; Ferrara 2005). Various pathological conditions also show elevated VEGF-A levels, such as proliferative retinopathies, arthritis, psoriasis and cancer (Ferrara 1999, 2002; Folkman 1995). In cancer, VEGF-A is crucial for tumor development since it stimulates the growth of new blood vessels from nearby capillaries (tumor angiogenesis), which allows tumor cells to acquire oxygen and nutrients and ultimately leads to metastasis.

The importance of keeping tight control of VEGF-A expression has been clearly demonstrated in transgenic mice. Both the deletion of a single VEGF-A allele or the modest overexpression of VEGF-A result in defective vascularization and subsequent embryonic lethality (Carmeliet et al. 1996; Ferrara et al. 1996;

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Miquerol et al. 2000). In addition, conditional gain and loss of function experiments in the erythroid lineage have demonstrated that alteration of VEGF-A levels during development significantly affects erythropoiesis in mouse embryos (Drogat et al. 2010). These studies emphasize the critical role of VEGF-A in developmental angiogenesis. In adult mice, tissue-specific overexpression of VEGF-A causes serious problems in processes such as angiogenesis and vascular hyperpermeability (Larcher et al. 1998), angioma formation and organ development (Dor et al. 2002), and can lead to severe proliferative retinopathy and retinal detachment (Ohno-Matsui et al. 2002). These results have been corroborated by conditional transgenic knock-out models of VEGF-A which also show impaired vascular functions (Eremina et al. 2008; Sivaskandarajah et al. 2011).

There are nine different isoforms of VEGF-A which execute their roles through binding to one of two tyrosine kinase receptors, VEGFR1 and VEGFR2 (Carmeliet 2005b; Ferrara et al. 2003). Each isoform is thought to have specific roles depending on its level of expression, and the same isoform can exert different effects at distinct anatomical sites. Expression of the VEGF-A isoforms is regulated at the transcriptional level but it is the post-translational level that provides the greatest display of regulatory diversity, involving mRNA stabilization, alternative splicing and translational regulation mechanisms.

Given that this level of complexity is possible from just one gene is impressive, and shows off the elaborate nature of gene regulation. It is for this reason that VEGF-A is used as a paradigm for the intricate regulation of gene expression, particularly at the post-transcriptional level.

2 VEGF-A Transcriptional Regulation

The human *VEGFA* gene is approximately 14 kb in length and is located on chromosome 6 at position 6p21.1 (Vincenti et al. 1996). Figure 8.1 shows the structure of the gene, which is composed of eight exons and seven introns (Tischer et al. 1991). In humans, the main *VEGFA* promoter sequence spans 2.36 kb (Buteau-Lozano et al. 2002) but the promoters for the mouse and rat genes are only 1.2 kb long (Levy et al. 1995; Shima et al. 1996). The human promoter contains several consensus-binding sites for transcriptional regulators such as AP1, AP2 and Sp1, all of which are themselves regulated by growth factors, cytokines, hormones, tumour suppressor genes and oncogenes. A hypoxia response element (HRE) is also present within the 5′ flanking region, but interestingly the VEGF-A promoter does not contain a consensus TATA box (reviewed in Pages and Pouyssegur 2005). An alternative promoter has also been found within the human *VEGF-A* 5′UTR (Akiri et al. 1998), with a transcription start site located 633 nucleotides downstream of the main starting site (Fig. 8.1). Unlike the main promoter this alternative promoter is insensitive to hypoxia, suggesting that there is no cross-regulation with the main promoter region. This therefore offers the potential of regulation under distinct conditions and different spatiotemporal expression patterns (Akiri et al. 1998).

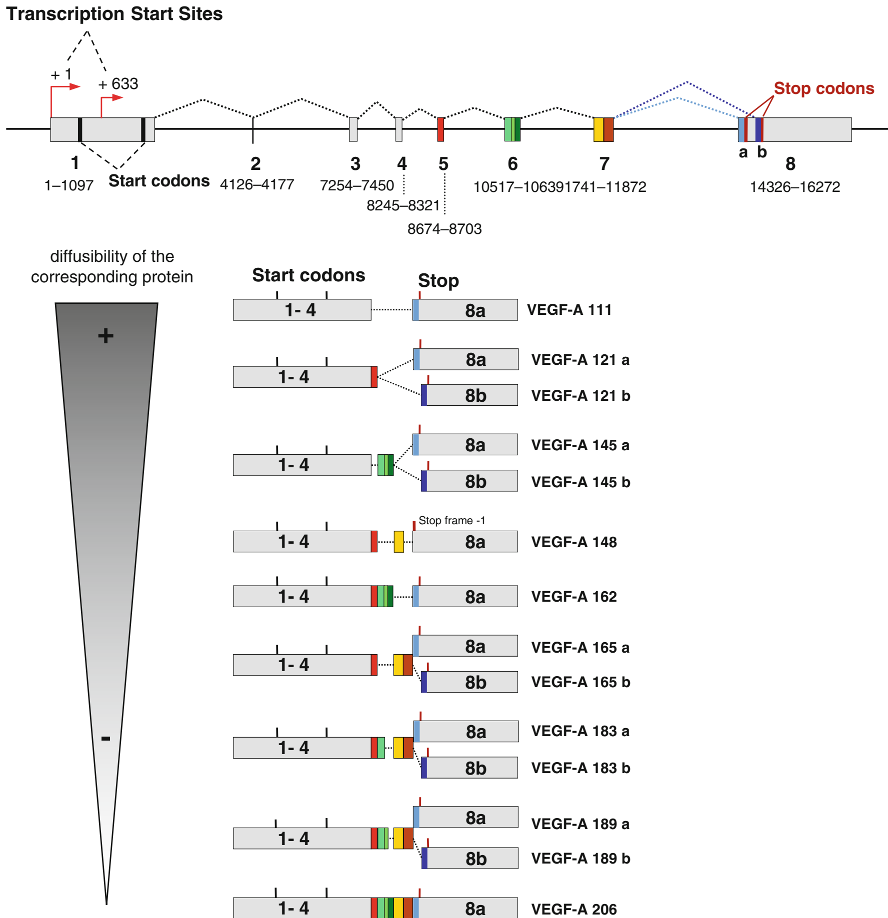

Fig. 8.1 Human *VEGFA* gene structure and exon composition of the isoforms generated by alternative splicing. (*Top*) The human *VEGFA* gene spans 16,272 bp of chromosome 6p21.1 and consists of eight exons and seven introns. In exon 1 *red arrows* show the start of the two promoter sites and *black lines* show the main translation start sites (AUG codons). In exon 8 the two alternative stop codons are indicated in *red*. (*Bottom*) Exon content of all known VEGF-A isoforms which are named according to the number of amino acids in the human protein. All isoforms contain exons 1–4 and one of two versions of exon 8 (8a or 8b) depending on which terminal splice site is used. Splice site 8a produces the VEGF-Axxx (or xxxa) pro-angiogenic isoforms and 8b produces the VEGF-Axxxb anti-angiogenic isoforms. Exons 6 and 7 encode heparin-binding domains so affect the diffusibility as an inverse relationship with their affinity for the extra cellular matrix

The most widely-studied physiological stimulus causing transcriptional up-regulation of the *VEGFA* gene is hypoxia. Hypoxic conditions initiate angiogenesis via VEGF-A throughout embryogenesis and during cancer progression. It is now well-established that the Hypoxia Inducible Factor (HIF) transcriptional activators are key mediators of the hypoxic response and the pathways leading to their activation

are well-characterised. Both HIF-1 and HIF-2 bind to the VEGF-A hypoxia response element (HRE) (Forsythe et al. 1996; Blancher et al. 2000).

Since its discovery in 1989, the transcriptional regulation of VEGF-A has been extensively studied and a wide range of factors are known to be at play. However, more recent research has focused on the post-transcriptional regulation of VEGF-A, and this next stage of regulation has proven to provide yet more complexity.

3 VEGF-A Post-transcriptional Regulation

3.1 Alternative Splicing

The splicing process provides an opportunity for regulation of both the final protein structure and the level of protein expression. Different combinations of exons can be retained or removed to create a diverse array of mature mRNAs from the single pre-mRNA. In addition, alternative splicing within the non-coding regions of the mRNA controls the final number of regulatory elements such as translation enhancers or RNA stability domains that can significantly affect protein expression levels.

Through alternative splicing, nine isoforms of VEGF-A have been first found which are named according to the total number of amino acids in the mature proteins: 111, 121, 145, 148, 162, 165, 183, 189 and 206. In addition, some of these isoforms exist in VEGF-Axxxa or VEGF-Axxxb versions (Fig. 8.1). The *VEGF-A* gene contains eight exons. 1–4 are constitutive, being present in all isoforms, with exon 1 encoding an N-terminal signal of 26 hydrophobic amino acids that is typical of secreted proteins. Exons 5–8 are alternatively spliced to produce the different sized isoforms, with exons 6 and 7 containing basic residues that confer an affinity for heparin-binding. Heparin sulphate proteoglycans are present at the cell surface and within the extracellular matrix, thus the presence or absence of exons 6 and 7 affects the ability of that secreted VEGF-A isoform to diffuse away from the cell. In this way the presence or absence of exons 6 and/or 7 controls the spatial distribution and bioavailability of the VEGF-A isoforms (Vempati et al. 2011). As an example, VEGF-A121 lacks exons 6 and 7 thus does not bind heparin and is free to diffuse away after being released from the cell (Fig. 8.1). In contrast the VEGF-A165 and 189 isoforms are able to bind to heparin sulfate on the cell surface and in the extracellular matrix (Houck et al. 1992). These different heparin binding affinities result in the formation of a VEGF-A gradient which is essential for the process of angiogenesis, with soluble isoforms acting at distal sites to promote vascular recruitment and the extracellular membrane-associated isoforms acting locally to promote the expansion of capillary beds (Grunstein et al. 2000). The fact that an identical isoform can have distinct activities at different anatomical sites suggests that the microenvironment of individual tissues dictates VEGF-A function (Guo et al. 2001).

The C-terminus of VEGF-A is encoded by exon 8 and contains an alternative 3′ splice site that gives rise to the VEGF-Axxxa or VEGF-Axxxb versions of the isoform. Even if there is still controversy regarding the existence of the VEGF-Axxxb isoforms (Harris et al. 2012), in vitro and in vivo studies have demonstrated that the

VEGF-Axxx (or -xxxa) isoforms are pro-angiogenic and are up-regulated in tumours whereas the VEGF-Axxxb isoforms are anti-angiogenic and are down-regulated in tumours (Bates et al. 2002; Woolard et al. 2004; Pritchard-Jones et al. 2007). To mediate their anti-angiogenic properties, the VEGF-Axxxb isoforms are thought to bind to the VEGF-A receptors but impair their downstream signaling (Woolard et al. 2004; Cebe Suarez et al. 2006), since when the VEGF-Axxxb and -xxx isoforms were co-expressed they acted in a dominant negative way and only partial receptor agonist activity was detected. This also suggests that the inhibitory function of the xxxb isoforms is mediated through competitive binding (Kawamura et al. 2008; Woolard et al. 2009). Further evidence for this has been found using recombinant human VEGF-A165b which possesses similar affinity towards the anti-Vascular Endothelial Growth Factor antibody (bevacizumab) as that of VEGF-A165, supporting the idea of inhibition through competitive binding. Interestingly, this also suggests that the balance between the expression of the anti-angiogenic and pro-angiogenic isoforms could regulate tumour growth and in the same way the anti-angiogenic xxxb isoforms could affect the sensitivity of tumours to bevacizumab through competitive binding (Varey et al. 2008). It was also demonstrated that the balance between the expression of the anti and pro-angiogenic isoforms could regulate follicle development (McFee et al. 2012), spermatogonial stem cell homeostasis in vivo (Caires et al. 2012) but also plays a critical role in the regulation of glomerular permeability (Oltean et al. 2012).

Recombinant human VEGF-A165b shows anti-angiogenic activity in eye hypoxia-driven angiogenesis (Konopatskaya et al. 2006). In addition, recombinant human VEGF-A165b possesses a similar affinity towards the anti-VEGF antibody (bevacizumab) as that of VEGF-A165, supporting the idea of inhibition through competitive binding. Interestingly, this also suggests that the balance between the expression of the anti-angiogenic and pro-angiogenic isoforms could regulate tumour growth and in the same way the anti-angiogenic xxxb isoforms could affect the sensitivity of tumours to bevacizumab through competitive binding (Varey et al. 2008).

The distal splice site used to generate the VEGF-Axxxb isoforms is 66 nucleotides further along the gene from the proximal splice site (Figs. 8.1 and 8.2) and results in a Ser-Leu-Thr-Arg-Lys-Asp C-terminal (Bates et al. 2002). This is very different from the Cys-Asp-Lys-Pro-Arg-Arg C-terminal generated by the proximal splice site in VEGF-Axxx isoforms and undoubtedly results in a distinct tertiary structure of the VEGF-Axxxb isoforms since they contain an acidic residue (Asp) in place of the Cys-160 present in the VEGF-Axxx isoforms, which forms a disulphide bond with the Cys-146 from exon 7. This was clearly proven for VEGF-A165 when the highly-charged C-terminal Pro-Arg-Arg tail of the VEGF-A165 isoform was replaced by the neutral Arg-Lys-Asp of the VEGF-A165b isoform and resulted in a profound alteration of the structure-function relationship of VEGF-A (Cui et al. 2004). VEGF-Axxxb isoforms have been identified for VEGF-A121, VEGF-A183, VEGF-A145, VEGF-165 (Perrin et al. 2005) and VEGF-A189 (Miller-Kasprzak and Jagodzinski 2008). VEGF-A165b was the first xxxb isoform identified and it is the most widely-studied (Bates et al. 2002). Its over-expression inhibits the growth of prostate carcinoma, Ewing's sarcoma and renal cell carcinoma in xenografted mouse

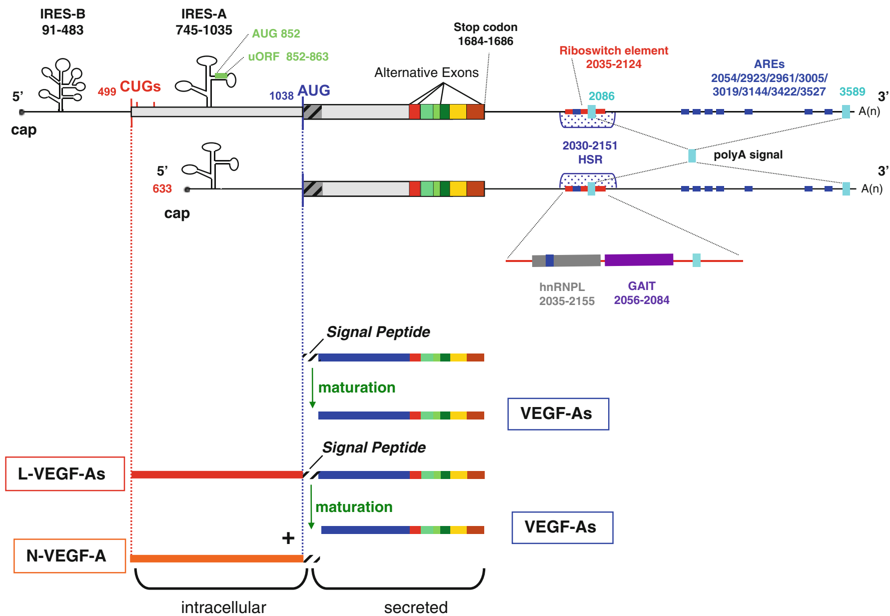

Fig. 8.2 Human VEGF-A mRNAs and their major regulatory elements. (*Top*) Schematic of VEGF-A mRNA transcribed from the two alternative promoters. Regulatory elements in the untranslated regions (UTR) and the coding region are indicated; positions are numbered according to the mRNA sequence of the 5′ end of VEGF-A189. In the 5′UTR, two AUG start codons and three non-canonical CUG start codons are shown. IRES-B: internal ribosome entry site B, which controls transcription initiation from the CUG start codon. The IRES-A controls transcription initiation from the AUG start codons. uORF: upstream open reading frame, located in the IRES-A. In the 3′UTR, the two alternative polyadenylation sites are shown (polyA signal). *AREs* AU rich elements, *HSR* hypoxia stability region. The area around the riboswitch element has been expanded to show the binding sites for hnRNPL and the GAIT translation complex, which compete with each other for binding to this element in the regulation of oxidative stress. (*Bottom*) Initiation of translation at the CUG start codon produces the L-VEGF-A isoforms. These can be cleaved during the maturation process to yield an intracellular N-VEGF-A product and the mature secreted VEGF-A isoform that resembles the VEGF-A isoforms translated from the AUG start codon

tumor models (Rennel et al. 2008) and inhibits tumor cell-mediated migration and the proliferation of endothelial cells (Rennel et al. 2008). Interestingly, mammary alveolar development during lactation is also inhibited by VEGF-A165b (Qiu et al. 2008) and endogenous VEGF-A165b contributes to survival of trophoblasts exposed to lower oxygen tensions through an autocrine pathway (Bills et al. 2014).

Although many VEGF-A isoforms have now been identified, most VEGF-A-producing cells are thought to preferentially express VEGF-A121, VEGF-A165 and VEGF-A189. These are known as VEGF-A120, 164 and 188 in the mouse since there is one less amino acid in each mouse VEGF-A isoform. To clearly demonstrate the crucial role of alternative splicing in the regulation of VEGF-A activity, and to investigate the specific roles of the individual isoforms, transgenic mice have been generated that express only a single VEGF-A isoform. Mouse embryos

expressing only VEGF-A120 (*VEGFA120/120*) show impaired post-natal cardiac angiogenesis, resulting in severe myocardial ischemia, early post-natal death and impaired lung vascular development (Mattot et al. 2002). Half of the embryos die in the perinatal period due to congenital birth defects, with the other half perishing within 2 weeks after birth, in part due to myocardial ischemia (Carmeliet et al. 1999). Similar studies with mice expressing only VEGF-A188 (*VEGFA188/188*) also show significant effects, with half of all embryos dying between embryonic stage E9.5 and E13.5 (Stalmans et al. 2002). Interestingly, mice expressing only VEGF-A164 (*VEGFA164/164*) are healthy (Stalmans et al. 2002).

Together these transgenic mouse studies suggest that different alternatively spliced isoforms are required at different stages in normal development. Other findings support this since it has been shown that different isoforms are expressed in distinct spatio-temporal patterns both during embryonic development and in adult tissues (Ng et al. 2001). However, it is also known that there is some functional redundancy between isoforms, for example during the initial formation of arch arteries (Stalmans et al. 2003).

The importance of alternative splicing of VEGF-A pre-mRNA in terms of VEGF-A regulation is now well-established. Despite this, very little is known about the mechanisms regulating alternative splicing and thus how the cell controls the levels of the different VEGF-A isoforms. Exonic splicing enhancer/silencer sequences are sequences of DNA that promote/reduce the recognition and use of splice sites. An exonic silencer sequence (ESS) has been proposed to be present in exon 6 of VEGF-A, but the regulatory proteins interacting with this sequence remain unknown (Wang et al. 2009). In the alternative splicing process, Serine/Arginine rich proteins (SR proteins) also play a key role in identifying splice sites. Under hypoxic conditions the expression and phosphorylation of the SR proteins ASF/SF2, SRp20 and SRp40 were shown to correlate with increased VEGF-A expression and a shift towards expression of the VEGF-A121 isoform in the endometrial cancer cell line RL95 (Elias and Dias 2008). Increased expression of VEGF-A121 over the longer isoforms is known to be mediated by two splicing factors of the U2AF65 protein family, namely CAPERalpha and CAPERbeta (Dowhan et al. 2005). SR proteins have also been implicated in the alternative splicing of exon 8, with the ASF/SF2 and SRp40 proteins favoring the use of the pro-angiogenic proximal splice-site, and the SRp55 protein promoting the use of the anti-angiogenic distal splice-site (Nowak et al. 2008). However, other factors are also known to play a role in the pro-/anti-angiogenic decision: the E2F1 transcription factor has been shown to favor the expression of anti-angiogenic VEGF-Axxxb isoforms through a mechanism involving the expression of the splicing factor SC35 (Merdzhanova et al. 2010), and now a link between the Wilms' tumour suppressor gene (WT1) and the regulation of VEGF-A pro-/anti-angiogenic alternative splicing has been established (Amin et al. 2011). WT1 binds to the promoter region of SRPK1 (Serine/Arginine-rich protein-specific kinase 1), a kinase that regulates the activity of SR proteins. This decreases the expression of the kinase, resulting in decreased AFS/SF2 phosphorylation and nuclear localization, and an increase in expression of anti-angiogenic VEGF-A165b. Conversely, mutation of the WT1 tumor suppressor gene

results in ASF/SF2 hyperphosphorylation and expression of pro-angiogenic VEGF-A isoforms (Amin et al. 2011). These data suggest that tumors lacking functional WT1 or containing enhanced SRPK1 expression may control angiogenesis through the regulation of alternative splicing of VEGF-A.

There is no doubt that our understanding of the mechanisms regulating alternative splicing is still in its infancy. A recent study proved that the alternative splicing field still has many more doors to open when it described a novel VEGF-A isoform in the lung tissue of a legally aborted female fetus. This alternatively spliced isoform possessed a 20 bases insertion in the third intron, inducing a frame shift mutation that introduced a stop codon in the middle of the fourth exon (Zhou et al. 2012). The mechanism involved in this splicing regulation and whether this alteration impact VEGF-A protein structure and/or function is still unknown but this discovery highlights the discrepancies in our understanding of this important process.

Reinforcing this biological control, a VEGF-A stop-codon readthrough mechanism has been recently discovered that leads to a 22 amino-acids extension generating new antiangiogenic isoforms (Eswarappa et al. 2014).

3.2 mRNA Stability

In cells different mRNAs have very different stabilities. The more stable an mRNA is the longer its lifetime and hence the more protein is produced from it. Short mRNA half-lives allow cells to alter protein synthesis rapidly when necessary (for example in response to environmental changes such as nutrient levels, cytokines, hormones or stress) and in this way the control of mRNA stability is an important regulator of protein expression (Mitchell and Tollervey 2000; Shim and Karin 2002).

VEGF-A mRNA is highly unstable under normal oxygen and nutrient conditions with a half-life of 15–40 min in vitro (Dibbens et al. 1999; Ikeda et al. 1995; Levy et al. 1996; Shima et al. 1995). Hypoxic conditions induce VEGF-A expression by increasing mRNA stability through a regulatory process that is independent of HIF1A-induced transcription (Ryan et al. 2000). Like most mRNAs with a short half-life, VEGF-A mRNA contains many AU-rich elements (AREs) which are well-known cis-sequences generally composed of different nonameric (AUUUA) or pentameric (AUUUAUUUA) consensus or U-rich sequences. They increase the rate of degradation of the mRNA and so by blocking or promoting access to these sites the cell can control the stability of the mRNA (Barreau et al. 2005; Gingerich et al. 2004). The VEGF-A AREs are clustered in the 3′UTR section of VEGF-A mRNA (Fig. 8.2). In humans, the stability of VEGF-A mRNA under hypoxic conditions is mediated through the 3′UTR (Claffey et al. 1998; Goldberg-Cohen et al. 2002; Levy et al. 1998), therefore research on the control of VEGF-A expression in response to hypoxia has focused on ARE-binding proteins. Interestingly, it has been shown that degradation of mouse VEGF-A mRNA under normoxic conditions requires the independent action of destabilizing elements from across the mRNA (in the 3′UTR, the coding region and the 5′UTR). In contrast to human VEGF-A mRNA, under hypoxic conditions mouse VEGF-A mRNA requires all three sections and these

coordinate to stabilize the mRNA (Dibbens et al. 1999). In humans a number of RNA-binding proteins have been shown to interact with the 3′-UTR elements of VEGF-A mRNA and increase its stability including: the CSD/PTB complex (Cold Shock Domain/Polypyrimidine Tract Binding Protein) (Coles et al. 2004); MDM2, a protein translocated from the nucleus to the cytoplasm under hypoxic conditions (Zhou et al. 2011); hnRNPL, which associates with hnRNP complexes to promote mRNA formation, processing and packaging (Shih and Claffey 1999); the double strand RNA binding protein DRBP76/NF90, which under hypoxic conditions facilitates VEGF-A expression by promoting mRNA loading onto polysomes and translation (Vumbaca et al. 2008); HSP70, which was found to bind and stabilize VEGF-A mRNA through a mechanism independent of its chaperone function (Kishor et al. 2012); and HuR (Levy 1998). HuR is a member of the ELAV protein family, which also includes the Hel-N1, HuC and HuD proteins (Fan and Steitz 1998; King 2000; Levine et al. 1993; Peng et al. 1998). ELAV proteins bind to AREs and are thought to regulate the stability of mRNA from splicing through to translation by generating multimeric "ribonucleosomes" due to their nucleation and cooperative binding properties. For example, HuR bound to the 3′UTR has been shown to interact directly with PAIP2 (Poly(A)-Binding Interacting Protein 2) that is bound to a distinct region of the mRNA, and it has been suggested that interaction between these proteins can influence the overall RNA structure and render it inaccessible to endonucleases (Onesto et al. 2004, 2006). Recently it was also proposed that ELAV/Hu proteins can displace miRNAs (which would otherwise silence the mRNA; see Sect. 3.4.3) and thus suppress their destabilizing properties (Simone and Keene 2013). This seems to be the case for mouse VEGF-A mRNA since the HuR and miR-200b binding sites overlap and it was shown that the miR-200b-induced suppression of VEGF-A expression was competitively antagonized by HuR at the 3′UTR (Chang et al. 2013). Finally, HuR has also been implicated in effective VEGF-A mRNA export from the nucleus and loading onto active polysomes under hypoxia. Along with hnRNPL and hnRNPA1, the extra-nuclear shuttling of these mRNA-binding proteins has also been shown to regulate VEGF-A mRNA stability (Vumbaca et al. 2008).

The 3′UTR ARE of VEGF-A mRNA can also be the target of proteins that destabilize mRNA in various mammalian cell types, for example AUF1 and tristetraprolin (TTP) (Bernstein and Ross 1989; DeMaria and Brewer 1996; Gorgoni and Gray 2004; Stoecklin et al. 2003; Zhang et al. 2002). AUF1 promotes the assembly of other factors necessary for recruiting the mRNA degradation machinery, such as translation initiation factor eIF4G, heat-shock cognate protein hsc70, lactate dehydrogenase and the poly(A)-binding protein. Interestingly, although the poly(A)-binding protein is a stabilizing factor for polyadenylated mRNAs, it has destabilizing effects on VEGF-A mRNA (Gorgoni and Gray 2004; Ma et al. 2006). It was recently shown that AUF1-mediated regulation of VEGF-A expression in mouse macrophage-like RAW-264.7 cells is mediated through its C-terminus domain that contains a region with three arginine-glycine-glycine (RGG) motifs (Fellows et al. 2012). AUF1 itself can be regulated by many signaling pathways and therefore is subjected to numerous post-translational modifications such as phosphorylation,

glycosylation, methylation and ubiquitination (for a review, see Gratacos and Brewer 2010). TIS11/Tristetraprolin (TTP) is another ARE-binding protein that regulates the destabilization of VEGF-A mRNA through a signaling pathway involving casein kinase 2 and p38 MAPK (Lee et al. 2011). These examples show the breadth of cellular signaling pathways involved in the regulation of VEGF-A mRNA stability and the depth of their control by the cell.

3.3 Alternative Polyadenylation

Polyadenylation is the addition of a poly(A) tail to the 3′UTR of an mRNA and is important for mRNA stability, nuclear export and translation. Before the poly(A) tail is added, the 3′end of the mRNA is cleaved at specific sites, thus if an mRNA contains more than one polyadenylation site this can produce alternative transcripts, as for alternative splicing.

Although the 3′UTR is known to play a pivotal role in the post-transcriptional control of VEGF-A expression (Bartel 2009), our knowledge of the use and regulation of poly(A) signals is very limited. VEGF-A cDNA sequencing has revealed the presence of two major polyadenylation sites: a consensus AAUAAA site and a non-canonical AUUAAA site, located 399 and 1902 nucleotides after the stop codon respectively (Fig. 8.2) (Claffey et al. 1998; Dibbens et al. 2001). The resulting mRNAs from both sites have been characterized (Leung et al. 1989; Keck et al. 1989), but only one study has investigated the use of VEGF-A alternative polyadenylation (Dibbens et al. 2001). Using a mouse model it reported that the majority of VEGF-A transcripts are processed at the distal polyadenylation site (resulting in mRNAs containing the longer form of the 3′UTR), and that the same site is used under both hypoxic and normoxic growth conditions (Dibbens et al. 2001). Given the large section of the regulatory 3′UTR that is missing in transcripts using the proximal polyadenylation site, it is possible that the resulting VEGF-A isoforms play distinct roles. Therefore, much more work is needed to ascertain the importance of this method of VEGF-A regulation.

3.4 Translational Regulation

3.4.1 Internal Ribosome Entry Site (IRES) Elements and Alternative Initiation at Non-AUG Codons

In eukaryotic cells translation usually depends on the presence of an m7G cap at the 5′ terminus of the mRNA. The cap is required for assembly of the initiation complex, and allows translation to begin at the start codon further downstream. Traditionally the start codon is an AUG coding for methionine; however, alternative start codons are now known to exist, such as CUG which codes for leucine and whose initiation pathways are thought to be independent of those of the AUG codon (Starck et al. 2012). In addition, translation of a number of cellular mRNAs can be initiated from the middle of an mRNA sequence through a cap-independent

mechanism. These mRNAs contain an internal ribosome entry site (IRES) within their 5′ UTR. IRESs are structural elements that are specifically required to maintain and/or activate the expression of specific proteins during cell stress situations when cap-dependent translation is compromised (Spriggs et al. 2008). They are thought to have an extensive predicted secondary structure and have been reported mostly in mRNAs containing long 5′UTRs with a high GC content. To date more than 70 viruses and more than hundred eukaryotic mRNAs are known to contain at least one IRES, including VEGF-A (Mokrejs et al. 2010).

VEGF-A mRNAs have a 1038-nucleotide-long GC-rich 5′UTR upstream of the classical AUG start codon which contains three in-frame alternative CUG start codons and two IRESs (Fig. 8.2) (Akiri et al. 1998; Huez et al. 1998; Miller et al. 1998). The first IRES to be identified, IRES-A, lies within the 300 nucleotides upstream from the AUG start codon and controls the initiation of translation at this point (Fig. 8.2). IRES-B is located closer to the 5′cap, 16 nucleotides upstream of the first CUG codon and controls initiation from the two alternative CUG start codons. This IRES-mediated control of AUG *versus* CUG has been demonstrated both in vitro (Akiri et al. 1998; Huez et al. 1998) and in vivo (Bornes et al. 2007). In vivo, VEGF-A IRESs are thought to be particularly important for responding to local environment stresses where cap-dependent translation is inhibited, such as hypoxia, and indeed it has been shown that IRES have a low activity in embryos and adult tissues but allow efficient translation at early time points in ischemic muscle (Bornes et al. 2007). Recent studies have now focused on identifying the regulatory factors involved in IRES-mediated translation. One study used a high-throughput screening approach that combined siRNA treatment with transfection of a VEGF-A IRES reporter mRNA, to identify and validate MAPK3 kinase as a novel positive regulator (Casanova et al. 2012). The DEAD-box RNA helicase 6 (DDX6) has also been identified as an IRES trans-acting factor. Using MCF-7 cell line extracts it was shown that under normoxic conditions recombinant DDX6 inhibits VEGF-A IRES-mediated translation whereas hypoxic conditions caused a decrease in DDX6 levels that led to the induction of VEGF-A expression (de Vries et al. 2013). VEGF-A IRES can also be controlled from within the mRNA and in this way they have been shown to play a role in dictating the use of the alternative start codons, AUG and CUG. For example, mRNA encoding the alternatively spliced VEGF-A165 and -189 sequences can be efficiently expressed through initiation events using both start codons. The choice of initiation site is determined through modulation of IRES-A activity by the alternatively spliced coding sequences (Bornes et al. 2004). It is thought that different splice variants contain different elements that, through long-range interactions within the VEGF-A mRNA, could regulate IRES-A activity to promote/inhibit the use of the AUG site, however the exact molecular mechanism of this control is still unknown.

Initiation of translation from the CUG codon is equally efficient regardless of which splice variant is expressed (Bornes et al. 2004). The first CUG start codon is located 539 nucleotides upstream of the coding sequence (Huez et al. 2001; Meiron et al. 2001; Tee and Jaffe 2001; Touriol et al. 2003). Initiation of translation at this CUG codon leads to the production of longer VEGF-A isoforms (L-VEGF-A)

containing an additional 180 amino acids (Fig. 8.2). During maturation, L-VEGF-A can be cleaved at the peptide signal sequence to generate both a shorter secreted VEGF-A isoform and an intracellular portion termed N-VEGF-A. Interestingly, the VEGF-A121 isoform is known to be exclusively expressed through initiation at the CUG codon and thus is a maturation product of L-VEGF-A121. Following L-VEGF-A cleavage, the N-VEGF-A fragment is released and translocates to the nucleus (hence the "N" prefix). It is a 23-kDa NH2-specific peptide containing 206 amino acids which are highly conserved among mammals, suggesting that N-VEGF-A has an important function, the details of which are yet to be identified (Fig. 8.2) (Huez et al. 2001; Tee and Jaffe 2001; Rosenbaum-Dekel et al. 2005). The role and purpose of L-VEGF-A is also not well-understood although it has been suggested that it may serve as a reservoir for the generation of shorter isoforms since an increase in the longer intracellular L-VEGF-A165 and L-VEGF-A189 protein isoforms has been linked to a reduction in shorter secreted isoforms (Chiarini et al. 2006). Despite our lack of understanding of the role of L-VEGF-A, its importance is demonstrated by a single nucleotide polymorphism in the human VEGF-A gene (-634 C-G) that causes IRES-B dysfunction and leads to a 17 % reduction in initiation at the CUG codon and thus a decrease in L-VEGF-A expression (Lambrechts et al. 2003). This polymorphism has been associated with a number of serious conditions, such as an increased risk of motor neuron degeneration in amyotrophic lateral sclerosis (ALS) (Lambrechts et al. 2003), the development of diabetic macular edema that correlates with macular retinal thickness in type 2 diabetes (Awata et al. 2005), an increased aggressiveness of breast cancer (Jin et al. 2005), and an increased risk of gastric (Guan et al. 2009) and prostate cancer (Sfar et al. 2006). These studies not only provide evidence of the crucial role of IRES function and the significance of the L-VEGF-A isoforms but also illustrate how these distinct regulatory elements are interdependent such that a single mutation in one element has such a serious downstream effect on many others.

3.4.2 RNA G-Quadruplex Structure

G-quadruplexes are guanine-rich regions of the mRNA that can fold up to form secondary structures organized in stacks of planar layers of guanine tetrad (or quartet) units. It is emerging that these elements have regulatory functions in different steps of RNA metabolism, including mRNA translation (Millevoi et al. 2012). G-quadruplexes are found in coding and non-coding mRNA. The majority of those that are located in 5′UTRs play an inhibitory role in cap-dependent translation (for example in Zic-1, ESR1, NRAS or MT3-MMP mRNA), probably through the recruitment of stabilizing proteins to prevent ribosome scanning (Bugaut and Balasubramanian 2012).

VEGF-A mRNA contains a 17 nucleotide-long element within the IRES-A site (nucleotides 774-790) that can fold into a two-G-quartet quadruplex structure. It is thought that this is important for IRES-A function since IRES-A activity was blocked by mutations that disrupted the intramolecular G-quadruplex structure (Morris et al. 2010). This finding is quite surprising since most naturally-occurring G-quadruplexes with the ability to regulate translation are at least three-G-quartet

quadruplex structures while artificially engineered two-G-quartet quadruplexes like that found in VEGF-A have shown only a moderate stability (Bugaut and Balasubramanian 2012). Thus, it is important to establish whether the VEGF-A G-quadruplex contributes to IRES-A activation under stress conditions such as hypoxia, endoplasmic reticulum stress or ischemia, since this is when the IRESs are active and required for the initiation of translation. If this structure affects IRES-A activation under these conditions then G-quadruplex formation will be confirmed as yet another mechanism of VEGF-A regulation.

3.4.3 Upstream Open Reading Frame (uORF)

Upstream open reading frames (uORFs) are generally short sequences located within the 5′UTR of an mRNA. They can regulate protein translation under normal and stress conditions, usually by inhibiting expression of the main transcript (Calvo et al. 2009; Spriggs et al. 2010). Generally, uORFs act as a constitutive barrier to the scanning ribosome and thereby reduce ribosome access to the main AUG codon. uORFs are found in the 5′UTR of proto-oncogenes, cytokines and many other eukaryote mRNAs, including human 5-HT3A (5-hydroxytryptamine receptor 3A), TPO, BACE1 [β-site APP (Amyloid Precursor Protein) cleavage enzyme 1] and the huntingtin protein (the protein associated with Huntington's disease) (Chatterjee and Pal 2009).

The VEGF-A 5′UTR has a unique, short uORF that is highly-conserved between species and begins at AUG 852, 186 nucleotides upstream of the main AUG start codon (Fig. 8.2). It is located within the IRES-A sequence and is translated through a cap-independent mechanism. It has been suggested that the VEGF-A uORF acts as a cis-regulatory element involved in the shut-off of the CUG codon in the control of VEGF-A121 expression since mutation of AUG 852 (Fig. 8.2) increased translation of the VEGF-A 121 isoform but had no effect on the expression of the VEGF-A165 or -189 isoforms (Bastide et al. 2008). VEGF-A121 translation occurs exclusively downstream of the CUG start codon, thus an active uORF could inhibit its translation and in that way contribute towards controlling the expression of the different VEGF-A isoforms. As a potentially constitutive inhibitor, the VEGF-A uORF may be regulated by specific sequences, trans-acting factors or conditions that have not yet been identified due to the difficulties in predicting the effect of uORF alterations. Despite this, the VEGF-A uORF clearly affects its expression and is another factor contributing to its fine-tuning.

3.4.4 miRNA-Mediated Regulation of VEGF-A

MicroRNAs (miRNAs) consist of a small (20–25 nucleotides) sequence of non-coding RNA that control the translation of genes through RNA silencing. They are generated from local hairpin structures by two RNA endonucleases, Drosha and Dicer (Kim et al. 2009). The importance of miRNA in VEGF-A regulation was first suggested when researchers mutated the dicer gene in mice. This led to retarded development, defective angiogenesis and the death of embryos between days 12.5 and 14.5 of gestation. This phenotype correlated with over-expression of VEGF-A, therefore it was concluded that the defect in angiogenesis was most likely due to a

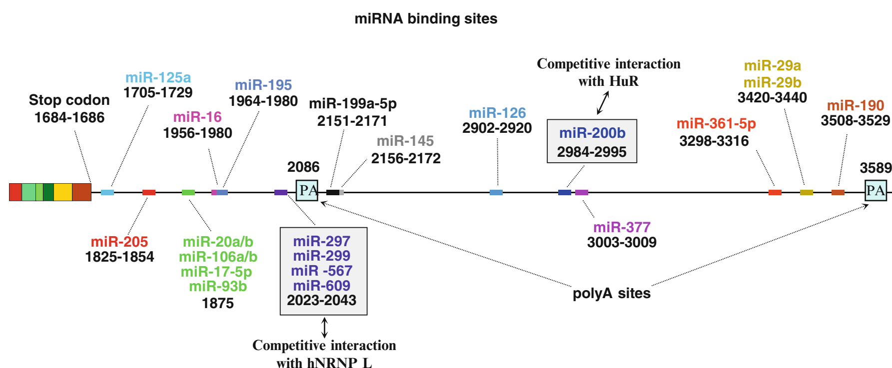

Fig. 8.3 miRNA-targeting sites in the human VEGF-A mRNA 3′UTR. The 3′ untranslated region of human VEGF-A mRNA is shown with the two alternative polyadenylation (PA) sites. The position of each miRNA target site is illustrated and numbered according to the 5′end of the VEGF-A 189 mRNA sequence

deficiency in VEGF-A mRNA processing (Yang et al. 2005). Since then many miRNAs have been identified that target VEGF-A mRNA, all of which bind to the 3′UTR region. Figure 8.3 summarizes the different miRNA binding sites found. Close to the end of the main ORF is a binding sequence for miR-125a, which has been shown to inhibit the proliferation and metastasis of hepatocellular carcinoma by targeting VEGF-A (Bi et al. 2012). This is followed by a sequence for miR-205, whose expression in glioma cell lines increases VEGF-A expression (Yue et al. 2012). Further along is a binding site for a number of miRNAs, including miR-20a and miR-20b. These were some of the first VEGF-A-binding miRNAs to be identified and, like miR-15b and miR-16, they were found to co-regulate other angiogenic factors in addition to VEGF-A regulation (Hua et al. 2006; Lei et al. 2009). miR-93b also binds to this site; it was shown to be down-regulated under hyperglycemic conditions which correlated with increased VEGF-A expression (Long et al. 2010). The miR-16 responsive element is further along, located 270 nucleotides downstream of the translation stop codon (Karaa et al. 2009), and affects angiogenesis in multiple myeloma (Sun et al. 2013). Interestingly, miR-195, which was demonstrated to suppress angiogenesis and metastasis of hepatocellular carcinoma (Wang et al. 2013), overlaps the miR-16 binding site, suggesting a mutually exclusive interaction of these 2 miRNAs. Close to the first poly(A) site lies a CA Rich Element (CARE) which is targeted by at least four miRNAs, miR-297, miR-299, miR-567, and miR-609. Their binding can induce a robust inhibition of protein synthesis under normoxic conditions (Jafarifar et al. 2011). miR-145 and miR-199a-5P then binds to an overlapping sequence further downstream. Through its post-transcriptional regulation of VEGF-A in endometrial mesenchymal stem cells miR-199a5P contributes to the pathogenesis of endometriosis (Hsu et al. 2014) while miR-145 has been shown to inhibit tumor angiogenesis, invasion and growth (Zou et al. 2012; Fan et al. 2012). Following this are sites for miR-126 and miR-200b binding; their respective down-regulation in lung cancer cells and in the diabetic

retina cause increased VEGF-A expression (Liu et al. 2009; McArthur et al. 2011). By targeting a sequence located just downstream of the miR-200b binding site, miR-377 regulates mesenchymal stem cell-induced angiogenesis in ischemic hearts (Wen et al. 2014). Finally, the miR-361-5p, miR-29a, miR-29b and miR-190-binding sites are close to the second poly(A) site. MicroRNA miR-361-5p level is inversely correlated with VEGF-A expression in human cutaneous squamous cell carcinoma (Kanitz et al. 2012), and miR-29a, miR-29b and miR-190 were demonstrated to repress metastasis and angiogenesis (Chen et al. 2014; Melo and Kalluri 2013; Hao et al. 2014).

At least two of the miRNA binding sites are known to control VEGF-A expression through competitive binding with regulatory proteins. The RNA binding protein HuR, which plays a role in mRNA stability (see Sect. 3.2), can also bind to the same site as miR-200b. Their competitive binding has been shown to control VEGF-A expression in bone marrow-derived macrophages (Chang et al. 2013) in a mechanism that is evolutionarily conserved, at least between mouse and zebrafish. At a separate location, the hnRNPL protein can bind to the CARE sequence of the 3′UTR when it is delocalized from the nucleus to the cytoplasm under hypoxic conditions. This presumably renders this element inaccessible to the RNA-Induced Silencing Complex with its incorporated miRNAs (the miRISC), which target the same element (Jafarifar et al. 2011). This mechanism also demonstrates the key role of hnRNPL during hypoxia since it is also able to promote VEGF-A mRNA stability (see Sect. 3.2) and plays a role in the VEGF-A mRNA riboswitch (see below).

