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Control of microRNA biogenesis and transcription by cell signaling pathways

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Summary

A limited set of cell-cell signaling pathways presides over the vast majority of animal developmental events. The typical raison d'etre for signal transduction is to control the transcription of protein-coding genes. However, with the recent appreciation of microRNAs, growing attention has been paid towards understanding how signaling pathways intertwine with microRNA-mediated regulation. This review highlights recent studies that uncover unexpected modes of microRNA regulation by cell signaling pathways. Not only can miRNA transcription be positively or negatively regulated by cell signaling, the TGF-β/BMP pathways and Ras/MAPK pathways have now been shown to directly influence microRNA biogenesis to mediate substantial cellular phenotypes.

Introduction

Fundamental to the organized development of all multicellular organisms is the ability of cells to communicate with each other. In animals, a handful of fundamental cell signaling systems are used reiteratively to determine cell fates and pattern tissues, including the Notch, Hedgehog, Wnt, TGF-β/BMP, receptor tyrosine kinase (RTK), Jak/STAT, nuclear receptor and Hippo pathways [1,2]. The typical view of these cell signaling pathways is to transduce inputs from the cell surface to the nucleus, to alter the transcriptional status of protein-coding target genes. However, other outputs of the fundamental cell signaling systems have been catalogued, including direct regulation of cytoskeletal dynamics, cell adhesion, cell polarity, and/or cell death. Since many diseases and cancers involve deregulation of these core developmental signaling pathways, a comprehensive view of their action is necessary.

The recognition of an extensive class of ~22 nucleotide (nt) RNAs generated from endogenous hairpin transcripts, collectively known as microRNAs (miRNAs), changed the playing field for understanding gene regulatory mechanisms [3]. The founding miRNAs were recognized in the 1990s upon cloning of the *C. elegans* temporal identity mutants *lin-4* and *let-7* [4,5], but the generality and the regulatory reach of the miRNA network was not appreciated until this past decade. Many animal genomes encode hundreds of miRNAs, and evidence suggests that they regulate a majority of protein-coding transcripts [6]. It may come as no surprise then, that there are many compelling links between developmental cell signaling pathways and miRNAs. We and others have reviewed how components of many

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signal transduction pathways are regulated by miRNAs [7,8]. Here, we highlight recent advances on how cell signaling regulates miRNAs, with an emphasis on unexpected intersections of cell signaling with miRNA biogenesis.

Basics of miRNA biogenesis and function

While a diversity of miRNA biogenesis schemes have been reported, a canonical mechanism governs the production of the majority of animal miRNAs [9]. Most miRNAs are transcribed by RNA polymerase II, as part of non-coding genes or from introns of protein-coding genes. Primary miRNA (pri-miRNA) transcripts contain one or more local hairpins that are cleaved by the nuclear RNase III enyzme Drosha and its dsRBD partner DGCR8. Drosha exists in multiple complexes, with larger complexes containing additional factors such as the RNA helicases p68 and p72 [10]; the latter are involved in maturation of a subset of miRNAs [11]. Drosha cleavage releases ~55-70 nt hairpins known as pre-miRNAs, which are exported to the cytoplasm and cleaved again by a Dicer RNase III enzyme to yield ~22 nt miRNA duplexes. One strand is preferentially incorporated into an Argonaute (AGO) protein, which serves as the core of an effector complex that is guided by the small RNA to targets [12]. Animal miRNAs can repress targets via surprisingly short complements of ~7 nt to the 5' ends of miRNAs (preferentially nucleotides 2-8), inducing mRNA destabilization and/or inhibiting productive translation [13]. Comparative genomics provides compelling evidence for purifying selection operating on tens of thousands target sites distributed amongst a majority of protein-coding genes [6], and both transcriptome [14,15] and proteome studies [16,17] provide experimental evidence that individual miRNAs can directly repress hundreds of targets.

Highly dose-sensitive nature of cell signaling pathways

The bulk of miRNA targets are rather subtly repressed at the transcript and protein level, leading to the notion that miRNAs serve on the whole to finely tune target levels [6]. However, the quantitative strength of miRNA-mediated repression does not necessarily predict phenotypically relevant gene regulation, since this must also take into account the biological function of the target genes [18]. While the level of many genes can be manipulated over a wide range without apparent effect to the organism, slight changes in the activity of some genes suffice to cause substantial phenotypes. In particular, members of cell signaling pathways are frequently dose-sensitive. This feature has been exploited in genetic screens for new signaling components. Specifically, in a background that is sensitized for pathway activity, one can easily identify loci for which loss of one gene copy enhances or suppresses the starting phenotype.

