

REVIEW

The MicroRNA-signaling-peroxisome proliferator-activated receptor gamma connection in the modulation of adipogenesis: bioinformatics projection on chicken

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ABSTRACT Meat quality and nutritional value hinge on many factors, including fat content in the adipose tissue. In the adipogenesis process, stem cells are first committed to become preadipocytes, followed by preadipocyte differentiation. In the later event, peroxisome proliferator activated receptor gamma (**PPAR γ**) is the gateway through which adipogenic genes are activated. This review focuses on clarifying the effects of signaling transduction on PPAR γ that have been experimentally established in the adipogenesis process. Furthermore, microRNA (**miRNA**) are validated to target the signaling factors and impact adipogenesis are appraised to establish the miRNA-signaling-PPAR γ regulatory

connection in adipogenesis. As opposed to red meat, chicken is white meat, which is increasingly appreciated for health and environmental reasons. Most works reported on the miRNA-signaling-PPAR γ network in adipogenesis used human and other laboratory and farm animal models. We show here that database interrogation and bioinformatics analysis may be applied to extrapolate reported findings to chicken based on evolutionary conservation. Understanding molecular modulation of adipogenesis may contribute to clinical treatment of lipid disorders and obesity in humans, and improved meat quality and commercial value in chicken farming, and possibly in the creation of artificial meat.

Key words: preadipocyte differentiation, PPAR γ , microRNA gene regulation, signal transduction, bioinformatics analysis

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INTRODUCTION

Meat is a major source of protein and lipid in food consumption by humans. Meat quality is affected by fat content, texture, hydration, acidity, pigmentation, and flavor (Claire D'Andre et al., 2013). Appropriate levels of intramuscular fat (**IMF**) content not only improve meat quality in terms of texture and taste but also enhance supplement of essentially fatty acids.

Besides pork, beef, and lamb, broiler chickens are also a major source of meat and protein, particularly for regions where pork and beef are forbidden for religious and cultural reasons. Chicken meat is considered “white”

meat, which is increasingly recommended by nutritionists for health purposes.

In chicken and other poultry, liver is the major site for lipid biosynthesis. Lipoproteins, cholesterol and triacylglycerol formed in the liver are transported to adipocytes in the adipose tissue for storage as an energy source (Ricoult and Manning, 2013; Wang et al., 2017). Excessive lipids are stored in adipocytes as triglyceride (Kwok et al., 2016). When other organs and tissues require energy, the lipid is hydrolyzed to release free fatty acids into circulation (Karpe et al., 2011).

THE MIRNA-SIGNALING-PPAR γ CONNECTION IN PREADIPOCYTE DIFFERENTIATION IN ADIPOGENESIS: A PREVIEW

Adipogenesis is a process in which progenitor cells, mainly the multipotent stem cells (**MSC**), differentiate to generate mature adipocytes (Zuk et al., 2002).

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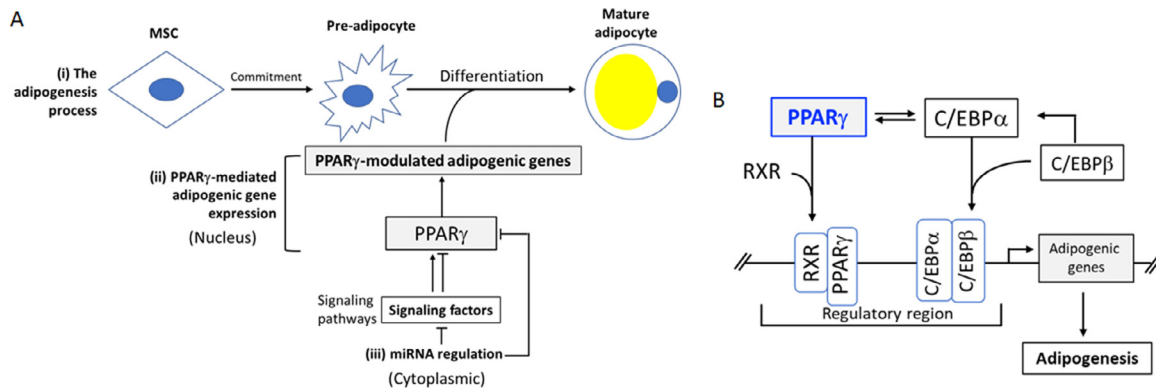