Interestingly, a miRNA has also been reported to affect the activity of the VEGF-A IRESs. MiR-16 can specifically negatively regulate IRES-B, thereby controlling the expression of the diffusible VEGF-A121 isoform. In contrast, IRES-A is insensitive to miR-16 inhibition (Karaa et al. 2009). This is the first study detailing miRNA-mediated control of the translation of specific VEGF-A isoforms, but it is unlikely to be the last.

3.4.5 Riboswitch

A riboswitch is an element usually found in the untranslated region of mRNA which binds to small molecules or proteins and alters their folding pattern to induce a change in the translation of the mRNA. Binding of the effector molecule thereby allows the mRNA to directly control its own expression in response to changes in the cellular environment. Riboswitches are most commonly known to exist in bacteria, fungi and plants but some have been identified in eukaryotes. One such example is found in the 3′UTR of VEGF-A mRNA (Fig. 8.2). This atypical riboswitch is metabolite-independent and undergoes a binary conformational change that is controlled by differential protein binding in response to environmental signals. Within the riboswitch element are binding sites for the interferon-γ-activated inhibitor of translation (GAIT) complex and the hnRNPL protein, the proximity of which suggests that interactions between these proteins and the riboswitch element are mutually exclusive (Fig. 8.2). In myeloid cells under hypoxic conditions, hnRNPL accumulates within the cell and promotes a conformational switch to promote translation, thereby increasing VEGF-A levels. Under different environmental

conditions the stimulus-dependent proteasomal degradation of hnRNPL allows GAIT to bind and repress translation by maintaining the mRNA in its alternative conformation (Ray and Fox 2007; Ray et al. 2009). Within the 3′UTR, the VEGF-A riboswitch sequence encompasses the first polyadenylation site, therefore it is possible that RNA conformational changes could influence the availability of this polyadenylation site and consequently affect the presence of other regulatory elements within the 3′UTR such as AU rich elements and miR-binding sites. The novelty and significance of these studies highlight the continuing expansion of our knowledge of the scope of eukaryotic ribo-regulation.

4 Discussion and Conclusion

The critical roles of VEGF-A during embryogenesis and in the adult are emphasized by the extraordinary levels to which evolution has gone to maintain control of its expression. From one gene, the presence of two promoters, two polyadenylation signals and 14 alternative splicing options mean that 56 potential VEGF-A mRNAs could be expressed. As well as the wide spectrum of post-transcriptional mechanisms described in this chapter, VEGF-A mRNA contains one of the largest varieties of regulatory elements of all known mRNAs. In addition, there are proteins which are involved in multiple regulatory processes (e.g. HuR, hnRNPL) and there is cross-talk between the processes (e.g. differential IRES sensitivity to miRNA inhibition). Moreover, the use of alternative initiation codons enables the expression of at least 29 putative pre-proteins (14 AUG-initiated forms, 14 CUG-initiated "L-VEGF-A" forms and the amino terminal extension N-VEGF-A), each of which exhibit distinct biological functions. It is for all these reasons that VEGF-A is a paradigm for the intricate regulation of gene expression at the post-transcriptional level.

VEGF-A is certainly not alone in its complex regulation. Alternative splicing has been shown to be used by more than 90 % of genes (Pan et al. 2008) and a recent study showed that around 79 % of transcripts have multiple polyadenylation sites (Hoque et al. 2013). On the other hand, translational control is less common and to the best of our knowledge there are only around 100 human mRNAs initiating translation from alternative start codons and only approximately 115 eukaryotic cellular mRNAs reported to contain IRESs, although there is controversy regarding the validity of some suggested IRESs (Gilbert 2010; Shatsky et al. 2010). As new approaches and technologies emerge, it is highly likely that the use of translational mechanisms to regulate gene expression will be found to be more widespread (Ingolia et al. 2009, 2011, 2012). Thus in the future there may be many genes with a level of regulation as complex as that of VEGF-A. There is also undoubtedly much more to learn about the post-transcriptional regulation of VEGF-A itself, but even in its incomplete state it is an impressive example of the depth of our understanding of the different post-transcriptional regulatory processes and provides us with an opportunity to appreciate the incredible complexity that underlies the day-to-day cellular control of just one of our 25,000 genes.

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9 Post-transcriptional Regulation of Prostaglandin Biosynthesis

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1 General Aspects of Prostanoid Synthesis

Prostaglandins represent a class of potent bioactive lipid mediators derived from arachidonic acid (AA), a 20-carbon tetraenoic fatty acid (C20:4ω6). Initially isolated from prostate, semen and seminal vesicles (Horton and Thompson 1964; Samuelsson 1963), the biosynthesis of prostanoids involves several enzymes including phospholipases, cyclooxygenases and prostaglandins synthases. The clinical relevance of prostaglandins are apparent in their regulation of homeostatic functions including reproductive physiology (Salleh 2014), whereas alteration of prostaglandin homeostasis is often associated with pathologies associated with inflammatory syndromes and cancer (Ricciotti and FitzGerald 2011; Wang and Dubois 2010).

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1.1 Phospholipases

Phospholipases represent a family of enzymes catalyzing the hydrolysis of phospholipids, especially glycerophospholipids (e.g., phosphatidylcholine), which contain glycerol, saturated fatty acid at sn-1 position and unsaturated fatty acid in the sn-2 position. Hydrolysis at the sn-2 position by members of the phospholipase A_2 (PLA_2) family, of which the Ca^{+2}-dependent cytosolic PLA_2 ($cPLA_2$) plays a dominant role, enables AA release from the cell membrane (Park et al. 2006; Simmons et al. 2004; Betz and Hansch 1984; Pniewska and Pawliczak 2013). Several isoforms of PLA_2 can be distinguished depending on their subcellular localization, such the secreted $sPLA_2$, cytosolic PLA_2 ($cPLA_2$), and lipoprotein-associated PLA_2 (lp-PLA_2) (Murakami and Kudo 2002; Hiraoka et al. 2005). The expression of PLA_2 is induced by pro-inflammatory cytokines through the activity of several transcription factors such as NFκB, PPAR, and C/EBP (Pfeilschifter et al. 1993; Touqui and Alaoui-El-Azher 2001). Additionally, Phospholipase C (PLC) can influence AA levels through metabolism of diacylglycerol (Rebecchi and Pentyala 2000; Tang et al. 2006; Pniewska and Pawliczak 2013).

1.2 Cyclooxygenases

Following its release, arachidonic acid is converted to PGH_2 by cyclooxygenase (COX) enzymes that are located at the luminal side of the endoplasmic reticulum and nuclear membrane (Chandrasekharan and Simmons 2004). COXs serve as bi-functional enzymes converting AA into prostaglandin G_2 (PGG_2) through cyclooxygenase activity, followed by synthesis of prostaglandin H_2 (PGH_2) via a peroxidase activity (Smith et al. 2000; Simmons et al. 2004; Chandrasekharan and Simmons 2004).

Two main isoforms of COXs have been identified. Cyclooxygenase-1 (COX-1) is constitutively expressed in most cell types and modulates several biological processes necessary for maintaining physiological homeostasis (Smith et al. 2000; Simmons et al. 2004; Chandrasekharan and Simmons 2004). Cyclooxygenase-2 (COX-2) represents the inducible isoform of COX, whose expression is induced by various growth factors as well as pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα (Ramsay et al. 2003). The promoter of COX-2 contains an NFκB response element as well as other cytokine-dependent response elements (Ramsay et al. 2003; Chandrasekharan and Simmons 2004). COX-2 protein displays 60 % homology with COX-1 (Hinz and Brune 2002), however possesses a C-terminal "pocket", which is preferentially targeted by COX-2 specific inhibitors (Kurumbail et al. 1996). While most noted for its role in various patho-physiological functions, such as inflammation and cancer (Ricciotti and FitzGerald 2011; Wang and Dubois 2010; Ristimaki 2004), COX-2 plays a prominent role in the various reproduction stages ranging from ovulation to implantation, to decidualization and subsequent delivery (Salleh 2014; Chan 2004). Within the vascular, constitutive low-level expression

of COX-2 contributes to the continuous generation of vasoprotective PGI_2 in endothelial cells (characterized by a low hydroperoxide tone) constantly exposed to mechanical forces resulting from steady physiological blood flow (Funk and FitzGerald 2007; McAdam et al. 1999; Inoue et al. 2002).

1.3 Prostaglandin Synthases and Receptors

Prostanoids (prostaglandins and thromboxanes) are synthesized from PGH_2 by various prostaglandin synthases. Following their synthesis, prostaglandins can act in an autocrine and/or paracrine manner through binding of specific membrane or nuclear receptors (Narumiya and FitzGerald 2001). Listed below are the major physiological prostanoids, along with a description of their respective synthases and receptors. Figure 9.1 illustrates the synthesis and signaling of the predominant prostaglandin PGE_2.

1.3.1 PGE_2

PGE_2 is the most abundant prostaglandin associated with pathologies involving inflammation, pain sensitivity and fever, and neoplasia (Park et al. 2006; Kawahara et al. 2014; Wang and Dubois 2010). PGE_2 also promotes gastric mucosa protection, renal hemodynamics, and stimulates ovulation and myometrium contraction during parturition (Takeuchi 2010; Stouffer et al. 2007; Yount and Lassiter 2013). Three PGE_2 synthases (PGES), cytosolic cPGES and two membrane-associated PGES, mPGES-1 and the microsomal mPGES-2, produce PGE_2 by using endoperoxide PGH_2 as a substrate (Regan 2003). mPGES-2 and cPGES are constitutively expressed, whereas mPGES-1 is induced by pro-inflammatory cytokines (Kudo and Murakami 2005; Ricciotti and FitzGerald 2011; de Oliveira et al. 2008). cPGES interacts with COX-1 and preferentially converts COX-1-dependent PGH_2, whereas mPGES-1 is the major enzyme involved in PGE_2 production from COX-2 derived PGH_2 (Regan 2003; Kudo and Murakami 2005). mPGES-2 is produced as a golgi membrane-associated protein and is associated with both COX-1 and COX-2 (Kudo and Murakami 2005).

PGE_2 signaling is mediated *via* the four membrane receptors EP1, EP2, EP3, and EP4 encoded by PTGER1, PTGER2, PTGER3 and PTGER4 genes, respectively (Funk 2001; Hull et al. 2004; Breyer et al. 2001; Sugimoto and Narumiya 2007). EP receptors are G protein-coupled receptors primarily localized to the cell surface, although evidence exists indicating nuclear membrane localization (Bhattacharya et al. 1998; Breyer and Breyer 2000; Funk 2001). EP1 is a $G\alpha_q$-coupled receptor coupled phospholipase C/inositol triphosphate signaling and free Ca^{2+} mobilization (Breyer et al. 1996). EP2 and EP4 are $G\alpha_s$-coupled receptors that stimulate adenylate cyclase to produce cAMP, which in turn activates kinases such as protein kinase A (Hull et al. 2004; Regan 2003). EP3 receptor consists of multiple splice variants and has been shown to couple to both $G\alpha_i$ and $G\alpha_s$ proteins, leading to reduced and increased cAMP levels, respectively (Namba et al. 1993; Kotani et al. 1997; Hatae et al. 2002).

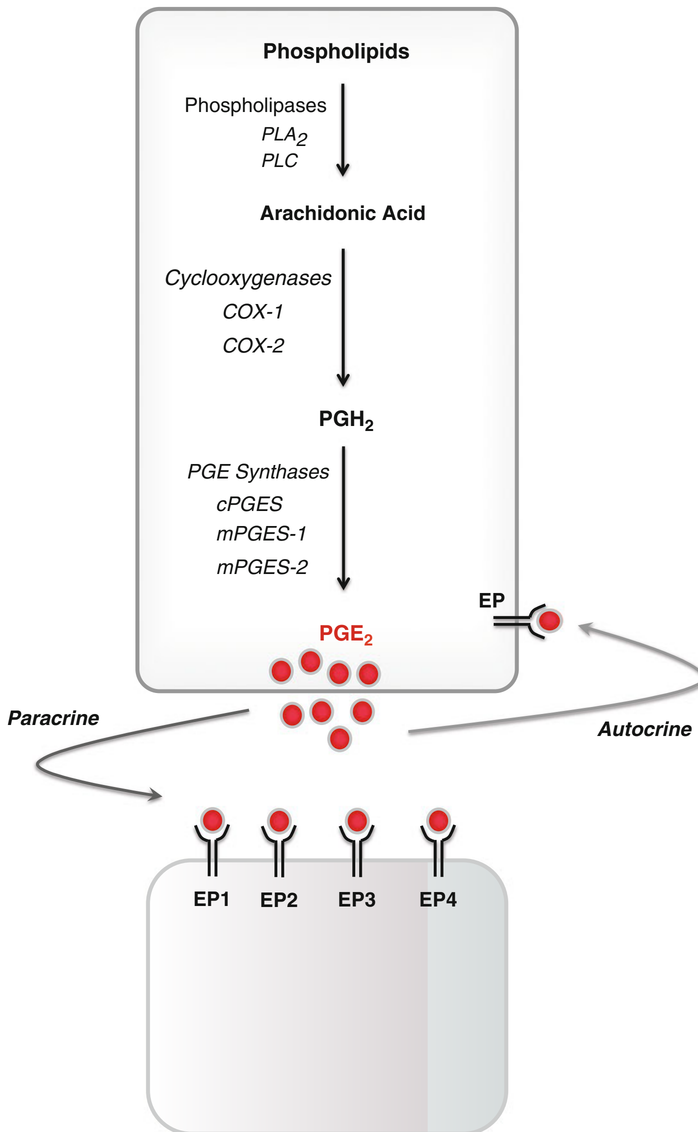

Fig. 9.1 Overview of the PGE_2 synthesis pathway. Arachidonic acid is liberated from phospholipids by phospholipase A_2 and phospholipase C activity. Free arachidonic acid can then be converted into the intermediate PGH_2 by either isoform of the cyclooxygenase enzymes, COX-1 and COX-2. PGE synthases cPGES, mPGES-1, and mPGES-2, convert PGH_2 into PGE_2 that signals through the G protein-coupled receptors EP1, EP2, EP3, and EP4 in both an autocrine and paracrine manner

The action of PGE_2 in a tissue-specific physiology predominantly depends on the cell-specific EP receptor expression and levels of the four receptor subtypes (Breyer et al. 2001). EP3 and EP4 receptors are the most widely expressed subtypes, observed in all tissues examined (Sugimoto and Narumiya 2007). Based on

observations using pharmacological antagonists and genetic knockout studies of EP receptors, it is of considerable interest to further explore their post-transcriptional regulation in order to control their expression levels (Kawamori et al. 2001; Yang et al. 2006; Keith et al. 2006).

1.3.2 PGI_2

PGI_2 (prostacyclin) is produced by PGI_2 synthase (PGIS) primarily in vascular endothelial cells and smooth muscle cells for maintenance of cardiovascular homeostasis (Dorris and Peebles 2012; Ricciotti and FitzGerald 2011). The biological effects of PGI_2 are mediated through the $G\alpha_s$-coupled IP receptor, whose expression has been found in platelets, heart, aorta, lung, kidney, and liver (Dorris and Peebles 2012). Similar to EP2 and EP4 receptors, the IP receptor promotes activation of adenylate cyclase and PKA (Dorris and Peebles 2012). PGI_2 is also involved in physiological functions such as gastric mucosa protection, smooth muscle relaxation, and vasodilatation (Williams et al. 1994; Harada et al. 1999; Tanaka et al. 2004). Moreover, PGI_2 is a potent inhibitor of platelet aggregation, leukocyte adhesion, and vascular smooth muscle cells proliferation (Dorris and Peebles 2012). In cancer cells, PGI_2 stimulates angiogenesis, through IP receptor expressed at the surface of tumor endothelial cells (Osawa et al. 2012). It has also been shown that PGI_2 can signal through the nuclear receptor PPARδ (peroxisome proliferator-activated nuclear receptor) and PGI_2-induced PPARδ activation can regulate arterial thrombus formation, blastocyst development, and limits inflammation (Kang et al. 2011; Barbieri et al. 2012; Chen et al. 2009; Dorris and Peebles 2012).

1.3.3 PGD_2

PGD_2 is mainly synthesized in the CNS, mast cells, dendritic cells, macrophages, and Th2 lymphocytes (Ricciotti and FitzGerald 2011). PGD_2 is implicated in platelet aggregation, smooth muscle contraction, broncho-constriction, immune cells chemotaxis, and regulation of sleep and wake cycles (Urade and Hayaishi 2011; Kanaoka and Urade 2003). PGD_2 is synthesized by two enzymes PTGDS and HPGDS. PTGDS is mainly expressed in brain tissues, male genital organs and in the heart (Urade and Hayaishi 2000), where HPGDS is hematopoietic in origin (Kanaoka and Urade 2003). PGD_2 plays a pro-inflammatory role through binding to the DP receptors DP1 and DP2 encoded by PTGDR1 and PTGDR2 genes, respectively. DP1 is a $G\alpha_s$-coupled receptor ubiquitously expressed and the activation of this receptor leads the activation of adenylate cyclase and PKA (Narumiya et al. 1999). This pathway is implicated in sex determination *via* activation and nuclear translocation of SOX-9 (Malki et al. 2005). DP2 is a $G\alpha_i$-coupled receptor, which enhances intracellular calcium and decreases cAMP leading to the migration of Th2 cells, eosinophils, and basophils (Hirai et al. 2001). PGD_2 released by mast cells is also implicated in asthma (Matsuoka et al. 2000) and possess tumor suppressor activities (Wu et al. 2012).

1.3.4 $PGF_2\alpha$

$PGF_{2\alpha}$ is implicated in reproduction by stimulating ovulation, luteolysis, contraction of uterine smooth muscle, and initiation of parturition (Yount and Lassiter 2013; Sugimoto et al. 1997; Saito et al. 2003; De Rensis et al. 2012). $PGF_{2\alpha}$ plays also a

role in renal salt transport, myocardial dysfunction, brain damage, and mechanical allodynia (Breyer and Breyer 2001; Kunori et al. 2009; Takayama et al. 2005; Saleem et al. 2009). $PGF_{2\alpha}$ is generated by PGF synthase and mediates its effects through FP receptors. Two variants of FP receptors have been described, FP_a and FP_b, and both of them are associated with a $G\alpha_q$ protein, which activates phospholipase C and leads to an increase of intracellular Ca^{+2} and PKC activation (Ito et al. 1994; Bos et al. 2004). $PGF_{2\alpha}$ is produced in human myometrium and ovary (Matsumoto et al. 1997; Sugimoto et al. 1997) and interestingly, its expression is stimulated by oxytocin in endometrial cells (Asselin et al. 1997).

1.3.5 TXA_2

TXA_2 (thromboxane) produced by TXA_2 synthase is a crucial mediator of platelet adhesion and aggregation, smooth muscle constriction, and activation of endothelial inflammatory response that promotes vasoconstriction (Bos et al. 2004; Ricciotti and FitzGerald 2011). TXA_2 is preferentially expressed in platelets but can also be expressed in macrophages (Bos et al. 2004; Ricciotti and FitzGerald 2011). Secreted TXA_2 mediates its effects through the binding to a membrane $G\alpha_q$-coupled receptor named "TP receptor", which is mainly expressed in platelets. Once activated, this $G\alpha_q$ protein activates phospholipase C, which in turn promotes Ca^{+2} release and PKC activation, leading to platelet aggregation. The production of TXA_2 is implicated in several cardiovascular patho-physiological processes including myocardial infarction, atherosclerosis, thrombosis, and stroke (Bos et al. 2004; Ricciotti and FitzGerald 2011).

1.4 Prostaglandin Transporter and 15-Hydroxyprostaglandin Dehydrogenase

The release of prostaglandins can be mediated by simple diffusion across the cell membrane, but recent findings have suggested that specific carriers are necessary for their transport. The prostaglandin transporter (PGT) is a broadly expressed transporter, which possess the ability to uptake various prostaglandins (i.e. PGE_2, $PGF_{2\alpha}$ and PGD_2) from the extracellular milieu (Kanai et al. 1995; Schuster 2002). Moreover, this uptake is a prerequisite for the inactivation of PGE_2 and $PGF_{2\alpha}$ in the cytosol, where 15-hydroxyprostaglandin dehydrogenase (15-PGDH) converts these prostaglandins into their stable 13,14-dihydro-15-keto-PGE_2 (PGEM) and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ forms (Holla et al. 2008; Wang and Dubois 2010). Non-enzymatic metabolism is attributed to turnover of other prostaglandins (Wang and DuBois 2007). Interestingly, it has been reported that 15-PGDH is reduced in human colorectal and gastric cancer, thus increasing the amount of PGE_2 in the tumor microenvironment and favors immune escape, cancer cell proliferation, and survival (Backlund et al. 2008; Yan et al. 2004).

2 Post-transcriptional Regulation of Prostaglandin Synthesis: Mechanisms of Messenger RNA Decay

Messenger RNA turnover is a highly regulated cellular process that can occur in a rapid, acute manner in response to intracellular and extracellular signals (Garneau et al. 2007). The necessity of post-transcriptional regulation is apparent as 40–50 % of global gene expression changes in response to cellular signals occur at the level of mRNA stability (Balagopal et al. 2012; Fan et al. 2002; Cheadle et al. 2005). Following transcription, eukaryotic mRNAs undergo splicing, RNA editing, and the addition of the 5′ 7-methylguanosine cap and 3′ poly(A) tail (Lutz and Cornett 2013). The 5′ cap and 3′ poly(A) tail, along with other transcript-specific, *cis*-acting regulatory elements, associate with RNA-binding proteins in order to promote mRNA nuclear export and translation initiation (Day and Tuite 1998; Garneau et al. 2007). Cytoplasmic mRNA decay initiates with the shortening of the poly(A) tail by a complex of enzymes known as mRNA deadenylases, with mammalian cells utilizing three major deadenylation complexes, the Ccr4/Caf1/Not (Caf1) complex, the poly A-specific ribonuclease (PARN) complex, and the Pan2/Pan3 complex (Sanduja et al. 2012; Chen and Shyu 2011; Fabian et al. 2013; Funakoshi et al. 2007; Goldstrohm and Wickens 2008). At this point, degradation initiating at the 5′ end involves removal of the 5′-7-methyl guanosine cap by the decapping complex Dcp1/Dcp2, leaving the mRNA body susceptible to degradation by the 5′-3′ exonuclease Xrn1 (Garneau et al. 2007; Schoenberg and Maquat 2012; Balagopal et al. 2012). Alternatively, the mRNA can be degraded by 3′-to-5′ exonucleolytic degradation through a complex of exonucleases known as the exosome (Garneau et al. 2007; Balagopal et al. 2012). Many mRNAs targeted for degradation are localized to processing (P)-bodies, which are small cytoplasmic foci that contain components of the 3′-to-5′ and 5′-to-3′ decay machinery along with factors involved in microRNA (miRNA) silencing, nonsense-mediated decay (NMD), and translational silencing (Eulalio et al. 2007; Kulkarni et al. 2010; Zheng et al. 2011; Blanco et al. 2014). Recent evidence demonstrating COX-2 mRNA localization occurring at P-bodies in a signal-dependent manner illustrates the functional significance these discrete cytoplasmic RNA granules have upon prostaglandin biosynthesis (Blanco et al. 2014). The pathways illustrating general cytoplasmic mRNA decay are shown in Fig. 9.2. This section will focus on the functional contribution of regulatory elements within the mRNA 3′ untranslated region (3′UTR) as well as the role of RNA-binding proteins, miRNAs, and P-bodies upon in prostaglandin biosynthesis.

2.1 Post-transcriptional Regulation and AU-Rich 3′UTR Elements

The mRNA 3′UTR is a critical region of the transcript that mediates the interaction with RNA-binding proteins and miRNAs. Considerable evidence exists to support the notion that highly conserved 3′UTR elements play a critical role in post-transcriptional regulation of prostaglandin biosynthesis, with a majority of studies

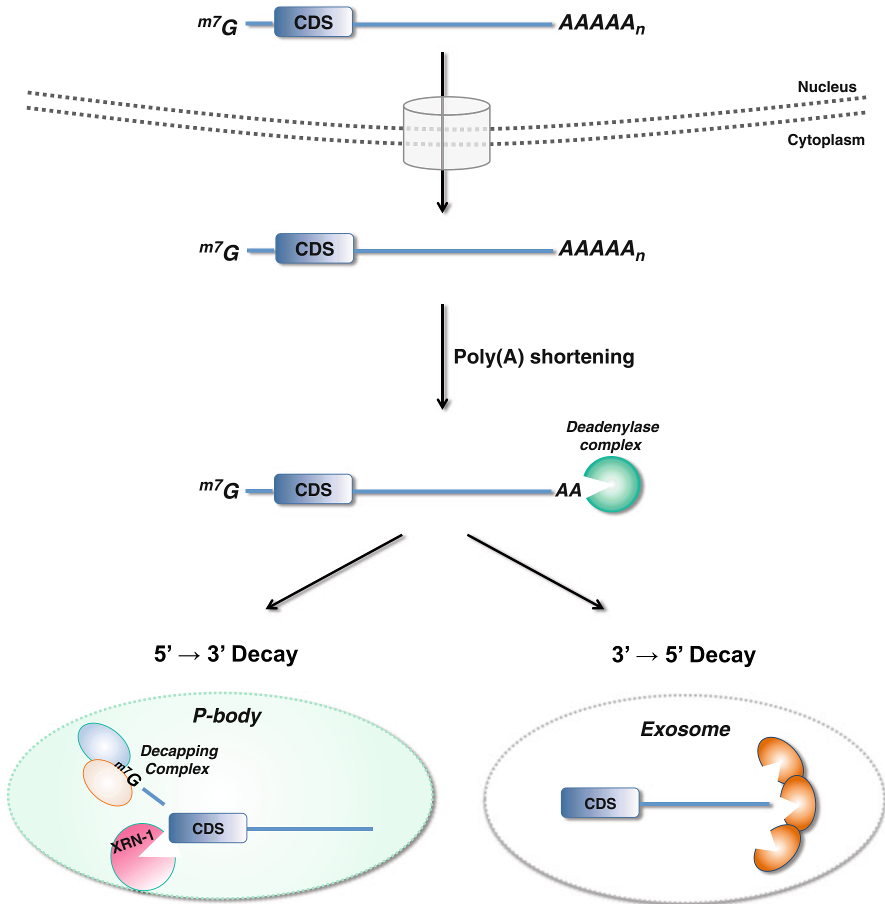

Fig. 9.2 Cytoplasmic mRNA decay pathways. A mRNA transcript undergoes nuclear splicing, and addition of a 5′ 7-methylguanosine cap (^{m7}G) and 3′ poly(A) tail, is exported to the cytoplasm. Degradation initiated through poly(A) shortening by the deadenylase complex is the rate-limiting step of mRNA decay. Once partially deadenylated to 10–15 adenosines, the mRNA transcript can be sequestered to P-bodies wherein mRNA decapping and subsequent Xrn-1-mediated decay occurs. Alternatively, mRNA transcripts may be degraded from the 3′ end by a complex of exonucleases that comprise the exosome

pertaining to regulation of COX-2 expression (Raz et al. 1989; Bailey and Verma 1990; Dixon 2003; Moore et al. 2011).

A commonly observed, well-characterized 3′UTR feature observed in many inflammatory cytokines, growth factors, and proto-oncogenes is the AU-rich element, or ARE (Barreau et al. 2005; Beisang and Bohjanen 2012). The presence of one or several AREs in the mRNA 3′UTR characterizes the short-lived lifespan of many immediate-early response genes and the importance of this particular RNA element is evident, since estimates ranging from 5 to 8 % and 16 % of coding

genes contain a 3′UTR ARE sequence (Bakheet et al. 2006; Gruber et al. 2010). The functional ARE is characterized by the 3′UTR consensus sequence AUUUA and is most often composed of multiple AUUUA motif copies. AREs are organized into several classes and clusters on the basis of the number and context of the AUUUA pentamer. For example, various immediate-early response genes (e.g. proto-oncogenes) have scattered repeats of the AUUUA motif, whereas mRNAs encoding inflammatory mediators and cytokines (e.g. COX-2) that have multiple repeats of the AUUUA pentamer clustered together (Beisang and Bohjanen 2012; Bakheet et al. 2006). The use of search engines such as AREsite (http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi) and ARED (ARE Database; http://brp.kfshrc.edu.sa/ARED/) have provided investigators online tools to identify the presence of AREs in eukaryotic mRNAs (Bakheet et al. 2006; Gruber et al. 2010), and the presence of this 3′UTR element was observed in a majority of PGE_2 pathway genes (Moore et al. 2011). The role of ARE-mediated post-transcriptional regulation in prostaglandin biosynthesis has been best described for COX-2 and $cPLA_2$ (Dixon 2004; Young and Dixon 2010; Tay et al. 1994). The functional ARE of COX-2 is comprised of an evolutionarily-conserved 116 nucleotide stretch containing six AUUUA clusters located proximal to the stop codon (Dixon et al. 2000). The COX-2 ARE mediates important interaction with ARE RNA-binding proteins that can either target the mRNA for rapid decay or enhance the half-life of the transcript, depending on the cellular context (Sengupta et al. 2003; Young et al. 2009). Similar to COX-2, AREs present in the $cPLA_2$ exert an mRNA-destabilizing effect and impact overall PGE_2 synthesis (Tay et al. 1994). A further refinement of our understanding of these post-transcriptional mechanisms may provide a novel therapeutic window for the development of innovative NSAIDs targeting aberrant ARE function for the treatment of pain, inflammation, and cancer.

2.2 ARE RNA-Binding Proteins

Through its presence, the ARE serves to target mRNAs for rapid degradation and/or translational suppression within the cytoplasm (Barreau et al. 2005; Beisang and Bohjanen 2012). AREs mediate this through association with RNA-binding proteins and miRNAs that bind with high affinity (Beisang and Bohjanen 2012; Stumpo et al. 2010; Young et al. 2012; Jing et al. 2005; Garneau et al. 2007). There are >20 known ARE-binding proteins with a majority being associated with promoting mRNA stabilization, mRNA decay, or controlling translation by directing ARE-containing mRNAs to P-bodies and stress granules (Beisang and Bohjanen 2012; Garneau et al. 2007). Through these mechanisms, ARE-binding proteins exhibit pleiotropic effects on gene expression, since a single ARE-binding protein can bind to multiple mRNAs and binding can occur among different classes of AREs (Lopez de Silanes et al. 2007).

Considerable evidence exists to support the integral role of *trans*-acting ARE-binding proteins in prostaglandin biosynthesis that allow for fine control of gene expression in response to various cellular cues and signaling within the

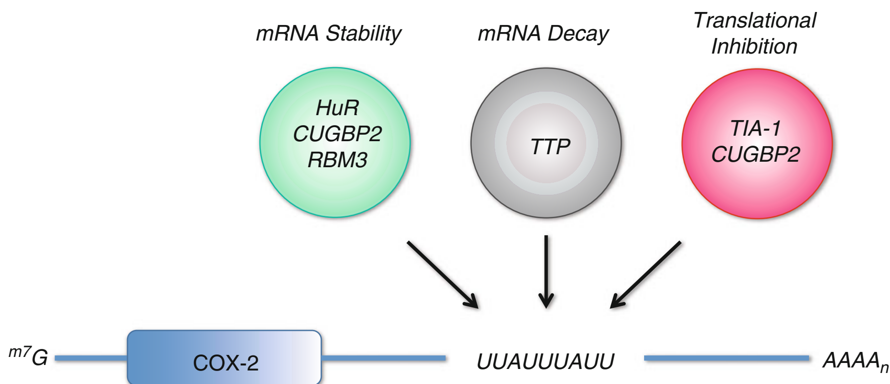

Fig. 9.3 ARE RNA-binding proteins mediating post-transcriptional regulation of COX-2. COX-2 expression is tightly regulated at the post-transcriptional level by RNA-binding proteins that promote mRNA stability (HuR, CUGBP2, and RBM3), mRNA decay (TTP), and translational inhibition (TIA-1 and CUGBP2) through their binding of the COX-2 AU-rich element (ARE)

microenvironment. Various cytoplasmic proteins have been detected to bind AREs and a majority of work has focused on COX-2 ARE-binding proteins with 16 different RNA-binding proteins identified to bind the COX-2 3′UTR (Young and Dixon 2010). This section will describe the most well characterized ARE RNA-binding proteins that regulate COX-2 expression and associated prostaglandin biosynthesis (Fig. 9.3).

2.2.1 HuR

The HuR protein (ELAVL1, Hu antigen R) is a ubiquitously expressed member of the ELAV (Embryonic-Lethal Abnormal Vision in *Drosophila*) family of RNA-binding proteins that consists of HuR and primarily neuronal-specific HuB, HuC, and HuD (Brennan and Steitz 2001; Hinman and Lou 2008). It is primarily nuclear localized (>90 %), where it assists in pre-mRNA splicing and cytoplasmic export of mRNA transcripts, followed by rapid nuclear re-localization (Brennan and Steitz 2001; Fan and Steitz 1998; Srikantan and Gorospe 2011). HuR is comprised of two tandemly arrayed RNA-recognition motifs (RRM), followed by a hinge region and a third RRM. The hinge region contains an HNS domain, which mediates nucleo-cytoplasmic shuttling of the HuR protein (Fan and Steitz 1998; Keene 1999).

The ability of HuR to function as an ARE-stability factor and enhance mRNA half-life appears to be linked to its subcellular localization (Brennan and Steitz 2001; Srikantan and Gorospe 2012). In response to cellular stress (e.g., inflammation, hypoxia, DNA damage), HuR translocates to the cytoplasm and enhances the stability of inflammation- and other survival-associated transcripts. Similarly, HuR overexpression observed in a variety of tumor types results in cytoplasmic localization and subsequent ARE-mRNA stabilization (Wang et al. 2013a; Abdelmohsen and Gorospe 2010; Srikantan and Gorospe 2012). Although the precise molecular underpinnings that regulate HuR nucleo-cytoplasmic localization are not entirely

known, a variety of cellular signals known to activate MAPK pathways (ERK and p38 kinases), PI-3-kinase pathway, and Wnt signaling pathway, have been shown to trigger HuR cytoplasmic localization and influence ARE-mRNA stabilization (Winzen et al. 1999; Yang et al. 2004; Tran et al. 2003; Ming et al. 2001; Briata et al. 2003).

HuR has been shown to bind and post-transcriptionally regulate numerous ARE-containing transcripts associated with cancer traits and inflammation (Wang et al. 2013a; Abdelmohsen and Gorospe 2010). Based on its ability to bind the COX-2 ARE region consisting of a highly conserved cluster of six AUUUA elements located near the stop codon, HuR has been identified as a *trans*-acting factor involved in regulating COX-2 expression (Dixon et al. 2001). The enhanced stabilization of COX-2 mRNA observed in colon cancer cells and inflamed myeloid leukocytes is, in part, due to elevated cytoplasmic levels of HuR (Dixon et al. 2001, 2006; Young et al. 2009). Furthermore, several studies indicate that HuR overexpression and cytoplasmic localization is a marker for elevated COX-2 that is correlated with advanced tumor stage and poor clinical outcome (Dixon 2003; Abdelmohsen and Gorospe 2010; Wang et al. 2013a). PLA_2 and mPGES-1 have also been identified as a direct targets of HuR, with inflammatory signaling enhancing the association of $cPLA_{2\alpha}$ mRNA with cytosolic HuR (Liao et al. 2011; Lopez de Silanes et al. 2004). These findings suggest HuR to play a distinct regulatory role by integrating expression of factors involved in prostaglandin synthesis through mRNA stabilization.

It is generally accepted that HuR stabilizes mRNAs by competing or displacing destabilizing factors from the ARE (Benjamin and Moroni 2007). Another mechanism indicates that HuR can inhibit mRNA association with the decay machinery or possibly protect the poly(A) tail from degradation (Linker et al. 2005; Lal et al. 2004; Ma et al. 1997). Current evidence now indicates that HuR's ability to influence stabilization of bound mRNAs can be mediated through HuR's interplay with miRNAs that associate with the same transcript (Srikantan et al. 2012), and recent findings demonstrate that HuR promotes COX-2 mRNA stabilization by outcompeting miRNAs that share the same ARE binding sites in the COX-2 3′UTR (Young et al. 2012). In line with these findings, HuR has been shown to rescue mRNA transcripts held translationally silenced by miRNAs in P-bodies to promote their translation (Bhattacharyya et al. 2006; Srikantan et al. 2012).

High-throughput based biochemical screens have identified small-molecule compounds with the ability to disrupt HuR/ARE interactions (Meisner and Filipowicz 2010; Meisner et al. 2007; Chae et al. 2009; D'Agostino et al. 2013). Work conducted by Meisner and coworkers had identified and characterized three compounds (okicenone, dehydromutacin, and MS-444) as specific, low-molecular-weight HuR inhibitors (Meisner et al. 2007). These compounds are polyketides purified from plant and microbial extracts and the latter, MS-444, has been extensively studied with regard to its mechanism of action and ability to inhibit HuR. Mechanistically, MS-444 inhibits HuR homodimerization and blocks its cytoplasmic export. The impact of this results in loss of HuR-dependent mRNA stabilization and disruption of HuR/miRNA interaction (Young et al. 2012; Meisner et al. 2007).

2.2.2 TTP

Tristetraprolin (TTP, ZFP36, TIS11, NUP475) is a member of a small family of Cys3His zinc finger proteins. This family is comprised of TTP, ZFP36L1 (BRF-1), and ZFP36L2 (BRF-2), all which have been shown to play a critical role in regulated mRNA decay (Sanduja et al. 2011; Blackshear 2002; Ciais et al. 2013). TTP is one of the best-characterized post-transcriptional regulators, whereby it's binding of AREs promotes rapid mRNA decay (Brooks and Blackshear 2013; Carballo et al. 1998). The binding of TTP to AREs targets the transcript for rapid degradation through the recruitment of mRNA deadenylases, translational repressors, and mRNA decapping proteins onto the mRNA transcript (Chen et al. 2001; Mukherjee et al. 2002; Fenger-Gron et al. 2005; Lykke-Andersen and Wagner 2005; Franks and Lykke-Andersen 2007; Hau et al. 2007). At this point, TTP promotes the nucleation of P-bodies by delivering its cargo mRNA for subsequent degradation (Kedersha et al. 2005; Blanco et al. 2014; Franks and Lykke-Andersen 2007) (Fig. 9.4a).

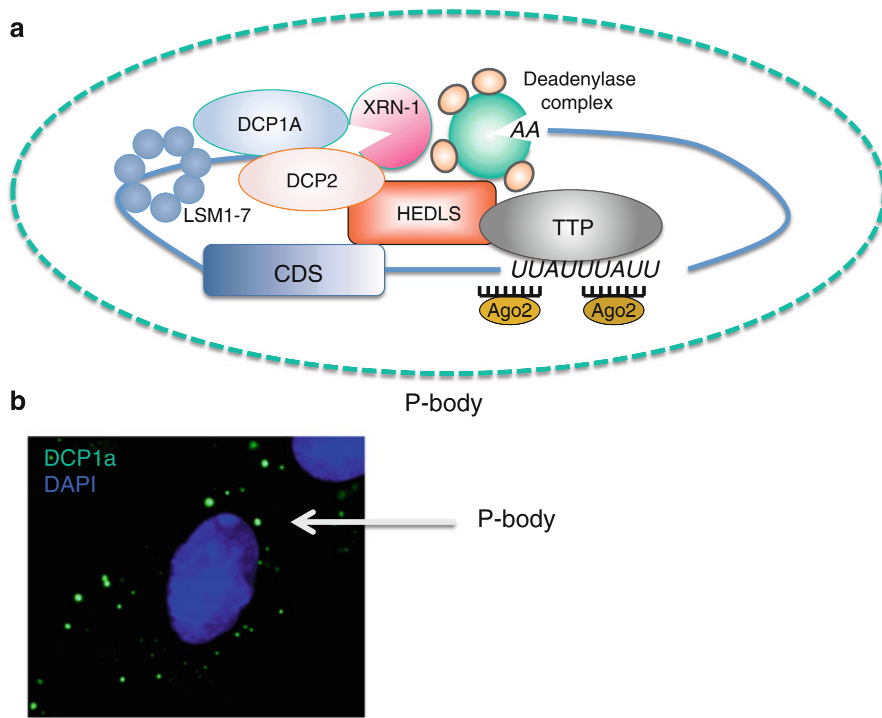

Fig. 9.4 P-bodies are sites of mRNA storage or decay. (**a**) Schematic of a P-body. Once an ARE-containing mRNA is targeted for decay and partially deadenylated, TTP recruits mRNA degradation machinery facilitating mRNA decapping (Dcp1a/Dcp2/Hedls/Lsm1-7) and further deadenylation. RNA degradation occurs in a 5′→3′ direction by the exonuclease Xrn-1. TTP also facilitates miRNA-mediated mRNA degradation through association with Ago proteins. (**b**) Example of P-bodies present in intestinal epithelial cells. P-bodies were detected by immunofluorescence staining for the P-body marker DCP1a (*green signal*). Cellular nuclei are visualized by DAPI (*blue signal*). Scale bar 10 μm

TTP has also been implicated as a novel mediator of miRNA-dependent post-transcriptional regulation through its ability to associate with the argonaute (Ago) protein family members and promote decay of ARE-containing mRNAs localized to P-bodies (Eulalio et al. 2007; Jing et al. 2005). Alternatively, TTP can promote exosome-mediated mRNA degradation through its ability to interact with the exosome and recruit it to the AU-rich mRNAs (Chen et al. 2001).

TTP plays a critical role in normal physiology as TTP knockout mice develop early on a severe syndrome of growth retardation, cachexia, arthritis, inflammation and autoimmunity (Taylor et al. 1996). Based on this severe inflammatory phenotype, inflammatory mediators such as TNF-α, GM-CSF and COX-2 have been identified as ARE-containing targets of TTP (Lai et al. 1999; Carballo et al. 2000; Brooks and Blackshear 2013; Sawaoka et al. 2003; Young et al. 2009). TTP preferentially binds the nonameric sequence motif, UUAUUUAUU, the core destabilizing element of many ARE-containing mRNAs (Blackshear et al. 2003; Brewer et al. 2004). As nonameric sequences are the second most abundant sequence motif present in genes involved in the PGE_2 pathway (Moore et al. 2011), TTP's role in the regulation of prostaglandin biosynthesis may extend beyond COX-2 regulation.

TTP is an inducible, immediate-early response gene whose expression can be induced by mitogenic stimuli such as insulin, growth factors (e.g., TGF-β), and pro-inflammatory signals (Blanco et al. 2014; Ogawa et al. 2003). However, a consistent loss of TTP expression occurs in a variety of human cancers such as breast, colon, cervix, prostate, and lung (Sanduja et al. 2009, 2012; Brennan et al. 2009; Young et al. 2009), and suppressed TTP expression can serve as a negative prognostic breast cancer indicator (Brennan et al. 2009). These findings indicate that the presence of TTP in normal tissues serves a protective role by controlling expression of various pro-inflammatory and prostaglandin synthesis mediators, while loss of TTP expression in tumors contributes to aberrant overexpression of these transcripts.

2.2.3 RBM3

RBM3 (RNA-binding motif protein 3) is a member of a family of glycine-rich RNA-binding proteins (Derry et al. 1995). RBM3 is comprised of a single RRM and has been shown to regulate COX-2 expression through ARE binding (Cok and Morrison 2001; Sureban et al. 2008). In addition to COX-2, RBM3 has been shown to bind and stabilize the IL-8 and VEGF mRNAs and prevents mitotic catastrophe in colon cancer cells (Sureban et al. 2008). Together with the findings that RBM3 is significantly upregulated in colorectal tumors and overexpression promotes oncogenic transformation in fibroblasts (Sureban et al. 2008), identify a pro-oncogenic role of this ARE-binding protein.