This strategy was first used to uncover components of RTK signaling during photoreceptor specification, including the small GTPase Ras1 [19]. Notably, activating mutations of Ras are amongst the most common features of human tumors. Later, an activated *Drosophila* Ras1 modeled on the common human V12 mutation was used for saturation-level screening of ~850,000 mutants [20]. This yielded an impressive collection of ~300 dominant enhancer and ~600 dominant suppressor mutations, indicating that heterozygosity can frequently alter the consequences of Ras1 overactivity. The *Ras1* modifiers proved to identify most components of this signaling pathway, including an extensive kinase cascade that is engaged by active Ras, culminating in the mitogen activated protein kinase (MAPK, also known as ERK).

Another conserved cell signaling pathway is mediated by ligands of the TGF-β/BMP family. The most broadly used homolog in *Drosophila* is Decapentaplegic (Dpp), which directs tissue patterning and growth throughout many developmental settings. Screening for dominant modifiers of *dpp* mutants revealed many such loci, including the *Mothers against*

dpp (*Mad*) and *Medea* loci [21]. Mad and Medea proved to encode related transcription factors, of which Mad is more broadly required for Dpp signaling; similarly, its orthologs Smad1/2/3/5/8 (the regulatory Smads, or R-Smads), are the major nuclear effectors of mammalian TGF-β/BMP signaling; Medea is classified with mammalian Smad4 as "co-Smads" [22]. A third example of a highly dose-sensitive cell signaling cascade is the Notch pathway [23]. Not only are phenotypic outputs of Notch signaling highly amenable to genetic modification [24], three core pathway components in *Drosophila* (the receptor *Notch*, the ligand *Delta*, and the nuclear corepressor *Hairless*) are haploinsufficient; that is loss of one allele confers fully penetrant morphological defects. This is not peculiar to insects, since haploinsufficient phenotypes have also been observed for mammalian Notch pathway components [25,26].

The dose-sensitive nature of the fundamental cell signaling pathways suggests that they may be enriched for compelling instances of miRNA targeting. In fact, studies conducted prior to the formal recognition of miRNAs revealed that multiple target genes of the *Drosophila* Notch pathway were critical targets of miRNA-mediated repression. In particular, two large families of Notch targets encoding bHLH repressor genes and Bearded genes bear conserved ~7 nt motifs termed Brd boxes (AGCUUUA), GY boxes (GUCUUCC) and K boxes (cUGUGAUa) in their 3' UTRs [27,28]. Their significance was hinted at by gain-of-function alleles in Bearded family and bHLH-R genes associated with loss of 3’ UTRs [29,30]. Indeed, these motifs mediated negative post-transcriptional regulation, including reduction of steady state transcript levels concomitant with loss of poly-A tails. More strikingly, mutation of these motifs within genomic transgenes recapitulated aspects of the original gain-of-function phenotypes. Years later with the first cloning of *Drosophila* miRNAs [31], it was shortly noticed that many bore perfect Watson-Crick complementarity to Brd, GY, or K boxes, specifically at the 5’ ends of the miRNAs [32]. Thus, the study of post-transcriptional regulation of Notch signaling laid a groundwork for understanding key features of miRNA:target interactions. More recent genetic studies now reveal an impact of miR-8/200 family members on Notch-mediated tumorigenesis [33,34]. Altogether, these observations provide strong rationale to study functional connections between cell signaling pathways and miRNAs in detail.

R-Smads: Transcription factors in the TGF-β pathway moonlight as miRNA biogenesis factors

Many studies have documented the regulation of TGF-β/BMP signaling by miRNAs [35-38], as well as the transcriptional control of miRNAs by TGF-β/BMP signaling [39,40]. However, recent work illuminates an unexpected influence of TGF-β/BMP signaling on miRNA biogenesis. Studies of the ability of TGF-β and BMPs to induce a contractile phenotype in vascular smooth muscle cells, revealed that both ligands caused rapid, post-transcriptional, upregulation of mature miR-21 [41]. This miRNA subsequently represses *programmed cell death protein 4* (*PDCD4*), an inhibitor of smooth muscle contractile genes. Functional knockdown of miR-21 activity prevented the ability of these ligands to induce the contractile phenotype, indicating that miR-21 is an important effector of TGF-β/BMP signaling.