Figure 1. The miRNA-signaling-PPAR γ connection that modulates the differentiation stage of adipogenesis. (A) An outline of the major steps in adipogenesis focusing on (i) preadipocyte differentiation modulated by (ii) PPAR γ -mediated expression of adipogenic genes and (iii) miRNA targeting the signaling factors leading to PPAR γ expression. See text for details. Lines with arrows or blunt ends indicate activation or inhibition, respectively; the same arrow/blunt-end configurations are applied to all other figures. (B) PPAR γ and C/EBP α coregulation of expression of PPAR γ -modulated adipogenic genes. In the simplified scheme, RXR dimerizes with PPAR γ while C/EBP α and C/EBP β also dimerize with one another. Subsequently, the two heterodimers act in concert in binding specific regulatory sites to coregulate expression of PPAR γ -modulated adipogenic genes, driving adipogenesis.

Adipocytes are developmentally derived from the mesodermal germ layer (Kaisanlahti and Glumoff, 2019). In the adipogenesis process, MSC first undergo a commitment step to become preadipocytes (Figure 1A, route (i)), a step promoted by Bcl6, ZFP467, and STAT5A (Quach et al., 2011; Stewart et al., 2011; Hu et al., 2016), to name a few. Subsequently a different set of genes is recruited to convert the committed preadipocytes to mature adipocytes in the differentiation process (Figure 1A, route (i); Ambele et al., 2020).

Subsequently, adipogenic proteins are synthesized under tight expression regulation by transcriptional factors in the nucleus, and post-transcriptionally by regulatory RNA in the cytoplasm via various signal transduction pathways (Figure 1A, routes (ii) & (iii)). At the end of the signaling process are the peroxisome proliferator-activated receptor (PPAR) family nuclear proteins, PPAR α , PPAR β (also called PPAR δ) and PPAR γ (Mirza et al., 2019). In particular, PPAR γ , the focus of the present review, is the crucial master controller of adipogenesis that modulates differentiation of preadipocytes into mature adipocytes (Rosen et al., 1999; Farmer, 2006).

PPAR γ expression is, in turn, modulated by other regulators of various signaling pathways (Figure 1A, route (ii)). On the other hand, transcripts of genes regulated by PPAR γ , including PPAR γ itself, are targeted and prevented from translation by microRNA (miRNA) post-transcriptionally in the cytoplasm (Figure 1A, route (iii)). Despite acting predominantly as negative regulators of targeted genes, miRNA involvement could exert both positive and negative impact on the adipogenesis process.

This review aims to clarify the role of the miRNA-signal transduction-PPAR γ connection in regulating the terminal stage of differentiation of preadipocyte into mature adipocytes in the adipogenesis process. Most studies on adipogenesis have been conducted using cell lines derived from human and mammals, but rarely in chicken. Based on the supposition that functionally crucial genes are conserved across species in evolutionary

terms, but keeping in mind that chicken and other poultry have their own unique metabolic system, we attempt in this review to extrapolate findings in adipogenesis in human and other mammals to chicken. This is done by combining literature survey and interrogation of appropriate databases followed by bioinformatics analysis. The outcome of the efforts is presented here.

PPAR γ IS A GATEWAY TO ADIPOGENESIS

The PPAR γ protein is composed of multiple functional domains. Amongst them is a highly conserved DNA-binding domain for recognition of and binding to the Peroxisome Proliferator Response Elements (PPRE), which are highly enriched in the regulatory regions of PPAR γ -activated adipogenic genes (Lemay and Hwang, 2006; Figure 1B).

For functional activation, PPAR γ first associates with retinoid X receptor (RXR) (Tontonoz et al., 1994; Tontonoz et al., 1997; Barak et al., 1999; Wafer et al., 2017). The PPAR γ -RXR heterodimer subsequently binds to PPRE to activate expression of the PPAR γ -modulated adipogenic genes (Figure 1B; Nielsen et al., 2008; Hamza et al., 2009; Siersbaek et al., 2010). On induction of preadipocyte differentiation, 7,700 PPAR γ -RXR binding sites are thought to be utilized, actively driving transcription of PPAR γ -modulated genes and adipogenesis (Hamza et al., 2009). Besides RXR, PPAR γ also binds to other lipid metabolite ligands for transcriptional activation. Notably, derivatives of linoleic acid have been proposed as authentic endogenous ligands in the PPAR γ -modulated adipogenesis process (Baker et al., 2005; Schopfer et al., 2005).