Previously, yeast two-hybrid screens identified the ability of RBM3 to interact with HuR (Anant et al. 2010). Although the significance of this interaction remains to be determined, RBM3, like HuR, is a nucleo-cytoplasmic shuttling protein whose cytoplasmic export is triggered by stresses such as hypothermia, hypoxia, serum deprivation (Wellmann et al. 2010). Through its ability to promote the translation of otherwise unstable mRNA transcripts, RBM3 is being recognized as a contributing factor promoting enhanced prostaglandin levels in pathogenic states.

2.2.4 CUGBP2

A member of the CUGBP-ETR-3-like factors family, CUG triplet repeat-binding protein 2 (CUGBP2) is a ubiquitously expressed RNA-binding protein comprised of two N-terminal RRMs and one C-terminal RRM (Mukhopadhyay et al. 2003; Murmu et al. 2004).

Like other members of this family of proteins, CUGBP2 regulates alternative splicing, RNA editing, and mRNA translation (Anant et al. 2001). Importantly, CUGBP2 displays high affinity for the COX-2 ARE and has been shown to regulate its expression in response to ionizing radiation in colon cancer cells and growth factor stimulation of colonic stromal cells (Mukhopadhyay et al. 2003; Walker et al. 2010). Studies in vascular smooth muscle cells demonstrated that the association between CUGBP2 and the COX-2 ARE occurs in response to platelet-derived growth factor (PDGF)-dependent phosphorylation of the CUGBP2 protein. To this extent, the kinases c-Src and c-Abl directly phosphorylate CUGBP2 thus mediating its ability to inhibit ribosomal loading of the COX-2 mRNA (Xu et al. 2007). In a model of cardiac hypertrophy, CUGBP2 was identified to control pro-inflammatory stimulus involving COX-2/PGE_2 and promote subcellular mRNA trafficking specific cytoplasmic stress granules to maintain homeostasis in cardiac cells (Moraes et al. 2013).

CUGBP2 exhibits a complex biology such that it promotes COX-2 mRNA stabilization coupled with translational inhibition. Although CUGBP2 and HuR share similar affinities for the same binding sites in the COX-2 ARE, CUGBP2 effectively outcompetes HuR and promotes translational silencing of COX-2 (Murmu et al. 2004). This dynamic, seemingly antagonistic role of CUGBP2 indicates a possible role for this protein in the early stages of inflammation-induced tumorigenesis by attenuating PGE_2 synthesis through repression of COX-2 expression.

2.2.5 TIA-1

T cell intracellular antigen 1 (TIA-1) is an RNA-binding protein originally identified in activated T lymphocytes and is comprised of three RRMs. TIA-1 displays high affinity for short repeats of uridylate rich regions in non-coding regions (e.g., 3′UTR) of many pro-inflammatory cytokines (Tian et al. 1991; Lopez de Silanes et al. 2005). TIA-1 acts as a translational repressor, and under conditions of stress, it sequesters its target mRNA transcripts to cytoplasmic RNA granules known as stress granules (SGs). There, mRNAs are held translationally silenced and are sorted to re-enter translation or proceed to ARE-mediated mRNA decay in P-bodies (Kedersha et al. 2005; Anderson and Kedersha 2008). Along with it recognized role in translational suppression, TIA-1 is also implicated in alternative splicing regulation of various pre-mRNAs through binding adjacent to exon/intron boundaries (Wang et al. 2010b, 2014).

TIA-1 has been shown to bind the COX-2 ARE and regulate its expression through translational inhibition without altering COX-2 mRNA turnover. However, TIA-1-mediated regulation of COX-2 is deficient in colon cancer cells, thus contributing to increased polysome association with the COX-2 mRNA (Dixon et al. 2003). This regulation of COX-2 expression was also observed in TIA-1-deficient

fibroblasts that produce significantly more COX-2 protein and PGE_2 than wild-type fibroblasts. The physiological role of TIA-1 was best described in studies with the TIA-1 knockout mice. Similar to TTP deficient mice, TIA-1 null mice exhibited aberrant expression of pro-inflammatory cytokines (e.g., COX-2), thus leading to the development of arthritis (Phillips et al. 2004). These findings indicate that loss of TIA-1-mediated regulation of COX-2 contributes to increase COX-2 protein expression and PGE_2 synthesis in cancer and inflammation.

2.3 MicroRNAs

MicroRNAs (miRNAs) represent a class of endogenous small non-coding RNAs involved in the post-transcriptional regulation of broad biological functions (Frankel and Lund 2012; Ambros 2004; Graves and Zeng 2012). MiRNAs are ≈22 nucleotides and regulate the expression of complementary target mRNA(s) primarily through 3′UTR binding by influencing translation and by causing degradation of target mRNAs (Fabian et al. 2010) and findings have implicated miRNA-mediated mRNA decay to be the predominant mechanism (Guo et al. 2010). MiRNAs are transcribed by RNA polymerase II to produce a longer primary precursor pri-miRNA (Graves and Zeng 2012). This pri-miRNA is maturated by the Drosha endonuclease microprocessor complex to release ≈60–70 nucleotides long stem-loop intermediate pre-miRNAs. Alternatively, the pre-miRNA can be generated in a Drosha-independent manner, through intronic pri-miRNAs, which are maturated by the spliceosome into pre-miRNAs (Bartel 2004). The pre-miRNA is then exported in the cytosol through by exportin 5 and processed by Dicer to release a ≈22 nucleotides mature miRNA (guide strand)/miRNA* (passenger strand) duplex (Bartel 2004). According to the current canonical model, the guide strand is associated with argonaute (Ago) proteins and incorporated in the RNA-Inducing Silencing Complex (RISC) where the miRNA binds its mRNA target(s), while the miR* is degraded (Yates et al. 2013; Bartel 2004). Current findings indicate that the miR* can also play an important role in biological processes by targeting different mRNAs than their respective guide strands (e.g. miR-21* in kidney fibrosis or miR-199* in PGs biosynthesis) (Rayner et al. 2011). Consequently, the role of the passenger strand needs to be considered with caution, especially in studies using knockout mice, where the guide and the passenger strands are both deleted.

miRNAs regulate more than 60 % of coding mRNAs and their biological effects are organized in a complex network (Friedman et al. 2009). This level of complexity occurs through the pleiotropic actions of miRNAs targeting numerous mRNAs and it is likely that the phenotype arising alteration of one particular miRNA is the consequence of a concerted action on several targets (Ambros 2004; Bartel 2004). A further degree of complexity is provided by other non-coding RNAs such as competitive endogenous RNAs (ceRNA including pseudogenes, circular RNAs or long non-coding RNAs), which are potent regulators of miRNAs by acting as a "sink" for miRNAs (Tay et al. 2014). Other post-transcriptional regulators need also to be taken into account in the miRNAs network, such as RNA-binding proteins

including ARE-binding proteins, which can promote or inhibit miRNAs function (Adams et al. 2014; Young et al. 2012; Srikantan et al. 2012).

miRNAs have been associated with a variety of pathologies including metabolic diseases, cardiovascular disease, and cancer (Farazi et al. 2013). Alterations in expression or activity of miRNAs regulating the expression of key enzymes involved in prostaglandin homeostasis are likely to contribute to the onset and progression of these pathologies. Numerous miRNAs have been predicted by different computational methods to bind directly to the 3′UTR of key enzymes involved in prostaglandin biosynthesis (e.g., TargetScan, miRanda, microRNA.org) (Moore et al. 2011). Several miRNAs targeting phospholipase A2, phospholipase C, COX-1, COX-2, as well as prostaglandin synthases have been identified, with a majority of efforts on investigating COX-2 regulation (Moore et al. 2011; Young and Dixon 2010). Furthermore, miRNAs can affect the expression of these enzymes through indirect mechanisms, for instance targeting transcription factors controlling the expression of the enzymes implicated in PG synthesis (e.g. NFκB) (Ma et al. 2011). Herein, we will primarily discuss functionally validated miRNAs regulating the expression of the different enzymes involved in PGs synthesis (Table 9.1).

2.3.1 miR-16-1 and miR-15a

The first reports implicating a role for miRNAs in cancer progression examined miR-16-1/15a cluster and its function in chronic lymphocytic leukemia (CLL) due to a commonly deleted genomic region in CLL accompanied with loss of miR-16-1 and miR-15a expression (Calin et al. 2002; Dohner et al. 2000). The expression of miR-16 is also reduced in other cancers, including colorectal, liver, prostate, lung, and gliomas (Young et al. 2012; Ge et al. 2014; Yang et al. 2014; Bottoni et al. 2005; Lu et al. 2005; Bonci et al. 2008; Navarro et al. 2011). Various mechanisms are responsible for the downregulation of miR-15a and miR-16 in cancers. MiR-16 is encoded within an intronic region of DLEU2 (deleted in lymphocytic leukemia) on chromosome 13q14, a region which is frequently deleted in CLL (Pekarsky and Croce 2014). Other evidence indicates downregulation of miR-16 can be also mediated by epigenetic events such as HDACs overexpression (Sampath et al. 2012). MiR-16-1/15a serves in a tumor suppressor capacity by regulating expression of anti-apoptotic factors such as Mcl-1 and Bcl-2 (Yang et al. 2014; Liu et al. 2014; Sanchez-Beato et al. 2003; Cimmino et al. 2005). MiR-16 in particular has additionally been shown to play a role in cell cycle maintenance through regulation of several cell cycle regulatory genes (Liu et al. 2008).

The initial connection between miR-16 and COX-2 was demonstrated in the work by Jing et al. that identified the ability of miR-16 to promote degradation of ARE-containing transcripts as miR-16-1 contains sequence homology to AU-rich elements (Jing et al. 2005). Other studies have validated miR-16-1 targeting of the COX-2 3′UTR in work examining COX-2 3′UTR-mediated post-transcriptional regulation by miR-16-1 in response to diabetic stimuli in leukocytes (Shanmugam et al. 2008). A similar link between miR-16 and COX-2 was observed in hepatoma and colorectal cancer cells (Agra Andrieu et al. 2012; Young et al. 2012). While these findings underscore the importance of miR-16 in PG synthesis, RNA-binding proteins also regulate miR-16 function. MiR-16-induced mRNA decay requires the

Table 9.1 Validated microRNAs targeting genes involved in prostaglandin synthesis

Gene	Validated miRNAs[a] (References)
$cPLA_2$-α (PLA2G4A) $cPLA_2$-β (PLA2G4B)	N.D. miR-338 (Montenegro et al. 2009)
Phospholipase C (PLCγ1)	miR-200b, miR-200c, miR-429 (Uhlmann et al. 2010) miR-218-2 (Guan et al. 2013)
COX-1 (PTGS1)	N.D.
COX-2 (PTGS2)	miR-16-1 (Shanmugam et al. 2008; Young et al. 2012; Agra Andrieu et al. 2012) miR-26b (Ji et al. 2010; Li et al. 2013) miR-101a (Chakrabarty et al. 2007; Daikoku et al. 2008; Strillacci et al. 2009; Tanaka et al. 2009; He et al. 2012; Wang et al. 2010a) miR-137 (Chen et al. 2012) miR-143 (Song et al. 2011; Pham et al. 2013; Wu et al. 2013) miR-146a (Sato et al. 2010; Cornett and Lutz 2014) miR-199a-5p (Chakrabarty et al. 2007; Daikoku et al. 2008; Akhtar and Haqqi 2012) miR-199a-3p/miR-214 (Williams et al. 2012) miR-542-3p (Moore et al. 2012) miR-558 (Park et al. 2013; Strillacci et al. 2009)
mPGES-1 (PTGES) mPGES-2 (PTGES2) cPGES (PTGES3)	N.D. miR-146a (Matysiak et al. 2013) N.D.
EP1 Receptor (PTGER1) EP2 Receptor (PTGER2) EP3 Receptor (PTGER3) EP4 Receptor (PTGER4)	N.D. N.D. N.D. miR-101 (Chandramouli et al. 2012)
Prostacyclin synthase (PTGIS)	N.D.
IP Receptor (PTGIR)	N.D.
PGD_2 synthase (PTGDS) PGD_2 synthase (HPGDS)	N.D. N.D.
DP Receptor (PTGDR) DP Receptor (PTGDR2)	N.D. N.D.
$PGF_{2\alpha}$ Synthase (PGFS)	N.D.
FP Receptor (PTGFR)	N.D.
TXA_2 Synthase (TBXAS1)	N.D.
TXA_2 Receptor (TBXA2R)	miR-765 (Nossent et al. 2011)
Prostaglandin Transporter (SLCO2A1, PGT)	N.D.
15-Hydroxyprostaglandin Dehydrogenase (HPGD, 15-PGDH)	miR-21 (Lu et al. 2014)

[a]Based on strong evidence criteria using MiRTarBase database (Hsu et al. 2014) or functionally validated in literature. *N.D.* not detected

mRNA-destabilizing factor TTP to promote decay of ARE-mRNAs (Jing et al. 2005). Other proteins able to stabilize COX-2 mRNA, such as HuR, can interfere with miR-16 binding of the COX-2 ARE by binding miR-16 (Young et al. 2012). Similarly, hnRNPK (heterogeneous nuclear ribonucleoprotein K) leads to a

decreased binding of miR-16 to COX-2 mRNA (Shanmugam et al. 2008). Interestingly, studies have shown miR-16 also directly regulates HuR expression. In breast cancer, miR-16-1 has been implicated to repress translation of HuR and loss of miR-16-1 was correlated with HuR overexpression (Xu et al. 2010). Consistent with this, miR-16 has been shown to decrease COX-2 expression in hepatocellular carcinoma by two distinct mechanisms. First, through direct binding of the COX-2 3′UTR and impeding translation and second, miR-16 downregulates HuR expression (Agra Andrieu et al. 2012).

2.3.2 miR-200 Family

The miR-200 family encompasses five miRNAs, including miR-200a, miR-200b, miR-200d, miR-141 and miR-429, which are encoded by two different polycistronic pri-miRNA transcripts (Moore et al. 2011). MiR-200a, b and miR-429 are located on Chr-1, while miR-200c and miR-141 are on Chr-12 (Gregory et al. 2008). Interestingly, the chromosomal regions encoding miR-200 family members are frequently deleted in cancers, leading to a downregulation of these miRNA (Mongroo and Rustgi 2010). MiR-200 family is also downregulated in non-cancerous diseases such as liver fibrosis (Murakami et al. 2011). The link between miR-200 family and PGs synthesis was highlighted in breast cancer cells, where overexpression of miR-200b, miR-200c and miR-429 promoted downregulation of PLCγ1 mRNA expression, and an increase of PLCγ1 mRNA was observed with a miR-200bc/429 inhibitor in MCF7 cells (Uhlmann et al. 2010). Moreover, the increase of miR-200bc/429 in breast cancer cells reduces cell viability and induces apoptosis (Uhlmann et al. 2010).

2.3.3 miR-137

MiR-137 loss has been linked with several diseases such as schizophrenia (Schmidt et al. 2013) and cancers including lung, ovarian, and neuroblastoma (Li et al. 2014; Guo et al. 2013; Althoff et al. 2013). Based on predicted binding sites for miR-137 in the COX-2 3′UTR, expression of miR-137 in glioma cells promoted COX-2 downregulation and inhibition of cell proliferation and invasion. These observations are coupled with an inverse correlation between the expression of miR-137 and COX-2 in glioblastoma cells, suggesting a potential role of miR-137 as a tumor suppressor in glioblastoma (Chen et al. 2012).

2.3.4 miR-21

MiR-21 is the only miRNA known to date to be upregulated in all human cancers (Pan et al. 2011; Volinia et al. 2006). A large amount of experimental data has demonstrated that overexpression of miR-21 impacts various aspects of tumorigenesis including tumor cell proliferation, evasion of apoptosis, and invasion and metastasis (Pan et al. 2011). In addition, miR-21 is implicated in metabolic disorders such as NAFLD (Non-Alcoholic Fatty Liver Disease), where its ability to downregulate PTEN affects insulin sensitivity (Vinciguerra et al. 2009; Ling et al. 2012).

MiR-21 is an important regulator of prostaglandin synthesis by directly targeting 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which converts PGE_2 into its inactive form, 15-keto-PGE_2 (Lu et al. 2014). In cholangiocarcinoma, where elevated expression of miR-21 is correlated with enhanced PGE_2 levels and loss of 15-PGDH (Lu et al. 2014). It has been found that nicotine stimulation upregulates the expression of miR-21 in gastric cancer cells and this effect was in an NFκB-dependent manner (Shin et al. 2011), indicating that ability of miR-21 to affect PGs synthesis can be also influenced by indirect effects. On these same lines, a connection between PTEN loss and the increase of $cPLA_2$ has been found in the prostate cancer cells (Vignarajan et al. 2014). Thus, the ability of miR-21 to downregulate PTEN may indirectly affect cPLA2 expression and impact PGs biosynthesis.

2.3.5 miR-101

The genomic locus encoding miR-101 is located on Chr 1p31.3 and loss of miR-101 has been observed in various tumor types including in 67 % of metastatic cancer cells, suggesting its involvement in tumor cell invasion (Varambally et al. 2008; Su et al. 2009; Liang et al. 2014; Hiroki et al. 2010; Zhang et al. 2011; Strillacci et al. 2009). In endometrial cancer, miR-101 is involved in regulating expression of oncogenes such as Mcl-1 and Fos (Konno et al. 2014). MiR-101 has been shown to regulate COX-2 expression in a number of models. Using a toxin-induced liver injury, it has been shown that miR-101 regulates COX-2 expression and prostaglandin synthesis (Yoshioka et al. 2011). Another study has also shown the downregulation of miR-101 and a concomitant upregulation of COX-2 in cervical cancer tissue from patients, indicating miR-101 loss as a biomarker of COX-2 elevation (Lin et al. 2014). Supporting these findings, it has been found that the expression of miR-101 is low in HeLa cells and the restoration of miR-101 expression downregulates COX-2 expression with decreased cell proliferation and migration and an induction of apoptosis (Huang et al. 2013). Similar results were observed in colorectal and gastric cancer cells where COX-2 downregulation occurred following miR-101 overexpression, where miR-101 was observed to interact with COX-2 3′UTR and reduce protein expression (Hao et al. 2011; Wang et al. 2010a; Strillacci et al. 2009). miR-101 also plays an important role in embryo implantation by targeting directly COX-2. It has been shown that miR-101 is induced in mouse uterus during embryo implantation, while the expression of COX-2 is reduced (Chakrabarty et al. 2007). Given the importance of COX-2-derived prostaglandins during embryo implantation (Kennedy et al. 2007; Lim et al. 1999), the ability of miR-101 to downregulate COX-2 expression may influence the implantation process.

Beside its ability to downregulate COX-2, it has been suggested that miR-101 downregulates also EP4 receptor expression. An inverse correlation between the expression of miR-101 and EP4 has been observed in colorectal cancer and the overexpression of miR-101 in colon cancer cells causes a reduction of EP4 expression and impairs cell proliferation and migration (Chandramouli et al. 2012). These findings show that miR-101 is a potent inhibitor of PGE_2 signaling by impacting PGE_2 synthesis and its transduction pathway.

2.3.6 miR-146a

This miRNA is induced by NFκB and is an important regulator of innate immune response by targeting directly mRNAs in the TNFα and IL-6 signaling pathways (Taganov et al. 2006). More recently, miR-146a was found to be an important regulator of PG synthesis. The link was shown in fibroblasts from chronic obstructive pulmonary disease patients (COPD) where increases COX-2/PGE_2 expression were observed (Sato et al. 2010). This effect was partially associated with reduced levels of miR-146a and inhibition of COX-2 mRNA degradation, and overexpression of miR146a prevented IL-1β or TNFα-induced COX-2 expression and PGE_2 synthesis (Sato et al. 2010). Interestingly, the expression of miR-146a is positively regulated by NFκB, thus suggesting that the induction of this miRNA may represent part of a negative feedback loop aiming at lowering PG synthesis (Ghose et al. 2011; Taganov et al. 2006).

Recent findings have shown that miR-146a can also affect other factors involved in PG synthesis. MiR-146a inhibits an immunoregulatory function of bone marrow stem cells (BMSCs) by targeting directly mPGES-2 mRNA and limiting PGE_2 synthesis (Matysiak et al. 2013). In this study, neuronal differentiated BMSC (nBMSC) express a higher level of miR-146a compared to non-differentiated BMSCs and inhibition of miR-146a with a selective antagomiR in nBMSC leads to an increase of PGE_2 synthesis (Matysiak et al. 2013). Alteration of miR-146a expression has been observed in a variety of pathologies such as lung cancer (Cornett and Lutz 2014), where the expression of miR-146a is lost. Paradoxically, herpes simplex virus-1 can induce the expression of miR-146a in neuronal cells, which leads to an induction of inflammation and particularly an induction of PLA2 and COX-2 (Hill et al. 2009). These findings suggest that different mechanisms may occur, depending on the disease etiology and the cellular context.

2.3.7 miR-26b

MiR-26b is encoded within the intronic region of genes encoding for proteins of carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase (CTDSP) family (Zhu et al. 2012). The expression of this miRNA is reduced in different neoplasias such as hepatocellular, nasopharyngeal, lung, and breast cancers (Shen et al. 2014; Ji et al. 2010; Gao et al. 2011; Li et al. 2013). MiR-26b is associated with obesity and its expression is downregulated by free fatty acids, glucose, or glucocorticoids in adipocytes, indicating a link between this miRNA and insulin resistance (Xu et al. 2014). It has been shown that miR-26b regulates COX-2 expression in desferrioxamine-treated nasopharyngeal cancer cells and overexpression of miR-26b leads to a reduction in cell proliferation associated with the COX-2 downregulation (Ji et al. 2010). In breast cancer, the expression of miR-26b contributes to decreased COX-2 mRNA and protein levels and inhibits cell proliferation (Li et al. 2013).

2.3.8 miR-199/miR-199*

Currently, few studies have documented the function of miR-199 in physiological processes but its overexpression has been associated with liver fibrosis (Murakami et al. 2011). miR-199 appears to play an important function during pregnancy given

that its downregulation is observed in laboring myometrium while COX-2 expression is increased (Williams et al. 2012). More strikingly, overexpression of miR-199-3p/miR-214 using miRNA mimics in myometrial cells reduced COX-2 protein expression (Williams et al. 2012). The passenger strand of miR-199, miR-199* is also a potent inhibitor of COX-2 expression. In human osteoarthritis chondrocytes, it has been shown that miR-199* inhibits IL-1β-induced COX-2 expression (Akhtar and Haqqi 2012). Interestingly, IL-1β-induced p38 MAPK activation is inversely correlated with the level of miR-199* and an induction of miR-199* was observed in chondrocytes treated with a p38 inhibitor (Akhtar and Haqqi 2012). COX-2-derived prostaglandins play an important role during embryo implantation (Kennedy et al. 2007; Lim et al. 1999). The role of miRNA in this process has been observed with the expression of miR-101 and miR-199* increased in the murine uterus during embryo implantation, while the expression of COX-2 is reduced (Chakrabarty et al. 2007). Consistent with these findings, the expression of miR-199a* are reduced in a model of endometrial cancer induced by PTEN loss, while the expression of COX-2 is induced (Daikoku et al. 2008).

2.3.9 miR-143/145

The miR-143/145 cluster is located on Chr 5q32 and current findings suggest that they originate from the same primary miRNA (Moore et al. 2011). Downregulation of miR-143/145 is observed in several disorders including colorectal, prostate, esophageal, bladder, osteosarcoma cancers, and hematologic malignancies such as B cell lymphoma or chronic lymphocytic leukemia (Luo et al. 2011; Kojima et al. 2014; Wu et al. 2011; Yoshino et al. 2013; Hu et al. 2012; Fabbri et al. 2008). The suppressive effect of miR-143 on COX-2 expression has been described in gastric, bladder, and pancreatic cancer cells through targeting the COX-2 3′UTR (Wu et al. 2013; Song et al. 2011; Pham et al. 2013). In addition, miR-143-3p and miR-143-5p decrease cell viability and proliferation and induce apoptosis in gastric cancer cells (Wu et al. 2013). MiR-143 expression is diminished in bladder cancer cells and its overexpression leads to COX-2 downregulation and suppression of metastasis (Song et al. 2011).

2.3.10 miR-542-3p

Very few studies have been performed on this miRNAs but current findings suggest that miR-542 acts as a tumor suppressor, through its ability to downregulate the pro-survival oncogene survivin (Althoff et al. 2014). More recently, it has been shown that miR-542-3p regulates PG synthesis by directly targeting the COX-2 3′UTR (Moore et al. 2012). Interestingly, the 3′UTR region targeted by miR-542-3p contains a common single nucleotide polymorphism (SNP) in the COX-2 gene at position 8473 in exon 10 (T8473C; rs5275) that is associated with increased risk and/or NSAID responsiveness in a number of cancers where COX-2 over-expression is a contributing factor. Mir-542-3p was identified to bind transcripts of the 8473T allele and promote mRNA decay, whereas the cancer-associated variant 8473C allele interfered with miR-542-3p binding. Colon cancer cells and tissue displayed COX-2 expression levels that were dependent on T8473C allele dosage and allelic-specific

expression of COX-2 was observed to be a contributing factor promoting COX-2 overexpression (Moore et al. 2012). These findings provide a novel molecular explanation underlying cancer susceptibility associated with COX-2 T8473C SNP and suggest that other SNPs might influence miRNA activity involved in PG synthesis.

2.3.11 miR-338

A relationship between miR-338 loss and several cancers including gastric, colorectal, and neuroblastoma has been found (Peng et al. 2014; Chen et al. 2013; Sun et al. 2014). MiR-338 plays an important regulatory role in PG synthesis by targeting directly PLA2G4B (Phospholipase A2 Group VI B), a member of the $cPLA_2$ family in human chorioamniotic membrane (Montenegro et al. 2009). The downregulation of miR-388 at term is associated with an induction of PLA2G4B and inhibition of miR-338 leads to an induction of PLA2G4B in decidual cells (Montenegro et al. 2009).

2.3.12 Targeting miRNAs as a Therapeutic Approach to Restore Prostaglandin Homeostasis

Altered prostaglandin homeostasis in various pathologies has driven various therapeutic strategies aimed at targeting the activity of the key enzymes involved in this process. In this context, several pharmacologic inhibitors specifically targeting the activity of COX-2 have been developed (e.g. celecoxib, rofecoxib). However, this therapeutic approach revealed also several off-target and severe side effects, such as an increased risk of myocardial infarction, gastric ulceritis or hepatic toxicity that limit clinical feasibility (Tegeder et al. 2001; Tan et al. 2007; Steffel et al. 2006; Dogne et al. 2006). More recently, it has been suggested that targeting the expression of COX-2, rather than its activity would be a better approach with less side effects (Cerella et al. 2010). An attractive alternative strategy may reside in modulating the expression or activity of pathogenic miRNAs involved in promoting altered prostaglandin homeostasis. Moreover, the ability of some miRNAs to target multiple players in the PG pathway would allow an efficient inhibition of PGs synthesis. Recent findings have shown that chemically modified synthetic nucleotides mimicking or inhibiting endogenous miRNAs can be used for therapeutic purpose (van Rooij et al. 2012; Stenvang et al. 2012; Thorsen et al. 2012; Garzon et al. 2010; Deiters 2010). Antisense modified oligonucleotides (AMOs) (2′-O-methoxyethyl phosphorothioate modified antisense oligonucleotide; 2′-Fluoro modified antisense oligonucleotide), antagomiRs (3′cholesterol-conjugated, 2′-O-Me oligonucleotides having terminal phosphorothioate), and locked nucleic acid (LNA) modifications display increased affinity for their targets and are stable in serum allowing for in vivo delivery (Stenvang et al. 2012). Delivery of synthetic oligonucleotides has shown in vivo feasibility through intravenous or intraperitoneal administration. Miravirsen, a locked nucleic acid–modified DNA phosphorothioate antisense oligonucleotide targeting miR-122, was the first miRNA-targeted drug to enter clinical trials in 2008 for the treatment of HCV by intravenous injection (Hu et al. 2012). Other studies have demonstrated the ability of new formulated

2′-O-(2-methoxyethyl) modified antisense oligonucleotide (2′-MOE ASO) combined with a permeation enhancer (sodium caprate) for oral administration in humans (Tillman et al. 2008). In addition, special formulations have been made in order to improve the delivery of oligonucleotides such as liposomal or oleic-based nanoparticles formulation (Trajkovski et al. 2011; Wang et al. 2013b).

Several molecules from natural origins and particularly dietary polyphenols have the ability to modulate the expression of miRNAs involved in various pathologies. These compounds, such as curcumin and coffee polyphenols, can be found in many fruits and vegetables and display beneficial effects in various disorders *via* oral delivery (Prasad et al. 2014; Murase et al. 2011). Some of these molecules can modulate the expression of specific miRNAs involved in PGs biosynthesis. For instance, epigallocatechin gallate (EGCG), from green tea upregulates miR-16 in hepatocellular carcinoma cells (Tsang and Kwok 2010).

2.4 Processing Bodies and Stress Granules

Processing bodies (P-bodies) and stress granules (SG) are RNA granules that play an integral role in mRNA turnover and translational regulation. Although P-bodies were originally identified as cytoplasmic sites of mRNA degradation, evidence indicates that it can also serve as sites of short-term mRNA storage (Eulalio et al. 2007; Garneau et al. 2007). Similarly, SGs serve as interim sites of mRNA storage in cells subjected to severe stress. Both P-bodies and SGs are non-membrane bound granula whose formation is dynamic and reversible. Immunofluorescence analysis demonstrates that, in response to stress, P-bodies and SGs exist in close proximity and partial co-localize. This aspect supports the hypothesis that transcripts traffic between P-bodies and SGs, and that irreversible stresses may result in shuttling of mRNA transcripts from SGs to P-bodies for subsequent decay (Kedersha et al. 2005; Anderson and Kedersha 2008).

Since their discovery, many studies have catalogued the core components that comprise these RNA granules. P-bodies harbor enzymes responsible for mRNA decapping (e.g., DCP1a, DCP2, HEDLS), mRNA deadenylation (CAF-1, CCR4) and degradation (XRN-1), miRNA decay machinery (e.g., AGO2), and components of nonsense-mediated mRNA decay (NMD) (Eulalio et al. 2007; Kulkarni et al. 2010; Zheng et al. 2011) (Fig. 9.4). SGs are comprised of largely stalled translational pre-initiation complexes, small ribosomal subunits (e.g., eIF3, eIF4E, eIF4G) and their core components (Kedersha and Anderson 2007). In addition to these core components, several ARE-binding proteins have been shown to transiently localize to P-bodies and SGs, allowing for delivering of their mRNA target for translational suppression and/or decay (Blanco et al. 2014; Franks and Lykke-Andersen 2007; Gilks et al. 2004). Recently it has been shown that ARE-binding proteins play an important role in the control of P-body formation. Induction of TTP expression by the growth-inhibitory cytokine TGF-β results in enhanced P-body formation, resulting in TTP-driven sequestration of the COX-2 mRNA transcript to P-bodies

(Blanco et al. 2014). This ability of TTP to promote P-body formation was also observed in vivo, where analysis of colonic epithelium in TTP knockout mice showed limited numbers of P-bodies per cell as compared to wild-type littermates. Similarly, fibroblasts derived from these mice indicate that absence of TTP expression dramatically impairs P-body formation (Blanco et al. 2014). In line with these findings, loss of TTP expression in epithelial tumors correlates with low cellular number of P-bodies. These findings provide mechanistic insights into how TTP promotes the decay of COX-2 mRNA and controls prostaglandin formation in normal intestinal epithelium.

Similar to P-bodies, ARE-binding proteins can deliver mRNA transcripts to SGs. Although many stressors have been shown to induce SG formation (nutrient deprivation, oxidative stress, heat-shock), and oxidative stress appears to enhance TTP localization to SGs (Kedersha and Anderson 2007). This indicates the ability of TTP may serve as a shuttling protein mediating a functional interplay between SGs and P-bodies. The translational repressor TIA-1 has been shown to localize to SGs. Similarly, HuR and CUGBP2 have been observed in SGs when cells are subjected to heat-shock and other stresses (Gilks et al. 2004; Kedersha and Anderson 2007). The presence of ARE-binding proteins may be indicative of a protective cellular mechanism against stress in an attempt to orchestrate post-stress cellular repair without the high-energy cost of de novo transcription.

3 Conclusions

The biological effects of prostaglandins are diverse in normal physiology and pathophysiological conditions. It is well established that increased PGE_2 synthesis and signaling is a contributing factor in inflammation and cancer. Substantial evidence has demonstrated the causal role of unregulated COX-2 expression in many chronic diseases and cancer, along with evidence indicating the benefit of inhibiting COX-2 activity. Due to the unwanted side effects associated with long-term COX-2 inhibition, potential alternatives may reside in pharmacological targeting of other key factors (i.e. mPGES and EP receptors) or using natural product-based inhibitors. The work reviewed here demonstrates targeting gene expression by influencing the activity of post-transcriptional regulators to be feasible approach and gaining momentum as our understanding of these ARE-binding proteins and miRNAs increases. MiRNAs have been linked to a variety of human diseases and are promising therapeutic targets due to their pleiotropic effects on a large number of genes. Developing therapeutic strategies that will substitute or restore miRNA expression controlling genes in prostaglandin biosynthesis may prove to be more comprehensive than targeting individual genes. Further identification and characterization of RNA-bindings proteins that interact with 3′UTR AU-rich elements present in genes discussed holds great promise for therapeutic targeting that can selectively inhibit RNA-binding protein function. Ultimately, these novel approaches will allow the nascent cellular post-transcriptional machinery to counteract the pathogenic effects of chronic mRNA stabilization.

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Translational and Post-translational Control of Leptin Production by Fat Cells

10

Konstantin V. Kandror

1 Introduction

Leptin, a 16 kDa product of the *ob* gene, is synthesized predominantly in adipocytes and targets central nervous system regulating food intake, energy expenditure, and several other important physiological functions of mammalian organisms (Dalamaga et al. 2013; Ahima and Flier 2000; Friedman 2009). Discovery of leptin 20 years ago (Zhang et al. 1994) opened a new era in obesity research. Originally, it has been thought that leptin works as a "lipostat", as food intake and accumulation of energy stores in fat cells result in an increase in leptin production thus leading to inhibition of appetite and elevation of energy expenditure. Conversely, when fat stores decline, adipocytes reduce leptin production, and food intake is increased. This simple or better, over-simplified model has nonetheless triggered a great interest to leptin as a potential anti-obesity medication. However, in spite of early impressive results obtained in rodents and several promising studies in humans (Friedman 2009) it appears that human obesity is, for the most part, accompanied by elevated circulating leptin levels and is often resistant to exogenous leptin. At the same time, other human studies have convincingly demonstrated that weight loss results in a decrease in plasma leptin concentrations, and that low leptin levels predispose human patients to weight gain. These and other experiments have suggested that although humans may be resistant to increased leptin levels, a fall in plasma leptin stimulates food intake, so that leptin's main role in humans may be to protect fat stores in order to improve survival when food is scarce (Unger 2004). In agreement with this idea, it was shown that in humans, feeding increases and starvation decreases leptin levels which may explain the high failure rate of dieting (Ahima and Flier 2000). Indeed,

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administration of low doses of leptin may help to lose weight without the anguish and distress that usually accompany this process (Rosenbaum et al. 2005).

Regardless of how leptin exerts its biological activity, it is clear that nutrient uptake/status is directly coupled to leptin production in adipocytes. The mechanism(s) of this phenomenon are only beginning to emerge. Since this understudied and inadequately discussed regulatory connection is central to all proposed mechanisms of leptin action, it will be the main focus of our review. The problem has at least two aspects: a short term and a long term connection. First, circulating leptin levels increase within hours after feeding and decrease shortly after food deprivation. Although in humans, this effect may not be as fast and robust as in rodents, all mammals studied thus far still demonstrate a direct link between food intake and circulating leptin (Ahima and Flier 2000). Second, leptin production depends on the adipocyte size, since larger cells contain and secret more leptin than smaller cells (Hamilton et al. 1995). Since adipocyte size is defined by the volume of the central lipid droplet that is not likely to change significantly in response to each meal, this phenomenon shows a long lasting correlation between the amount of stored energy and leptin expression. At the physiological level, this translates into elevated leptin levels in obesity (Ahima and Flier 2000; Friedman 2009). At present, it is unknown whether or not the acute regulation of leptin expression by feeding has the same molecular basis as the long-term regulation by fat accumulation and/or adipocyte volume. We suggest that post-transcriptional mechanisms may be responsible for the regulation of leptin production by nutrients.

2 An Acute Connection between Food Intake and Leptin Production

2.1 Expression of Leptin in Adipocytes is Regulated at Multiple Levels

As circulating leptin and insulin levels increase after feeding and decrease after food deprivation (Ahima and Flier 2000; Levy et al. 1997; Frederich et al. 1995), the predominant hypothesis in the field has been that leptin production is controlled by insulin (Ahima and Flier 2000). In particular, it has been proposed that insulin activates leptin expression at the level of transcription (Becker et al. 1995; Saladin et al. 1995; MacDougald et al. 1995) via an as yet unclear mechanism(s). This idea, however, has not been confirmed by experiments in vitro. Indeed, the effect of insulin on leptin production in isolated rat adipocytes is resistant to the inhibitor of transcription actinomycin D (Bradley and Cheatham 1999; Roh et al. 2003). In addition, regulation of leptin production by insulin and nutrients is sustained in isolated adipocytes and samples of fat tissue (Bradley and Cheatham 1999; Roh et al. 2003; Lee and Fried 2006; Lee et al. 2007a; Barr et al. 1997; Levy and Stevens 2001; Mueller et al. 1998) where neither insulin (Bradley and Cheatham 1999; Roh et al. 2003; Lee et al. 2007b) nor nutrient status (Lee and Fried 2006; Lee et al. 2007a) affect the levels of leptin mRNA. This suggests that insulin and nutrients control the expression of the ob gene not (or not exclusively) at the level of transcription.

2.2 Regulation of Leptin Production at the Level of Translation

Alternatively, it has been suggested that leptin expression is regulated at the level of translation via mTORC1. This is an attractive hypothesis, as the activity of mTORC1 depends not only on insulin levels but also, on nutrient and energy availability (Dibble and Manning 2013; Sengupta et al. 2010) providing an additional physiological dimension to the regulation of leptin production.

A brief description of regulation and signaling of mTOR is warranted here. mTOR is an evolutionarily conserved serine/threonine protein kinase which is distributed between the two functionally distinct protein complexes, TORC1 and less studied TORC2. The former is composed of mTOR, raptor, mLST8, PRAS40, and deptor (Sengupta et al. 2010; Foster and Fingar 2010; Howell and Manning 2011) and is regulated by the TSC protein complex. One of its subunits, TSC2, is phosphorylated by a variety of hormone- and nutrient-dependent protein kinases, such as Akt, ERK and AMP-activated protein kinase (AMPK) (Huang and Manning 2008). These phosphorylation events control the ability of TSC2 to serve as a GTPase activating protein for the low molecular weight GTPase Rheb that directly binds to and activates mTORC1 (Foster and Fingar 2010; Huang and Manning 2008; Inoki et al. 2003). Phosphorylation of TSC2 by Akt and ERK increases, while phosphorylation of TSC2 by AMPK decreases levels of GTP-bound Rheb thus leading to up- and down-regulation of mTORC1 activity. In addition, mTORC1 is activated by amino acids (in particular, by branched chain amino acids, such as leucine) via recently described mechanisms (Efeyan et al. 2012). Although additional pathways of mTORC1 regulation exist, it is clear that insulin and nutrients activate while lack of energy rapidly inhibits mTORC1. Therefore, this complex has emerged as a central sensor of energy- and hormone-dependent metabolic regulation in metazoans that could potentially control leptin expression.

Early reports have demonstrated that leptin expression in adipocytes is not only regulated by insulin but also depends on the intracellular energy metabolism (Levy and Stevens 2001; Mueller et al. 1998). Leptin expression correlates very well with the level of intracellular ATP (Levy et al. 2000), and is rapidly up-regulated by leucine (Roh et al. 2003) indicating the involvement of mTORC1 in its regulation. In parallel, it has been shown that the activity of mTORC1 in adipocytes in vitro and in vivo is increased by insulin and by nutrient signals (in particular, by leucine) in the meal (Lynch et al. 2002, 2006). We and others have found that insulin- and leucine-induced increase in leptin production in isolated primary adipocytes and in cultured 3T3-L1 adipocytes is blocked by the mTORC1 inhibitor, rapamycin (Bradley and Cheatham 1999; Roh et al. 2003; Lee et al. 2007a). Finally, stable expression of the constitutively active form of Rheb increases leptin expression in 3T3-L1 adipocytes (Chakrabarti et al. 2008). Activation of mTORC1 by stable over-expression of the dominant-negative form of AMP-activated protein kinase has a similar effect (Chakrabarti et al. 2008). Thus, it is very likely that insulin and nutrients activate leptin expression in adipocytes via the mTORC1-mediated pathway. Quantitatively, plasma leptin levels in rats rise 2.5–3 fold within 3 h after food intake (Levy et al. 1997). Activation of mTORC1 in rat adipocytes by leucine administration (Roh et al. 2003) or by over-expression of constitutively active Rheb

or dominant-negative form of AMPK (Chakrabarti et al. 2008) causes a comparable activation of leptin biosynthesis. This suggests that regulation of leptin expression at the level of translation via mTORC1 plays a predominant role in the post-prandial control of leptin levels irrespective of potential changes in transcription of the *ob* gene.

How exactly mTORC1 regulates leptin translation is not clear. mTORC1 exerts its biological effects on protein biosynthesis via phosphorylation of the protein kinase S6K1 and translational repressor 4E-BP which leads to activation of the former and inhibition of the latter. Importantly, phosphorylation of S6K1 and 4E-BP may represent two independent pathways of translational control in the cell (Sonenberg and Hinnebusch 2009). In particular, phosphorylation of 4E-BP is required for the initiation of translation of mRNAs with double stranded regions in the 5′-UTR. The effect of S6K1 on translation is more complicated and may or may not require the presence of a 5′-terminal oligopyrimidine tract in target mRNAs.

Leptin mRNA lacks a 5′-terminus oligopyrimidine tract, but contains predicted hairpins in its 61-nt long (in case of humans) 5′-UTR. However, addition of leptin 5′-UTR to the reporter message does not inhibit, but rather stimulates its basal level of translation and, in addition, renders this mRNA resistant to regulation by mTORC1 (Lee et al. 2007a; Chakrabarti et al. 2008). This suggests that mTORC1 controls translation of leptin mRNA via a novel mechanism that may be somehow related to multiple structural elements localized in its long (ca. 3 kb) 3′-UTR (Zhang et al. 1994). Fried's laboratory has explored the role of leptin's 3′-UTR in translation and found that it decreases the efficiency of translation of luciferase mRNA under basal conditions, whereas insulin administration stimulates its translation (Lee et al. 2007a).

Importantly, specific sequences in the 3′-UTR are known not only to regulate the efficiency of mRNA translation but also, to define its intracellular localization (Kloc et al. 2002). In primary adipocytes, ca. two thirds of leptin mRNA is associated with heavy membrane fraction (primarily, endoplasmic reticulum) and only one third is found in the cytosol in a form of free 80S mRNP (Roh et al. 2003). Since this ratio is not significantly changed in response to activation of mTORC1, it is likely that mTORC1 specifically activates expression of leptin messages that are compartmentalized on the endoplasmic reticulum (i.e. the site where secreted proteins are synthesized). Thus, it is possible that the long 3′-UTR of leptin mRNA contains elements that are responsible for its compartmentalization on the endoplasmic reticulum and for the regulation of its translation there. However, the detailed structure-functional analysis of this potentially highly important part of the leptin message has not yet been carried out.