Surprisingly, the mechanism of miR-21 induction involved the formation of a direct protein-protein interaction between different R-Smads (Smad 1/3/5) and the RNA helicase p68, which associates with Drosha/DGCR8 to promote cleavage of specific pre-miRNA transcripts, including *pri-mir-21* and *pri-mir-199a* (Figure 1). Interestingly, they observed some ligand specificity to the response, in that Smad1 was recruited to *pri-mir-21* upon BMP4 stimulation, whereas Smad2 and Smad3 were recruited upon TGF-β stimulation; Smad4 (the co-Smad) was not recruited to pri-miRNAs by either stimulus [41].

Recent follow-up work expanded the generality of this response, and illuminated the underlying mechanism [42]. miRNA profiling following BMP4 and TGF-β treatment revealed elevation of the levels of 20 mature miRNAs, including the originally studied miR-21 and miR-199a. Analysis of their hairpin sequences revealed that 17 contained a CAGAC motif located ~10 bp from the terminal loop. Curiously, this is very similar to the known DNA binding site of Smads, and the corresponding miRNA hairpin motif was termed the RNA Smad binding element (R-SBE). Mutational analysis showed that CAGAC motifs were necessary for Smad-mediated enhancement of Drosha processing (Figure 1). More significantly, such a motif was also sufficient to bring a non-targeted pri-miRNA under Smad control. Finally, they demonstrated that the known DNA binding MH1 domain of Smad was sufficient to interact with double stranded R-SBE, while the MH2 domain of Smad interacted with p68. In summary, TGF-β/BMP stimulation induces recruitment of R-Smads into R-SBE containing pri-miRNAs to enhance Drosha processing of a broad set of pri-miRNAs (Figure 1).

The concept that cell signaling pathways can influence miRNA biogenesis is broadened by studies of the nuclear receptor, estrogen receptor alpha (ERalpha). Upon activation by the steroid hormone estrogen, ERalpha can also associate with the Drosha complex and inhibit the processing of certain miRNAs [43]; the molecular mechanism remains to be elucidated. More generally, the notion that transcription factors might have dual DNA- and RNA-binding capacities is food for thought [44]. For example, the eminent p53 tumor suppressor exerts its transcriptional role not only by the coordinate regulation of many protein-coding genes, but also by direct activation of tumor suppressor locus *mir-34* [45,46]. On the other hand, p53 can also promote the cleavage of certain pri-miRNAs by a mechanism similar to the Smads, i.e. via recruitment to the Drosha microprocessor complex via direct interaction with p68 [47]. Notably, p53 has been documented to have RNA-binding activity [48,49], although it is not yet known whether this is involved in its ability to modulate miRNA processing. Such studies open a window onto the expanding possibilities for post-transcriptional regulation of miRNA processing [50].

Ras signaling: multiple mechanisms to affect miRNA transcription and biogenesis

Studies of Ras/MAPK signaling reveal many functional connections to miRNAs. One of the most compelling early observations was that the let-7 miRNA directly targets *Ras* in both *C. elegans* and mammals [51], suggesting that this miRNA could be a tumor suppressor. This has since been shown to be the case [52,53], in no small part due to the fact that let-7 can target many genes, including additional oncogenes such as *HMGA2* [54,55]. Other miRNAs have also been shown to repress Ras members, suggestive of other tumor suppressor activities [56–59] (Figure 2).

We first consider new advances in the regulation of miRNA transcription by Ras/MAPK signaling. Investigation of miRNA responses to oncogenic K-ras showed that the *mir-143/145* cluster was consistently downregulated in mammalian and fish model systems, suggesting a highly conserved mechanism [60]. This was not just a correlation, since re-expression of *mir-143/145* at physiological levels suppressed K-ras-mediated transformation. The mechanism by which K-ras signaling represses *mir-143/145* transcription involves RREB1, a transcription factor downstream of Ras, directly via RREB1 binding sites in the *mir-143/145* promoter (Figure 2). In a further twist, miR-143/145 themselves directly repress *K-ras* and *RREB1* via their 3' UTRs. This establishes a mutually exclusive, feed-forward paradigm by which K-ras promotes an oncogenic state [60]. Reciprocally, miR-143/145 target other pro-growth factors such as *Myc*, *Insulin Receptor Substrate 1*, and *ERK5*, and to enforce an anti-proliferative state. The

fact that loss of *mir-143/145* is crucial for K-ras-mediated transformation suggests its potential as an anti-cancer therapy.