2.3 Regulation of Leptin Production at the Level of Secretion

Studies both in vivo and in vitro have demonstrated that secretion of leptin from adipose cells has two components: constitutive and regulated. In other words, adipocytes continuously release leptin into the medium, but this process may be

acutely and substantially stimulated by insulin without any marked changes in the constitutive secretory pathway. The acute effect of insulin on leptin secretion precedes major changes in its biosynthesis and is preserved, at least partially, in the presence of cycloheximide (Bradley and Cheatham 1999; Lee and Fried 2006; Barr et al. 1997; Roh et al. 2000; Zeigerer et al. 2008). This suggests that fat cells may possess a regulatable pool of pre-synthesized leptin that can be discharged by insulin. In agreement with this idea, it has been shown that the major pool of the intracellular leptin in adipocytes is localized in small membrane vesicles (Barr et al. 1997; Roh et al. 2000, 2001; Bornstein et al. 2000; Ye et al. 2010) that are rapidly depleted by insulin and may thus represent a regulated secretory compartment. However, the storage capacity of this compartment is limited. It has been estimated that the size of the leptin storage pool in rat adipocytes is roughly equivalent to 1 h of constitutive secretion (Lee and Fried 2006; Roh et al. 2000), although this number may be underestimated as adipocytes tend to loose leptin in the process of cell isolation and, especially, during collagenase treatment (Barr et al. 1997). It is possible that regulation of leptin production at the level of translation and at the level of secretion have different biological functions: the former is likely to couple leptin production to food intake as described in the previous section while the latter may be responsible for the rapid pulsatile oscillations in circulating leptin levels that take place in vivo (Licinio et al. 1997, 1998; Sinha et al. 1996).

In general, an important functional feature of the adipocyte is that, unlike highly specialized endocrine and exocrine cells, it secretes a large variety of the biologically active protein products that include dozens of hormones, cytokines, acute-phase reactants, and enzymes (Adamczak and Wiecek 2013). The ability of fat cells to produce and to secrete adipokines in an orderly fashion is based on the unique structure of the secretory machinery that is clearly different from ones present in "classical" secretory and "constitutive" cells. Typically, secreted proteins are transported by specialized vesicular carriers through either the constitutive secretory pathway that exists in all cell types or through the regulated secretory pathways that are thought to be restricted to neuronal, exocrine and endocrine cells only (Burgess and Kelly 1987; Miller and Moore 1990). Secretion from adipose cells, however, does not fit this model. Although adipose cells continuously secrete their protein products, this process does not meet the definition of constitutive secretion. Firstly, fat cells accumulate substantial intracellular amounts of adipokines while typical constitutively secreted proteins are normally not stored inside the cell in any significant quantities (Miller and Moore 1990). Secondly, the release of such adipokines as leptin, adiponectin (Bogan and Lodish 1999; Scherer et al. 1995; Xie et al. 2008), adipsin (Kitagawa et al. 1989; Millar et al. 2000), and resistin (Ye et al. 2010) may acutely be stimulated by insulin and, possibly, by other secretagogues. However, leptin is the only adipokine for which the existence of a separate "storage compartment" has been demonstrated so far. Although the cell biological nature of leptin-containing secretory vesicles in adipocytes remains largely unexplored, they are different from the peptide-containing secretory granules present in neuronal, endocrine, and exocrine cells in buoyant density, sedimentational behavior, and kinetics of secretion (Roh et al. 2000). In this regard, it has been long known that

"constitutive secretory" cells may, in fact, possess regulated secretory pathways as well (Saucan and Palade 1994; Chavez et al. 1996). However, such pathways have not been systematically studied.

Secretion of another major adipocyte product, lipoprotein lipase or LPL, from fat cells follows its own non-canonical pathway. It has been demonstrated that adipocytes accumulate large amounts of active LPL (Enerback and Gimble 1993) suggesting that this enzyme is not efficiently targeted to the constitutive secretory pathway but is stored in intracellular compartment(s) that represents/includes the endoplasmic reticulum (ER). It has also been reported that insulin and serum may acutely stimulate the release of LPL activity from adipose cells (Fried and DiGirolamo 1986; Pradines-Figueres et al. 1988). However, this effect of insulin is completely blocked by cycloheximide (Eckel et al. 1984). Thus, insulin may or may not have a direct effect on secretion of LPL; rather, insulin may acutely increase its biosynthesis and/or activity.

The major intracellular pool of leptin does not overlap with that of LPL (Roh et al. 2001), adiponectin (Xie et al. 2008), and resistin (Ye et al. 2010) suggesting that leptin is localized in its own specialized storage vesicles. On the contrary, most of LPL and adiponectin reside in the ER (Roh et al. 2001; Qiang et al. 2007; Wang et al. 2007; Karki et al. 2011). Thus, the secretory process in adipose cells may be regulated at different levels: at the exit from the ER, as may be the case for LPL and adiponectin (Qiang et al. 2007; Wang et al. 2007) and at the level of the downstream vesicular storage compartment (leptin). Constitutively secreted proteins should be able to pass freely through all checkpoints in the secretory pathway. The exit of LPL and adiponectin from the ER may be controlled by different mechanisms. LPL requires a specialized chaperone, lipase maturation factor 1 (Peterfy 2012) and the adaptor protein Sel1L (Sha et al. 2014), whereas the release of adiponectin from the ER takes place in a thiol-mediated fashion and depends on the interplay between the two ER chaperones, ERp44 and Ero1-Lα (Qiang et al. 2007; Wang et al. 2007). Also, exit from the ER does not seem to be the only regulatable step in the intracellular trafficking pathway of adiponectin as this protein bypasses the endosomal compartment as well (Xie et al. 2008). Secretion of another adipokine, adipsin, also seems to follow the endosome-mediated pathway (Millar et al. 2000). Unfortunately, the molecular details of this process are not known.

In addition to secretion, adipocytes possess other routes of regulated protein traffic to the plasma membrane. In particular, these cells acutely translocate intracellular Glut1- and Glut4-containing membrane vesicles to the cell surface in response to insulin stimulation (Bogan 2012; Kandror and Pilch 1996). However, the integral membrane protein Glut4 and free soluble adipokines, such as leptin, adiponectin, adipsin, resistin, and LPL, are localized in different vesicular carriers (Barr et al. 1997; Roh et al. 2000, 2001; Ye et al. 2010; Bogan and Lodish 1999; Millar et al. 2000) strongly suggesting that the "Glut4 pathway" is different from secretory pathway(s) in adipocytes.

In summary, we are still far from the complete understanding of the cell biology of adipocyte secretion, although there is little doubt that the mechanistic dissection

of this process represents an urgent biomedical problem. Eventually, we should learn how to facilitate the release of adipokines of our choice and to inhibit secretion of unwanted proteins.

2.4 Regulation of Leptin Production at the Level of Degradation

Lee and Fried have found that up to 50 % of newly synthesized leptin molecules is rapidly degraded in lysosomes instead of being secreted (Lee and Fried 2006). This phenomenon is not unique for leptin. In adipocytes, a large fraction of de novo synthesized secreted proteins undergoes immediate lysosomal degradation. For example, up to 80 % of newly synthesized LPL molecules (Appel and Fried 1992; Olivecrona et al. 1987; Semb and Olivecrona 1987; Vannier and Ailhaud 1989) and a non-specified but significant part of newly synthesized adiponectin (Karki et al. 2011) entry the degradative pathway instead of being secreted. It is yet hard to tell why the cell decides to operate in such a way; it is clear, however, that "futile cycles" of biosynthesis and degradation are often seen in metabolism. One possibility is that such a mechanism provides a faster response to rapid changes in metabolic conditions. According to this idea, the cell may prefer to re-route secreted proteins from degradation to secretion and vice versa rather than to induce/suppress protein expression that takes a much longer time. Other factors, however, may also play a role. For example, it is feasible that the secretory capacity of the adipocyte is limited so that intracellular adipokines compete for being released outside the cell. In any case, interfering with lysosomal targeting/degradation may be a promising way to increase levels of circulating leptin and to improve metabolism in obese and insulin resistant patients.

3 A Long-Term Connection between Obesity and Leptin Secretion

The problem of leptin production has another important aspect. In addition to the short-term connection with food intake, circulating leptin levels are known to be steadily elevated in obesity (Ahima and Flier 2000; Friedman 2009). At the cellular level, larger adipocytes contain and secrete more leptin than smaller cells (Hamilton et al. 1995; Russell et al. 2001). Since the adipocyte size is defined primarily by the volume of the central lipid droplet, this phenomenon may show a cell-autonomous connection between the amount of stored energy (i.e. obesity at the molecular level) and leptin expression. The correlation between the size of the adipocyte and the level of leptin production was recognized for a long time but its mechanism remained obscure. Clearly, a single act of food intake cannot possibly change the size of the adipocyte in a significant fashion, so there should be another explanation for this phenomenon. We suggest that a simultaneous increase in the

adipocyte size and leptin production may represent two independent consequences of the same stimulus.

Recent work has revealed that fat storage in adipocytes is also controlled by mTORC1. In particular, Polak et al. have found that adipose-specific raptor null mice have less adipose tissue and are protected against diet-induced obesity (Polak et al. 2008). The authors attributed this effect to elevated energy expenditure due to the mitochondrial uncoupling. However, this model contradicts data from several research groups who have reported that the mTORC1 pathway positively regulates mitochondrial respiration (Ramanathan and Schreiber 2009; Schieke et al. 2006; Cunningham et al. 2007; Bentzinger et al. 2008). Furthermore, knock down of raptor in 3T3-L1 adipocytes does not uncouple mitochondria in vitro (Polak et al. 2008), suggesting that the latter phenomenon may not be directly linked to mTORC1 inhibition, but rather, reflect some secondary physiological effects the molecular mechanisms of which have yet to be elucidated.

It has also been shown that inhibition of mTORC1 signaling suppresses early adipogenesis (Polak et al. 2008; Kim and Chen 2004; Carnevalli et al. 2010; Zhang et al. 2009; Yeh et al. 1995) and/or lipogenesis (Lamming and Sabatini 2013; Ricoult and Manning 2013; Porstmann et al. 2008; Li et al. 2010; Chakrabarti et al. 2010; Laplante and Sabatini 2009) which may represent an alternative connection between the mTORC1 activity and fat storage. Simultaneously with stimulation of lipogenesis, mTORC1 inhibits lipolysis in adipocytes likely via the transcriptional regulation of the rate-limiting lipolytic enzyme, ATGL (Chakrabarti et al. 2010, 2013). The latter effect of mTORC1 may be especially significant since mTORC1-mediated inhibition of lipolysis contributes to overall triglyceride accumulation in adipocytes at least ten times more than stimulation of de novo lipogenesis (Chakrabarti et al. 2010).

Thus, the same mTORC1-mediated signaling pathway is responsible not only for the regulation of leptin expression at the level of translation but also, controls triglyceride storage in adipocytes via several different mechanisms (Fig. 10.1). Importantly, chronic over-nutrition and obesity lead to continuous activation of mTORC1 (Chakrabarti et al. 2013; Khamzina et al. 2005; Wang et al. 2009; Um et al. 2004) which should promote triglyceride storage (thus increasing the size of adipocytes) on one hand and stimulate leptin production on the other. In other words, the size of the adipocyte may serve as an indicator of the cumulative mTORC1 activity that may explain the correlation between the adipocyte size and the levels of leptin production. Normally, this situation should create a feedback regulatory loop that terminates eating and limits the expansion of fat stores. In humans, years of elevated leptin may lead to the classical desensitization response which may explain resistance to leptin in human obesity (Friedman 2009). However, more studies are needed to determine the molecular nature of leptin resistance and to discover safe ways to overcome it.

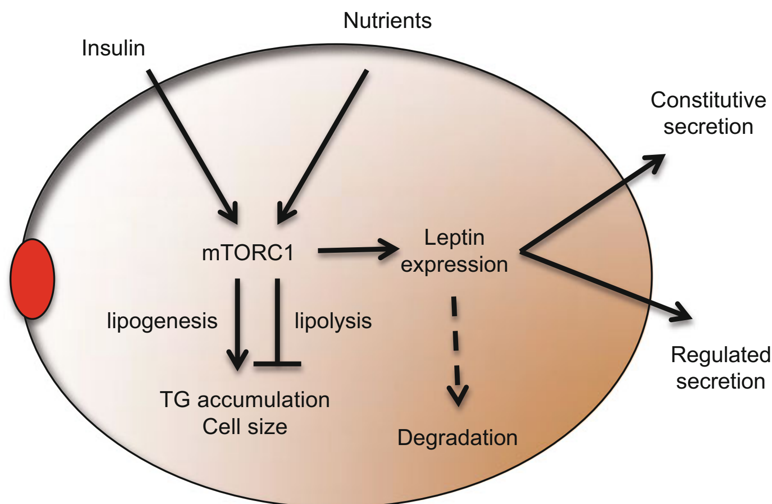

Fig. 10.1 mTORC1 couples leptin secretion to the adipocyte size. Insulin and nutrients activate leptin biosynthesis via the mTORC1-mediated pathway that also promotes storage of triglycerides and increases the size of the cell. De novo synthesized leptin molecules are partitioned between constitutive and regulated secretion and immediate degradation

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Post-transcriptional Regulation of Parathyroid Hormone Gene Expression in Health and Disease

11

Tally Naveh-Many

1 Introduction

Parathyroid hormone (PTH) regulates serum calcium and phosphate levels and bone strength. Changes in serum calcium are sensed by the seven *trans* membrane G-protein coupled calcium sensing receptor (CaR) on the parathyroid cell membrane. The parathyroid is unique in that the trigger for PTH secretion is a low extra-cellular calcium rather than high calcium as for other hormones. Small decreases in serum calcium and more prolonged increases in serum phosphate stimulate the parathyroid to secrete PTH which then acts on its target organs the kidney and bone to correct serum calcium and phosphate levels (Fig. 11.1) (Silver and Naveh-Many 2009). Dietary induced hypocalcemia or uremia lead to secondary hyperparathyroidism (SHP) with increased serum PTH, PTH mRNA levels and parathyroid cell proliferation (Moallem et al. 1998; Naveh-Many et al. 1995). SHP is a common disorder in patients with CKD and is characterized by excessive serum PTH levels, parathyroid hyperplasia and an imbalance in calcium and phosphorus metabolism. SHP develops early in the course of CKD and becomes more prominent as kidney function declines. SHP of CKD is associated with an increased morbidity and mortality (Silver et al. 2002).

Parathyroid cells have few secretory granules as compared to other endocrine cells and therefore PTH production is regulated largely at the levels of PTH gene expression and parathyroid cell proliferation (Habener et al. 1984). The changes in PTH gene expression by calcium phosphate and CKD are due to post-transcriptional mechanisms affecting PTH mRNA stability. The parathyroid also responds to changes in serum 1,25$(OH)_2$ vitamin D (1,25D) which decreases PTH levels. PTH

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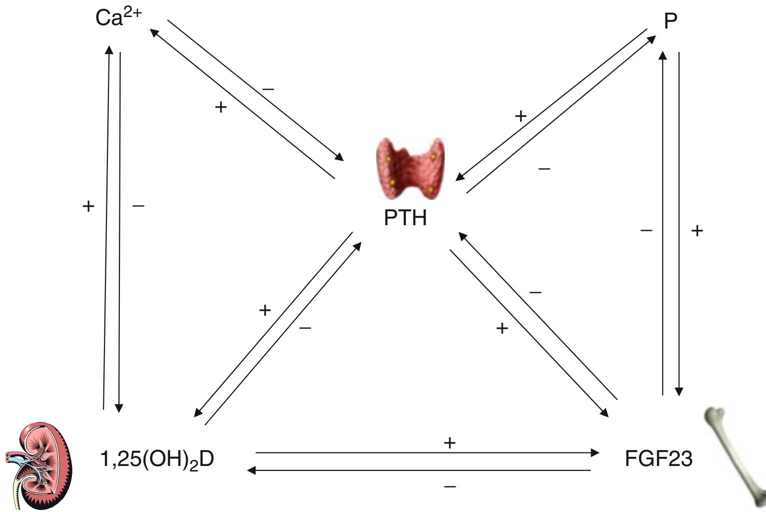

Fig. 11.1 Calcium, phosphate, PTH, FGF23, 1,25D and FGF23 interactions. There are endocrinological feedback loops that govern mineral homeostasis. FGF23, fibroblast growth factor 23; PTH, parathyroid hormone, P, serum phosphate (Silver and Naveh-Many 2009)

in turn, increases the renal synthesis of 1,25D. 1,25D then increases blood calcium largely by increasing the efficiency of intestinal calcium absorption (Fig. 11.1). The increased serum calcium would shut down PTH secretion by activating the parathyroid calcium sensing receptor (CaR) (Brown et al. 1993). In contrast to the post-transcriptional regulation of PTH gene expression by changes in serum calcium and phosphate levels and CKD, 1,25D decreases PTH gene transcription in vitro and in vivo (Silver et al. 1985, 1986; Russell et al. 1986). The 1,25D receptor (VDR) is expressed in the rat parathyroid, confirming that the parathyroid is indeed a target organ for vitamin D (Naveh-Many et al. 1990). Several groups have identified DNA sequences in the 5′-flanking region of the PTH gene that may mediate the negative regulation of PTH gene transcription by 1,25D (Demay et al. 1992; Fujiki et al. 2005; Murayama et al. 2004). The action of 1,25D to decrease serum PTH is used therapeutically in the management of CKD patients. They are given 1,25D or its prodrug 1α(OH)-vitamin D_3 to treat or prevent the secondary hyperparathyroidism (2HPT) of CKD. Fibroblast growth factor 23 (FGF23) is a bone-derived phosphaturic hormone that acts on the kidney to increase phosphate excretion and suppress biosynthesis of 1,25D (Fig. 11.1). FGF23 signals through the fibroblast growth factor receptor 1 (FGFR1) bound by the transmembrane protein Klotho (Kurosu et al. 2006). We have identified the parathyroid as a target organ for FGF23 (Ben Dov et al. 2007a). Recombinant FGF23 decreases PTH gene expression and secretion in vivo and in vitro by activation of the MAP kinase pathway (Fig. 11.1). In CKD there are very high levels of serum FGF23 together with increased serum PTH levels, due to down regulation of the kloth-FGFR1 FGF23 receptor complex in the parathyroid, leading to resistance of the parathyroid to FGF23 (Galitzer et al. 2010; Komaba

et al. 2010). In this chapter we discuss the molecular mechanisms of the post-transcriptional regulation of PTH gene expression by calcium, phosphate and CKD.

2 Post-transcriptional Regulation of PTH Gene Expression by Calcium, Phosphate and CKD

Hypocalcemia, hypophosphatemia and CKD regulate PTH mRNA stability by post-transcriptional mechanisms. Dietary induced hypocalcemia and experimental CKD markedly increase PTH secretion, mRNA levels and after prolonged stimulation, parathyroid cell proliferation (Moallem et al. 1998; Naveh-Many et al. 1995). In the rat, hypocalcemia leads to a >10-fold increase in PTH mRNA levels and this increase is post-transcriptional affecting mRNA stability (Moallem et al. 1998). Serum phosphate also has a direct effect on PTH secretion, PTH mRNA levels and parathyroid cell proliferation (Kilav et al. 1995; Naveh-Many et al. 1995). Careful in vivo studies showed that the regulation of PTH gene expression by dietary induced hypophosphatemia is independent of changes in serum calcium and 1,25D (Fig. 11.1) (Kilav et al. 1995). In vitro studies, when tissue architecture was maintained, confirmed the direct effect of phosphate on the parathyroid (Almaden et al. 1996, 1998; Nielsen et al. 1996; Rodriguez et al. 1996; Slatopolsky et al. 1996). In vivo studies showed that phosphorus depletion leads to a dramatic decrease in rat PTH mRNA levels and that this effect is post-transcriptional as is the effect of hypocalcemia to increase PTH mRNA levels (Kilav et al. 1995; Moallem et al. 1998). There is a ~60-fold difference in PTH mRNA levels between hypocalcemic and hypophosphatemic rats and these dietary models were used as tools to define the mechanism of the post-transcriptional regulation of PTH gene expression (Moallem et al. 1998; Nechama et al. 2008).

Secondary hyperparathyroidism is a common disorder in patients with CKD and in experimental models where there are increases in PTH gene expression, secretion and parathyroid hyperplasia (Naveh-Many and Silver 1990; Silver et al. 2002). In CKD patients with 2HPT, calcimimetics and oral phosphate binders are effective drugs used to control the high serum PTH levels (Block et al. 2004; D'Haese et al. 2003; Joy and Finn 2003; Moe et al. 2005). In a rat model of CKD induced by an adenine high phosphorus diet, PTH mRNA levels were increased already after 7 days of the diet and more so at 21 days (Levi et al. 2006). The addition of the calcimimetic R568 or an oral phosphate binder, lanthanum carbonate (La), decreased PTH mRNA and serum PTH levels in the CKD rats (Ben Dov et al. 2007b). The effects of CKD and the calcimimetic or the phosphorus binder were post-transcriptional as were those of changes in serum calcium and phosphate (Moallem et al. 1998; Nechama et al. 2009a; Yalcindag et al. 1999).

Therefore, PTH gene expression is regulated post-transcriptionally by serum calcium, phosphate, CKD and its management by calcimimetics and oral phosphorus binders. The changes in PTH mRNA stability are mediated by protein-PTH mRNA interactions that determine the susceptibility of PTH mRNA to the degradation machinery (Kilav et al. 1995; Levi et al. 2006; Moallem et al. 1998; Nechama et al.

2008). The balanced interaction of stabilizing and destabilizing proteins with the PTH mRNA determines PTH mRNA stability and levels, serum PTH and the resultant response of the parathyroid to calcium, phosphate and CKD (Nechama et al. 2008, 2009c).

3 The PTH mRNA *cis*-Acting Protein Binding Element

For many mRNAs, post-transcriptional regulation involves critical *cis*-acting elements, mostly in the untranslated regions (UTR) that are targets for *trans*-acting proteins regulating mRNA stability and translation (Barreau et al. 2006). Adenine and Uridine-rich elements (ARE) are a well-defined family of *cis*-acting elements critical for the expression of many unstable mRNAs that code for cytokines, transcription factors, proto-oncogenes and other mRNAs (Fig. 11.2) (Brewer 2002). Three classes of AREs have been identified, two of which contain several scattered or overlapping copies of the pentanucleotide AUUUA, while class III AREs lack this motif but contain A and U rich sequences and possibly other unknown determinants. A number of ARE binding proteins (ARBPs) have been identified. K-homology splicing regulator protein (KSRP) is an examples for decay promoting factors (Barreau et al. 2006; Gherzi et al. 2004). KSRP interacts with the large multiprotein 3′–5′ exoribonuclease complex, the exosome, and recruits it to target ARE containing mRNAs thereby promoting their rapid degradation (Chou et al. 2006; Linker et al. 2005). Other ARE binding proteins, such as the ELAV protein family members (mainly HuR), are stabilizing factors and AU rich binding factor 1 (AUF-1) promotes either decay or stabilization, depending on the mRNA and cell type (Fig. 11.2) (Wilusz and Wilusz 2004).

PTH mRNAs are typical eukaryotic mRNAs that contain a 7-methylguanosine cap at the 5′ terminus and a poly adenylic nucleic acid (poly A) stretch at the 3′ terminus (Naveh-Many 2005; Kemper 1986). PTH mRNA consists of three exons

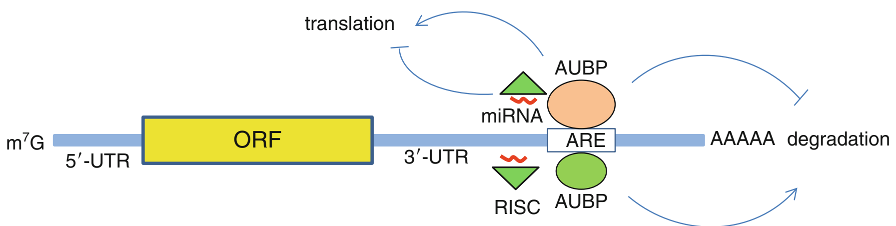

Fig. 11.2 Regulation of mRNA fate. mRNAa are comprised of the 5′ methyl cap (5^7G), open reading frame (ORF), 5′ and 3′-untranslated regions (UTRs) and poly A tail. Following transcription, Adenylate/uridylate (AU) rich binding proteins (ARBPs) bind to the mRNA and determine mRNA stability, localization and translation. AU-rich elements (AREs) within the mRNA 3′-UTR act to stabilize or destabilize the mRNA. Stabilized RNA undergoes translation in ribosomes, whereas destabilized RNA undergoes deadenylation, decapping, and degradation by the exo- or endo-nucleases. microRNAs (miRNAs) as part of the RNA-induced silencing complex (RISC), bind to target mRNAs at their 3′-UTR and target them for translational inhibition or degradation

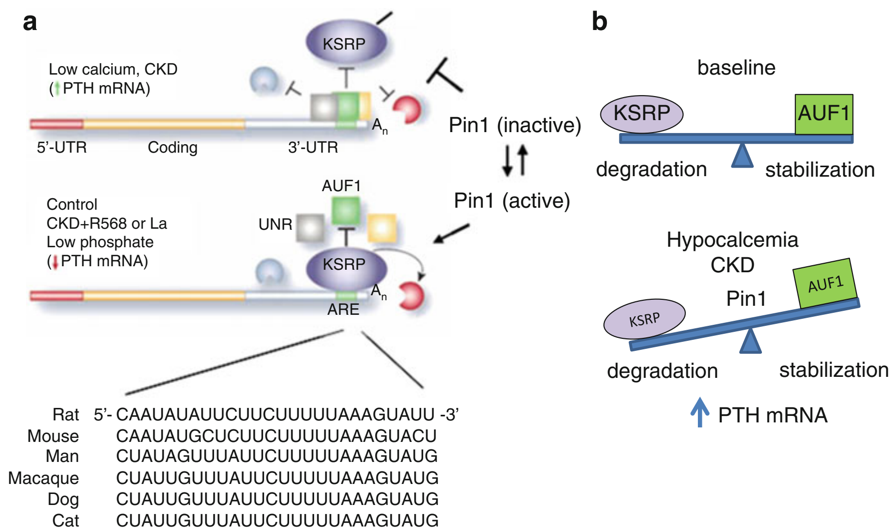

Fig. 11.3 Model for the regulation of PTH mRNA stability by changes in calcium and phosphate levels and CKD. **a**. Schematic representation of the PTH mRNA including the 5′-UTR (*red*), coding region (*orange*) the 3′-UTR (*gray*) and the 26 nucleotide *cis* acting AU rich element (ARE) (*green*). The nucleotide sequence of the element in different species is shown. Nucleotides that differ from the rat sequence are in bold. In hypocalcemic rats and in rats with experimental chronic renal failure (CKD) there is increased PTH mRNA stability and levels and this is associated with decreased binding of the destabilizing protein KSRP and increased binding of the protective proteins, AUF1 (AU rich binding factor) and Unr (Up-stream of N-ras), to the PTH mRNA 3′-UTR ARE. In control and more so in rats fed a low Pi diet, or treated with a calcimimetic (R568) or a phosphate binder (La) there is increased interaction of PTH mRNA with KSRP and decreased interaction with AUF1. KSRP recruits the exosome to PTH mRNA leading to PTH mRNA decay. The peptidyl prolyl *cis/trans* isomerase Pin1 is upstream of KSRP and leads to KSRP dephosphorylation and activation thus increasing KSRP-PTH mRNA interaction and mRNA degradation. In hypocalcemia or renal failure, Pin1 is inactive and KSRP is phosphorylated and hence less potent to bind PTH mRNA. AUF1 then binds the PTH mRNA 3′-UTR ARE with a greater affinity leading to increased PTH mRNA stability. Reproduced from Nechama et al. (2009c) and Silver and Naveh-Many (2009). **b**. Model for ARE-directed PTH mRNA decay. Basal PTH levels are obtained when the mRNA destabilizing ARBPs, such as KSRP and the stabilizing proteins such as AUF1, provide a balance of mRNA degradation and stabilization. In hypocalcemia or CKD, Pin1 is less active, resulting in KSRP phosphorylation and decreased binding to PTH mRNA, shifting the balance to mRNA stabilization

coding for the 5′-untranslated region (5′-UTR) (exon I), the prepro region (exon II) and the structural PTH hormone together with the 3′-UTR (exon III) (Fig. 11.3a). The PTH mRNA 3′-UTR in all species is rich in A and U nucleotides (Bell et al. 2005b).

Protein-PTH mRNA binding experiments demonstrated specific interaction of rat and human parathyroid extracts with transcripts for the rat and human PTH mRNA 3′-UTR terminal region. This binding was regulated by calcium or phosphorus depletion and correlated with PTH mRNA levels and stability in vivo (Moallem et al. 1998). A 26 nucleotide ARE at the 3′ end of the PTH mRNA 3′-UTR is the

minimal protein binding region and is highly conserved in the PTH mRNA 3′-UTRs of rat, mouse, man, dog and cat (Fig. 11.3a). The conservation of sequences within a region that does not code for protein (UTR) suggests that the binding element is a functional unit that has been evolutionarily conserved (Bell et al. 2005a; Kilav et al. 2001). This PTH mRNA 3′-UTR element is a *cis*-acting type III ARE that determines PTH mRNA stability and its regulation by changes in serum calcium, phosphate and CKD (see below) (Kilav et al. 2001; Nechama et al. 2008).

There is no parathyroid cell line, therefore a cell-free mRNA in vitro degradation assay (IVDA) was utilized to identify the factors involved in PTH mRNA decay. In the IVDA, extracts from parathyroids were incubated with in vitro transcribed RNA probes for the PTH mRNA to estimate mRNA decay mediated by the parathyroid extracts. IVDA has been shown to reproduce differences in mRNA stability that occur in vivo and specifically the differences in PTH mRNA stability induced by calcium, phosphate and CKD (Fritz et al. 2000; Moallem et al. 1998; Nechama et al. 2008). Parathyroid extracts from hypocalcemic and CKD rats stabilized and extracts from hypophosphatemic rats destabilized transcripts for the PTH mRNA compared to parathyroid extracts from control rat, correlating with steady state PTH mRNA levels in vivo. These effects were dependent upon the terminal 60 nucleotides of the PTH mRNA 3′-UTR that contain the ARE (Moallem et al. 1998; Nechama et al. 2009a; Yalcindag et al. 1999). A 63 nucleotide transcript containing the conserved 26 nucleotide ARE and flanking regions was both necessary and sufficient to regulate PTH mRNA stability and to confer responsiveness of reporter mRNAs to changes in calcium and phosphate (Kilav et al. 2001). Structural analysis showed that the PTH mRNA 3′-UTR and in particular the ARE is dominated by significant open regions with little folded base pairing (Kilav et al. 2004).

4 The PTH mRNA *trans* Acting Stabilizing Proteins

Two PTH mRNA binding and stabilizing proteins have been identified by PTH RNA affinity chromatography. These *trans* acting proteins are AUF1 and Up-stream of N-*ras* (Unr) (Dinur et al. 2006; Sela-Brown et al. 2000). AUF1 consists of four isoforms (p37, p40, p42, and p45) that are generated by alternative pre-mRNA splicing of AUF1 mRNA (Wagner et al. 1998). These proteins bind the PTH mRNA 3′-UTR and are part of the PTH mRNA-parathyroid protein binding complex. Addition of recombinant AUF1 isoforms to parathyroid extracts from phosphorus depleted rats prevented the rapid degradation of PTH transcripts in IVDAs (Sela-Brown et al. 2000). Interestingly, calcium and phosphorus depletion as well as kidney failure lead to post-translational modifications of the PTH mRNA stabilizing protein AUF1, with no change in AUF1 protein levels (Bell et al. 2005c; Levi et al. 2006). These modifications may lead to the differences in AUF1-PTH mRNA binding affinity and PTH mRNA stability. Over-expression of AUF1 in human embryonic kidney (HEK) 293 cells co-transfected with expression plasmids for the PTH gene or a chimeric GH gene containing the PTH mRNA 3′-UTR 63 nucleotide *cis*-acting element, stabilized PTH mRNA and chimeric reporter mRNA, but not a PTH mRNA lacking the *cis* element nor a reporter mRNA containing a truncated PTH element

(Bell et al. 2005a; Dinur et al. 2006). Unr over-expression had a similar effect. Knock-down of all four AUF1 isoforms or Unr by siRNA led to the opposite effect, decreasing PTH mRNA levels (Bell et al. 2005c; Dinur et al. 2006). AUF1 also stabilized a reporter mRNA containing the bovine PTH mRNA protein-binding element that is different from the one characterized in the rat (Bell et al. 2005a). These studies identified AUF1 and Unr as PTH mRNA stabilizing proteins (Fig. 11.3). However, the half-life of mRNAs is determined by the coordinate association of both stabilizing and destabilizing factors with the specific mRNA in the cytoplasm (Fig. 11.3b).

5 The PTH mRNA *trans* Acting Destabilizing Protein KSRP

The decay promoting protein KSRP was identified as a PTH mRNA *trans* acting destabilizing protein. KSRP is a RNA-binding protein implicated in a variety of cellular processes, including transcription, alternative pre-mRNA splicing, and editing as well as mRNA localization and stability (Gherzi et al. 2004; Lellek et al. 2000; Min et al. 1997; Snee et al. 2002). KSRP binds to ARE containing mRNA and promotes rapid mRNA decay of several inherently labile mRNAs, recruiting the 3′ to 5′ exoribonucleolitic complex exosome to the RNAs (Gherzi et al. 2004). The central part of the KSRP contains 4 adjacent K homology (KH) domains that are required for AREs recognition and interaction with the mRNA degradation machinery to promote decay of target mRNAs (Gherzi et al. 2004).

KSRP binds the PTH mRNA 3′-UTR ARE both in vivo in the parathyroid glands and in vitro in transfected cells (Nechama et al. 2008). This binding is decreased in glands from calcium-depleted or experimental CKD rats in which PTH mRNA is more stable compared to parathyroid glands from control and phosphorus-depleted rats in which PTH mRNA is less stable. KSRP also interacts with the PTH mRNA 3′-UTR ARE in vitro in transfected cells. The KH domains 3–4 of KSRP were sufficient for this association, as reported for other mRNAs (Gherzi et al. 2004; Nechama et al. 2008). KSRP over-expression and knock down experiments showed that KSRP decreased co-transfected PTH mRNA steady-state levels through the PTH mRNA ARE. Overexpression of KSRP specifically decreased both rat and human PTH mRNA levels in cotransfected HEK293 cells and accelerated PTH mRNA decay. This effect of KSRP was dependent on the PTH mRNA ARE. Conversely, KSRP knock-down increased both PTH mRNA stability and PTH mRNA steady-state levels. Moreover, PTH mRNA decay was dependent on the KSRP-recruited exosome in parathyroid extracts (Nechama et al. 2008). By its interaction with PTH mRNA ARE, KSRP would recruit the exosome to degrade PTH mRNA. These findings suggested that KSRP-PTH mRNA interactions control PTH mRNA $t_{1/2}$ by recruitment of a degradation complex to PTH mRNA. AUF1 binding to the same region in the PTH mRNA 3′-UTR competes with the binding of KSRP and thus protect PTH mRNA from degradation (Fig. 11.3).

Of interest, KSRP interacts with the endoribonuclease polysomal ribonuclease 1 (PMR1) (Chernokalskaya et al. 1998; Nechama et al. 2009b). PMR1 facilitates PTH mRNA degradation, adding an additional layer of complexity to the regulation

of PTH mRNA stability. PMR1 mediated decrease in PTH mRNA levels involves the PTH mRNA 3′-UTR ARE, KSRP and the exosome. KSRP recruits a degradation complex, comprising both exo- and endo-ribonucleases to PTH mRNA, thus controlling its mRNA half-life (Nechama et al. 2009b).

6 The Balanced Interactions of Stabilizing AUF1 and Destabilizing KSRP Determine PTH mRNA Stability and Levels

The pattern of interactions of KSRP and AUF1 with PTH mRNA suggest that these proteins have opposing roles in the regulation of PTH gene expression in vivo (Fig. 11.3). KSRP and AUF1 protein-PTH mRNA interactions in the parathyroid were studied using a RNA immunoprecipitation (RIP) assay which provides a snap-shot of protein-mRNA interactions at a specific time point in vivo. In this assay the parathyroid glands were cross-linked, AUF1 or KSRP containing complexes immunoprecipitated and the amount of PTH mRNA associated with each of the proteins determined by qRT-PCR analysis. KSRP-PTH mRNA interaction was decreased in glands from calcium depleted or CKD rats, where PTH mRNA is more stable, and increased in parathyroids from phosphorus depleted rats, where PTH mRNA is less stable. In contrast, AUF1-PTH mRNA interactions were increased by hypocalcemia and CKD and decreased in the phosphorus depleted rat parathyroids (Nechama et al. 2008). Both proteins bind to the same *cis* ARE in the PTH mRNA 3′-UTR (Fig. 11.3a). Consequently, the differential interactions of KSRP and AUF1 suggest that these proteins compete for their binding to the PTH mRNA 3′-UTR element, having antagonistic effects on PTH mRNA stability (Fig. 11.3) (Naveh-Many and Nechama 2007). Indeed, in vitro, over-expression of the PTH mRNA stabilizing protein AUF1 isoform p45 blocked KSRP-PTH mRNA binding and partially prevented the KSRP mediated decrease in PTH mRNA levels (Nechama et al. 2008). Therefore, the balanced interaction of these proteins with the PTH mRNA 3′-UTR determines basal PTH mRNA levels and the regulation of PTH mRNA levels (Fig. 11.3b).

Calcimimetics and oral phosphorus binders that are widely used to treat secondary hyperparathyroidism decrease PTH mRNA levels post-transcriptionally in CKD rats (Nechama et al. 2009a). KSRP-PTH mRNA interaction was increased by both the calcimimetic R568 and the oral phosphorus binder La which decreased PTH mRNA levels. IVDAs showed that PTH mRNA is destabilized by parathyroid extracts from CKD rats treated with R568 or La compared to parathyroid extracts from untreated CKD rats. This destabilizing effect of R568 and La was dependent upon KSRP and the PTH mRNA 3′-UTR. Therefore, the calcimimetic R568 and correction of serum phosphate by La determine PTH mRNA stability through KSRP-mediated PTH mRNA decay, thereby decreasing PTH expression (Nechama et al. 2009a). Changes in binding of the *trans* acting factors to the PTH mRNA therefore determine PTH gene expression in CKD and after management of the 2HP of CKD by both calcimimetics or oral phosphorus binders as well as after changes in calcium and phosphate (Fig. 11.3) (Nechama et al. 2009a).

6.1 The Peptidyl-Prolyl Isomerase Pin1 Determines PTH mRNA Levels and Stability in Secondary Hyperparathyroidism

The above results indicate that KSRP and AUF1 directly or indirectly respond to changes in serum calcium and phosphate concentrations and CKD by altering their association with PTH mRNA leading to differences in PTH mRNA stability and levels (Nechama et al. 2008, 2009a). These changes could be a result of post-translational modifications of these ARE binding proteins, affecting their binding affinity to the PTH mRNA. Indeed, as stated above, AUF1 is post-translationally modified in the parathyroids of 2HPT rats and this is at least in part due to differences in protein phosphorylation (Bell et al. 2005c; Levi et al. 2006). KSRP is also a phospho-protein with two identified phosphorylation sites at serine 193 (S193) and threonine residue 692 (T692). Phosphorylation at these sites prevents KSRP association with the ribonuclease degradation complex exosome (S193) or compromises KSRP binding to ARE-containing target mRNAs (T692) and hence their decay (Gherzi et al. 2006; Ruggiero et al. 2007). Therefore, the differential interaction of KSRP and AUF1 with PTH mRNA after changes in serum calcium and phosphate may involve KSRP and AUF1 post-translational modifications.

The peptidyl prolyl *cis/trans* isomerase Pin1 specifically binds phosphorylated serine/threonine-proline protein motifs and catalyzes the *cis/trans* isomerization of the peptide bonds thereby changing the biological activity, phosphorylation and turn-over of its target proteins (Wulf et al. 2002; Zhou et al. 2000). Pin1-catalysed conformational regulation has a profound impact on many key proteins involved in various cell functions (Winkler et al. 2000; Lu et al. 1999). Pin1 was shown to regulate the turnover of ARE containing mRNAs, mainly cytokine mRNAs, through the interaction and isomerization of ARE binding proteins. Pin1 interacts with AUF1 and thereby stabilizes both GM-CSF and TGFβ mRNAs (Shen et al. 2005, 2008). These observations led us to speculate that Pin1 may be involved in PTH gene expression through AUF1 and/or KSRP interaction and isomerization. Indeed, Pin1 is a PTH mRNA destabilizing protein in vivo and in vitro (Nechama et al. 2009c). The regulation of PTH mRNA stability by Pin1 was mediated by the PTH mRNA 3′-UTR ARE and by the mRNA destabilizing protein KSRP. We showed for the first time that KSRP is a Pin1 target protein. Pin1 interacts with phosphorylated KSRP at S181, leading to KSRP dephosphorylation and activation. Importantly, Pin1 enzymatic activity was decreased in parathyroid extracts from rats with 2HPT due to either a calcium depleted diet or CKD. Pharmacological inhibition of Pin1 increased PTH mRNA levels and stability and decreased KSRP-PTH mRNA interaction in the parathyroid. This decreased interaction would increase PTH gene expression after Pin1 inhibition. Furthermore, *Pin1*$^{-/-}$ mice display increased serum PTH and PTH mRNA levels. Therefore, Pin1 determines basal PTH expression in vivo and in vitro and decreased Pin1 activity correlates with increased PTH mRNA levels in rats with 2HPT. These results demonstrate that Pin1 is a key mediator of PTH mRNA stability and indicate a role for Pin1 in the pathogenesis of the 2HPT of CKD (Nechama et al. 2009c). Our data suggest that phosphorylated KSRP at S181 is inactive. Upon interaction with Pin1, *cis–trans* isomerization of

the proline bond in KSRP leads to conformational change, exposing the phosphorylated S181 residue and possibly additional phosphorylation sites. This leads to KSRP dephosphorylation by a still unidentified phosphatase. KSRP then interacts with PTH mRNA and enhances its decay. A low calcium diet and CKD lead to decreased Pin1 isomerase activity in the parathyroids of these rats. This decreased Pin1 activity would prevent KSRP dephosphorylation, resulting in decreased KSRP-PTH mRNA interaction, inhibition of PTH mRNA degradation, and increased PTH mRNA levels (Fig. 11.3a). The trigger for the reduced Pin1 activity in the parathyroid glands of 2HPT rats is not known. Post-translational modifications of Pin1 protein itself may play a role in this regulation. Pin1 is post-translationally modified by phosphorylation that affects its ability to interact with target proteins and its activity. PKA mediated phosphorylation at Ser residue 16 affects the interaction of Pin1 with its target proteins. Phosphorylation at Ser residue 71 by the protein kinase DAPK1, inhibits Pin1 isomerization activity (Lu et al. 2002; Lee et al. 2011). Future studies may identify the factors that decrease Pin1 activity in the hyper-functioning parathyroid glands of 2HPT. Another main challenge will be to unravel the cell signaling cascade and the particular kinases responsible for KSRP phosphorylation at S181 that determine PTH mRNA-KSRP interactions and PTH mRNA decay.

7 Parathyroid miRNAs Are Essential for the Response of the Parathyroid to Hypocalcemia and Uremia

miRNAs are abundant, in part cell type-specific short non-coding RNA molecules that affect gene expression by sequence-specific translation repression and/or mRNA degradation. miRNAs have crucial roles in developmental processes and disease states. miRNAs are transcribed as long pri-miRNA transcripts that undergo two processing reaction steps (Gurtan and Sharp 2013). Drosha cleavage releases a hairpin precursor miRNA (pre-miRNA) of ~70 nucleotides that is exported to the cytoplasm and further processed by Dicer. Dicer, along with AGO and TRBP is a component of the RISC (RNA-induced silencing complex) loading complex which is guided by the functional strand of the mature (~22 nt) miRNA to target mRNAs (Fig. 11.4) (Bartel 2009). Lack of functional Dicer blocks pre-miRNA processing of to their mature form, thus perturbing any regulatory circuit in which miRNAs are involved (Bernstein et al. 2003). Total body inactivation of *Dicer1* in both mice and zebrafish results in early embryonic lethality indicating that miRNAs are crucial for normal development (Giraldez et al. 2005; Wienholds et al. 2003). Conditional knock-out of *Dicer1* in various organs or cell lineages has shown that Dicer is required for normal function of many cell types or organs, suggesting an essential role of Dicer-dependent miRNAs in normal physiology.

There is no information about miRNA function in parathyroid physiology. miRNA profiling was recently reported from parathyroid glands of patients with the rare parathyroid carcinoma with almost no information data on SHP (Corbetta et al. 2010; Rahbari et al. 2011; Vaira et al. 2012). Three miRNAs (miR-26b, miR-30b and miR-126) were significantly dysregulated between parathyroid carcinoma and parathyroid adenoma with down-regulation of miR-126 being the most significant

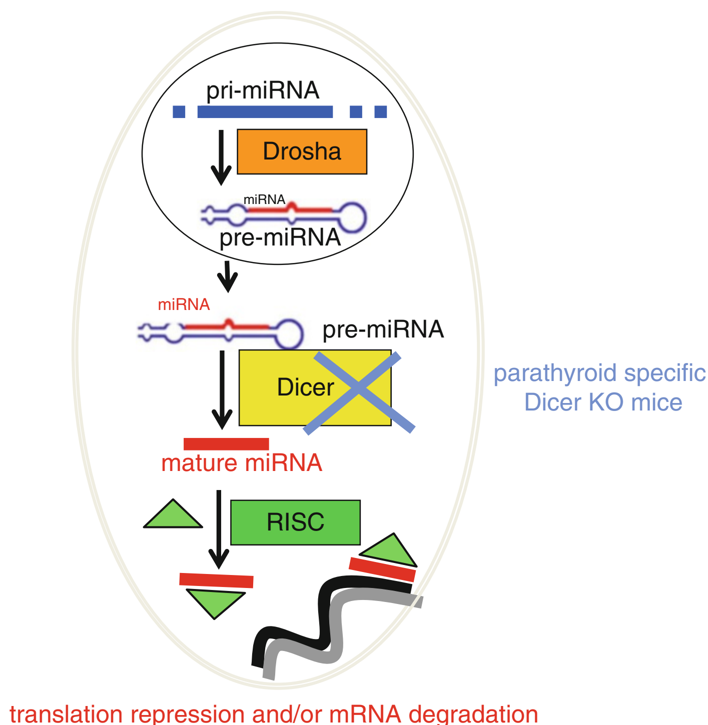

Fig. 11.4 miRNA maturation. Simplified presentation of miRNA maturation. Primary miRNA (primiRNA) is transcribed and cleaved by Drosha in the nucleus. The resulting precursor miRNA (pre-miRNA) exits to the cytoplasm and is processed by Dicer to form the mature miRNA. The miRNA enters the RNA-induced silencing complex (RISC) and leads to sequence-specific translational suppression or degradation of target mRNAs. Conditional tissue specific knock-out of *Dicer* in a specific organ is a useful approach to study the function of Dicer-dependent miRNAs in vivo

(Rahbari et al. 2011). Multiple Endocrine Neoplasia type 1 (MEN1) syndrome which is characterized by tumors of the parathyroids, neuroendocrine cells, cells of the gastro-entero-pancreatic tract and of the anterior pituitary is due to mutations in the *MEN1* gene. *MEN1* tumorigenesis may be under the control of a negative feedback loop between miR-24 and menin protein expression (Luzi et al. 2012).

To study the significance of miRNAs in parathyroid physiology and the development of SHP we have generated parathyroid specific *Dicer1* knock-out (PT-*Dicer*$^{-/-}$) mice that do not express miRNAs specifically in their parathyroids (Fig. 11.4). The mice developed normally and are fertile. Remarkably, the PT-*Dicer*$^{-/-}$ mice did not increase serum PTH after acute hypocalcemia. In addition, in vitro in parathyroid organ culture, PTH secretion was markedly impaired in parathyroids from the PT-*Dicer*$^{-/-}$ mice when the glands were incubated in a calcium depleted medium. Similarly, PT-*Dicer*$^{-/-}$ mice failed to increase serum PTH, PTH mRNA levels and parathyroid cell proliferation after the stimuli of dietary induced prolonged hypocalcemia. Moreover, adenine induced uremia led only to a moderate increase in serum PTH in the PT*Dicer*$^{-/-}$ mice compared to controls. In contrast, the

PT-*Dicer*$^{-/-}$ mice responded normally to activation of the parathyroid calcium receptor (CaR) by both hypercalcemia and a calcimimetic. These findings show that miRNAs are essential for the response of the parathyroid to both acute and chronic hypocalcemia and uremia which are the major stimuli for PTH secretion. Therefore, miRNAs are crucial to activation of parathyroid gland function at the levels of PTH secretion, gene expression and parathyroid cell proliferation, providing a further level of the post-transcriptional regulation of PTH gene expression.

8 Conclusions

The parathyroid is regulated at the levels of PTH secretion, gene expression and parathyroid cell proliferation (Fig. 11.5). Dietary induced hypocalcemia, hypophosphatemia and CKD determine PTH gene expression post-transcriptionally by the interaction of RNA binding proteins to the PTH mRNA 3′-UTR ARE. Pin1 enzymatic activity affects these protein-PTH mRNA interactions and consequently PTH mRNA decay. Changes in Pin1 enzymatic activity alter KSRP phosphorylation status and KSRP-AUF1-PTH mRNA interactions in the parathyroid cell and hence PTH mRNA stability and levels (Figs. 11.3 and 11.5). In addition, miRNAs are essential for activation of parathyroid gland function at the levels of PTH secretion, gene expression and parathyroid cell proliferation after acute and chronic hypocalcaemia and CKD (Fig. 11.5).

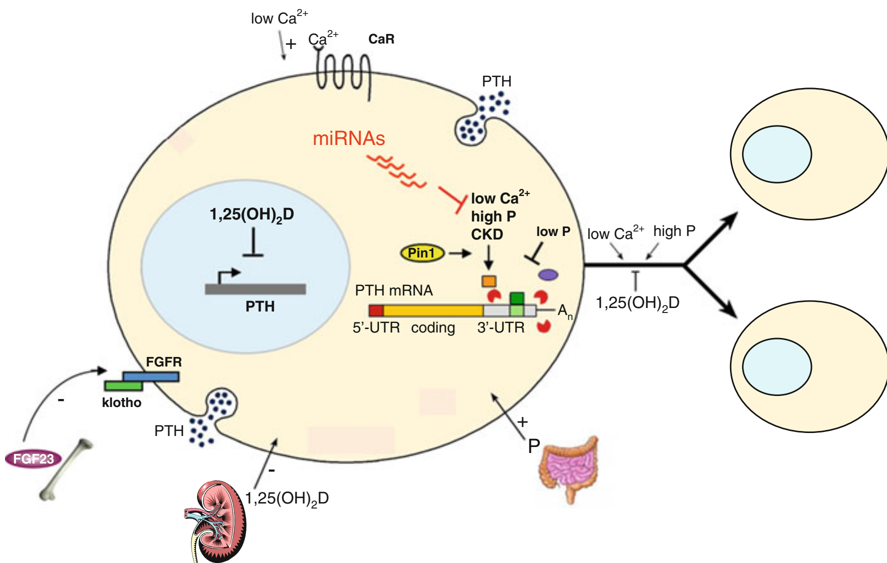

Fig. 11.5 Regulation of PTH gene expression, secretion and parathyroid cell proliferation. The parathyroid cell synthesizes and secretes PTH unless it is restrained by the parathyroid calcium receptor (CaR) which senses extracellular serum calcium levels. A low serum calcium leads to increased PTH mRNA stability and levels, PTH secretion, and parathyroid cell proliferation. A high serum phosphate and chronic kidney disease (CKD) lead to similar changes in all these parameters. PTH mRNA stability is regulated by the balanced interactions of the protective

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Fig. 11.5 (continued) parathyroid *trans* acting factors AUF1 and Unr and the decay promoting protein KSRP, that bind to a defined *cis* element in the PTH mRNA 3′-UTR. These interactions are regulated by the activity of the peptidyl prolyl *cis/trans* isomerase Pin1. In addition, miRNAs are essential for activation of parathyroid gland function at the levels of PTH secretion, gene expression and parathyroid cell proliferation after both acute and chronic hypocalcaemia and CKD. 1,25D (1,25$(OH)_2$D) decreases PTH gene transcription and parathyroid cell proliferation. Fibroblast growth factor 23 (FGF23) is a bone derived hormone that decreases PTH gene expression and secretion by acting through its Kloth FGFR1 (fibroblast growth factor 1) receptor complex. Reproduced from Silver (2001)

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Post-transcriptional and Post-translational Regulation of Steroidogenesis

12

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Abbreviations

ACAT1	Acyl-coenzyme A:cholesterol acyltransferase 1
ACTH	Adrenocorticotropic hormone
AII	Angiotensin II
ANC	Adenine nucleotide transporter

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AALO	Allopregnanolone
CEH	Neutral cholesteryl ester hydrolase
CE	Cholesteryl ester
CEs	Cholesteryl esters
ECD	Extracellular domain
ER	Endoplasmic reticulum
FRET	Quantitative fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone
hCG	Human chorionic gonadotropin
HDL	High-density lipoprotein
HSL	Hormone-sensitive lipase
IMM	Inner mitochondrial membrane
LDL	Low-density lipoprotein
LH	Luteinizing hormone
OMM	Outer mitochondrial membrane
NSF	*N*-ethylmaleimide-sensitive factor
CYP11A1	P450c11A
CYP11B1	P450c11
CYP11B2	Aldosterone synthase
CYP17	P450c17
CYP21A2	P450c21
PBR	Peripheral-type benzodiazepine receptor
PDZ	PSD-95, DglA, ZO-1
PKA	cAMP-dependent protein kinase
SR-BI	Scavenger receptor Class B, type I
StAR	Steroidogenic acute regulatory protein
TSPO	Translocator protein

1 Introduction

Steroid hormones play important roles in virtually every aspect of cellular metabolism, including the regulation of carbohydrate, lipid and protein metabolism and immune function (glucocorticoids), as well as salt and water balance and blood pressure regulation (mineralocorticoids). They are also critically involved in the maintenance of secondary sex characteristics, reproductive functions and muscle and bone growth (testosterone, progestins and estrogens) (Hu et al. 2010). The common precursor of steroid hormone biosynthesis is cholesterol, and steroids are synthesized most prominently in the steroidogenic cells of the adrenal gland, gonads and placenta (Payne and Hales 2004; LaVoie and King 2009; Miller and Bose 2011; Strauss et al. 1996). Brain also synthesizes steroids, which are commonly referred to as “neurosteroids”. However, circulating steroid hormones (progesterone, deoxycorticosterone, testosterone, estradiol), and not cholesterol, serve as precursors for neurosteroids, which are produced locally in the hippocampus and other brain regions (Mellon and Griffin 2002; Reddy 2010; Giatti et al. 2012).

1.1 Overview of Steroidogenesis

In steroidogenic cells of the adrenal gland, ovary and testis, the overall rate of steroid hormone production is controlled by tropic (peptide) hormones. Therefore, the type of steroid hormone that can be synthesized by a particular cell type is dictated by its complement of peptide hormone receptor, its response to peptide hormone stimulation and its genetically expressed complement of steroidogenic enzymes. Thus, adrenocorticotropic hormone (ACTH) stimulates cortisol/corticosterone in adrenocortical fasciculata-reticularis cells; angiotensin II (AII) and potassium regulate aldosterone synthesis in adrenal glomerulosa cells; follicle-stimulating hormone (FSH) controls the progesterone and estrogen synthesis in ovarian granulosa cells, whereas luteinizing hormone (LH) regulates progesterone synthesis in luteinized ovarian granulosa-luteal cells, androgen production in ovarian theca-interstitial cells and testosterone synthesis in testicular Leydig cells. The adrenal gland is also responsible for the synthesis of adrenal androgens (McKenna et al. 1997; Miller 2002). Tropic hormones (LH, FSH or ACTH) induce adrenocortical and gonadal steroidogenesis by binding to their respective G protein-coupled receptors, leading to activation of adenylate cyclase, which generates cAMP and activates cAMP-dependent protein kinase (PKA) (Marsh 1976; Simpson and Waternan 1983; Sanorn et al. 1980; Strauss et al. 1988). Stimulation of the cAMP-PKA signaling cascade exerts both acute and chronic effects on the regulation of steroid hormone production. Angiotensin (AII) stimulation of aldosterone biosynthesis in adrenal glomerulosa cells is primarily mediated by the protein kinase C signaling cascade, whereas potassium stimulation of aldosterone production also involves Ca^{2+}-calmodulin-dependent kinase (Spat and Hunyady 2004).

The trophic hormone (ACTH, LH, FSH) regulation of adrenal and gonadal steroidogenesis is subject to both acute (Miller and Bose 2011; Pon et al. 1986; Pon and Orme-Johnson 1988; Epstein and Orme-Johnson 1991a; Stocco and Clark 1996) and chronic regulation (LaVoie and King 2009; Miller and Bose 2011; Miller 1988; Simpson and Waterman 1988; Payne et al. 1992; Simpson et al. 1992). Acute steroid synthesis that occurs over minutes in response to trophic hormone stimulation is controlled at the level of cholesterol delivery to the inner mitochondrial membrane (IMM) for the first enzymatic step in the pathway, the conversion of cholesterol to pregnenolone by the P450scc. This rate limiting step, i.e., cholesterol transfer from the outer mitochondrial membrane (OMM) to the IMM, is dependent upon the trophic hormone stimulated rapid increase in transcription, as well as post-transcriptional modification, of the steroidogenic acute regulatory protein (StAR) (Hu et al. 2010; Miller and Bose 2011; Stocco 2001; Manna et al. 2009; Rone et al. 2009). Chronic stimulation (hours to days) occurs through the induction of P450scc gene transcription leading to increased P450scc and consequent increased steroidogenic capacity (Miller and Bose 2011; Miller 1988; Simpson and Waterman 1988; Payne et al. 1992; Simpson et al. 1992).

1.2 Steroid Biosynthetic Pathways

The process of steroid hormone synthesis, or steroidogenesis, represents a complex multistep and multi-enzyme process by which precursor cholesterol is converted to pregnenolone and subsequently metabolized into other biologically active steroids in a tissue specific manner (Hu et al. 2010; Payne and Hales 2004; LaVoie and King 2009; Miller and Bose 2011). This process can be broadly divided into five major steps: (1) acquisition of cholesterol from exogenous (lipoproteins) and endogenous (de novo synthesis) sources for storage in the form of cholesterol esters (CEs) in lipid droplets, (2) mobilization of cholesterol from lipid droplet stored CEs, (3) transport of cholesterol to and from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where cytochrome P450 side chain cleavage enzyme (P450scc, encoded by CYP11A1) is localized, (4) P450scc catalyzed cleavage of a 6-carbon unit from the cholesterol side chain producing pregnenolone, the common precursor for the synthesis of all of the other steroid hormones, and (5) efflux of pregnenolone from the mitochondria to the endoplasmic reticulum (ER), where it is converted by ER enzymes into intermediate precursors, which further shuttle between mitochondria and ER for the tissue specific production of progestins, estrogens, androgens, glucocorticoids or mineralocorticoids (LaVoie and King 2009; Miller 2008). In rodents, the majority of cholesterol needed for steroidogenesis in adrenal and ovary (and testicular Leydig cells under certain conditions) is obtained via SR-BI mediated selective delivery of HDL-cholesterol (Fig. 12.1), whereas in humans the bulk of the cholesterol needed for steroid synthesis is supplied by the LDL-receptor/endocytic pathway.

The major synthetic pathways for steroid hormones in the adrenal gland and gonads are shown in Fig. 12.2. Although the final steroid product differs in a tissue-specific manner, the first committed reaction in the biosynthetic pathway is the same, i.e., the conversion of cholesterol to pregnenolone by the cytochrome P450scc enzyme (CYP11A1). P450scc is an enzyme complex consisting of a flavoprotein (NADH-adrenodoxin reductase), a ferredox (adrenodoxin) and a cytochrome P450 localized on an inner mitochondrial membrane (Miller 1988, 2008). P450scc catalyzes three distinct reactions: 20α-hydroxylation, 22-hydroxylation and scission of 20, 22 carbon–carbon bonds, thus converting cholesterol to pregnenolone (Miller 1988, 2008). Next, the pregnenolone is converted to the final hormone product by sequential steps along the pathway depending on the enzymes that are present in that tissue (Fig. 12.2). Thus, adrenal zona fasciculata-reticularis and zona glomerulosa cells produce cortisol (corticosterone in rodents) and aldosterone, respectively. Likewise, ovarian theca cells secrete androgens, while granulosa cells synthesize progesterone and estradiol. Testicular Leydig cells primarily synthesize and secrete testosterone.

1.3 Post-transcriptional/Post-translational Regulation of Steroidogenesis

While much is known about the transcriptional regulation of steroidogenesis, relatively little is known about the post-transcriptional and post-translational regulation of this process. Below we summarize what is currently known about the post-transcriptional/post-translational modulation of steroidogenesis:

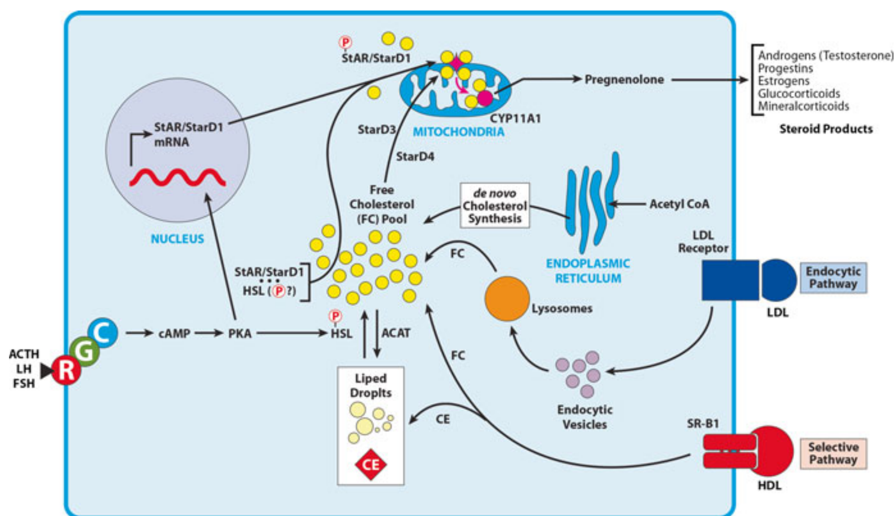

Fig. 12.1 Overview of steroidogenesis. Tropic hormones bind to their respective G protein-coupled receptors leading to activation of the cAMP-PKA signaling cascade which in turn induces acute and/or chronicle effects on the regulation of steroidogenesis. Upon hormone stimulation, cholesterol acquired from endocytic or selective uptake pathways or from de novo synthesis is mobilized from lipid droplets and transported to the inner membrane of mitochondria through the collective actions of HSL, StAR and their associated proteins. At the IMM, cytochrome P450 side chain cleavage enzyme catalyzes the cleavage of a 6-carbon unit from cholesterol to produce pregnenolone, which is effluxed from mitochondria to the endoplasmic reticulum and used as the precursor for the synthesis of all of the other steroid hormones

1.3.1 Phosphorylation/Dephosphorylation-Dependent Regulation of Steroidogenesis

Reversible phosphorylation of proteins, which can result in a conformational change in the structure in many enzymes and receptors, and in turn switches enzymes and receptors “on” and “off”, is an important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms (Krebs and Fischer 1964; Fischer and Krebs 1966; Burnett and Kennedy 1954; Bahler et al. 1990). Phosphorylation commonly occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins (Ciesla et al. 2011; Hanks et al. 1988; Cohen 2000). Several key transcription factors and enzymes that contribute to the regulation of steroidogenesis, including steroidogenic transcription factors, SF-1, DAX-1, CREB and TORC (Babu et al. 2000; Desclozeaux et al. 2002; Sands and Palmer 2008; Takemori and Okamoto 2008), StAR (Arakane et al. 1997), HSL (Kraemer and Shen 2002) and downstream enzymes such as CYP11A1 and CYP17A1 (Miller 2008; Vilgrain et al. 1984), have been shown to be regulated via phosphorylation/dephosphorylation mechanisms.

(1a) Phosphorylation of StAR:

Orme-Johnson and colleagues first identified StAR in ACTH treated primary rat adrenocortical cell suspensions (Pon et al. 1986; Krueger and Orme-Johnson 1983;

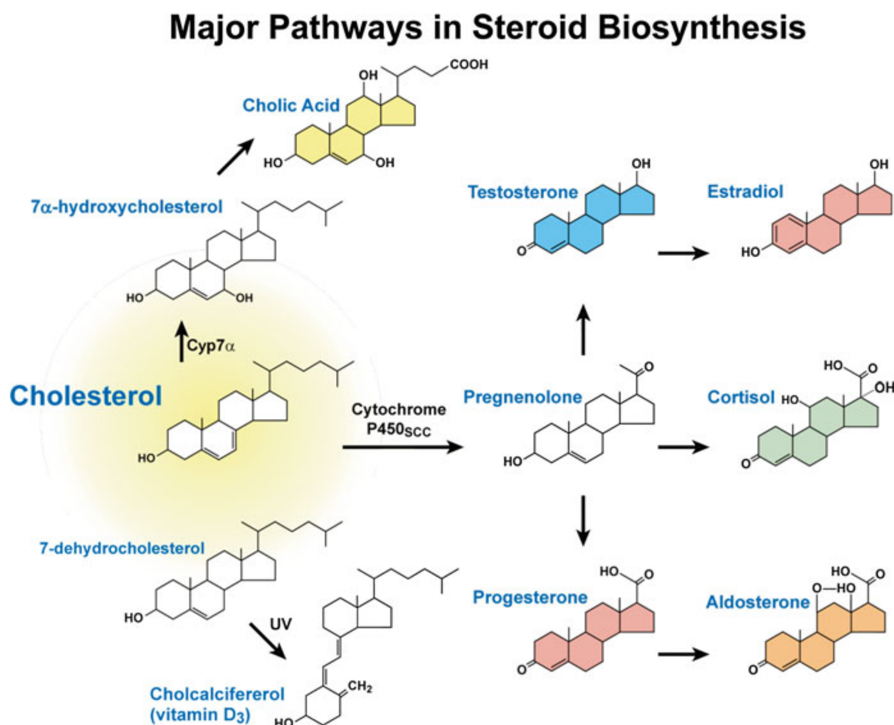

Fig. 12.2 The major synthetic pathways for steroid hormones

Epstein and Orme-Johnson 1991b). Using a two-dimensional gel electrophoresis of ^{35}S-radiolabeled proteins, a hormone-induced phosphoprotein was identified by radioautography, termed i_b and later as pp30. The synthesis of this protein was shown to occur with similar kinetics and dose response as corticosterone production after trophic hormone stimulation (Mellon and Griffin 2002; Stocco 2001; Manna et al. 2009). Subsequently, these seminal findings were further confirmed in the steroidogenic cells of the ovary and testis (Epstein and Orme-Johnson 1991a; Pon and Orme-Johnson 1998). Stocco and colleagues cloned this protein and renamed it steroidogenic acute regulatory protein, StAR (Clark et al. 1994). A number of in vitro studies have established that cAMP-dependent phosphorylation of murine StAR at serine residue 194 (Ser^{194}; Ser^{195} in human StAR) is required for its maximal biological activity (Arakane et al. 1997; Fleury et al. 2004). Very recently, investigation into the functional importance of Ser^{194} phosphorylation in vivo using transgenic mice expressing either wild-type (WT) StAR or StAR mutation S194A to rescue StAR deficient (knockout) mice has been reported (Sasaki et al. 2014). The results showed that, despite protein expression comparable to or higher than amounts seen with control animals or rescued with WT StAR, mutant S194A StAR did not rescue the neonatal lethality and only partially rescued the sex reversal in male mice observed uniformly in StAR KO mice. Like the StAR KO mice, the

adrenal cortex and testicular Leydig cells contained abundant lipid droplets when tissue sections were stained with Oil red O for lipids. Moreover, adrenal StAR from S194A rescued animals lacked an acidic species, which was detectable in response to ACTH treatment in animals rescued with WT StAR. These results are consistent with defective StAR phosphorylation. On the other hand, the WT-StAR transgene consistently restored viability and steroidogenic function in StAR-deficient (−/− mice) (Sasaki et al. 2008). These findings further establish that phosphorylation of Ser^{194} is essential for normal functioning of StAR protein in the adrenal cortex, testes and possibly ovaries of mice.

(1b) Phosphorylation of HSL:

Hormone sensitive lipase (HSL) was shown to be the neutral cholesterol esterase for the hormone-induced mobilization of stored CEs to supply precursor cholesterol for steroidogenesis (Kraemer and Shen 2002; Kraemer et al. 2002, 2004). Investigation into the functional significance of HSL in steroid production using *HSL−/−* mice showed that the cholesteryl ester content was substantially elevated in adrenals of *HSL*−/− mice, and basal corticosterone production was reduced approximately 50 %. The maximum corticosterone production induced by dibutyryl cAMP and lipoproteins was approximately 75–85 % lower in adrenal cells from *HSL*−/− mice compared with control. There is no intrinsic defect in the conversion of cholesterol into steroids in *HSL*−/− mice. Dibutyryl cAMP-stimulated conversion of high-density lipoprotein CEs into corticosterone was reduced 97 % in *HSL*−/− mice (Kraemer et al. 2004). The cholesteryl esterase activity of HSL is regulated by reversible phosphorylation/dephosphorylation (Yeaman 1990). A number of publications have reported PKA catalyzed phosphorylation of HSL at Ser^{563}, Ser^{659} and Ser^{660} in rat HSL, resulting in increases in HSL hydrolytic activity (Yeaman 1990; Shen et al. 1998; Anthonsen et al. 1998). In addition, ERKs can phosphorylate adipocyte HSL on Ser^{600} and stimulate its activity (Greenberg et al. 2001). In contrast to activation of activity seen with PKA or ERK phosphorylation, other kinases, such as glycogen synthase kinase-4, Ca^{++}/calmodulin-dependent protein kinase II, and AMP-activated protein kinase, phosphorylate HSL at a secondary basal site Ser^{565} in rat HSL (Yeaman 1990). Phosphorylation at Ser^{565} interferes with the phosphorylation of Ser^{563} by PKA (Yeaman 1990). HSL activity can be inactivated by protein phosphatases as well. The most active phosphatases against Ser^{563} are phosphatase 2A and 2C, while Ser^{565} is predominately dephosphorylated by phosphatase 2A (Wood et al. 1993).

(1c) Phosphorylation of Key Steroidogenic Transcription Factors and Enzymes:

SF-1, DAX-1, CREB and TORC have all been shown to be important transcription factors involved in steroidogenic pathways. SF1 can be phosphorylated by Erk2 at Ser^{203} in response to multiple components of the MAPK pathway and results in upregulated transcriptional activity (Babu et al. 2000; Desclozeaux et al. 2002). CREB can be phosphorylated by PKA as well as salt induced kinase in response to

cellular stimuli and results in transcriptional activation of multiple targets involved in steroidogenesis (Sands and Palmer 2008; Takemori and Okamoto 2008). A few of the downstream enzymes, such as CYP11A1 and CYP17A1 (Miller 2008; Vilgrain et al. 1984), have been shown to be regulated via phosphorylation/dephosphorylation mechanisms.

1.3.2 Modulation of Steroidogenesis through Protein–Protein Interactions

In living organisms, cellular functions are often conveyed, at both cellular and systemic levels, by a large number of protein complexes through specific interactions between protein partners. Protein complex assembly can result in the formation of homo-oligomeric or hetero-oligomeric complexes. During steroidogenesis, many proteins, including SR-BI, StAR, HSL, are involved in formation of unique protein complexes for their functions.

(2a) SR-BI: Dimerization and interaction with PDZ domain containing proteins

In response to hormone stimulation, SR-BI, the HDL receptor responsible for selective CE uptake, changes to oligomeric form to facilitate CE uptake (for simplicity, we use the term dimerization here to include the multiple forms of the SR-BI protein; i.e., dimers and higher order oligomers). In one of the earliest direct demonstrations of protein–protein interactions involving SR-BI, it was shown to exist as homodimers in microvilli-enriched adrenal plasma membranes from 17α-ethinyl estradiol (17α-E_2) primed rats (Azhar et al. 2002). Subsequent studies demonstrated that SR-BI exists in dimeric and high order oligomeric forms in all steroidogenic and non-steroidogenic cells and tissue which are active in 'selective' uptake of HDL-CEs (Reaven et al. 2001, 2006). Direct functional evidence for SR-BI dimerization came from the observation that SR-BI exists primarily in a monomeric form with some dimer formation in normal rat adrenal tissue. ACTH stimulation increased the dimerization of SR-BI in this tissue along with increased selective CE uptake, and dexamethasone-induced loss of ACTH led dramatically to the loss of SR-BI, SR-BI dimers and selective HDL-CE uptake. These results, coupled with striking architectural changes of the microvillar compartment at the adrenocortical cell surface, suggest that SR-BI dimers may, in a very basic way, be associated with SR-BI sites of action and function.

Additional functional studies further revealed a strong correlation between the levels of SR-BI dimers and increased selective HDL-CE uptake in cells and tissues. Moreover, co-immunoprecipitation studies of epitope-tagged SR-BIs (SR-BI-cMyc and SR-BI-V5) confirmed that SR-BI can exist as homodimers (Reaven et al. 2006). The use of cross-linking agents provided additional evidence that SR-BI forms dimers in native steroidogenic cell lines (endogenous), as well as in a heterologous insect cell expression system (Reaven et al. 2001). Also, analysis of cellular extracts from SR-BI transfected HEK-293 cells or ACTH-treated Y1-BS1 cells by size-exclusion chromatography and sucrose density centrifugation demonstrated that a significant portion of SR-BI exists in dimeric and oligomeric forms.

Morphological analysis by immunoelectron microscopy provided independent confirmation of SR-BI homodimerization. More specifically, when double tagged-SR-BI proteins (SR-BI-cMyc and SR-BI-V5) were co-expressed in HEK-293 cells and the different proteins were subsequently immunostained and identified with two differently stained gold particles, there was mixing and clustering of gold particles suggesting (1) that the proteins travel to the same cell location, and (2) that many of the gold particles are in exceedingly close physical contact, i.e., within the distance accepted for protein dimers by fluorescent resonance energy transfer (FRET) technique. Similar results were obtained when Y1-BS1 mouse adrenocortical cells were transfected with V5 and/or cMyc tagged-SR-BI proteins. Interestingly, SR-BI transfected Y1-BS1 demonstrated major architectural changes along with the formation of double membranes in flower like arrangements. Gold-labeled secondary antibodies against V5 or cMyc antibody localized SR-BI to these sites, and revealed substantial dimer formation of this protein–shown by close contact between gold particles (Reaven et al. 2004, 2006).

Further investigations concentrated on the contribution of the cysteine residues in the extracellular domain (ECD) of SR-BI either independently or in cooperation with the *C*-terminal domain on SR-BI dimerization. SR-BI contains a total of eight cysteine (C) residues (C21, C251, C280, C321, C323, C334, C384, and C470) and six of them are located in the ECD. Mutagenesis studies showed that C280, C321, C323 and C334 residues in the ECD are necessary for preserving normal SR-BI (HDL) binding activity, selective CE uptake, and/or cell surface expression. Interestingly, mutation of any of these four cysteine residues to serine resulted in a robust induction of SR-BI dimer formation, but they were rendered non-functional because these residues are most likely essential for optimal HDL binding and, hence, selective CE uptake (Hu et al. 2011).

(2b) SR-BI interaction with accessory proteins

Emerging evidence now indicates that accessory proteins are also required for the proper cellular expression of SR-BI and SR-BI-mediated HDL-CE transport and other functions (Ikemoto et al. 2000; Silver 2002; Kocher et al. 2003; Komori et al. 2008; Robichaud et al. 2008; Yuhanna et al. 2008; Zhu et al. 2008; Fenske et al. 2009; Eyre et al. 2010; Kocher and Krieger 2009). For example, it has been shown that PDZK1/NHERF3 regulates hepatic SR-BI stability and steady state protein levels (Hu et al. 2013a). Interestingly, PDZK1/NHERF3 is neither expressed nor essential for SR-BI abundance or its cellular localization in steroidogenic cells of the adrenal gland, ovary and testis (Kocher et al. 2003). Recently, our laboratory made an important discovery that two other NHERF family members, NHERF1 and NHERF2, negatively regulate the expression and function of SR-BI in steroidogenic cells of adrenal and gonads, as well as the liver (Hu et al. 2013a). More specifically, we showed that NHERF1 and NHERF2 mRNA levels decrease in response to cAMP stimulation, whereas mRNA levels of SR-BI are upregulated. Co-immunoprecipitation, colocalization, bimolecular fluorescence complementation, and mutational analysis all indicated that NHERF1 and NHERF2 form complexes with

SR-BI protein and, as a result, inhibit SR-BI-mediated selective CE transport and steroidogenesis. The PDZ1 or PDZ2 domain of NHERF1, the PDZ2 domain of NHERF2, or the MERM domains of NHERF1/2 and an intact COOH-terminal PDZ recognition motif (EAKL) in SR-BI is needed for the interaction. Both NHERF1 and NHERF2 also inhibit the de novo synthesis of SR-BI (Hu et al. 2013a). In contrast, no effect of NHERF4 was noted on selective HDL-CE uptake or steroidogenesis. Collectively, these data establish NHERF1 and NHERF2 as SR-BI protein binding partners that play a negative role in the regulation of SR-BI expression, selective CE transport, and steroidogenesis (Hu et al. 2013a).

(2c) StAR-TSPO interaction

StAR facilitates the rate limiting step in steroidogenesis, i.e., delivery of substrate cholesterol from the outer to the inner mitochondrial membrane where P450scc resides for the production of pregnenolone. Although the exact mechanism of action of StAR protein in mediating cholesterol transfer across the mitochondrial membranes is not known, limited evidence has previously suggested that StAR might work in concert with several other proteins including peripheral benzodiazepine receptor (PBR)/18-kDa transporter protein (TSPO), voltage-dependent anion channel 1 (VDAC1), phosphate carrier protein, cAMP-dependent protein kinase 1α (PKA-RIα) and TSPO-associated acyl-coenzyme A binding domain containing 3 (ABCD3) protein by forming a protein complex on the OMM (Stocco 2001; Rone et al. 2009; Miller 2007; Bose et al. 2008). However, two recent reports have provided evidence that PBR/TSPO is not involved in the steroidogenic process (Tu et al. 2014; Morohaku et al. 2014).

(2d) StAR-HSL interaction and HSL oligomerization

A significant amount of cholesterol transferred by StAR to mitochondria for steroidogenesis is hydrolyzed through HSL from CEs stored in steroidogenic cells. Using in vitro glutathione S-transferase pull-down experiments, we have demonstrated a direct interaction between HSL and StAR via the N-terminal as well as a central region of StAR (Shen et al. 2003). In addition, the 37-kDa StAR was co-immunoprecipitated with HSL from adrenals of animals treated with ACTH. Co-expression of HSL and StAR in Chinese hamster ovary cells resulted in higher cholesteryl ester hydrolytic activity of HSL. Transient overexpression of HSL in Y1 adrenocortical cells increased mitochondrial cholesterol content under conditions in which StAR was induced. It is proposed that the interaction of HSL with StAR in cytosol increases the hydrolytic activity of HSL and that together HSL and StAR facilitate cholesterol movement from lipid droplets to mitochondria for steroidogenesis.

In a separate study, using sucrose gradient centrifugation and in vivo and in vitro protein–protein interactions, we have demonstrated that HSL exists as a functional dimer composed of homologous subunits. Dimeric HSL displayed approximately 40-fold greater activity against cholesteryl ester substrate when compared with monomeric HSL without any differences in affinity for the substrate. Truncations of

HSL identified the importance of the N-terminal 300 amino acids, as well as other regions, in participating in the oligomerization of HSL (Shen et al. 2000).

(2c) StAR-14-3-3γ interactions

Recently, the adaptor/scaffold protein 14-3-3γ was shown to interact with StAR by binding to Ser^{194} of StAR and thus retain StAR in the cytosol and delay maximum steroidogenesis in a pattern opposite to 14-3-3γ homodimerization (Aghazadeh et al. 2012, 2014; Liu et al. 2006; Papadopoulos et al. 2007). Under basal conditions, StAR resides in the cytosol and 14-3-3γ exists as homodimers; upon hormone stimulation, phosphorylation and acetylation of 14-3-3γ on residue Ser^{58} and Lys^{49} increase its binding with StAR, which delays maximum steroidogenesis. After 2 h, this interaction falls apart, allowing induction of StAR activity and steroid production to proceed at a maximal rate.

1.3.3 Modulation of Steroidogenesis by miRNAs

Recently, we and others have shown that SR-BI is post-transcriptionally regulated by microRNAs (Hu et al. 2012, 2013b). In addition, miRNAs have been identified that regulate LH/hCG receptor and steroidogenic enzymes in a tissue specific manner (Menon et al. 2013).

MicroRNAs (miRNAs) comprise a novel class of endogenous non-protein-coding single-stranded small RNAs approximately 22–25 nucleotides long that have emerged as key post-transcriptional regulators of gene expression (Ambros 2004; Bartel 2004, 2009; Kim 2005; Fabian et al. 2010). They are transcribed in the nucleus by RNA polymerase II or RNA polymerase III into primary transcripts (pri-miRNAs) (Schwarz et al. 2003; Du and Zamore 2005, 2007), which are generally capped, polyadenylated and contain a hairpin stem of 33 bp, a terminal loop and two single-stranded flanking regions (Cai et al. 2004). These pri-miRNAs are then processed sequentially in the nucleus and cytoplasm by a complex of RNase III-endonucleases Drosha and Dicer to generate pre-miRNAs, and mature miRNAs, respectively (Siomi and Siomi 2010; Finnegan and Pasquinelli 2013). miRNAs cause post-transcriptional repression of protein synthesis by pairing with partially complementary seed sites in the 3′-untranslated regions (UTRs) of target mRNAs, leading to either deadenylation and subsequent mRNA degradation and/or translational inhibition (Bartel 2004, 2009; Fabian et al. 2010; Bushati and Cohen 2007; Eulalio et al. 2008; Filipowicz et al. 2008; Ghildiyal and Zamore 2009). Importantly, a single miRNA can regulate expression of hundreds of target genes (Krishnan et al. 2013; Venkataraman et al. 2013), whereas the expression of a single gene can be regulated by multiple miRNAs (Hu et al. 2012; Gillen et al. 2011). Since the their original discovery in 1993, miRNAs has been shown to be key post-transcriptional regulators of gene expression in metazoan animals, plants, protozoa and viruses (Bushati and Cohen 2007). In mammals miRNAs are suggested to control the activity of more than 60 % of all protein coding genes (Friedman et al. 2009), and to participate in diverse cellular processes including development, cell-cycle control, metabolism, stem-cell differentiation, inflammation and immunity, oncogenesis, and diseases (Bushati and Cohen 2007; Barter 2009; Ambros 2004;

Fabian et al. 2010; O'Connell et al. 2010; Yi and Fuchs 2011; Sayed and Abdellatif 2011; O'Connell et al. 2012; Abe and Bonini 2013; Di Leva et al. 2013; Fernández-Hernando et al. 2013; Flowers et al. 2013; Hata 2013; Szabo and Bala 2013; Rottiers and Naar 2012). Accumulating evidence now suggests that miRNAs also participate in the regulation of steroidogenesis (Menon et al. 2013; Yao et al. 2010; Romero et al. 2008; Robertson et al. 2013; Schmitz et al. 2011; Velazquez-Fernandez et al. 2014; Sirotkin et al. 2009; Yin et al. 2012; Yin et al. 2014; Dai et al. 2013; Xu et al. 2011; Kitahara et al. 2013).

Here we review the expression of miRNA in different steroidogenic tissues and their potential involvement in the regulation of steroid biosynthesis (Table 12.1).

Table 12.1 Summary of reported roles of miRNA during steroidogenesis

miRNA	Tissue	Process(es)/target(s)	Ref.
(1) Adrenal			
miRNA-132, miRNA-212	Adrenal	Aldosterone secretion	Hu et al. (2013b)
miRNA-21	Adrenal	Aldosterone secretion	Romero et al. (2008)
	Adrenocortical cells		
miR-24	Adrenal	Cortisol and aldosterone production	Robertson et al. (2013)
	Adrenal cortical cells	CYP11B1 and CYP11B2	
Human studies with pathophysiological implications			
miR-675, miR-139-3p	Adrenal	Discriminating ACCs from ACAs	Schmitz et al. (2011)
miR-335			
miR-21, miR-10b	Adrenal	Diagnose for ACC development, progression	Chabre et al. (2013), Chen et al. (2013), Wang et al. (2013b)
miR-1395p, miR-let-7f			
(2) Ovary			
miR-21, miR-23a, miR-145, miR-503			
miR-224, miR-383, miR-378, miR-132, and miR-212	Equine follicle development		
miR-122, miR-136-3p	Ovary	LRBP, LHR	Menon et al. (2013), Kitahara et al. (2013)
miR-455, miR-125a	Ovary	SR-BI	Hu et al. (2012)
miR-133b	Ovary, granulosa cells	Foxl2, changes StAR and CYP19A level	Dai et al. (2013)

(continued)

Table 12.1 (continued)

miRNA	Tissue	Process(es)/target(s)	Ref.
miR-513a-3p	Ovary	LHCGR	Troppmann et al. (2014)
miRNA-143	Ovary, follicle development		
miRNA-145	Ovary, granulosa cells	Activin receptor 1B, Smad 2	Yan et al. (2012), Yang et al. (2013)
miRNA-181a	Ovary, granulosa cells	Activin receptor IIA	Zhang et al. (2013)
miRNA-224	Ovary, granulosa cells	TGF-b, Smad4	Yao et al. (2010)
miRNA-320, MiRNA 383	Ovary, granulosa cells	Targets E2F, SF1, suppress granulosa cell proliferation	Yin et al. (2012, 2014)
miR-383	Ovary, granulosa cells	RBMS1, c-Myc	
Human studies with pathophysiological implications			
miR-132, miR-320, miR-miR-24, miR-222	Ovary, follicular fluid	Regulates estradiol concentration	Sang et al. (2013)
miR-24, miR-193b, miR-483-5p		Regulates progesterone concentration	
miR-132, miR-320	Follicular fluid	Down-regulated in follicular fluid of polycystic ovary patients	
miR-320, miR-383	Follicular fluid	Up-regulated in POC	
(3) Testis			
miR-132, miR-212	Testicular Leydig cells, MLTC-1		Hu et al. (2013b)
miR-34a,miR-181b, miR-122a	Mouse testis	In response to oxidative stress	Fatemi et al. (2014)
Human studies with pathophysiological implications			
miR-367-3p, miR-371a-3p	Human serum	Plasma biomarker for TGCT	Syring et al. (2014)
miR-372-3p, miR-373-3			

2 MiRNA Regulation of LH/hCG and SR-BI Receptors

Mevalonate kinase (Mvk) has been identified as a novel luteinizing hormone receptor (LHR) mRNA binding protein (LRBP), which binds to the coding region of LHR mRNA and causes translational suppression, leading to accelerated degradation of ovarian LHR mRNA in response to treatment with trophic hormones (Menon et al. 2013; Nair et al. 2002). Recently, it was demonstrated that miR-136-3p

and miR-122 contribute to LH/hCG-induced down-regulation of ovarian LHR mRNA (Menon et al. 2013; Kitahara et al. 2013). In addition, miR-513a-3p was shown to target the LH/hCG receptor and control the level of LHCGR expression by an inversely regulated mechanism at the post-transcriptional level (Troppmann et al. 2014).