Going in the other direction, Ras signaling induces the transcription of certain miRNAs. Amongst its targets is *mir-21*, which is directly activated by the downstream transcription factor AP-1 [61,62]. The expression of miR-21 was earlier shown to be universally elevated in hundreds of human solid tumors across a panel of tissue origins [63], and a variety of tests in cancer cell lines suggested it to have oncogenic activity, since it represses a number of tumor suppressor genes [62,64], inhibits apoptosis, and can compromise the DNA damage-induced cell cycle checkpoint [65]. Recently, the cancer relevance of miR-21 has been addressed in animal models. *In vivo* overexpression of *mir-21* enhanced lung tumorigenesis in concert with activated K-ras, but perhaps more significantly, the genetic deletion of *mir-21* suppressed K-ras-driven tumors [62]. As with miR-143/145, oncogenic miR-21 may involve a feed-forward loop, since amongst its direct targets are multiple repressors of Ras signaling including *Btg2* (which reduces the active GTP-bound Ras state), *Sprouty* genes (which are MAPK inhibitors) and *PDCD4* (an inhibitor of AP-1) [62] (Figure 2).

Beyond these typical (although certainly complex and intertwined) mechanisms of miRNA-regulated cell signaling and signaling-regulated miRNA transcription, a biochemical approach recently revealed an unexpected influence of MAPK signaling on miRNA biogenesis. As with many other proteins, many components of the miRNA processing pathway are subject to post-translational modification. For example, there exist hyperphosphorylated forms of TRBP, a dsRBD cofactor for Dicer [66]. Identification of 4 serine phosphorylation sites provided the opportunity to investigate functional alterations exhibited by phospho-mutant and phospho-mimetic variants. These studies showed that phosphorylation increases the level of stable TRBP protein, which in turn enhances both general miRNA processing and miRNA-mediated silencing (Figure 3).

The relevant kinase was found to be MAPK/ERK, which associates with TRBP, and whose chemical inhibition blocked phosphorylation of TRBP [66]. As well, this reaction could be reconstituted in vitro using recombinant TRBP, Erk2, and an activated form of the kinase upstream of Erk activation, MAP kinase kinase (MAPKK). Perhaps more importantly, this work assigned the miRNA generating machinery as a functional effector of MAPK signaling in promoting cell proliferation and survival. Phosphomimetic TRBP could promote both of these properties, while the presence of phospho-mutant TRBP could partially block the mitogenic effects of activating MAPK. Curiously, global profiling of miRNA expression changes induced by phosphomimetic TRBP showed a general increase in miRNA levels, including many pro-growth miRNAs, but repression of the tumor-suppressive miRNA let-7. Therefore, the differential coordination of miRNA biogenesis caused by MAPK-mediated phosphorylation of the Dicer cofactor TRBP represents a phenotypically substantial aspect of MAPK signaling.

These selected examples illustrate the varied ways in which EGFR/Ras/MAPK signaling can affect miRNA expression: they can be modulated by transcriptional activation or repression, and the activity of a core miRNA biogenesis component (TRBP) can be enhanced by signaling, with complex effects on miRNA biogenesis (Figure 3). These findings highlight the intricacy of gene regulatory programs unleashed by Ras signaling.

Concluding remarks

It is clear that beyond the association of protein-coding components of cell signaling pathways as miRNA targets, the control of miRNA transcription by signaling pathways is also of great importance. More surprisingly, we now appreciate that several key cell signaling systems can directly regulate miRNA biogenesis at post-transcriptional levels. In

general, one can imagine diverse possibilities for the regulation of miRNA biogenesis, including at the level of Drosha cleavage, at nuclear export of pre-miRNAs, Dicer cleavage, Argonaute loading, removal from Argonaute, and probably other steps not currently appreciated. The study of post-transcriptional control of miRNA biogenesis has proven to be a rich field of study the past few years [50], and will undoubtedly continue to grow in the future.

We also touched upon the principle that miRNA genes are often involved in feed-forward or feed-back loops within a given signaling system, and cross-regulatory loops between different signaling systems undoubtedly occurs. For example, the transcription and biogenesis of miR-21 is under complex control by Ras/MAPK and TGF-β/BMP signaling. Since the studies to date were in different cell systems, it might be that these are separate regulatory events. On the other hand, it seems eminently possible that these signaling pathways may cooperate in some settings. Going beyond the study of individual regulatory interactions, systems approaches will be needed in the future to fully understand the complexity of gene regulatory networks initiated by signaling pathways, at both transcriptional and post-transcriptional levels [67].