In a search for miRNAs regulating the expression of SR-BI, we recently provided evidence that miRNAs, such as miR-125a and miRA-455, target SR-BI mRNA, thus post-transcriptionally and negatively regulate SR-BI-mediated selective delivery of HDL-cholesterol in steroidogenic cells and consequently inhibit SR-BI-mediated and HDL-supported steroidogenesis (Hu et al. 2012). In addition, miR-185, miR-96, and miR-223 have been implicated in the negative regulation of hepatic SR-BI (Wang et al. 2013a).

3 miRNA Expression in Steroidogenic Cells of Adrenal Gland, Ovary and Testis

(3a) Adrenal

In an effort to examine the role of miRNAs in steroidogenesis, the expression profiling of miRNAs in rat adrenals in response to in vivo treatment of animals with hormones, ACTH, 17α-ethinyl estradiol (17α-E2) or dexamethasone (Hu et al. 2013b), was examined. Forty five out of the 72 expressed rat miRNAs showed significant changes in the adrenal in response to ACTH treatment. Among these, 27 mature or precursor miRNAs were up-regulated and 18 mature or precursor miRNAs were down-regulated in ACTH-exposed versus control adrenals. Among the various adrenal miRNAs whose expression was altered in response to treatment of rats with any of these three hormones, the most robust effect was on the expression of miR-132 and miR-212 (3–4 fold induction) in response to ACTH treatment (Hu et al. 2013b). The precursor for miRNA-212 was also up-regulated. Real-time PCR (qRT-PCR) confirmed that miRNA-212, miRNA-183, miRNA-182, miRNA-132 and miRNA-96 were up-regulated by ACTH, and miRNA-466b, miRNA-214, miRNA-503 and miRNA-27a were down-regulated by ACTH. MiR-132, together with miR-212, comprises the evolutionary conserved miR-132/212 family, encoded from the same intron of a small non-coding gene that is located on chromosome 11 in mice, chromosome 10 in rats and chromosome 17 in humans. Mature miRNA-132 and miRNA-212 share the same seed sequence (Wanet et al. 2012; Remenyi et al. 2013). In cardiac fibroblasts, miRNA-132/212 can modulate the angiotensin II signaling pathway (Eskildsen et al. 2014). In adrenal, angiotensin II is an important modulator of adrenal zona glomerulosa cell function, including aldosterone production and cell proliferation. The hormone-mediated increases in miR-132 and miR-212 levels suggest the possibility that they might mediate some of the trophic hormone regulation of steroidogenesis. Likewise, the level of miR-21 has also been shown to be specifically up-regulated by angiotensin II, and its expression levels

were correlated with increased aldosterone secretion and proliferation in adrenocortical cells (Romero et al. 2008). In a screen using non-diseased human adrenal and aldosterone-producing adenoma samples, miR-24 was shown to be differentially expressed. Further analysis showed that miR-24 was able to modulate CYP11B1 and CYP11B2 expression, as well as cortisol and aldosterone production in human adrenal cortex (Robertson et al. 2013).

Apart from their involvement in a variety of biological processes, microRNAs are gaining recognition as potential pathogenic biomarkers for a number of metabolic and genetic diseases (Iorio and Croce 2012; Maegdefessel 2014). To study the clinical implications of miRNA expression in human adrenocortical carcinomas, an analysis of normal adrenal tissue, adrenocortical adenomas (ACAs), adrenocortical carcinomas (ACCs) and metastases showed that ACCs exhibited significantly lower levels of miR-139-3p, miR-675 and miR-335, and miRNA expression, suggesting that profiling miR-675 and miR-335 helps in discriminating ACCs from ACAs (Schmitz et al. 2011). In a different study, more than 40 miRNAs were shown to be differentially expressed in hyperfunctioning adrenocortical adenomas (Velazquez-Fernandez et al. 2014). Mir-21, mir-10b, mir-139-5p and mir-LET-7f have been shown to be involved in ACC development, progression, and aggressiveness (Chabre et al. 2013; Chen et al. 2013; Wang et al. 2013b).

(3b) Ovary

A study designed to identify miRNAs affecting the release of the major sex steroids progesterone, androgen and estrogen by human ovarian cells showed that 36 out of 80 tested miRNA constructs resulted in inhibition of progesterone release in granulosa cells, and ten miRNAs promoted progesterone release. Fifty-seven miRNAs tested inhibited testosterone release, and only one miRNA enhanced testosterone output. Fifty-one miRNAs suppressed estradiol release, while none of the miRNAs tested stimulated it (Sirotkin et al. 2009).

Many studies have focused on ovarian granulosa cells and have shown that multiple miRNAs, including miR-21, miR-23a, miR-145, miR-503, miR-224, miR-383, miR-378, miR-132, and miR-212, are involved in follicle proliferation and granulosa cell function (Yin et al. 2012, 2014; Yao et al. 2010; Schauer et al. 2013). miR-224 was shown to target Smad4 and to regulate the TGF-β1 signaling pathway (Yao et al. 2010). Overexpression of miR-224 enhanced TGF-β1 induced granulosa proliferation, whereas inhibition of endogenous miR-224 attenuated TGF-β1 induced proliferation of granulosa cells. Both miR-224 and TGF-β1 can function to increase CYP19A1 mRNA levels and promote estradiol release from granulosa cells. miR-133b was shown to target the 3′ UTR of Fox12 and inhibited the transcriptional repression activity of FOXl2 against StAR and CYP19A1, thereby regulating estradiol production in granulosa cells (Dai et al. 2013). The expression levels of both miR-132 and miR-212 were up-regulated in primary rat ovarian granulosa cells by the second messenger of the trophic hormone, cAMP

(Hu et al. 2013b). miR-383 has been shown to have multiple targets in its role in regulating steroidogenesis. On the one hand, miR-383, which is itself trans-activated by SF-1, affects the stability of RBMS1, suppresses the level of c-Myc and regulates estradiol release from granulosa cells (Yin et al. 2012). On the other hand, miR-383 has also been shown to increase the expression of miR-320 and to suppress granulosa cell proliferation. The targets of miR-320 were shown to be E2F1 and SF-1, which result in suppression of follicle development and steroid production (Yin et al. 2014). In a study searching for regulators of estradiol production, micro-RNA378 (miR-378) was shown to be spatiotemporally expressed in porcine granulosa cells, and to regulate ovarian estradiol production by targeting aromatase (Xu et al. 2011).

With respect to clinical relevance, evidence has been presented that the expression levels of miR-320 and miR-383 are up-regulated in the follicular fluid of patients with polycystic ovarian syndrome. Additional analysis of human follicular fluid led to the identification of miR-132, miR-320, miR-520c-3p, miR-24 and miR-222, which have been implicated in the regulation of estradiol levels, whereas miR-24, miR-193b and miR-483-5p have been suggested to regulate progesterone production. Finally, miR-132 and miR-320 are expressed at significantly lower levels in the follicular fluid of patients with polycystic ovarian syndrome (Sang et al. 2013).

(3c) Testis

To date, most of the miRNA studies in testis have been directed towards their potential involvement in the spermatogenic process and other testicular functions rather than steroidogenesis. Many miRNAs have also been identified that specifically target germ-cell specific genes (for more information, please consult some recent reviews McIver et al. 2012; Papaioannou and Nef 2010; Sree et al. 2014; Kotaja 2014).

With respect to testicular Leydig cells, most of the studies have been focused on documenting changes in the expression profile of miRNAs using Leydig tumor cell lines. In a screen profiling the expression of miRNAs, the expression levels of both miR-132 and miR-212 were upregulated in mouse testicular Leydig tumor cells, MLTC-1, by the second messenger cAMP (Hu et al. 2013b). Another study reported changes in the expression of miRNA-34a, miRNA-181b and miRNA-1221 in mouse testis in response to hydroperoxide (TBHP)-induced excessive oxidative stress in vivo (Fatemi et al. 2014). These miRNAs have been implicated in the regulation of various cellular signaling pathways associated with inflammation, antioxidant responses and spermatogenesis.

A recent clinical study demonstrated that miR-367-3p, miR-371a-3p, miR-372-3p, miR-373-3 could potentially serve as biomarkers for the diagnosis of testicular germ cell tumor (TGCT) (Syring et al. 2014). In particular, miR-371a-3p showed a better correlation with TGCT than either AFP or hCG, and could potentially be employed as a biomarker in clinical management of TGCT, especially monitoring surveillance therapy and residual disease after chemotherapy.

4 Conclusions

During the past six decades, the understanding of the complex process of steroidogenesis has progressed rapidly in the steroidogenic cells of the adrenal gland, ovary and testis. At the cellular level, this represents a multistep and multienzyme process. In the past two decades or so, with the introduction of state-of-the-art molecular, cellular and biochemical techniques, considerable progress has been made in understanding the various steps involved in the acquisition of cholesterol substrate, both from external and internal sources, for its utilization by mitochondrial CYP11A1 (P450scc) for pregnenolone production, and subsequent enzymatic conversion of pregnenolone into tissue specific steroids. Moreover, events connected with the trophic hormone stimulation of steroidogenesis both at the acute and chronic levels have been reasonably defined. In addition, ample information is currently available about the transcriptional regulation of key steroidogenic enzymes, as well as StAR protein, which plays a pivotal role in the transport of cholesterol to mitochondria for steroid production. During the past few years, it is becoming increasingly clear that the steroidogenic process in general and certain steroidogenic proteins in particular are also subject to post-transcriptional and post-translational regulation. Broadly, these post-transcriptional and post-translational regulations include processes such as phosphorylation/dephosphorylation and protein–protein interactions, as well as the involvement of specific miRNAs. A further mechanistic understanding of these events in the near future should greatly aid in delineating the underlying mechanisms involved in cellular cholesterol trafficking, cholesterol transport to mitochondria and steroid production.

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Post-transcriptional Regulation of Glucocorticoid Function

13

Faoud T. Ishmael and Cristiana Stellato

1 Post-transcriptional Gene Regulation in Inflammation as Target of GC Anti-inflammatory Action

1.1 Mechanisms of PTR in Inflammation: Rationale for Therapeutic Anti-inflammatory Targeting

Post-transcriptional gene regulation (PTR) is a critical control mechanism of the inflammatory response integrated with transcriptional control of gene expression. By implementing changes in mRNA turnover and translation rates, PTR mechanisms adapt the amplitude and timing of protein expression to endogenous or environmental changes (Stoecklin and Anderson 2006a; Hollams et al. 2002). Transcriptional and post-transcriptional regulation are coordinately mediated by common signaling pathways—chiefly MAP kinases (Winzen et al. 1999; Frevel et al. 2003; Gaestel 2006; Gao et al. 2013). Rapid mRNA transcript degradation of cytokines, chemokines, enzymes and other mediators contribute to successful

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cessation of an acute inflammatory reaction; conversely, aberrant stabilization of the targeted transcripts can support overexpression of inflammatory genes during chronic inflammation. Stress- and inflammation-driven signals can also rapidly adapt protein translation rates to changing extracellular environment (Stoecklin and Anderson 2006a; Hollams et al. 2002). Genome-wide studies indicate that up to 50 % of genes induced during a stress response are mainly regulated post-transcriptionally (Fan et al. 2002). RNA-binding proteins (RBP), microRNA (miRNA) and other classes of small noncoding RNAs (sncRNA) constitute the heterogeneous group of regulatory factors conveying PTR through binding to conserved sequences mainly present in the untranslated regions (UTR) of their mRNA targets, with which they form ribonucleoprotein (RNP) complexes ultimately conveying PTR action. The activation of regulatory factors and their assembly in multimeric RNP complexes is a dynamic process susceptible of regulation by inflammatory signaling (Winzen et al. 1999; Frevel et al. 2003; Gaestel 2006; Gao et al. 2013) and therefore, potentially amenable to therapeutic targeting. Small variations in mRNA half-life, in the range of two- to fourfold changes, can rapidly lead to over a 1000-fold difference in mRNA levels (Ross 1995). With additional regulation at the level of protein translation, the rate-limiting control provided by PTR is critical in inflammatory and immune responses, where changes in concentration and length of expression for dangerous and protective genes are in dynamic balance and become critical in determining either successful resolution of acute inflammation or chronic overexpression of the adaptive immune response (Shyu and Wilkinson 2000; Anderson et al. 2004; Baltimore et al. 2008).

The key importance of PTR as regulatory hub in inflammation was clearly illustrated early on by two independent studies in mouse models whose design was complementary, as one component of the RNP complex regulating TNFα mRNA expression was disrupted in each study: in the work by Kontoyannis et al. by mutation of the adenylate-urydilate-rich elements (ARE) present in the mRNA 3′UTR regulating the transcript's turnover and translation rates (Kontoyiannis et al. 1999) and, in the study by Taylor et al., by ablation of tristetraprolin (TTP), the ARE-binding protein necessary for rapid degradation of TNFα and many other ARE-bearing inflammatory transcripts (Taylor et al. 1996). In both models, circulating TNFα levels were aberrantly increased without changes in transcription rates in the TNFα-producing macrophages. Strikingly, both models displayed strong inflammatory phenotypes that were largely overlapping: in mice carrying the deletion in the TNFα ARE, high circulating levels of TNF-α associated with early onset of inflammatory cell infiltration within the joints and the bowel, closely resembling the clinical features of human rheumatoid arthritis and Crohn's disease, respectively (Kontoyiannis et al. 1999). Along the same lines, mice lacking TTP displayed early onset of cachexia, severe inflammatory arthritis, autoimmune dysfunction and myeloid hyperplasia through the overexpression of TNFα and GM-CSF (Taylor et al. 1996). Thus, disruption of the RNP complex regulating post-transcriptionally TNFα recapitulated major features of human inflammation and identified

conclusively the pathogenic role that PTR can play when aberrantly regulated. Along the same lines, mice lacking the ARE-RBP heterogeneous nuclear ribonuclear protein D (*Hnrnpd*), also named AUF-1, a regulator of inflammatory cytokine mRNA stability, displayed another important inflammatory phenotype. The animals developed a chronic pruritic dermatitis with eczematous lesions closely resembling atopic dermatitis, with increased IgE levels, hypereosinophilia and a Th2-skewed immune profile (Sadri and Schneider 2009). Also in this model, deregulation of TNFα was partially accountable for the skin manifestations, together with CCL27 chemokine-driven mechanisms. This model further points at the essential participation of specific RBPs in skewed immune responses, which should be more closely scrutinized for therapeutic purposes.

Studies on the miRNA component of PTR have also clearly indicated, in many in vitro and animal models, their role in shaping the immune response and their participation in pathophysiological mechanisms of those chronic inflammatory and autoimmune diseases that are treated with GCs (Chen et al. 2013; Foster et al. 2013; Ivanov and Anderson 2013; Lu and Rothenberg 2013). Taken together, these data clearly indicate the importance of PTR mechanisms in controlling the inflammatory response and thus lend themselves to be studied as potentially key determinants of GC anti-inflammatory action.

1.2 Mechanisms of GC Action: A Brief Overview

Systemic or topical GCs are the first-line therapy in immune-mediated and inflammatory diseases such as persistent eosinophilic asthma, moderate to severe COPD, rheumatoid arthritis, systemic lupus erythematosus (Stellato 2007). Their anti-inflammatory action is generated through multiple mechanisms mediating coordinate changes in gene expression. While a thorough review of the molecular mechanisms of GC action is beyond the scope of this chapter and is provided by recent reviews (Barnes and Adcock 2009; Oakley and Cidlowski 2013), a brief overview with a focus on the pathways discussed herein is presented in Fig. 13.1. Binding of GC activates the glucocorticoid receptor (GR), which disassociates from a multimeric complex with chaperone proteins and translocates in the nucleus. Ligand-activated GR homodimerizes in the nucleus and acts as a transcription factor, controlling gene expression at transcriptional level (green boxes) by either DNA-dependent mechanisms, mediated by binding to GC responsive elements (GRE)—or in rare instances to negative GRE (nGRE)—or by DNA-independent mechanisms. In the latter case, GR engages in protein:protein interactions with subunits of NF-κB, AP1 and other transcription factors and cofactors, producing either transrepression of inflammatory genes, or cooperation in the induction of innate host defense (HD) genes such as Toll-like Receptor (TLR)-2 and others, together with genes carrying non-immune and metabolic functions. GC-induced genes relevant to anti-inflammatory action also act as transcriptional inhibitors (as in the case of Glucocorticoid-induced leucine

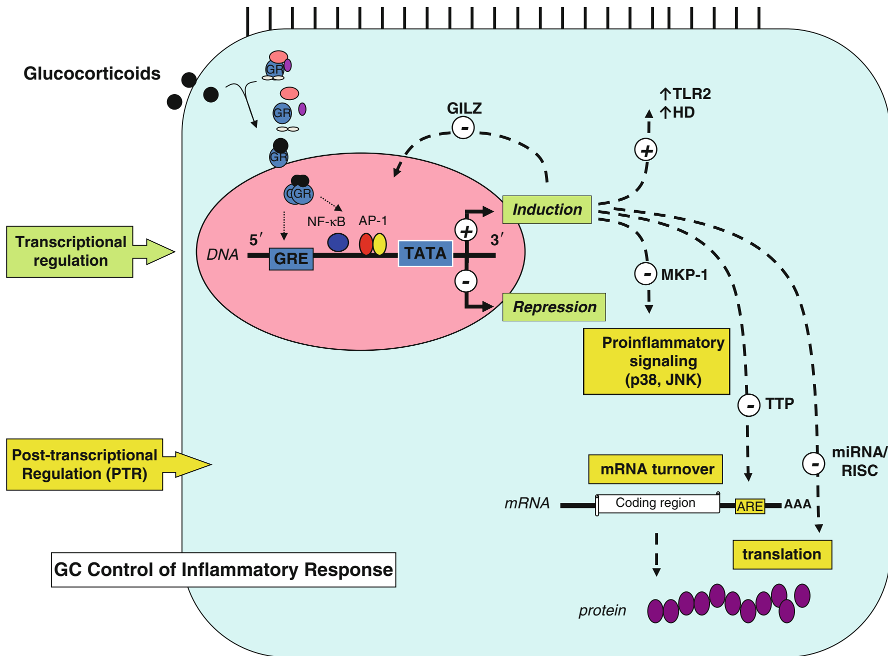

Fig. 13.1 Mechanisms of GC action discussed in this review. GCs exert their anti-inflammatory action through multiple mechanisms mediating coordinate changes in gene expression (see text). Regulation of inflammatory responses by GC is the result of integrated transcriptional and post-transcriptional pathways. The genes described so far to mediate post-transcriptional control involved in GC action modulate (*yellow boxes*) inflammatory signaling impacting mRNA turnover and translation, in part by the function of MKP-1, or regulate components that participate directly to the RNP complex conveying changes in mRNA turnover and translation, such as the RNA-binding protein TTP, several miRNAs and their processing enzymes

zipper: GILZ), or mediate inhibitory GC effects downstream of transcription. The GC-induced—or more generally, the GC-regulated genes—so far identified as affecting mRNA turnover and translation rates are involved in controlling inflammatory signaling or regulate directly the RNP complex, such as RBPs, miRNAs and their processing enzymes.

1.3 Inflammation-Related Genes Targeted by GC through PTR Effects

Alteration of mRNA turnover or translation has been documented for a large number genes involved in inflammation and immunity, such as TNF-α, IL-1α , IL-6, IL-8, interferon-β, granulocyte macrophage colony-stimulating factor (GM-CSF), cyclooxygenase (COX)-2, IL-2, IL-3, inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and many chemokine genes

Table 13.1 Genes regulated by glucocorticoids through alteration of mRNA stability: mRNA binding sequences and RBP found to be involved in GC action

Gene	mRNA stability	mRNA region (and element) involved	RBP	References
COX-2[a]	Decreased	3′UTR (ARE)	Unknown	Lasa et al. (2001)
Cyclin D3	Decreased	3′UTR	Unknown	Garcia-Gras et al. (2000)
CCL2	Decreased	3′UTR, 5′UTR (G/C rich region)	TTP, GR	Ishmael et al. (2008, 2010)
CCL7	Decreased	3′UTR, 5′UTR (G/C rich region)	TTP, GR	Ishmael et al. (2008, 2010)[b]
CXCL1	Decreased	3′UTR	TTP	Ishmael et al. (2008)
Fibronectin	Increased	Intronic (unspecified)	Unknown	Ehretsmann et al. (1995)
GM-CSF	Decreased	Unknown	Unknown	Tobler et al. (1992)
IFNβ	Decreased	ARE	Unknown	Peppel et al. (1991)
IL-1	Decreased	Unknown	Unknown	Amano et al. (1993)
IL-4Rα	Decreased	Unknown	Unknown	Mozo et al. (1998)
IL-6	Decreased	3′UTR	TTP	Tobler et al. (1992); Ishmael et al. (2008)
CXCL8	Decreased	Unknown	Unknown	Tobler et al. (1992)
iNOS	Decreased	Unknown	Unknown	Korhonen et al. (2002)
LIF	Decreased	Unknown	Unknown	Grosset et al. (1999)
SP-1	Increased	3′UTR, 7.6S region	Unknown	Huang et al. (2012)
TNFα	Decreased	ARE	TTP	Smoak and Cidlowski (2006)
VEGF	Decreased	Unknown	Unknown	Gille et al. (2001)

Modified and extended from Stellato (2004)

[a]*Abbreviations*: *COX* Cyclooxygenase, *GM-CSF* Granulocyte-Monocyte Colony Stimulator Factor, *IFN* Interferon, *IL* Interleukin, *IL-4Rα* IL-4 Receptor α, *iNOS* inducible Nitric Oxide Synthase, *LIF* Leukemia Inhibitory Factor, *VEGF* Vascular Endothelial Growth Factor

[b]Complete list of TTP-dependent and GR-dependent GC sensitive genes, respectively, in these references

such as CXCL1, CCL2, CCL7, CCL11, CCL13, and many others (Amano et al. 1993; Tobler et al. 1992; Shaw and Kamen 1986; Peppel et al. 1991; Carballo et al. 2000; Sawaoka et al. 2003; Stoecklin et al. 2003; Ogilvie et al. 2005; Fechir et al. 2005; Fan et al. 2011; Hamilton et al. 2010)—a comprehensive list is in recent reviews (Anderson 2010). The majority of these genes have been demonstrated to be GC-sensitive in many experimental and therapeutic settings and, at the same time, it is known that GC sensitivity can vary in different cell types and can be hampered by inflammatory conditions, leading to GC resistance or at least loss of full therapeutic control. However, the list of GC-regulated genes for which the existence of a PTR component is demonstrated is much shorter (see Table 13.1), though studies utilizing genome-wide approaches are accelerating the pace of the identification of PTR-dependence of GC-mediated gene regulation (Ishmael et al. 2008, 2010). Knowledge of the participation of aberrant PTR response as mechanism of GC resistance is even more ill-defined.

2 Post-transcriptional Determinants of GC Anti-inflammatory Action

Mechanistic knowledge on how immune responses rely on PTR for coordinate expression of cytokines, chemokines and other inflammation-related factors is lending a strong rationale to investigate GCs' ability to target PTR mechanisms as part of their anti-inflammatory activity. Following are studies that have started unraveling the specific PTR determinants—the signaling mediators, specific regulatory functions, RNA binding sequences, RBPs and miRNA functions, etc.—involved and necessary for GC action. Further studies are necessary to fully characterize GC-driven PTR components and to develop experimental systems that could establish and measure the overall impact of PTR in GC's anti-inflammatory therapeutic action.

2.1 GC Effect on Signaling Pathways Regulating PTR Functions

According to transcript-, stimulus- and cell-specific-mechanisms, multiple signaling pathways can regulate—through different post-translational modifications—the trafficking, binding affinity, compartmentalization and other functional aspects of RNA-binding proteins and miRNAs. Members of the mitogen-activated protein kinases (MAPK) family, such as the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38/SAPK2 and extracellular regulated kinase (ERK) are essential—though obviously not exclusive—regulators of PTR, in full integration with their upstream control on gene transcription (Kracht and Saklatvala 2002; Gaestel 2006). Post-transcriptional activities of MAP kinases have been increasingly well-characterized in mechanistic terms and recognized as necessary for inflammation-driven overexpression of a large number of inflammatory targets (Kracht and Saklatvala 2002; Dean et al. 2004; Anderson 2008; Gaestel 2013). At the same time, inhibition of MAPK signaling constitutes a major and powerful component of GC anti-inflammatory action, better characterized with regard to the MAPK signaling control of transcriptional mechanism (Kracht and Saklatvala 2002; Clark et al. 2003; Clark and Lasa 2003). Although MAPK-dependent transcript stabilization by inflammatory stimulation has been demonstrated for many transcripts that are described as susceptible to GC inhibition through acceleration of mRNA decay—among these IL-1, VEGF, COX-2, TNFα, IL-6 and IL-8 and many chemokines (Amano et al. 1993; Gille et al. 2001; Lasa et al. 2001; Swantek et al. 1997; Tobler et al. 1992; Ishmael et al. 2008; Stellato 2004), relatively few studies to date have *directly* investigated the PTR regulatory sequences and binding partners targeted by GC as part of the inhibitory effect on MAPK-dependent gene regulation, and attempt to measure the overall anti-inflammatory impact of this regulatory mode. Such experimental evidence would be relevant for identifying specific pathways potentially targetable for a selective anti-inflammatory therapeutic intervention. The most relevant studies on GC effect to this end have focused, in

several experimental models, on p38-regulated PTR showing that treatment with specific p38 inhibitors in activated cells antagonized kinase-induced mRNA stabilization (or increased translation) and promoted acceleration of mRNA decay or decreased translation, effects that were found to be mediated by the ARE-bearing 3′-UTRs of these genes (Kracht and Saklatvala 2002; Zhang et al. 2002; Dean et al. 2004). One of the first studies to characterize the involvement of p38 MAPK-mediated PTR by GC action used COX-2 mRNA turnover as model (Lasa et al. 2000, 2001). The decay of a β-globin reporter including the ARE-bearing 3′-UTR of COX-2 mRNA was transiently transfected in HeLA cells and examined upon stimulation by a constitutively active form of MAPK kinase-6 (MKK-6), an upstream activator of p38, which increased the reporter's mRNA stability. This effect was inhibited both by cell pretreatment with dexamethasone (Lasa et al. 2001) and p38 antagonists (Lasa et al. 2000). As in multiple systems (Clark and Lasa 2003), GC cell treatment inhibited stimulus-induced p38 phosphorylation, supporting the conclusion that GC effect on the reporter mRNA turnover occurred through the inhibition of p38 MAPK activity, in turn responsible for COX-2 mRNA stabilization (Lasa et al. 2001).

The effect of GC on MAPK-driven PTR control—that mediated by p38 in particular—was later found to be significantly linked to GC-induced transcriptional activation of the MAPK phosphatase 1 gene (MKP-1), which antagonized the p38-mediated stabilization of COX2 mRNA (Lasa et al. 2002). GC-driven MKP-1 induction was confirmed in several cell types including macrophages and airway epithelium (Imasato et al. 2002). Earlier data showed that GC effects on mRNA stability of COX-2 and other inflammatory transcripts did require de novo gene expression (Newton et al. 1998), supporting the hypothesis that MKP-1 contributed to the post-transcriptional mechanisms of GC action observed for COX-2 regulation. More recent studies using either transgenic or KO models of MKP-1 gene have shown its non-redundant role as negative regulator of inflammation and as important factor of GC anti-inflammatory mechanism, as suggested by the significant impairment of GC response in macrophages of MKP-1$^{-/-}$ mice (Abraham and Clark 2006; Liu et al. 2007). Recently, overexpression or ablation of MKP-1 in LPS-stimulated rat macrophages was found to regulate levels of IL-6, IL-10 and TNFα mRNA through changes in mRNA stability in conjunction with regulation of nucleocytoplasmic shuttling of AUF-1, the ARE-binding RNP promoting the mRNA decay of those cytokines (Yu et al. 2011) and, as discussed earlier, shown in KO model to be involved in control of TNF-α, chemokine and Th2-driven inflammation (Sadri and Schneider 2009). Studies on GC effects in similar systems will be necessary to identify the specific molecular species downstream of MAPK through which GC exert PTR control of inflammatory signaling—and how much of it is dependent on MKP-1—in order to dampen the inflammatory process.

The PTR effects by GC documented to be ARE-dependent and mediated by MAP kinases indicate that such mechanism can be cell-specific (Lasa et al. 2001, 2002) and, possibly, stimulus- and transcript-specific. As not all ARE-containing genes are p38-dependent (Tebo et al. 2003; Frevel et al. 2003), the signaling

pathways conveying ARE-dependent, GC-driven PTR requires further scrutiny. It is clear, in fact, that additional mechanisms of control by GC on PTR signaling may very well exist outside the p38-MKP1 axis. For example, dexamethasone inhibited cytokine-induced IL-8 in human airway epithelial cell lines by acceleration of mRNA decay, in parallel with induction of MKP-1 expression; however the effect on IL-8 mRNA stability was not recapitulated by overexpression of MKP-1 nor influenced in opposite way by its silencing, suggesting that the post-transcriptional effect of GC on IL-8 expression can be conveyed by molecular species downstream of MKP-1 (Dauletbaev et al. 2011). In particular, the effect of GC on MAPK-activated protein kinases (MAPKAP) 2 and 3, which are master regulators of mRNA decay and translation through phosphorylation of TTP and other major RBPs deserves further experimental testing (Moens et al. 2013; Gaestel 2013).

2.2 Sequence Elements for Regulations and RNA-Binding Proteins Conveying GC Action

Genes required for a specific biological process—a specific pool of transcription factors, cytokines, chemokines, enzymes required for a TLR-mediated activation, for example—can be regulated post-transcriptionally in a coordinate fashion via a specific RBP that binds to common regulatory sequences present in those mRNAs, as a post-transcriptional regulon (Anderson 2010; Keene 2007). Therefore, control of expression or function of the RNP components by GC may convey a large, yet rather specific impact on gene networks involved in specific pathophysiological processes, by acting on a regulatory step well downstream of transcription. In keeping with the immunomodulatory function of GC action, it can be expected however that components of post-transcriptional gene control may change in sensitivity to GC according to tissue- and cell-specific issues, and according to the type of inflammatory event and the ensuing signaling. Characterization of GC action on the components of the RNP complex—the RNA regulatory sequences and their binding partners—is a growing area of investigation aimed at identifying more specific anti-inflammatory strategies.

GC and Sequence Elements for Regulations The interaction of regulatory factors—RNA-binding proteins, microRNAs and potentially other sncRNAs—with sets of relatively specific sequences present on the targeted mRNA is at the core of post-transcriptional gene regulation (Bakheet et al. 2006). The targeted sequences—heterogeneous in sequence, secondary structure, position and specific functions—are more frequently represented in the 5′ and 3′ untranslated regions (UTR) of the mature cytoplasmic mRNA, and have been collectively denominated USER (Untranslated Sequence Elements for Regulations) (Wilusz and Wilusz 2004; Keene 2007). The majority of inflammation-driven, MAPK-dependent PTR as well as many of the described post-transcriptional effects by GC occur through association of regulatory factors to adenylate/uridylate-rich elements (AREs) within the 3′-UTR of mRNAs. This evolutionarily conserved, heterogeneous group of sequences have

been the first to be described as central in PTR events (Shaw and Kamen 1986) and in immune-mediated PTR processes (Chen and Shyu 1995; Anderson 2010), as indicated by the fact that immune-related genes are highly enriched in AREs (see http://rc.kfshrc.edu.sa/ared, http://rna.tbi.univie.ac.at/AREsite) (Bakheet et al. 2006; Gruber et al. 2011). In one of the first demonstrations of ARE-dependent GC effect, macrophages from mice expressing the TNF-α gene carrying the deletion of the ARE (ΔARE) overexpressed TNF-α protein due to decreased decay rate of TNF-α mRNA as well as to loss of translational inhibition (Kontoyiannis et al. 1999). In these cells, LPS-induced increase in TNF-α became unresponsive to the inhibitory effect that a p38 inhibitor displayed on WT cells, showing dependence of p38/JNK-mediated activation of TNF-α translation. Conversely, cell treatment with dexamethasone of ΔARE macrophages retained in part its ability to inhibit TNF-α secretion, suggesting that the ARE-dependent inhibition of TNF-α is an important—but not exclusively p38/JNK-mediated- PTR mechanism of inhibition of TNF-α by GC. There are currently no data on GC regulation on ARE-BP acting as repressors of TNF-α translation, such as T-cell intracellular antigen (TIA) and TIA-related protein (TIAR) (Zhang et al. 2002). As further experimental proof of ARE-dependent GC action, the dexamethasone-induced acceleration of an mRNA reporter expressed by a construct containing COX-2 3′UTR was suppressed by deletion of a small ARE cluster within the 3′-UTR, which bears a large number of AREs (Lasa et al. 2001). Dependence from 3′UTR of GC action on mRNA stability, though not specifically addressing ARE-dependence, has been described for several inflammatory genes (Stellato 2004) (see Table 13.1). No data are so far available on the participation to GC action of other classes of 3′UTR USER, such as the GU-rich elements (Halees et al. 2011) and is so far unknown whether endogenous GC would support PTR mechanisms keeping inflammatory transcripts highly unstable at baseline conditions, as documented through binding of the RBP Roquin to the constitutive decay element (CDE) present as well in the 3′UTR for TNFα mRNA and other transcripts (Leppek et al. 2013). Other sequences outside the 3′UTR have been found to mediate the effect of GC on mRNA turnover and/or translation. In particular, GC-mediated PTR has been recently linked to sequences present in the 5′UTR of CCL2 mRNA and other transcripts. Initial data generated in rat smooth muscle cells (SMC) indicated that the increase in decay rate of CCL2/MCP-1 mRNA induced by cell incubation with GC was not dependent on the ARE-bearing 3′-UTR but was rather mediated by a unique sequence in the 5′-UTR (Poon et al. 1999). Recently, GC-induced increased decay of CCL2 mRNA in human airway epithelial cells has been ascribed to a Guanidine/Cytidine (GC)-rich motif identified in the 5′UTR region in the CCL2 mRNA (Ishmael et al. 2010). This sequence mediated the transcript association of a complex containing the GC receptor (GR), which operated in this experimental setting as a bona-fide RNA-binding protein (see next paragraph).

An important non-immune gene induced by GC in alveolar type 2 epithelial cells encodes the surfactant protein B (SP-B), necessary for appropriate function of the surfactant fluid lining the alveolar space in the lungs. Together with transcriptional control, GC increase SP-B mRNA stabilization by a 3′UTR-dependent mechanism requiring a 126-nt-long segment denominated 7.6S region (Huang et al. 2008).

Mutagenesis studies indicated that within this region, a 30-long element predicted to form a stem-loop structure was sufficient for GC-mediated mRNA stabilization (Huang et al. 2012).

Considering the number of regulatory sequence elements within 5′-UTR and 3′-UTR of eukaryotic mRNAs besides ARE (see http://utrdb.ba.itb.cnr.it/) (Grillo et al. 2010), the interactions that could occur among the regulatory factors assembling over multiple USERs present in the same transcript, together with specific changes brought to the RNP complexes by stimulus-dependent signaling, it is evident that much more remains to be discovered on the identity and characteristic of additional USER supporting GC action and their binding partners.

RBPs as Targets/Mediators of GC Function RNA-binding proteins are among the most abundant and well-conserved eukaryotic genes (Gerstberger et al. 2014). They regulate several aspects of the mRNA metabolism, such as its maturation, nucleocytoplasmic transport, subcellular localization, rate of decay, and translation (Dreyfuss et al. 2002; Anderson 2008, 2010) and their key regulatory roles in immune responses is now well established (Ivanov and Anderson 2013; Yuan and Muljo 2013). Target-bound RBPs implement their functions by forming dynamic multimeric complexes with miRNAs, other RBPs and RNA-degrading enzymatic proteins not directly interacting with the mRNA (Jiang and Coller 2012; Ciafre and Galardi 2013; Srikantan et al. 2012; Steitz and Vasudevan 2009), often co-localized in cytoplasmic foci of mRNA metabolism (P bodies and stress granules) (Anderson and Kedersha 2009; Bhattacharyya et al. 2006; Jing et al. 2005). Numerous ARE-binding proteins (ARE-BPs) have been functionally characterized as regulatory factors of mRNA decay and translation of inflammatory and immune genes [for review see (Dreyfuss et al. 2002; Stoecklin and Anderson 2006a; Anderson 2008, 2010)], but very few have been investigated with regard to their potential function in GC action. While numerous proteins—among them TIA-1, TIAR, FXR1P, CUGBP2—can exert translational repression, changes in mRNA stability leading to prolonged half-life and increased expression of a vast number of inflammatory genes have been ascribed mostly to HuR, the ubiquitous member of the Hu family of RBPs (Brennan and Steitz 2001; Meisner and Filipowicz 2011; Simone and Keene 2013) and to YB1, described for a more limited number of transcripts, among which IL-2 mRNA (Chen et al. 2000; Lyabin et al. 2014). In contrast, numerous RBPs have been characterized as involved in destabilizing ARE-bearing inflammatory mRNAs, such as AUF1, KSRP, RHAU, and members of the TTP/BRF family of proteins (for review see Stoecklin and Anderson 2006b). Despite their documented role as regulatory factors for established GC-sensitive genes—cytokines, chemokines and enzymes involved in inflammation—as well as their strong functional dependence from MAPK signaling pathways (Sugiura et al. 2011) that are as well GC-sensitive, the biology of RNA-binding proteins in human inflammatory diseases is presently ill-defined and knowledge on the mechanisms of control by GC, or by anti-inflammatory intervention in general, on their expression and function is lagging behind in translational research settings. Following are the main observations of studies that have so far addressed directly the role of RBPs in GC-mediated PTR.

Tristetraprolin: A Mediator of Post-transcriptional GC Action Tristetraprolin (TTP) is the product of the ZFP-36 gene (also known as TIS11, Nup475, and GOS24) and member of a family of CCCH zinc finger proteins that include TTP, Butyrate-response factor (BRF)-1 and BRF-2 (Carrick et al. 2004). These proteins mediate deadenylation-dependent mRNA decay (Raineri et al. 2004) through binding of zinc finger domains to adjacent UUAU/UUAU half-sites, which trigger complex interactions with components of the exosome, the decapping/Xrn1 complex, and with the RISC complex (Worthington et al. 2002; Blackshear et al. 2003a; Brewer et al. 2004; Stoecklin and Anderson 2006b). Tristetraprolin is induced as an immediate early response gene by inflammatory mediators, phorbol esters, LPS and growth factors in a number of immune and structural cell types, including T cells, macrophages and fibroblasts where it displays a predominantly cytoplasmic localization (Blackshear 2002). TTP is also expressed in lung, liver, and intestine tissues (Cao 2004; Lu and Schneider 2004). As previously mentioned, the importance of TTP as endogenous rate-limiting factor of the inflammatory response has been convincingly demonstrated in TTP$^{-/-}$ mice, which develop severe inflammatory arthritis, autoimmune dysfunction and myeloid hyperplasia through the deregulated expression of TNF-α and GM-CSF (Taylor et al. 1996). Tumor necrosis factor α is both a major target and regulator of TTP, as TNF-α induces TTP synthesis, which in turn leads to destabilization of TNF-α mRNA (Carballo et al. 1998). TTP also modulates mRNA decay rates of GM-CSF, COX-2, INOS, IL-2, IL-3, IL-10, and several chemokines, such as CCL2, CCL7, CXCL1, CXCL8 and others (Lai et al. 2006; Carballo et al. 2000; Sawaoka et al. 2003; Stoecklin et al. 2003; Ogilvie et al. 2005; Fechir et al. 2005; Ishmael et al. 2008). Additional transcripts whose decay is regulated by TTP have been identified in a recent genome-wide study of mouse embryonic fibroblasts (MEFs) isolated from TTP-knockout (TTP$^{-/-}$) mice (Lai et al. 2006), and in mouse macrophages in which TTP expression was silenced (Jalonen et al. 2006). Importantly, phosphorylation of TTP by p38-MAPK and the downstream MAPKAP, MK2 (Carballo et al. 2001; Mahtani et al. 2001) is an important mechanism of post-transcriptional control during inflammation, as it leads to functional inactivation of TTP due to an inhibitory complex with the adaptor protein 14-3-3 (Chrestensen et al. 2004; Johnson et al. 2002; Stoecklin et al. 2004), though this could be dependent by contextual stimuli (Rigby et al. 2005). Furthermore, TTP has a key role as MK2/3-regulated RBP in limiting TNFα translation (reviewed in Gaestel 2006, 2013). Overall, the function, regulation and targeted genes of TTP make this molecule an ideal mediator of GC anti-inflammatory activity.

Indeed, expression of TTP and BRF-1 has been reported to be induced by GC in primary human airway epithelial cells and epithelial cell lines (Pawliczak et al. 2005; Ishmael et al. 2007). Induction of TTP by GC has been shown in vivo by treatment with dexamethasone in adrenalectomized rats, which caused a three- to fourfold increase of TTP mRNA expression in the lung, liver and thymus (Smoak and Cidlowski 2006). Based on these results, the mechanism of TTP induction by GC was further defined using the airway human epithelial cell line A549. An important characteristic of TTP regulation by GC found in this system is the extended length of induction of TTP mRNA, which was detectable up to 8 h following GC

stimulation. This contrasts with the rapid but transient induction by TNFα, where TTP mRNA peaked after 2 h and returned to baseline within 4 h from stimulation. At the protein level, both TTP and TNFα sustained in A549 cells a prolonged expression of TTP up to 24 h post-stimulation. Concordance of the action on TTP expression by TNFα and GC may appear counterintuitive, yet it can be reconciled viewing TTP as a molecule involved in homeostatic limitation of an acute inflammation, a process which 'begins with the end in mind' (Anderson 2010), with induction of TTP being advantageous as a negative feedback mechanism in both settings. From a mechanistic standpoint, GC-induced TTP expression was found to be transcription—and GR binding—dependent, as it was abolished by cell pretreatment with the transcriptional inhibitor actinomycin D and by GR antagonist RU486, respectively; paired with experiments showing no change in TTP mRNA stability, run-on experiments confirmed that TTP induction by GC was driven in A549 cells by increased transcription, though a *bona fide* GRE was not identifiable from the published hTTP sequence. Chromatin immunoprecipitation (ChIP) experiments in GC-treated cells showed enrichment of GR recruitment in two regions of the TTP gene: a 5′ flanking portion of the promoter containing binding sites for STAT, Smad, and NF-κB—all transcription factors known to engage protein–protein interaction with GR—and a portion of the 3′ flanking region which contains a half-GRE sequence. Silencing of TTP expression in A549 cells significantly impaired inhibition of its major target, TNF-α by GC, demonstrating a functional role of GC-induced TTP. This process was dependent on the TNF-α 3′UTR, as GC treatment selectively decreased, in transfected A549 cells, the expression of a luciferase reporter carrying the TNF-α 3′UTR. These results indicate for the first time that GC inhibition of TNF-α is mediated by 3′UTR-mediated post-transcriptional mechanisms critically dependent by induction of TTP. However, additional tissue- and/or stimulus-specific factors regulating GC influence on TTP expression are also suggested by a study reporting that LPS-induced TTP expression is instead inhibited by GC in mouse macrophages (Jalonen et al. 2005).

The work by Ishmael et al. (2008) extended GC sensitivity of TTP expression also to human primary bronchial epithelial cells and examined the role of TTP in GC anti-inflammatory action on a genome-wide scale. Mouse embryonic fibroblasts (MEFs) cell lines developed from TTP KO mice, in comparison to MEFs of wild-type (WT) littermates were treated the topical GC budesonide or diluent control for 3 h and then treated in the absence or presence of 10 ng/ml TNF-α for 1 h. The number of genes regulated in WT cells was largely reduced in $TTP^{-/-}$ cells, with the greatest loss of response (97 %) accounted for genes that in WT were down-regulated by GCs: only 5 of the 145 GC-responsive genes in WT were still comparably repressed in $TTP^{-/-}$ cells (Fig. 13.2a). Interestingly, also GC-induced genes were diminished in $TTP^{-/-}$ cells by 89 % compared to WT cells, suggesting a vast network of indirect effects. TNF-α-dependent responses were also globally affected in the absence of GC treatment, pointing again to the role of TTP in limiting the amplitude of acute inflammation either in response to triggering stimuli or as part of the endogenous anti-inflammatory mechanisms. Of particular relevance, the chemokines CCL2, CCL7, CXCL1, CXCL5 and CXCL7 were among the genes

whose inhibitory response to GC was significantly lost in $TTP^{-/-}$ MEF. Such targeting reinforces the importance of TTP-mediated function in the anti-inflammatory mechanism of GC action: chemokines are in fact key determinants of recruitment and activation of inflammatory cells within the inflamed mucosa and as such, they are main targets of GC therapy in chronic inflammatory diseases such as rhinosinusitis, asthma and COPD (Stellato 2007; Stellato and Schleimer 2000). Genome ontology analysis confirmed that regulated genes were functionally enriched in immunity and signal transduction, processes that are chiefly targeted by GCs for their anti-inflammatory action (Fig. 13.2b). Involvement of TTP also on genes important for GC control of metabolism and cell development indicate that TTP is likely to mediate other homeostatic and nonimmune functions driven by GCs. Loss of GC response was validated in the same study for selected targets in WT MEFS, as well as in primary human epithelial cells (unpublished data, C. Stellato), following transient TTP silencing, validating the general hypothesis beyond the $TTP^{-/-}$ mouse model.

Probing the mechanism of TTP function in GC response revealed a high level of complexity involving direct, ARE-mediated effects as well as indirect effects. For a group of 11 transcripts selected among those that lost their GC sensitivity in $TTP^{-/-}$ cells, those bearing TTP- compatible sequences such as AUUUA pentamers or UUAUUUAUU nonamers (Stoecklin et al. 2008)—CCL2, CCL7, CXCL1, CXCL5 as well as IL-6 and MMP9—showed association with TTP, documented through selective immunoprecipitation of ribonucleoprotein complexes (RNP-IP) using anti-TTP ab and subsequent real-time PCR; the transcripts showing no association with TTP by RNP-IP, despite profound changes in GC sensitivity in $TTP^{-/-}$ cells—IL1RL1, CXCL7, CCL5, Serpin3n, Egr1—presented instead only one or no AREs in the 3′-UTRs, with the exception of EGR-1 mRNA, which bears two UAUUUAU heptamers in an A/U-rich setting (Fig. 13.2c). However, among the transcripts found to be associated to TTP, GC-dependent changes in mRNA decay rate in $TTP^{-/-}$ cells—assessed by actinomycin D treatment—were heterogeneous, challenging the assumption that TTP would mediate GC action exclusively by accelerating ARE-mediated mRNA decay (Fig. 13.2d). For CCL7 and CCL2 mRNA, the acceleration of mRNA decay induced by GC in WT MEFs was no longer present in $TTP^{-/-}$ cells, in line with the more straightforward hypothesis. On the contrary, IL-6 mRNA decay was not modified by either GC treatment or by lack of TTP, despite significant mRNA enrichment in the RNP-IP assay and the presence of a 3′-UTR extremely rich in AREs. CXCL5 mRNA became unstable in $TTP^{-/-}$ cells compared to WT cells already in the absence of GC, indicating that TTP supported in this case a relative stabilization of CXCL5 basal mRNA turnover rate and that the inhibitory effect of GC seen in $TTP^{-/-}$ cells was likely a consequence to the loss of this function, rather than the GC action in itself. Further remarking the complexity of TTP biology—and possibly also the limitation of the Actinomycin D-based experimental system used in the study—these data implicate that besides ARE-mediated mRNA decay, TTP could form RNPs through protein interaction with other RBPs (Anderson 2008) and/or by establishing cooperative or antagonistic function with miRNAs (Jing et al. 2005; van Kouwenhove et al. 2011)

Fig. 13.2 GC-mediated gene regulation is significantly altered in $TTP^{-/-}$ murine fibroblasts. (**a**) Differential regulation of gene expression in WT and $TTP^{-/-}$ MEFs. *Left*, Heat maps of the array data sets from WT or $TTP^{-/-}$ cells and *right*, changes in GC response for selected genes (expressed as Z ratio) in WT vs $TTP^{-/-}$ cells upon exposure to budesonide (Bud) versus diluent-treated cells. (**b**) Genome ontology analysis by Gene Set Matrix Analysis (GSMA) showing loss of GC response for genes involved in asthma pathogenesis in $TTP^{-/-}$ MEFs. The Gene Set Matrix Analysis (GSMA) algorithm allows the identification of coordinated changes in gene expression. Experimental conditions are examined against "gene lists" pooled according to a biologically relevant grouping system instead of against each gene present on the array. (**c**) Association of TTP with GC-regulated mRNA targets identified by immunoprecipitation of endogenous mRNA-TTP complexes. *Upper right*, Representative real-time PCR amplification plot of fluorescence intensity over background (ΔRn) against PCR cycle (C_T) showing enrichment of CXCL1, but not of 18S RNA transcripts, in lysates of GC-treated MEFs subjected to IP with an anti-TTP Ab (filled symbols) vs the IP with the isotype-matched Ab (open symbols). *Bargraph*, enrichment of immunoprecipitated transcripts by the anti-TTP relative to IP with isotype Ab control (fold values indicated). Of the 11 transcripts tested, 6 (*grey bars*) contain multiple AREs. (**d**) Differential effect of TTP loss in GC-induced CCL7 and IL-6 mRNA decay. Results are shown as the percent of mRNA at time 0 in actinomycin-treated cells. The *bar graph insets* indicate the half-life ($t_{(1/2)}$, in h) in each condition. Modified from Ishmael et al. (2008), reprinted with permission

(see following dedicated paragraph). Prototypic of this context-driven, rather than strictly ARE-dependent, function is how TTP promote stabilization of the ARE-rich INOS mRNA by interacting with the ARE-bound KH-type splicing regulatory protein (KSRP). Under proinflammatory stimulation, TTP association with KSRP would remove this mRNA decay-inducing factor and the associated exosome and allow binding for the RBP HuR, resulting in INOS mRNA stabilization (Fechir et al. 2005). Many of the transcripts found to be up-regulated upon GCs treatment in TTP$^{-/-}$ cells could be similarly susceptible of TTP regulation through critical changes within the regulatory RNP complexes. In addition, GC could modulate TTP function also through changes in TTP phosphorylation, thus affecting functional aspects (change its localization, function or affinity for its binding site) rather than solely its synthesis.

TTP-mediated effect of GC can indirectly affect a larger gene pool when targeting a regulatory protein: an example from the study by Ishmael is the TTP-dependence of GC inhibition of the transcription factor Egr-1, which would also affect downstream expression of Egr-1-dependent proinflammatory genes (Cho et al. 2006; Ingram et al. 2006) underscoring how regulation of mRNA turnover can indirectly affect transcriptional control of multiple genes.

The effect and mechanisms of GC action on TTP-mediated translational control of gene expression, whose importance is documented for TNFα, remains to be explored as for that exerted on other RBPs known to regulate translation—such as TIA, TIAR and others (Gaestel 2013).

Glucocorticoid-Receptor (GR)-Mediated Chemokine Decay: A Novel Mechanism of GC-Mediated PTR The glucocorticoid receptor (GR) is a steroid hormone receptor belonging to the superfamily of nuclear receptors (NR), which exert gene regulatory functions as transcription factors (Gronemeyer et al. 2004). Transcription factors, such as the GR, and RBPs share important functional characteristics, such as sequence-specific interaction with nucleic acids, activation-dependent nucleocytoplasmic shuttling, and the ability to affect multiple targets through binding to conserved nucleic acid sequences. Taking this similarity even further, some protein factors can bind to both DNA and RNA—as in the case of the zinc-finger protein NF-90 that regulates IL-2 transcription through binding to the NFAT site (Shi et al. 2007) as well as mRNA turnover and translation of a vast group of transcripts through AU-rich-specific interactions (Kim et al. 2007). Other two zinc-finger proteins, Yin-Yang (YY)1 and TTP, as well as nucleolin participate in both nuclear and cytoplasmic regulatory events by ribonucleoprotein- and protein–protein interactions either with DNA and transcription factors or with RNA, miRNA and RBPs in the cytoplasm (Chen et al. 2000; Singh et al. 2004; Mongelard and Bouvet 2007; Liang et al. 2009; Stoecklin et al. 2008).

Whether the GR may have a similar ability was hypothesized based on the growing data on PTR contribution to GC action (Stellato 2004). The specific role of GR in mRNA stability was tested for the first time in a model of rat aortic smooth muscle cells (SMC) (Dhawan et al. 2007), based on earlier observation by the same group of the ability of the synthetic GC Dexamethasone (Dex) to accelerate mRNA decay for CCL2/MCP-1 mRNA (Poon et al. 1999). Experiments using RNA electromobility

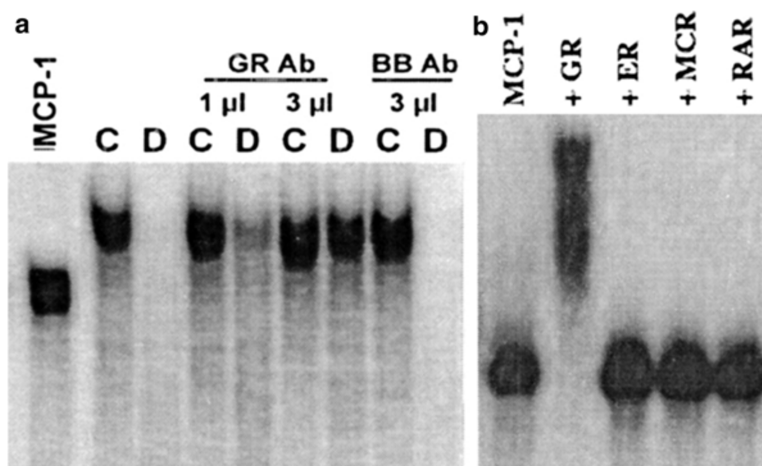

Fig. 13.3 The Glucocorticoid Receptor (GR) associates with MCP-1/CCL2 mRNA and promotes its degradation. Rat aortic endothelial cells were treated with diluent control (C)/dex (D) for 3 h and cytoplasmic lysates was incubated with full length, in vitro transcribed CCL2/MCP-1 mRNA for 30 min ± GR Ab for EMSA essay. (**a**) Incubation with control cell lysates (C) shows a probe shift indicating formation of an RNP complex; CCL2 probe is degraded in dex-treated cells (D), an effect prevented specifically and concentration-dependently by lysate preincubation with an anti-GR ab. (**b**) Direct binding of GR with in vitro transcribed CCL2/MCP-1 mRNA was demonstrated by complex formation following probe incubation with recombinant GR; the interaction was specific with respect to other nuclear receptors. Modified from Dhawan et al. (2007), reprinted with permission

shift assay (R-EMSA) showed an upward mobility shift of the complex formed by cytoplasmic extracts of Dex-treated SMC after a short incubation (5 min) with radiolabeled full length CCL2/MCP1 mRNA compared to control cytoplasmic extracts, suggesting that additional proteins bind to the transcript under Dex control. Using a cell-free mRNA stability assay, in which degradation of a labeled synthetic CCL2/MCP-1 transcript was detected over time following incubation with cytoplasmic SMC lysates, the authors showed that incubation for 30 min of the Dex-treated SMC extracts with the CCL2/MCP-1 probe led to its complete degradation, while the extracts from control SMC did not change its detection; strikingly, the probe degradation was specifically inhibited by preincubation of Dex-treated SMC extracts with multiple anti-GR antibodies (Fig. 13.3a). Direct GR-mRNA interaction was confirmed by R-EMSA using recombinant human GR and an in vitro transcribed, human CCL2/MCP-1 mRNA probe (Fig. 13.3b). This interaction was hormone receptor—and mRNA—specific, as neither incubation with other NRs or usage of three alternative transcripts reproduced the results obtained with GR-CCL2 coupling. It is also noteworthy that the shift observed with extracts from Dex-treated cells was higher than that obtained by rhGR alone, suggesting that in intact cells, GR is likely part of a multimeric RNP complex; this was further suggested by the ability of recombinant GR to inhibit transcript degradation by Dex-treated extracts

and by enrichment of MCP-1 mRNA detected by immunoprecipitation of RNP complexes (RNP-IP) with an anti-GR specific antibody. These data indicated a previously unknown cytoplasmic function for the GR as well as the existence of novel regulatory sequences, in addition to the conserved ARE, as determinants of CCL2 mRNA stability.

The study by Ishmael et al. (2010) aimed to study whether this novel mechanism was in place in a human cellular model of GC response and tested the hypothesis that in functioning with an RBP-like function, GR-mediated control of mRNA decay would have a more global role in GC-mediated PTR action. First, R-EMSA experiments conducted with cytoplasmic extracts from human primary airway epithelial cells and the epithelial cell line BEAS-2B indicated that biotin-labeled CCL2 and CCL7 mRNA, but not CCL5, associated with GR upon cell treatment with the synthetic GC budesonide and were degraded by GC treatment, an outcome prevented by GR neutralization by a specific anti-GR ab in epithelial cell lysates. The association was validated by RNP-IP (Helou et al. 2008) in which cytoplasmic lysates from control and GC-treated epithelial cells were incubated with anti-GR antibody—or with an IgG isotype control to measure background binding—to immunoprecipitate *endogenous* GR-mRNA complexes, and identified bound CCL2 and CCL7 by PCR in GC-treated samples, similarly to what the same group reported for chemokine transcripts interaction with TTP (Ishmael et al. 2008). Biotin pulldown experiments probed for the binding site of GR using truncated transcripts containing the 5′UTR, coding region (CR) and 3′UTR sections (Fig. 13.4a). In the study of GC-induced decay of rat CCL2 mRNA, transcript stability was not dependent from the ARE or other sequences in the 3′UTR but relied instead on the presence of three stem loops in the transcript 5′-UTR (Poon et al. 1999). As in the rat CCL2 system, association of GR was observed predominantly with the 5′UTR construct, with minimal signal with the 3′UTR construct. The binding site was further mapped through cross-linking of purified recombinant GR with truncated 5′UTR regions (indicating actual nucleic acid-protein interaction) and ultimately localized to a 15-mer fragment localized between nucleotide 44 and 60 (Fig. 13.4b).

As for transcription factors, RBPs interact with nucleic acids over specific cis-regulatory sequences to implement common regulatory function over multiple genes. Therefore, validation of GR function as an RBP would entail the ability to associate with multiple transcripts through a specific sequence and regulate their decay/translation rate. This paradigm was tested in the human airway epithelial cell line BEAS-2B employing RIP-ChIP analysis in which transcripts bound to the endogenous GR were identified by gene arrays after specific IP (Tenenbaum et al. 2002; Keene et al. 2006). Statistically significant mRNA enrichment in GR-IP arrays versus IgG control-IP arrays was found for approximately 500 transcripts, supporting the hypothesis of a broader RBP function for the GR besides interaction with CCL2 and CCL7 mRNAs. The common sequence motif shared by the transcripts associated with the GR, searched using a specific algorithm for RNA sequences (Lopez de Silanes et al. 2004; Kim et al. 2007; Kuwano et al. 2010), consisted in a Guanidine/Cytidine (GC)-rich sequence (78 % of the signature motif), spanning 27–31 nucleotides and forming two contiguous loops on a short stem (Fig. 13.4c), that was proven

Fig. 13.4 The GR binds to CCL2 mRNA and to multiple targets in human epithelial cells via a G/C-rich Motif present in the 5′ UTR. (**a**) *Top*: schematic of CCL2 mRNA, with nucleotide numbers corresponding to untranslated regions (UTR) and coding region (CR). *Below,* detection of GR by Western blot by biotin pulldown assay from unstimulated BEAS-2B cell lysate, using biotinylated CCL2 full-length RNA (FL), the 5′ and 3′ UTRs, or the CR. The RBP HuR is detected as positive control for association with CCL2 3′UTR. (**b**). UV cross-linking of purified GR protein to 15-nt fragments of the CCL2 5′UTR, followed by detection by Western blot of the covalently-linked GR via mobility shift of the RNA-bound GR protein (GR-RNA). (**c**) *Left*, graphic logo representing the probability matrix of the GR motif, showing the relative nucleotide frequency for each position within the motif sequence. *Right, s*econdary structure of the GR motif comprising the nucleotides with highest frequency for each position within the motif shown in A. (**d**) Biotin pulldown assay showing association of recombinant GR from unstimulated BEAS-2B cell lysates with the GR motif shown in (**c**) (*sequence shown below*), compared to GR association with the full-length 5′UTR of CCL2, CCL7 and CCL5 mRNA (the latter as negative control). Modified from Ishmael et al. (2010); Panganiban et al. (2014), reprinted with permission

to bind to recombinant GR via biotin pulldown assay. The entire UniGene database was queried based on this motif and 7889 transcripts displayed a compatible sequence in their 5′UTR (45 % of the entire database) (Fig. 13.4d).

Collectively, the data from these three studies convincingly indicate that the GR can mediate GC action beyond its core function in transcriptional control, and that it may participate in cytoplasmic regulatory mechanisms of gene expression via association with mRNA and protein complexes mediating PTR. Unquestionably, much remains to be tested to understand the molecular basis of this cytoplasmic, PTR-related function of GR and ultimately, its relevance in the global anti-inflammatory action of GC. On the structural level, it will be important to identify the GR domain interfacing with the GC-rich motif and the stoichiometry of this binding, as the GR lacks a conserved RNA-binding domain (RBD) that for other transcription factors/RBPs, like NF-90 and nucleolin, mediates association with mRNA (Ginisty et al.

1999; Shi et al. 2005). Furthermore, it will be necessary to define potential interactions with other RBPs and miRNAs that could mediate GR participation to RNPs, in addition to that mediated by RNA-bound mechanisms, or change its affinity for RNA binding. From a functional standpoint, it remains to be tested if acceleration of mRNA decay occurs for most of the putative GR motif-bearing targets identified, whether it is the only RNA-associated action of GR and whether other GR motifs, or RNP interactions, can mediate alternative cytoplasmic functions of the GR. It can be postulated that GR association with mRNAs can be modified by stimulus-induced changes in the composition of the RNP complex, and/or by action upon the association of GR-bound RNA with cytoplasmic foci of regulated mRNA decay and translation, such as P bodies and stress granules, where these functions are critically regulated during inflammation (Anderson and Kedersha 2009).

2.3 GC Effects on miRNA Expression, Metabolism and Function

MicroRNAs (miRNAs), central components of post-transcriptional gene regulation, are now recognized to play important roles in mediating and modulating the effects of GCs. MiRNAs are effectors of essential biological processes such as cell activation, development, differentiation, and apoptosis. As such, miRNAs may participate in the effects of GCs to regulate inflammatory pathways which could convey, enhance, or even inhibit GC effects.

MiRNAs are small, non-coding RNAs (20–25 nucleotides long) which are transcribed in a Pol II-dependent manner from primary transcripts or introns of genes. A number of transcription factors have been identified as stimulators of miRNA synthesis, including inflammatory transcription factors such as AP-1 and NF-kB as well as the glucocorticoid receptor. Primary miRNA transcripts (pri-miRNAs) are trimmed by a complex of proteins that includes the nuclear RNAse III Drosha and the RBP DGCR8/Pasha into a pre-miRNA hairpin which includes the miRNA sequence and a complementary guide stand. RNA-binding proteins such as KSRP, TTP, and Lin28 can bind to pri-miRNAs to enhance or retard processing to pre-miRNAs. Pre-miRNA hairpins are transported into the cytoplasm where they are cleaved by the RNAse III enzyme DICER into the mature miRNA and incorporated into the multiprotein RNA-induced silencing complex (RISC). The RISC binds to transcripts, usually in the 3′UTR, and promotes degradation and/or translation inhibition. GCs have been shown to regulate the expression and processing of miRNAs at multiple points in the miRNA biosynthetic pathway, as discussed below.

Effect of GC on miRNA Expression Our understanding of how miRNAs are regulated and how they exert their effects on cellular pathways has been greatly accelerated by high throughput technologies such as deep sequencing, microarrays, and qPCR analyses. Though these means, it has been revealed that GCs alter miRNA expression in a wide variety of cells and tissues (Table 13.2). The targeted miRNAs may convey anti-inflammatory, apoptotic, and metabolic effects of glucocorticoids and serve as potential therapeutic targets in multiple disease processes.The pro-apoptotic effect of GCs on lymphocytes constitutes a large component of its

Table 13.2 Studies on glucocorticoids effects on miRNAs expression and functional outcomes in inflammation and metabolism

miRNA(s)	Effect	Mechanism	Cell type/disease process
Inflammation			
Let7a, miR-146a, miR-155	Anti-inflammatory effect	miRNA down-regulation	Leukocytes, lupus mouse model (Chafin et al. 2013)
miR-155	Anti-inflammatory effect	miRNA down-regulation via NFkB inhibition	Mouse macrophages (Zheng et al. 2012)
miRs-1246, -663a, -1275, -92b-5p, -24-30, -34a-c-5p, Let7c	Altered expression after inhaled GCs	Up- and down-regulation of miRNAs	Airway epithelium of asthmatics (Solberg et al. 2012)
miR-98	T-cell inhibition	Up-regulation of miR-98	Human T-cells (Davis et al. 2013)
miR-29b-c	Apoptosis	Down-regulation of miRNAs	Human plasmacytoid dendritic cells, lupus (Hong et al. 2013)
miRs-223, -16,-15b	Apoptosis	Up-regulation of miRNA	Human lymphocytes, acute lymphocytic leukemia (Rainer et al. 2009)
miR-17-92 cluster	Apoptosis, development	miRNA down-regulation by inhibition of Dicer, Drosha, DGCR8	Human lymphocytes (Smith et al. 2010), acute lymphocytic leukemia (Harada et al. 2012) chondrocytes (Xing et al. 2014)
Metabolic			
miR-29a	Differentiation	Transcriptional down-regulation of miRNA	Human osteoclasts (Ko et al. 2013)
miR-27b	Fat accumulation	GR-dependent transcriptional up-regulation of miRNA	Adipocytes (Kong et al. 2014)
miR-1	Skeletal muscle atrophy	GR-dependent transcriptional up-regulation of miRNA	Mouse muscle cells (Kukreti et al. 2013)
miRs-1, -147,-322,-351, -503*, -708	Skeletal muscle atrophy	Up- and down-regulation of miRNAs	Mouse muscle cells (Shen et al. 2013)

anti-inflammatory actions and is a central target in lymphoid malignancies. Deep sequencing and miRNA microarray analysis revealed that the miR-17-92 cluster of miRNAs was repressed in apoptotic rat lymphocytes following treatment with dexamethasone (Smith et al. 2010). These miRNAs were confirmed to be repressed by GCs in human lymphocytes by qPCR analysis, and shown to be necessary for cell survival in acute lymphocytic leukemia cell lines (Harada et al. 2012).

One of the first studies examining the effect of GC therapy on an inflammatory disease-driven miRNA signature was implemented in eosinophilic esophagitis (EoE), a chronic eosinophilic inflammatory disease of the esophagus with a strong Th2-driven pathophysiology (Lu et al. 2012). The miRNA expression profile obtained from esophageal biopsies in patients with EoE showed specific changes from those obtained from healthy control subjects and patients with chronic, noneosinophilic esophagitis; importantly, 27 of the 32 differentially expressed miRNAs were normalized in patients with EoE that were responding to glucocorticoid therapy (Fig. 13.5a). This study also identified miR-675 as a marker of response to GC therapy, as this miRNA resulted to be upregulated only in GC-responsive patients compared with the

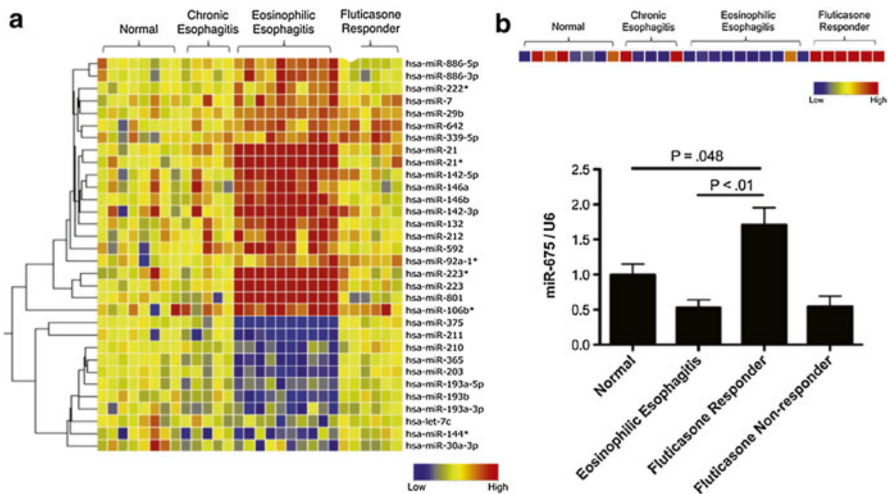

Fig. 13.5 GC treatment in patients with eosinophilic esophagitis (EoE) reveals a GC-sensitive miRNA signature and identifies miR-675 as biomarker of response to GC treatment. (**a**) Heat map showing the expression level of 32 differentially expressed miRNAs in patients with EoE compared with patients with chronic esophagitis and patients with EoE in remission after GC therapy. Genes are upregulated (*red*) and downregulated (*blue*) in comparison with healthy control subjects. (**b**) *Above,* heat map showing changes in expression of miR-675, the only miRNA differentially regulated in patients with EoE who responded to glucocorticoid therapy, compared with that seen in healthy control subjects, patients with chronic esophagitis, and patients with EoE. *Below,* normalized expression of miR-675 detected by RT-PCR in healthy control subjects, patients with EoE, fluticasone propionate-responsive patients, and fluticasone propionate-nonresponsive patients. Modified from Lu et al. (2012), reprinted with permission

other subsets of patients and controls, but its levels were not increased in biopsies of patients with poor clinical and histological response to GC (Fig. 13.5b).

In bronchial asthma, where GC treatment is the therapeutic mainstay, expression of nine miRNAs was found to be altered in the airway of patients after GC treatment (Solberg et al. 2012). The miRNAs upregulated by GCs include Let7, which has been shown to have anti-inflammatory properties in the airway (Kumar et al. 2011). In this report, miRNA profile was also investigated in IL-13-treated airway epithelial cells from healthy donors. Of 262 regulated miRNAs, members of the miR-34/449 family (miR-34c-5p, miR-34c-5p, miR-449a, and miR-449b-5p) were significantly suppressed. Interestingly, in vitro treatment with GC of IL-13-stimulated cells did not correct this suppression. Considering that IL-13-driven changes are pathogenic in asthma, it can be hypothesized that the lack of GC sensitivity of miR-34/449 family in this setting may play a role in some of the severe asthma phenotypes showing poor response to GC therapy. Lack of regulation by GC treatment, however, may also have a different implication: for example, in keeping with the concept that activation of innate immune pathways are spared by GC regulation (Schleimer 2004), TLR4-mediated changes in miRNA profile in the lungs of mice following exposure to LPS was not affected by GC treatment (Moschos et al. 2007). The extent of GC action in regulating miRNA expression and mechanisms of action is now being realized, and is clear that GCs may regulate both the transcription and the subsequent processing of miRNAs.

GC-Mediated Transcriptional Regulation of miRNA Expression Numerous miRNAs have been shown to contain the GRE in their promoters, and transcriptional regulation of these miRNAs can occur via GRE-mediated action (Ko et al. 2013; Kong et al. 2014; Kukreti et al. 2013; Guan et al. 2011). Some miRNAs, such as Let7a, have anti-inflammatory functions and their induction may convey some of the anti-inflammatory GC effects. However, it is also becoming evident that many of the miRNAs transcriptionally regulated by GC may play roles in GR-dependent metabolic effects, which constitute the major limiting factor of GC therapeutic applications. GC-induction of miR-27b in adipocytes and miR-1 in skeletal muscle are mediated by GR-GRE interactions, and results in fat accumulation and skeletal muscle atrophy in the respective tissue. As weight gain and muscle atrophy are main adverse effects of GCs which limit their systemic use in patients, these pathways have important therapeutic implications.

The transcription factors NFκB and AP-1 are required for transcription of many primary miRNA transcripts involved in inflammation (Cheng et al. 2013; Fujita et al. 2008; Gatto et al. 2008; Indraccolo et al. 2014; Kumar et al. 2014; Madhyastha et al. 2014; Taganov et al. 2006; Yin et al. 2008; Zheng et al. 2014; Zhou et al. 2014) that can be stimulated by infection, toll-like receptor activation, or cytokine signaling (Seddiki et al. 2014). Inflammatory gene transrepression through ligand-activated GR interaction with NFκB or AP-1 is a major mechanism of GR action, documented also for GC inhibition of important proinflammatory miRNAs such as miR-155 (Zheng et al. 2012). MiR-155 has been identified to play a central role in immunity and inflammation, and controls differentiation of T-cells into Th1, Th2, and Th17 cells (Seddiki et al. 2014).

GC-Mediated Regulation in the Processing of miRNAs Microarray analysis and deep sequencing performed in dexamethasone-treated primary rat thymocytes, a model of

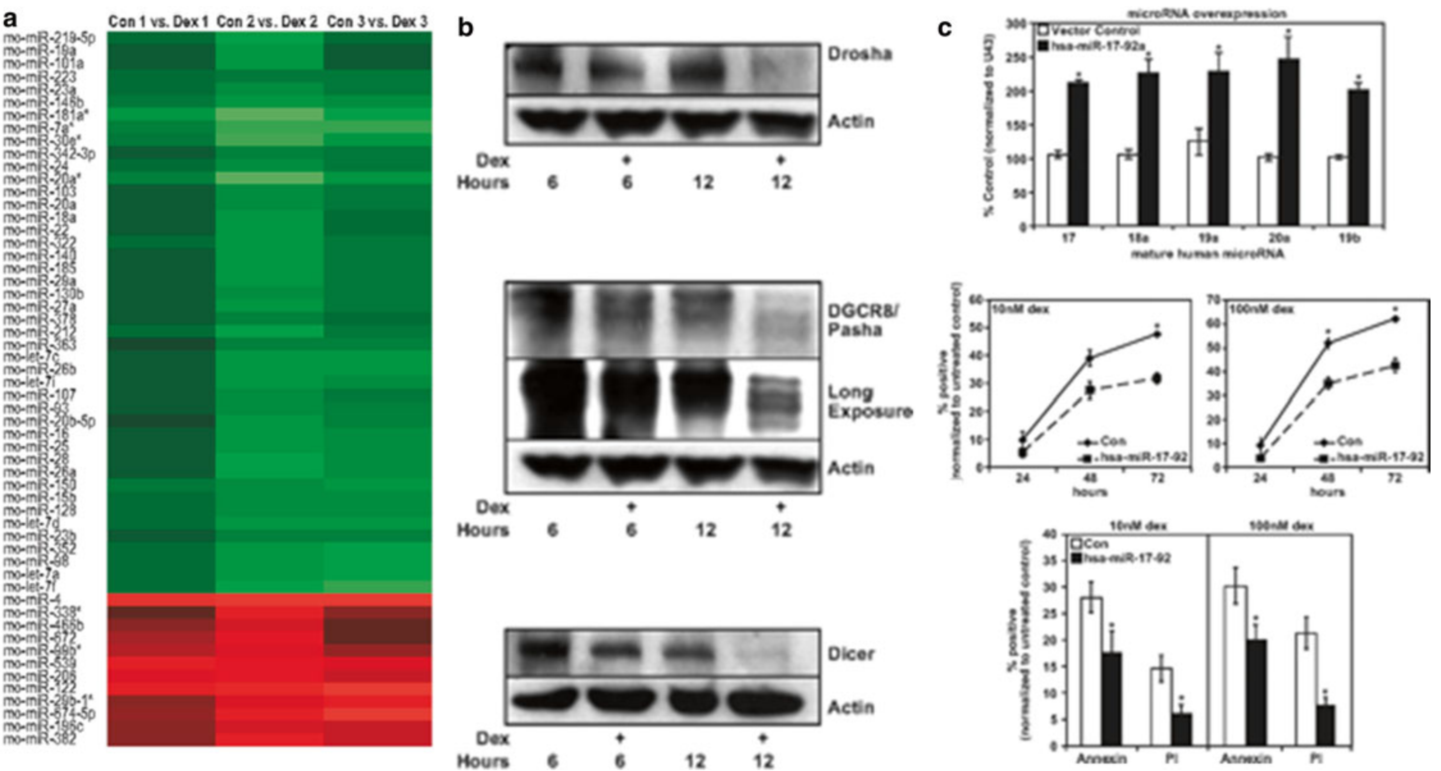

Fig. 13.6 Repression of miRNA processing enzymes and of miR 17-92 contributes to GC-induced lymphocyte apoptosis. (**a**) Microarray analysis of microRNA expression in dexamethasone- (Dex)-treated primary rat thymocytes. Heat map representation of microRNAs differentially expressed in control (Con) and Dex-treated primary thymocytes, induced (*red*) or downregulated (*green*) versus control. (**b**) MicroRNA processing enzymes are repressed during GC-induced

Fig. 13.6 (continued) apoptosis of primary thymocytes. Western blot of microRNA processing enzymes Drosha, DGCR8/Pasha, and Dicer in thymocytes unstimulated or treated with dexamethasone for 6 or 12 h. (**c**) Stable overexpression in Jurkat cells of individual mature hsa-miR-17-92 polycistron members (*upper panel*), found in the study to be downregulated by Dex treatment, blunts glucocorticoid- induced apoptosis. *Middle panel*, flow cytometric analysis of propidium iodide (PI) uptake in empty vector or hsa-miR-17-92 stable overexpressors showing blunted apoptosis in response to 10 and 100 nM dexamethasone treatment. *Lower panel*, similar results were obtained in primary thymocytes from transgenic hsa-miR-17-92 mice. Shown is apoptosis monitored by flow cytometric analysis of annexin-FITC/propidium iodide staining. Modified from Smith et al. (2010), reprinted with permission

GC-induced lymphocyte apoptosis, indicated that 79 % of affected miRNAs were *down-regulated* (Fig. 13.6a), which raised the question of whether GCs could regulate proteins involved in miRNA processing (Smith et al. 2010). The repression of these miRNAs resulted to be primarily linked to a GC-dependent inhibition of components of the miRNA microprocessor complex, the RNAse Drosha and the RBP DGCR8/Pasha, and of DICER expression (Fig. 13.6b). Reduction of these proteins results in the accumulation of unprocessed pri-miRNAs in the nucleus and a decrease in mature cytoplasmic miRNAs. Restoring exogenously the expression of the targeted regulatory proteins relieved the block in miRNA processing. Furthermore, overexpression of the miRNAs repressed by GC action, which include the miR-17-92 cluster, blunted the ability of GCs to induce apoptosis in mouse thymocytes and in Jurkat T-cells (Fig. 13.6c). These results suggest that GC-mediated down-regulation of miRNA-processing complexes could be a mechanism to induce lymphocyte apoptosis, by reducing the levels of mature miRNAs required to maintain cell survival.

Other RBPs also modulate the processing of miRNAs and may be regulated by GCs (Trabucchi et al. 2009; Bhattacharyya et al. 2013). Pri-miRNA sequences may contain AU-rich elements which can be bound by RBPs in the nucleus (Trabucchi et al. 2009). For instance, the primary transcript of miR-155 contains eight class I AREs. The RBPs TTP and KSRP recognize AREs and can regulate the processing of pri-miRNAs (Bhattacharyya et al. 2013; Trabucchi et al. 2009). KSRP enhances the conversion of pri-miR-155 to pre-miR-155 in airway cells, while TTP inhibits this process (Bhattacharyya et al. 2013; Trabucchi et al. 2009). In the airway, miR-155 promotes the production of IL-8 (Bhattacharyya et al. 2013), an important cytokine in airway inflammation which is repressed by GCs (Nakagome et al. 2012). As TTP is a regulatory target of GCs, it could play a major role in the effects of GCs on miRNA processing.

In some cases, TTP may promote miRNA biogenesis to enhance mature miRNA formation. TTP has been shown to inhibit expression of the RBP Lin28 by destabilizing its transcript (Kim et al. 2012; Lee et al. 2013). As Lin28 inhibits Let7 biogenesis in multiple cancer cells by interfering with the pri- to pre-miRNA processing, TTP is capable of removing this repression (Kim et al. 2012; Lee et al. 2013). In the case of Let7a, GCs exert its effect both transcriptionally and post-transcriptionally. Let7a contains a GRE in the promoter which results in GR-dependent transcriptional up-regulation (Guan et al. 2011), and the TTP-dependent effects on Lin28 promotes maturation of pri-Let7a to pre-Let7a (Kim et al. 2012).

The role of miRNAs in the actions of GCs is beginning to be unraveled. As miRNAs play crucial roles in biological processes, the actions of these molecules could convey many of the effects of GCs. Deregulation of these pathways could produce a lack of clinical GC-response, and GC-resistance is emerging as a major issue in diseases such as asthma, autoimmunity, and cancer. As such, these pathways may represent mechanisms of disease pathogenesis and serve as potential target for generation of novel therapeutics.

3 Clinical Implications of PTR in GC Function

The immunomodulatory and anti-inflammatory actions of glucocorticoid hormones are used by the potent synthetic GCs used in therapy for a large spectrum of malignancies, autoimmune conditions, allergic and inflammatory diseases. However, their chronic systemic use is compounded by severe systemic side effects and by the occurrence, in a subset of patients, of incomplete clinical efficacy even with high-dose regimens, leading to a GC-dependent status, or to full GC resistance (Adcock and Barnes 2008). The search for anti-inflammatory therapies carrying the efficacy and potency of this class of compounds, but devoid of their significant side effects on metabolism and growth is an important but so far elusive therapeutic goal. Increasing understanding of how PTR pathways coordinately regulate gene expression and growing appreciation of their involvement in the pathophysiology of GC-targeted diseases is indicating that therapeutic interventions exploiting PTR mechanisms and effector molecules can carry significant therapeutic effects. Understanding the PTR mechanisms and mediators conveying GC action, therefore, may provide valuable insight to this endeavor. In support of this approach, overexpression of TTP in a rat model of experimental LPS-mediated periodontitis prevented the local bone loss induced by LPS, significantly decreased inflammatory cell and osteoclast recruitment to the inflammatory site, and decreased levels of the TTP targets TNFα, IL-6 and COX2 compared to the control animals (Patil et al. 2008). This study highlights the potential value of TTP-mediated action—and at large, of targeting PTR mechanisms—as a self-standing anti-inflammatory strategy. Closer to clinical setting, development of small molecule inhibitors of signaling pathways regulating RBP function, as in the case of p38 and MK2/3 (Gaestel 2013), or generation of miRNA-based technology to attack specific pathologic processes (Krutzfeldt et al. 2005; Lu and Rothenberg 2013; Mattes et al. 2008) are examples of how GC-driven/GC-sensitive PTR mechanisms, once identified, can independently provide additional or alternative strategies for controlling overexpressed or aberrant immune responses. In addition to studies on the mechanisms of PTR-mediated GC action, it is pressing to better identify altered PTR regulation in clinical settings where GCs are administered and uncover mutations that could affect regulatory binding sequences, or levels and functionality of RBPs and miRNAs, which could impair PTR-mediated GC response. To this end, multiple single nucleotide polymorphisms have been identified, for example, in the human *TTP* gene (Blackshear et al. 2003b), some of which could be associated with rheumatoid arthritis and autoimmune diseases (Carrick et al. 2006).

4 Summary and Conclusions

The studies discussed in this review indicate that the impact of post-transcriptional regulation in the mechanism of anti-inflammatory action of GCs is larger than previously appreciated (summarized in Fig. 13.7), and yet ill-defined. They provide

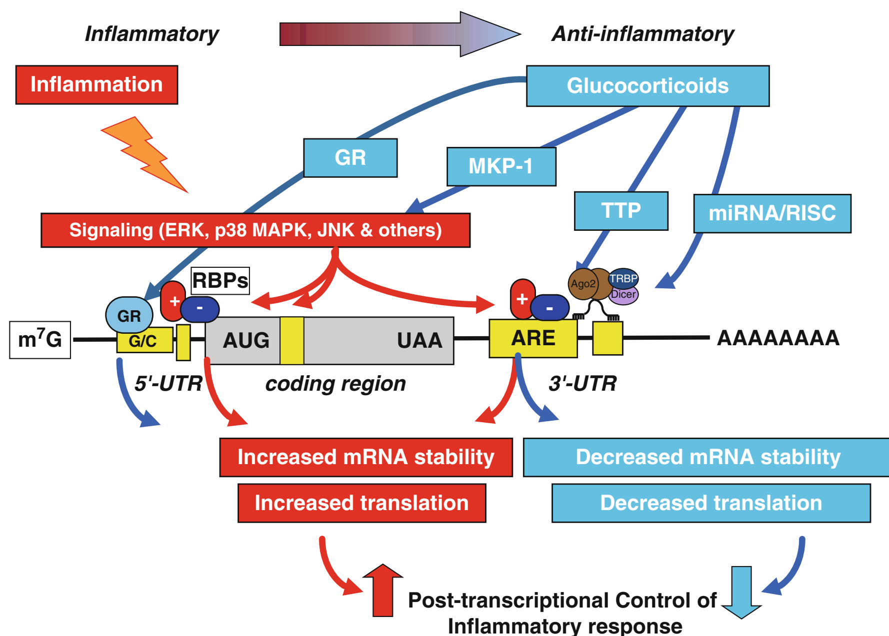

Fig. 13.7 Mechanisms of Post-transcriptional control of inflammatory response by GC, as discussed in this chapter. Heterogeneous sequence elements for regulation (indicated as *yellow boxes*, ARE and G/C rich elements discussed in the text) present in the coding or—more frequently—in untranslated regions (UTR) mediate binding of cognate regulatory factors such as RBPs and miRNA. Inflammatory stimuli trigger kinase-dependent binding, association and activation of factors inducing mRNA stabilization and/or increased protein translation (in *red boxes* and *arrows*). In balance with this action, binding of multimeric regulatory complexes functionally supporting mRNA decay and suppressing, or lowering, translation rates are able to limit the amplitude and the duration of the inflammatory response—and regulate as well appropriate baseline levels in homeostatic conditions. These mechanisms are regulated by GC at multiple levels (in *blue boxes* and *arrows*), supporting—either directly or indirectly—processes inducing a net decrease in mRNA stability and/or translation rates as means of downregulating overexpressed inflammatory genes. GC-induced MAPK phosphatase (MKP-1) indirectly lead to a PTR-mediated anti-inflammatory outcome by dephosphorylation of MAPK members, which chiefly support increased mRNA stability and translation of inflammatory genes. A more direct influence by GC on PTR is through the induction of TTP, an ARE-binding protein conveying accelerated mRNA decay and translational suppression of immune-related genes. Influence of GC on PTR relies heavily on changes in miRNA expression, through transcriptional regulation and regulation of the miRNA processing enzymes. Lastly, upon GC stimulation the GR has been described in cellular models to associate with mRNAs in the cytoplasm and mediate acceleration of mRNA decay through specific interactions with a G/C-rich sequence present in the 5′UTR

compelling arguments towards testing a paradigm of anti-inflammatory approach based on targeting of post-transcriptional mechanisms. Regulatory factors of PTR dynamically assembling in specific, context-driven RNP complexes are increasingly scrutinized as therapeutically targetable. Considering that these molecular species coordinately regulate large subsets of functionally related transcripts

through common regulatory elements, a better understanding of GC ability of intervening at the structural and functional interface between RBPs, miRNAs and their targeted transcripts may uncover anti-inflammatory and immunoregulatory outcomes potentially not subjected to the limitations of GC systemic side effects and incomplete therapeutic response due to GC resistance.