A final key point to the studies discussed regards the phenotypic contribution of miRNAs and miRNA pathway components to signaling-mediated phenotypes, especially in light of the fact that miRNAs are often perceived to have subtle or fine-tuning effects. During induction of the contractile phenotype in vascular smooth muscle cells by BMP and TGF-β signaling, mammalian miR-21 is a critical and non-redundant effector gene. During oncogenic Ras/MAPK signaling, the post-translational activation of the miRNA biogenesis factor TRBP by phosphorylation, the transcriptional repression of *mir-143/145*, and the transcriptional activation of *mir-21*, all make substantial phenotypic contributions. In fact, recent studies of an inducible *mir-21* transgene showed that continuous miR-21 activity is needed for maintenance of an oncogenic state, providing first in vivo evidence of tumor addiction to a miRNA [68]. These findings provide promise that the knowledge of miRNA modulation under conditions of dysfunctional cell signaling, so frequently known to be causal for disease and cancer, may eventually lead to new therapeutic strategies.

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Saj and Lai Highlights

- A set of fundamental cell signaling pathways controls most aspects of animal development.
- These pathways regulate expression of protein-coding genes and non-coding genes, including miRNAs.
- Several cell signaling pathways also directly regulate miRNA biogenesis at a posttranscriptional level.

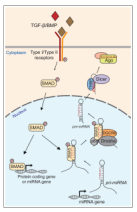

Figure 1.
Control of transcription and miRNA biogenesis by TGF-β/BMP signaling. At the core of this pathway, ligand binding to Type I/II heterodimeric receptors induces phosphorylation and nuclear translocation of SMAD transcription factors, which directly regulate the expression of protein-coding and miRNA genes. Mammalian SMAD proteins can also bind to the double-stranded stems of certain pri-miRNAs that bear CAGAC motifs, and promote their cleavage by the RNase III enzyme Drosha and its dsRBD partner DGCR8. This enhances the production of CAGAC-bearing pre-miRNAs, which are exported to the cytoplasm, cleaved by Dicer, and loaded into Argonaute (Ago) complexes to repress target genes.

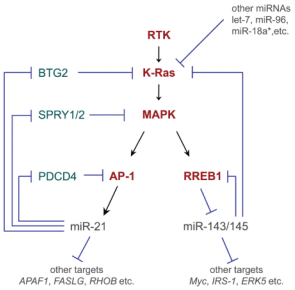

Figure 2.

Extensive feedback and feedforward loops between Ras signaling and miRNAs. In this simplified view of receptor tyrosine kinase (RTK) signaling, extracellular stimulation activates a Ras small GTPase (e.g. K-ras) and a kinase cascade including MAP kinase (MAPK), which regulates downstream transcriptional activators such as AP-1 and transcriptional repressors such as RREB1. In addition to regulating the expression of protein-coding genes, these transcription factors also regulate miRNA genes. AP-1 directly activates *mir-21*, an oncogenic miRNA that blocks multiple inhibitors of Ras signaling, including BTG2, SPRY1/2, and PDCD4, thereby establishing a potent feed-forward loop. RREB-1 directly represses the *mir-143/145* cluster, which in turn have tumor suppressor activity by feedback repression of Kras and RREB1. These miRNAs also have other targets, many of which contribute to their oncogenic or tumor suppressive activities; as well, other miRNAs regulate Ras pathway components.

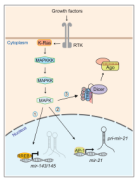

Figure 3.

Control of transcription and miRNA biogenesis by RTK/Ras signaling. Stimulation of receptor tyrosine kinases (RTK) by extracellular growth factors results in activation of the small GTPase Ras and a downstream kinase cascade including MAP kinase kinase kinase (MAPKKK), MAPKK and MAPK. Translocation of MAPK to the nucleus mediates the activity of downstream transcription factors, including (1) RREB1, a repressor of *mir-143/145* and (2) AP-1, an activator of *mir-21*. Naturally, there are also many protein-coding genes whose transcription is regulated by RTK/Ras signaling. In addition, activation of MAPK results in phosphorylation of TRBP, a dsRBD cofactor of the Dicer RNase III enzyme. Phospho-TRBP mediates enhanced biogenesis of most miRNAs, including many growth-promoting miRNAs, although its activation is also associated with lower levels of the tumor suppressive miRNA let-7. For simplicity, Drosha cleavage of *pri-mir-21* is not shown. Saj and Lai Highlights A set of fundamental cell signaling pathways controls most aspects of animal development. These pathways regulate expression of protein-coding genes and non-coding genes, including miRNAs. Several cell signaling pathways also directly regulate miRNA biogenesis at a post-transcriptional level.