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Post-transcriptional Regulation by Brain-Derived Neurotrophic Factor in the Nervous System

14

Alexandra M. Amen, Daniel L. Pham, and Mollie K. Meffert

1 Introduction

Brain-derived neurotrophic factor (BDNF) is a secreted protein that belongs to the neurotrophin family of growth factors and influences the growth and survival of neurons in both the central and peripheral nervous systems. BDNF signaling is crucial in early development where it plays significant roles in neuronal progenitor proliferation and differentiation, cell survival, the outgrowth of neuronal processes, and both the formation and pruning of synaptic connections. Mice homozygous for loss of BDNF (BDNF knockout) often die post-natally, and surviving mice show significant nervous system dysfunction (Jones et al. 1994; Ernfors et al. 1995; Patterson et al. 1996). Additionally, throughout adulthood, secretion of BDNF in response to neuronal activity functions to promote synaptic strengthening and enhance learning and memory, as well as to provide general trophic support for neuronal health and survival. These critical roles mean that abnormal BDNF signaling is closely associated with a range of mental health disorders, including autism spectrum disorder, depression, schizophrenia, bipolar disorder, and anxiety disorders, as well as neurodegenerative diseases, including Alzheimer's Disease and Parkinson's Disease (McAllister et al. 1999; Muglia et al. 2002; Tyler et al. 2002; Zuccato and Cattaneo 2009; Santos et al. 2010; Nagahara and Tuszynski 2011; Andero and Ressler 2012).

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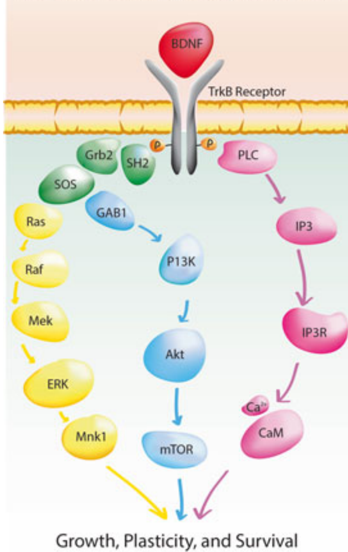
BDNF expression is highest in the brain and is present in all brain regions, with the highest BDNF mRNA levels generally observed in the hippocampus and cerebral cortex (Wetmore et al. 1990; Conner et al. 1997). Despite its name, BDNF is also expressed and functional in a range of tissues outside the brain, including the peripheral nervous system, retina, skeletal muscle, kidneys, bone, and ovaries (Johnson et al. 1986; Jones et al. 1994; Ernfors et al. 1990; Griesbeck et al. 1995; Huber et al. 1996). In each of these tissues BDNF has been reported to play trophic roles in cell growth, survival, or repair (Fig. 14.1), but this chapter will focus particularly on BDNF in the central nervous system (CNS) where knowledge of downstream signaling events is the most comprehensive. BDNF has been well-characterized as a critical regulator of learning and memory in the hippocampus, and these studies have played a prominent role in elucidating the post-transcriptional mechanisms of BDNF action reviewed in this chapter.

The subcellular localization and release sites of BDNF in CNS neurons has been investigated in multiple reports, but studies have often been hampered by the low levels of endogenous BDNF message and protein in vivo and in vitro, making detection difficult. *Bdnf* mRNA has been reported in both the neuronal soma and dendrites depending on the length of its 3′ UTR. However, recent research using deep-sequencing and high-resolution in-situ hybridization approaches indicates that all forms of *bdnf* mRNA appear to be restricted to the soma and proximal dendrites (Will et al. 2013). Similarly, BDNF has been observed in the synaptic compartment and was thought to be released from both axonal and dendritic sites, while recent research indicates that endogenous BDNF is primarily stored in pre-synaptic vesicles (Andreska et al. 2014). Conflicting data regarding the subcellular location and release sites of BDNF protein and message may in part arise from discrepancies between the behavior of endogenous BDNF and the exogenous BDNF expression constructs sometimes employed. Additionally, post-endoplasmic reticulum trafficking of secretory cargo, presumably including BDNF, has been shown to be subject to both developmental and acute spatial regulation in dendrites, where synaptic activity acts to restrict long-range transport of cargo (Hanus et al. 2014).

BDNF interacts with the high affinity tropomyosin-related kinase B (TrkB) tyrosine kinase receptors to activate multiple intracellular signaling cascades, including the MAPK, mTOR, and PLCγ pathways. Signaling downstream of TrkB receptors mediates the trophic effects of BDNF on neurons and synaptic connections, and blocking the BDNF/TrkB interaction results in significant impairments in synaptic plasticity and learning (Takei et al. 2001; Tanaka et al. 2008; Lai et al. 2012). Enduring effects of BDNF on growth and plasticity require changes in gene expression, and BDNF has been shown to regulate both transcription and translation. The precise complement of proteins present in neurons and at synapses fundamentally shapes neuronal growth and activity, ultimately determining synaptic response. Through the activation of select transcription factors such as serum response factor (SRF), nuclear factor kappa B (NF-κB), and CREB (cAMP response element binding protein, Finkbeiner et al. 1997; Kalita et al. 2006; Riccio et al. 2006; Kajiya et al. 2009), BDNF is able to specifically upregulate trophic targets at the level of transcription. However, recent high-throughput approaches have shown that the



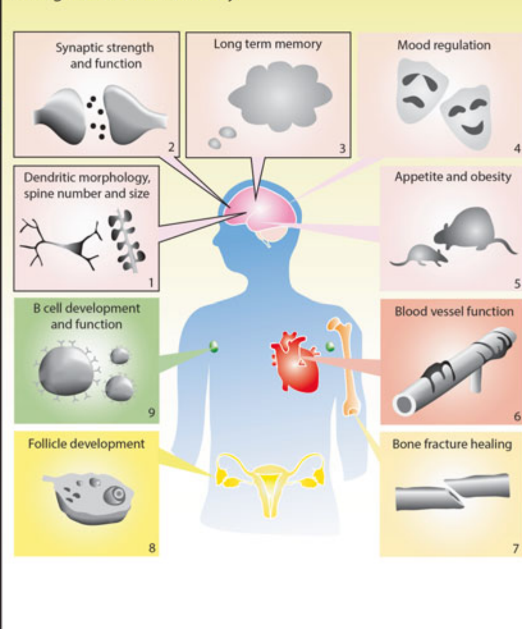


Fig. 14.1 BDNF signaling impacts biological processes throughout the human body. BDNF binds to the TrkB receptor, resulting in receptor dimerization and autophosphorylation. Binding to the TrkB receptor activates multiple downstream pathways, including PLCγ, PI3K/AKt, and Mapk/Erk. Through PLCγ, BDNF increases intracellular calcium concentration and activates calcium-binding molecules such as CaMKIIα, leading to CREB activation and regulation of gene transcription. BDNF-dependent Erk activation also phosphorylates CREB. Both Erk and mTOR regulate translation by binding to the cellular translational machinery. Through these pathways, BDNF regulates cell function, growth, and survival. In the brain, BDNF increases dendritic arborization (Horch and Katz 2002; Jaworski et al. 2005; Cheung et al. 2007; Takemoto-Kimura et al. 2007; Tanaka et al. 2008; Je et al. 2009; Lazo et al. 2013) and spine growth and number (Tyler and Pozzo-Miller 2001, 2003; Alonso et al. 2004) in neurons (*1*). Synaptic strengthening through long-term potentiation has been shown to require BDNF (*2*) (Korte et al. 1995; Kang et al. 1997; Rex et al. 2007). BDNF is also required for the formation and maintenance of memory (*3*) (Tyler et al. 2002; Heldt et al. 2007; Pardon 2010; Bekinschtein et al. 2007). Mechanisms underlying the effects of BDNF in the brain have been an active area of study, and critical roles for post-transcriptional regulation by BDNF have been demonstrated in the biological processes illustrated in boxes 1–3. BDNF-dependent modulation of dopaminergic and serotinergic networks regulates mood, anxiety, and depression (*4*) [see reviews by Martinowich and Lu (2007) and Nikulina et al. (2014)]. BDNF regulates energy intake and body weight through its signaling in numerous hypothalamic nuclei [see review by Rios (2013)], and modulates stress response through the hypothalamic-pituitary axis (Givalois et al. 2001; Givalois et al. 2004; Jeanneteau et al. 2012 (*5*). Outside of the central nervous system, BDNF expression is low under basal conditions in mature bone cells and is upregulated in osteoblasts responding to bone fracture (*6*) (Kilian et al. 2014). BDNF also is known to promote the development and survival of B cells (*7*) in the immune system (Schuhmann et al. 2005; Fauchais et al. 2008). BDNF expression in the ovary has been shown to regulate follicular development and oocyte maturation (*8*) (Paredes et al. 2004; Spears et al. 2003; Jensen and Johnson 2001). In the vasculature, BDNF plays a role in the maintenance of blood vasculature and pericyte function (*9*) (Donovan et al. 2000; Anastasia et al. 2014). As novel functions of BDNF are discovered in multiple biological systems, our knowledge of the post-transcriptional mechanisms contributing to BDNF action is expected to continue to expand

cellular transcriptomes appear to correlate only moderately with corresponding proteomes (Tian et al. 2004; Schwanhausser et al. 2011; Ideker et al. 2013), revealing that the levels of many proteins are predominantly controlled post-transcriptionally. These studies collectively highlight the importance of understanding mechanisms of post-transcriptional regulation of gene expression. Given the dramatic role that BDNF plays in neuronal health, survival, and plasticity, understanding how BDNF signaling can generate trophic programs of gene expression at a post-transcriptional level has the potential to reveal critical regulatory points in both normal and abnormal brain function, and will be the central theme of this chapter.

2 BDNF Regulation of Global Protein Synthesis

Activity-dependent alterations in neuronal protein synthesis have long been known to be required for the endurance of synaptic changes and memory consolidation (Pfeiffer and Huber 2006; Gal-Ben-Ari et al. 2012). For example, secretion of BDNF during neuronal activity regulates translation, which allows for modulation of the neuronal proteome and accounts in part for the important role of BDNF in synaptic plasticity and cognition (Klann and Denver 2004; Soule et al. 2006; Santos et al. 2010). BDNF signaling through its TrkB receptor increases global cellular protein synthesis as measured by metabolic label incorporation in rodent cortical or hippocampal cultured neurons (Takei et al. 2001, 2009; Huang et al. 2012). The role of BDNF in upregulating total translation has also been demonstrated in studies using brain slices from transgenic mice engineered to either lack or overexpress the gene encoding BDNF. Brain slices from BDNF-deficient mice exhibit a significant decrease in basal translation, whereas basal translation is increased in brain slices from transgenic mice that overexpress BDNF (Takei et al. 2009).

The three major steps of eukaryotic translation involve translation initiation, elongation, and termination, and BDNF has been shown to enhance total cellular protein synthesis by influencing molecular components of both the initiation and elongation phases. In eukaryotic cells, translation is believed to be primarily regulated at the initiation phase, which begins when eukaryotic initiation factor 4E (eIF4E) binds to the 7-methyl-guanosine residue that constitutes the 5′ cap of the mRNA. eIF4E binding to the mRNA allows recruitment of additional initiation factors, ultimately leading to ribosomal association with the eIF-mRNA complex. In the mammalian brain, eIF4E itself is regulated by eIF4E-binding protein 2 (4EBP2), which binds to and negatively regulates eIF4E (Banko et al. 2005). BDNF signaling through TrkB results in phosphorylation of 4EBP by the PI3K/mTOR pathway, which causes 4EBP to dissociate from eIF4E, leading to increased eIF4E activity and generally enhancing mRNA translation. Additionally, eIF4E itself may be phosphorylated by BDNF-mediated activation of the MAPK pathway, which appears to lead to stabilization of eIF4E and, again, enhanced total translation (Gingras et al. 1999, 2004; Takei et al. 2001; Kanhema et al. 2006). BDNF also decreases phosphorylation of eukaryotic initiation factor 2a (eIF2a) and increases phosphorylation of its guanine exchange factor eIF2B, both of which have been shown to enhance binding of tRNA to the ribosome (Takei et al. 2001).

BDNF acts on the elongation step of translation primarily by regulating the activity of eukaryotic elongation factor 2 (eEF2). eEF2 is a GTP-binding protein that is responsible for translocation of the tRNA from the A to P site in the ribosome, and is therefore a crucial regulator of the rate of elongation. Following treatment with BDNF, the translation elongation rate in neurons has been reported to be twice that of baseline, as measured by ribosomal transit time (Inamura et al. 2005). Phosphorylation of eEF2 is inactivating and thought to arrest translation, although translation of some mRNAs may be selectively upregulated when eEF2 is phosphorylated (Scheetz et al. 2000). BDNF appears to increase total levels of eEF2 protein (Takei et al. 2009), while decreasing phospho-eEF2 in an mTOR-dependent manner (Inamura et al. 2005; Takei et al. 2009). In contrast, infusion of BDNF into the dentate gyrus of adult rats lead to a rapid, transient increase in eEF2 phosphorylation in whole cell homogenates but not synaptodendrosomes (a subcellular fraction containing a portion of the dendritic shaft and associated spines; Kanhema et al. 2006). Such results suggest that while BDNF-mediated control of translation initiation may be widespread, control via translation elongation could be more compartment or transcript-specific. Collectively, these alterations in both initiation and elongation contribute to the modest BNDF-mediated upregulation of protein synthesis observed by global measures such as radiolabel incorporation.

3 BDNF Regulates the Local Protein Composition in Dendrites and Synapses

Neurons are structurally complex cells with elaborate dendritic and axonal processes that extend significant distances from the cell soma. Both passive and active mechanisms can mediate transport of proteins and mRNAs to distal processes. However, following exposure to an appropriate incoming stimulus, the levels of selective proteins are also specifically and rapidly upregulated at post-synaptic sites, as a component of synaptic plasticity. The finding of both mRNAs and polyribosomes in dendrites and dendritic spines, the major site of excitatory synaptic input, provided the initial suggestion of a role for local translation in dendrites and synapses (Steward and Levy 1982; Steward and Schuman 2001; Ostroff et al. 2002). Activity-dependent local translation is now well-established and is known to be required for multiple forms of plasticity (Sutton and Schuman 2006; Wang et al. 2009; Holt and Schuman 2013). A number of studies have been aimed at compiling a profile of the synaptic transcriptome, with over 2500 transcripts reported to be localized to the synapse via deep sequencing (Cajigas et al. 2012). As might be expected, a number of mRNAs that code for proteins involved in such processes as neuronal signaling, synaptic plasticity, and synapse organization are preferentially enriched in synaptic and dendritic compartments (Poon et al. 2006; Zhang et al. 2006; Cajigas et al. 2012). The mRNAs for some important plasticity-related proteins, such as CamKIIα, were reported to be present, although not enriched, in the dendritic cytoplasm, suggesting the possibility that preferential local translation of these mRNAs could contribute to the abundance of the proteins at synapses (Poon et al. 2006; Zhong et al. 2006).

Mechanisms through which BDNF increases global protein synthesis, as previously discussed, can also function to increase protein synthesis in dendrites and synapses (Takei et al. 2004; Kanhema et al. 2006). For example, BDNF leads to phosphorylation of 4EPB1 in both the dendrite and synapse in an mTOR–dependent manner (Takei et al. 2004; Kanhema et al. 2006). Additionally, in isolated synapses, BDNF causes the inhibitory protein CYFIP1 to dissociate from eIF4E, leading to enhanced translation (Napoli et al. 2008). Similarly, phosphorylation of p70S6K via BDNF activation of mTOR results in phosphorylation of ribosomal S6 protein, increasing translation activation at the synapse (Takei et al. 2004). In other cell types, it is thought that the association of ribosomal subunits, translation factors, and mRNAs with F-actin functions to hold the molecular players in an ordered state and facilitate translation. BDNF has been reported to cause the translocation of eIF4E into spines where it associates with cytoskeleton-bound RNA granules, an effect that is blocked by inhibiting F-actin polymerization (Smart et al. 2003).

Not surprisingly, neuronal studies on local protein regulation by BDNF have often focused on the upregulation by BDNF of proteins that are known to play important roles in synaptic function and plasticity. Increased translation of multiple specific mRNAs such as glutamate receptor subunits, Homer2, and CamKIIα is observed at synapses in response to BDNF (Schratt et al. 2004; Caldeira et al. 2007; Guire et al. 2008; Fortin et al. 2012). In a study performed by Aakalu et al. (2001), the authors constructed a GFP reporter containing the 5′ and 3′ UTRs of CamKIIα, to reveal that stimulation with BDNF led to translational "hot spots" in dendrites that may correlate with active synapses. BDNF has also been shown to increase the levels of certain mRNAs in dendrites (Ying et al. 2002; Rao et al. 2006; Messaoudi et al. 2007), supporting the function of BDNF as a regulator of local gene expression. It is worth noting that BDNF has also been implicated in modulating synaptic protein content through post-translational modifications. For example, BDNF-induced phosphorylation of both NDMA and AMPA receptor subunits, leads to increased surface trafficking and activity of these receptors (Suen et al. 1997; Lin et al. 1998; Caldeira et al. 2007). Evidence also suggests that BDNF may post-translationally regulate synaptic protein stability through the ubiquitin proteasome pathway (Jia et al. 2008), and that neuronal activity leads to increased proteasomes in the synapse, which can function to remodel synaptic protein content (Bingol and Schuman 2006).

4 Effect of BDNF on Translational Specificity

4.1 BDNF Increases Translation of a Select Group of mRNAs

Long-term changes in synaptic and circuit function are known to require changes in gene expression. Enduring strengthening or weakening of synapses each necessitate organized alterations in programs of gene expression in order to ensure that the appropriate proteins are coordinately regulated to enhance or reduce synaptic

responses. BDNF effectively promotes synapse growth and plasticity by coordinating a response that specifically and selectively increases plasticity-related proteins. Despite the ability of BDNF to affect general translational machinery and total cellular translation, the modest increase in global translation that is observed in response to BDNF has actually been attributed to a robust effect on a relatively small number of specific transcripts. Using a candidate-based approach, several initial studies demonstrated that BDNF can increase the translation of specific plasticity-related proteins—such as CamKIIα, Arc, and glutamate receptor subunits—in both dissociated neuronal culture and synaptic preparations (Aakalu et al. 2001; Yin et al. 2002; Kelleher et al. 2004; Takei et al. 2004; Kanhema et al. 2006; Jourdi et al. 2009). In contrast, BDNF has been observed to decrease translation of certain mRNAs, such as potassium channels and co-transporters (Rivera et al. 2002; Raab-Graham et al. 2006). Radiolabelled synapses stimulated with BDNF and subjected to 2D electrophoresis also revealed a robust increase in a very specific set of proteins, while some proteins were decreased and the majority were unchanged (Yin et al. 2002).

The development of high-throughput techniques enabled an appreciation of the truly impressive extent to which BDNF mediates target specificity. In a comprehensive study from the Greenberg lab (Schratt et al. 2004), the authors used polysome profiling to show that only roughly 4 % of transcripts present in neurons were increased by BDNF stimulation, and that these increases were sensitive to mTOR signaling. Additionally, multidimensional protein identification technology (MudPIT) demonstrated that a brief, 30 min BDNF stimulation of isolated synapses was sufficient to specifically increase proteins involved in synaptic vesicle formation and trafficking, translation, and synaptic components (Liao et al. 2007). In line with previous studies, the translation from highly selective pools of mRNAs were specifically increased as well as decreased by BDNF, while most mRNAs remained unchanged (Liao et al. 2007). Similarly, gel based proteome profiling revealed that a long-term (12 h) BDNF stimulation could increase levels of a number of proteins involved in cellular metabolism and proliferation, while most protein levels were decreased or unchanged (Manadas et al. 2009). Interestingly, the authors reported that BDNF was capable of affecting both the mRNA and protein levels of its targets, but that these changes were not always correlated, again implicating post-transcriptional roles for BDNF in regulating *de novo* protein synthesis or protein stability (Manadas et al. 2009).

4.2 Mechanisms Enabling BDNF-Mediated Translational Specificity

The ability of BDNF to rapidly and significantly increase the translation of a restricted subset of mRNAs is in accordance with its role as a positive modulator of growth and excitatory synaptic function, but how is BDNF able to confer such a remarkable degree of specificity to translational processes?

4.2.1 RNA-Binding Proteins

One well-established method of achieving translational specificity is through RNA-binding proteins, which play important roles in post-transcriptional regulation of mRNAs by enhancing or repressing the translation of certain mRNAs. Several different RNA-binding proteins have been observed both to be present at synapses, and to regulate the translation of major targets of BDNF (Huang et al. 2002; Napoli et al. 2008; Wu et al. 1998; Bhakar et al. 2012). For example, the RNA-binding protein CPEB (cytoplasmic polyadenylation element-binding protein) regulates mRNA translation by mediating cytoplasmic polyadenylation. CPEB binds to a cytoplasmic polyadenylation element (CPE) present in the 3′ UTR of multiple BDNF target mRNAs, such as CamKIIα (Huang et al. 2002; Wu et al. 1998). CPEB also associates with an inhibitory eIF4E binding protein, Maskin, which when present in the CPEB/Maskin/eIF4E complex specifically prevents translation of bound CPE-containing mRNAs. Activity-dependent phosphorylation of CPEB causes increased cytoplasmic polyadenylation, which disrupts the Maskin/eIF4E interaction, leading to increased translation of CPE-containing mRNAs (Richter and Sonenberg 2005). In this regulatory pathway, CPEB phosphorylation in response to synaptic activity has been shown to result in an accumulation of CamKIIα at the synapse (Huang et al. 2002). Although CPEB regulation has not been directly studied in relation to BDNF-mediated post-transcriptional gene target specificity, the fact that BDNF initiates several major phosphorylation cascades and increases translation of many CPE-containing mRNAs suggests that BDNF could potentially regulate cytoplasmic polyadenylation in the dendrite and synapse.

As discussed, BDNF regulates translation both locally and globally through mTOR-dependent phosphorylation of 4EBP2, leading to enhanced eIF4E-dependent initiation. Though this pathway might initially be expected to non-selectively increase translation of mRNAs, recent research has shown that 4EBP2 knockout does not significantly alter the neuronal polysome profile, but instead leads to increased translation of specific mRNAs such as neuroligins (Gkogkas et al. 2013). In addition, several studies have demonstrated a specific requirement for 4EBP2-dependent modulation of eIF4E activity in LTP and memory. These results indicate an important role for such molecules in translation regulation and might suggest selectivity for proteins involved in synaptic plasticity (Banko et al. 2005; Costa-Mattioli et al. 2009). Ultimately these investigations imply that 4EBP2-mediated regulation of cap-dependent initiation could contribute to translational specificity for a subset of transcripts in response to BDNF rather than mediating a general increase in translation.

BDNF signaling has also been linked to another RNA-binding protein, the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein that is enriched at CNS synapses and could participate in conferring specificity to BDNF-mediated translation. Loss of FMRP results in human Fragile X Syndrome, which is an autism spectrum disorder and the most common monogenic inheritable form of intellectual disability. FMRP is generally thought to function as a repressor of protein synthesis, but participates in the regulation of translation through multiple incompletely characterized mechanisms (Bhakar et al. 2012). Loss of FMRP leads to exaggerated

translation of a variety of synaptic proteins including many major targets of BDNF, such as CamKIIα, Arc, Map1B, and APP (Napoli et al. 2008; Bhakar et al. 2012). FMRP function and expression has been directly linked to BDNF signaling in multiple studies (Castren et al. 2002; Napoli et al. 2008; Wang et al. 2012b; De Rubeis et al. 2013). BDNF decreased FMR1 mRNA levels in cultured hippocampal neurons, and both FMR1 mRNA and FMRP protein levels were found to be decreased relative to controls in transgenic mice overexpressing the BDNF receptor TrKB (Castren et al. 2002). The phosphorylation status of FMRP may play a role in its inhibition of translation, and dephosphorylation of FMRP has been suggested to enhance translation of FMRP target mRNAs (Ceman et al. 2003; Narayanan et al. 2007). One study directly examining the relationship of BDNF to FMRP phosphorylation showed that BDNF caused calcineurin-mediated dephosphorylation of FMRP, leading to increased translation of FMRP targeted mRNAs (Wang et al. 2012b). Similarly, PP2A, another phosphatase that is reported to dephosphorylate FMRP (Niere et al. 2012), is itself increased by BDNF (Takei et al. 2009). Research aimed at understanding the mechanism by which FMRP regulates translation has suggested that FMRP may work in part by binding to the protein CYPIP1 to harbor FMRP mRNA targets in the FMRP-CYPIP1 complex. CYPIP1 itself also binds to and sequesters eIF4E, preventing translation of these targets (Napoli et al. 2008; De Rubeis et al. 2013). BDNF was shown to decrease co-immunoprecipitation of CYPIP1 and eIF4E, suggesting that eIF4E is released and able to initiate translation of FMRP target mRNAs (Napoli et al. 2008; De Rubeis et al. 2013).

4.2.2 microRNAs

Although the discovery of microRNA-mediated regulation of protein synthesis is relatively recent (Lee et al. 1993), in the past 20 years many ground-breaking studies have demonstrated the importance of microRNA (miRNA) function in all cell types. miRNAs are short (19–25 nucleotide), non-coding RNA molecules that are endogenously expressed. They are able to recognize and bind partially-complementary sites in their target mRNAs, causing translational suppression and/or degradation of these targets. miRNA function in the brain has been shown to be crucial for normal neuronal development and plasticity in both mammalian and non-mammalian species (Krichevsky et al. 2003; Sempere et al. 2004; Giraldez et al. 2005; Smalheiser and Lugli 2009) Given that a single miRNA can regulate expression of an entire suite of proteins, miRNAs are attractive candidates for coordinating complex responses such as neuronal development, plasticity and synaptic remodeling at a post-transcriptional level. There is strong evidence for the presence of both miRNAs and miRNA processing machinery at the synapse (Schratt et al. 2006; Ashraf et al. 2006; Wayman et al. 2008; Huang et al. 2012; Lugli et al. 2012), further supporting a potential role for miRNAs in synaptic plasticity. The miRNA processing enzyme Dicer has been found to be present and active in synaptosomes, and there is additional evidence for enrichment of both precursor (pre-miRNAs) and mature miRNAs at synapses (Lugli et al. 2005, 2008). Interestingly, enrichment of mature miRNAs has been correlated with the presence of the corresponding pre-miRNA, again suggesting that miRNA processing might happen locally at the

synapse (Lugli et al. 2008). Though it is still not known how miRNAs could be directed or trafficked to a particular synapse, it has been suggested that synaptic pre-miRNAs might be enriched for pre-miRNA structures with shorter stems and more bulges, offering a potential structural basis for their transport as opposed to immediate processing (Smalheiser 2008).

Initial studies addressing the potential for miRNA regulation by BNDF examined the brain-specific miRNA miR-134, which is associated with learning and memory in rodents and negatively regulates spine size by inhibiting Limk1 mRNA in the synapse (Schratt et al. 2004; Gao et al. 2010). Limk1 undergoes increased translation in response to BDNF, and the authors found that BDNF prevents miR-134-mediated repression of Limk1 through an unknown mechanism (Schratt et al. 2004, 2006). BDNF was shown to decrease levels of miR-9, relieving suppression of MAP1b and leading to increased axon branching (Dajas-Bailador et al. 2012). In addition to negative regulation of miRNAs, BDNF was also reported to upregulate the transcription of several miRNAs. For example, miR-134 transcription was enhanced by BDNF, promoting inhibition of the RNA-binding protein Pumilio2 (Pum2). This caused an increase in dendritic arborization of young cultured neurons (Fiore et al. 2009; Vessey et al. 2010). Additionally, BDNF is thought to increase transcription of miR-132, a regulator of dendrite outgrowth and morphology, in a MAPK-dependent manner (Vo et al. 2005; Wayman et al. 2008; Kawashima et al. 2010). Interestingly, inhibition of miR-132 was shown to reduce BDNF-mediated upregulation of several synaptic glutamate receptor proteins such as NR2A, NR2B, and GluA1 (Kawashima et al. 2010).

In addition to these reports of BDNF effects on individual miRNAs, recent work from our laboratory has shown that BDNF can act upstream in processing steps to coordinately regulate the biogenesis of multiple miRNAs. Precursor miRNAs undergo several well-established processing steps to produce the functional mature miRNA. The canonical miRNA biogenesis pathway starts with transcription of a larger precursor miRNA (called a pri-miRNA) in the nucleus, where it is processed into a pre-miRNA stem-loop structure (of ~70 nucleotides) by the enzyme Drosha. This pre-miRNA hairpin is then exported into the cytoplasm, where it undergoes a second processing step by the RNaseIII enzyme Dicer and its binding partner Tar-RNA Binding Protein (TRBP) into a mature miRNA duplex. Regulation of either of these processing steps has the potential to dramatically affect the levels of functional mature miRNAs, and the translation of their corresponding mRNA targets. We demonstrated that BDNF achieves gene target specificity in protein synthesis by regulating the miRNA biogenesis pathway (Huang et al. 2012). BDNF stimulation of cultured hippocampal neurons elevates both Dicer and TRBP proteins in a rapid, transcription-independent manner, which enhances pre-miRNA processing and is associated with a concurrent increase in many mature miRNAs. This finding might initially appear somewhat counterintuitive, given that an increase in mature miRNAs could suggest a decrease rather than the modest increase in total translation produced by BDNF. However, we also observed that BDNF selectively decreases the processing of a specific class of miRNAs that are inhibited by the RNA-binding protein Lin28. The Let-7 family of miRNAs are a highly abundant group of these

Lin28-targeted miRNAs, which repress a range of pro-growth mRNAs that are crucial for synaptic function, such as CamKIIα and GluA1 (Huang et al. 2012). The Let-7 class of miRNAs has received widespread attention due to their role in development and cancer. Because they inhibit translation of a range of important pro-growth proteins, the Let-7 miRNAs are virtually absent from stem cells, and are substantially upregulated during developmental progression (Moss et al. 1997; Loohuis et al. 2012). Additionally, Let-7 miRNAs function as tumor suppressor genes and are downregulated in roughly 15 % of cancers (Wang et al. 2012a). The Let-7 miRNAs are one of the most highly expressed classes of miRNAs in mature cell types, and high-throughput data suggests that Let-7 miRNAs make up at least 50 % of miRNAs present in mature neurons (Juhila et al. 2011; Shinohara et al. 2011). By reducing Let-7 levels and relieving Let-7 miRNA-mediated repression, BDNF is able to produce enhanced translation of a restricted subset of targeted mRNAs which presumably underlies the modest global increase in translation caused by BDNF.

Regulation of Let-7 miRNA biogenesis by Lin28 has been well documented (Moss et al. 1997; Heo et al. 2008, 2009; Nam et al. 2011). Lin28a and its mammalian paralog Lin28b are RNA-binding proteins and pro-growth pluripotency factors that are highly expressed in stem cells and a range of cancer cells and are downregulated during development, mirroring the increase in Let-7 miRNAs (Moss et al. 1997; Loohuis et al. 2012). This opposing expression pattern occurs through negative feedback between Lin28 and Let-7 miRNAs. Lin28 proteins recognize and bind a 'GGAG' motif in the terminal loop of Let-7 miRNA precursors, which causes degradation or inhibits processing of the precursor Let-7 miRNAs into mature miRNAs (Heo et al. 2008, 2009; Nam et al. 2011). Thus, Lin28 proteins are able to prevent Let-7 miRNA maturation prior to Dicer/TRBP processing. Lin28 mRNAs are, in turn, subject to repression by Let-7 miRNAs which can create feed-forward derepression when Lin28 proteins are elevated. Though Lin28 is generally considered to be absent from differentiated cell types such as neurons, we found that Lin28a protein was substantially elevated by BDNF in a rapid, transcription-independent manner, and that this accounted for the specific decrease in mature Let-7 miRNA expression following BDNF stimulation (Huang et al. 2012). Collectively, BDNF positively regulates general miRNA biogenesis by upregulating Dicer and TRBP proteins, and specifically negatively regulates Let-7 miRNA biogenesis in particular by increasing Lin28a protein. In this way, BDNF increases miRNA-mediated repression of many mRNAs while selectively relieving translational repression of mRNAs that are targets of Let-7 miRNAs. Increased miRNA biogenesis by Dicer/TRBP induction could account for the transcripts that have been shown to undergo decreased translation in response to BDNF (Rivera et al. 2002; Raab-Graham et al. 2006), and indeed knockdown of Dicer protein in cultured neurons could prevent BDNF-mediated decrease in such targets (Huang et al. 2012). In contrast, BDNF leads to increased translation of specific pro-growth mRNAs that harbor Let-7 miRNA binding sites by increasing Lin28a protein levels to inhibit Let-7 miRNA processing. Knockdown of Lin28a was validated to prevent a BDNF-mediated increase in multiple important Let-7 containing targets, such as CamKIIα, that are known to

contribute to the synaptic and cognitive effects of BDNF (Huang et al. 2012). Further, synthetic insertion of a Let-7 miRNA binding site in an mRNA 3′UTR, was shown to be sufficient to create a BDNF-upregulated target gene.

5 Physiological Effects of Post-transcriptional Regulation by BDNF

5.1 Neuronal Morphology

Alterations in neuronal structure, such as dendritic outgrowth and spine maturation, are physiological components of enduring plasticity that have been shown to require BDNF-dependent changes in protein synthesis (Jaworski et al. 2005; Tanaka et al. 2008). In a translation-dependent manner, BDNF signals through TrkB receptors to enhance dendritic complexity and branching in both hippocampal and cortical neurons (Horch and Katz 2002; Jaworski et al. 2005; Cheung et al. 2007; Takemoto-Kimura et al. 2007; Tanaka et al. 2008; Je et al. 2009; Lazo et al. 2013). BDNF released from one neuron can enhance dendritic arborization of a closely neighboring neuron by binding to its TrkB receptors, and pyramidal neurons lacking TrkB receptors have significantly reduced pyramidal dendritic arbors (Horch and Katz 2002; Xu et al. 2000) This ability of BDNF to increase dendritic arborization is clearly linked to its regulation of translation. For example, Jaworski et al. (2005) demonstrated that enhanced dendritic complexity in response to BDNF expression was dependent on the mTOR signaling pathway, and could be blocked by inhibiting translation initiation. Studies from our laboratory have shown that the gene target specificity of BDNF-mediated translation is also crucial for its promotion of dendritic arborization. In particular, enhanced dendritic growth requires BDNF-induced reductions in Let-7 miRNA levels mediated by the RNA-binding protein Lin28a, presumably due to the many plasticity and growth-related mRNAs that are targeted by Let-7 miRNAs. In hippocampal neurons expressing a Lin28-resistant precursor of Let-7 miRNA, BDNF is no longer able to enhance dendritic arborization (Huang et al. 2012).

BDNF is also a well-characterized regulator of spine dynamics, and can induce changes in dendritic spine size and number. In cultured hippocampal neurons, BDNF has been shown to robustly increase spine density on the apical dendrite of pyramidal neurons in a MAPK/ERK-dependent manner (Tyler and Pozzo-Miller 2001, 2003; Alonso et al. 2004). Research has also suggested a differential effect of acute (one time) vs gradual (low-level repeated) BDNF stimulation on spine morphology. Specifically, acute BDNF stimulation of cultured hippocampal neurons produced transient TrkB activation and resulted in spine head enlargement, indicative of a more mature spine with a larger post-synaptic density. Alternatively, gradual BDNF stimulation led to long-term TrkB elevation and spine neck elongation, suggesting spine destabilization and remodeling. Both types of BDNF stimulation caused an increase in overall spine density (Ji et al. 2010).

Again, the effect of BDNF on spine plasticity is dependent on its regulation of protein-synthesis. In general, activity-induced upregulation of dedritic spines can be

blocked by treating cultured neurons with the drug anisomycin, an inhibitor of protein synthesis (Fifková et al. 1982; Srivastava et al. 2012). More directly, long-term increases in hippocampal spine density mediated by BDNF have been shown to require new translation utilizing a system that allows inducible cell-autonomous inactivation of protein synthesis (Je et al. 2009). BDNF signaling through TrkB was also demonstrated to be necessary and sufficient for LTP-mediated spine head enlargement, and this effect was entirely blocked by anisomycin (Tanaka et al. 2008). Collectively, this research indicates that post-transcriptional regulation of gene expression by BDNF can induce structural changes in both synaptic and dendritic morphology that participate in plastic responses.

5.2 LTP and Memory

Synaptic plasticity is widely considered the cellular correlate of learning and memory, and involves the selective strengthening and weakening of synapses in response to incoming stimuli. The strengthening of specific synapses occurs through a phenomenon known as long-term potentiation (LTP), and is the most widely studied and best understood form of synaptic plasticity. LTP involves sequential phases that include both early-LTP (E-LTP), which lasts 1–2 h and is transcription and translation independent, and late-LTP (L-LTP) which is of longer duration and is known to require novel transcription and translation (Krug et al. 1984; Stanton and Sarvey 1984; Costa-Mattioli et al. 2009). Interestingly, L-LTP can be blocked locally by restricted dendritic administration of protein synthesis inhibitors to prevent local translation (Bradshaw et al. 2003).

BDNF is stored near synaptic sites and is released during neuronal activity, including paradigms of LTP induction (Hartmann et al. 2001; Aicardi et al. 2004; Matsuda et al. 2009). LTP requires the expression and activity of BDNF (Korte et al. 1995; Kang et al. 1997; Rex et al. 2007). Exogenous administration of BDNF has been reported to induce LTP in multiple brain regions including the hippocampus (Kang and Schuman 1996; Tanaka et al. 2008), though the sufficiency of BDNF for this effect may depend upon experimental context. In line with the ability of BDNF to control translation both locally and cell-wide, BDNF has been shown to regulate LTP in a global but also synapse-specific manner (Kang and Schuman 1996; Tanaka et al. 2008). Generally it is thought that initial phosphorylation cascades initiated by BDNF-TrkB signaling lead to changes associated with E-LTP, and that subsequent BDNF-induced protein synthesis is required for the enduring late phase of LTP (Kang and Schuman 1996; Messaoudi et al. 2007; Tanaka et al. 2008).

Consistent with the endurance of LTP requiring new protein synthesis, the persistence of long-term memory (LTM) also relies upon a consolidation period that is protein-synthesis dependent (Bailey et al. 2004; Lamprecht and LeDoux 2004). Consolidation of LTM leads to memories that can persist for days, weeks, or years. The hippocampus is thought to be a major locus of memory consolidation, and in-vivo hippocampal injection of protein synthesis inhibitors prevents hippocampal-dependent memory formation (Tronel et al. 2005; Power et al. 2006).

The requirement for BDNF in tests of hippocampal-dependent learning and memory is also well established (Tyler et al. 2002; Heldt et al. 2007; Pardon 2010). While few studies have directly attempted to link BDNF function with novel translation in hippocampal-based learning, recent research has shown that LTM in the hippocampus requires multiple cycles of translation that occur following learning paradigms, and that BDNF is elevated concurrent with the initiation of these cycles (Bekinschtein et al. 2007). Additionally, inhibition of BDNF prevented LTM formation in the same timecourse as intrahippocampal anisomycin injection (Bekinschtein et al. 2007). These findings are consistent overall with a role for BDNF in translation-dependent changes of neuronal and synaptic function that are associated with learning and memory consolidation.

5.3 Post-transcriptional Regulation by BDNF in Autism Spectrum Disorder

Multiple cognitive disorders have been associated with dysregulated protein synthesis (for a review, see Buffington et al. 2014), and several have also been strongly linked to dysregulated BDNF signaling. In particular, there is abundant evidence for aberrant BDNF signaling and downstream protein synthesis in autism spectrum disorder (ASD) arising from diverse genetic causes. ASD has a global prevalence of about 1 % and is characterized primarily through impairments in social interaction and communication. ASD is largely a genetic disorder, however it can arise as a result of monogenic or polygenic causes, and frequently involves *de novo* mutations and gene copy number variants. Abnormalities in activity-dependent neuronal signaling and synapse development and function are thought to be shared features of ASD arising from known monogenic causes, as well as other syndromic disorders with features of autism (for a review, see Ebert and Greenberg 2013).

Fragile X Syndrome (FXS) is the most common monogenic cause of autism, and occurs as a result of mutation in the *FMR1* gene promoter leading to loss of fragile X mental retardation protein (FMRP), a negative regulator of protein synthesis. Using *FMR1* knockout mice as a model of the human syndrome, researchers have observed that exogenous BDNF application is able to rescue significant hippocampal LTP deficits in FXS, and that genetic reduction of BDNF alongside FMRP knockout further impairs cognitive deficits (Lauterborn et al. 2007; Uutela et al. 2012). As was previously discussed, FMRP regulates translation of a number of mRNAs that are also regulated by BDNF, such as Arc and CamKIIα (Napoli et al. 2008) as well as *bdnf* and *TrkB* mRNA themselves (Louhivuori et al. 2011). BDNF signaling through its TrkB receptor has also been shown to decrease *FMR1* mRNA as well as FMRP protein (Castren et al. 2002). Together, this research suggests that BDNF expression, function, and the subsequent post-transcriptional regulation of FMRP target mRNAs is abnormal in FXS.

Tuberous sclerosis (TSC) is another autism spectrum disorder with a known genetic cause, resulting from loss-of-function mutations in the *TSC1* or *TSC2* genes.

The TSC1–TSC2 protein complex functions to inhibit mTOR signaling via Rheb activity. BDNF binding to TrkB leads to Akt or Erk-mediated phosphorylation of TSC1-TSC2, causing its inactivation and allowing for appropriate mTOR function (Han and Sahin 2011). Loss of *TSC1* or *TSC2* results in misregulation of mTOR and abnormal protein synthesis, which can be reversed through inhibition of mTOR (Ehninger et al. 2008). Similarly, multiple cases of syndromic autism have been linked to mutations in phosphatase and tensin homolog (PTEN), which also results in overactivation of the PI3K/Akt/mTOR pathway and is decreased in response to BDNF (Briz et al. 2013). Given that Akt, Erk, and mTOR are all major targets of BDNF signaling, it is likely that BDNF-mediated control of protein synthesis could be impaired in neurons with deficiency in major regulators of this pathway. Mutations affecting the methyl-CpG-binding protein 2 (MeCP2) transcriptional regulator results in an additional monogenic ASD, termed Rett Syndrome. MeCP2 is known to bind methylated CpG sites in the BDNF gene (Klose et al. 2005), and MeCP2's function as either an activator or a repressor has been shown lead to complex misregulation of BDNF expression and evoked secretion linked to Rett Syndrome phenotypes (Chen et al. 2003; Martinowich et al. 2003; Chang et al. 2006; Li et al. 2012; Ebert and Greenberg 2013). Future studies will be aimed at determining whether regulating important targets of BDNF signaling could ameliorate neuronal abnormalities observed in diverse forms of ASD.

6 Concluding Remarks

BDNF has been well-established as an important regulator of both global and local protein synthesis in neurons. The equally well-established and consistent roles of BDNF in promoting pro-growth responses, whether studied at the level of molecules, synapses, or cognition, highlight the significance of the remarkable pro-growth gene target specificity that BDNF confers to translation. Physiological effects of BDNF such as enhanced spine density, synaptic function, and cognitive performance necessitate that BDNF enhances translation not of all mRNAs, but instead of a subset of mRNAs that support growth and plasticity. Recent research has begun to reveal the mechanisms allowing BDNF-mediated specificity in protein synthesis, but links between translational specificity in response to BDNF and the physiological and behavioral requirements of BDNF are not yet well studied. A better understanding of the post-transcriptional mechanisms allowing BDNF to selectively generate trophic responses has the potential to reveal novel control points in both normal physiology and disease states. Future research should also aim to help us determine whether the mechanisms behind BDNF-mediated post-transcriptional regulation can be generalized across brain regions and tissue types. Overall, further elucidation of BDNF signaling pathways and mechanistic understanding of how they impact structural and functional effects of BDNF will be vital to understanding of the role of BDNF in brain function, and for potential development of therapeutics aimed at treating the wide variety of diseases and disorders associated with BDNF misregulation.

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