PPAR γ often acts in concert with CCAAT-enhancer-binding proteins alpha (C/EBP α) to regulate expression of adipogenic proteins (Figure 1B; Hu et al., 1995; Rosen et al., 1999; Wang et al., 2015). Studies have shown that the C/EBP α consensus site is also over-represented in the same neighborhood as the PPAR γ -RXR

binding sites in the genomic sequence, suggesting C/EBP α involvement in the activation of adipogenic genes (Lefterova et al., 2008). C/EBP α needs to form a heterodimer with C/EBP β for regulatory functions (Figure 1B; Lefterova et al., 2008). However, C/EBP α , but not C/EBP β , is crucial to promoting differentiation of mouse fibroblast cells into adipocytes (Freytag et al., 1994). C/EBP α is expressed at high levels in the liver and the adipose tissue for triggering differentiation of pre-adipocytes into mature adipocytes, and for the in vivo development of white adipose tissues (Linhart et al., 2001). C/EBP α by itself is insufficient in promoting adipogenesis (Clarke et al., 1997; Wu et al., 1999; Elberg et al., 2000; Rosen et al., 2002), but needs to collaborate with PPAR γ for regulatory functions (Figure 1B). When both factors are present, PPAR γ and C/EBP α cooperate to gain access to the respective binding sequences in the regulatory region of the adipogenic genes (Lefterova et al., 2008; Nielsen et al., 2008; Siersbaek et al., 2010; Madsen et al., 2014). Besides inducing preadipocyte differentiation, C/EBP α and PPAR γ collaboration is also important in maintaining the differentiated state of the adipocyte (Wu et al., 1999).

It is important to note here that there are 2 major isoforms of PPAR γ , namely PPAR γ 1 and PPAR γ 2, generated by differential promoter usage and splicing. In chicken, the 2 isoforms appear at the different stages of adipogenesis but perform the same functions albeit with different efficiencies (Mu et al., 2020). However, most studies on PPAR γ in relation to adipogenesis do not differentiate between the two isoforms. Hence, PPAR γ is used in this review, unless stated otherwise.

INVOLVEMENT OF MICRORNA IN GENE REGULATION

The primary site of gene regulation is at the level of gene transcription in the nucleus, followed by translation in the cytoplasm. In higher living species, a post-transcriptional regulatory system is found in the cytoplasm in the form of short single-stranded RNA, designated as microRNA, that serves to degrade the targeted mRNA or block translation (Humphreys et al., 2005; O'Brien et al., 2018).

In the miRNA biogenesis process, a precursor miRNA, or pre-miRNA, intermediate is formed. Pre-miRNA has a hair-pin structure composing of a leading 5p (5-prime) and a passenger 3p (3-prime) strands in the double-stranded stem. Further pre-miRNA processing generates either a 5p or 3p mature miRNA species, targeting different sets of transcripts; miRNA species of both the 5p and 3p strands often co-exist in the cytoplasm (Choo et al., 2014).

The miRNA-Mediated Gene Regulation Process

The most important part of a mature single-stranded miRNA is the “seed sequence”, which acts as a guide to

recognize and bind to the complementary sequence of the targeted mRNA. The seed sequence of a miRNA is a heptameric sequence situated at nucleotide positions 2-9 from the miRNA 5'-end. Functionally important miRNA and the targeted mRNA are highly conserved across species, as illustrated in this review. The miRNA seed sequence most frequently forms a perfect 6- to 8-basepair complementarity with the targeted mRNA sequence although the 3'-segment of a miRNA seldom forms homologous matches with the mRNA sequence. Once the miRNA-mRNA association has occurred, the targeted transcript is degraded (O'Brien et al., 2018). In the event of imperfect seed sequence complementarity, the mRNA is not degraded but translation is blocked (Humphreys et al., 2005; Mathonnet et al., 2007). miRNA have been shown or predicted to be involved in the regulation of most, if not all, known biological processes. Not surprisingly, miRNA also play important roles in regulating preadipocyte differentiation in the adipogenesis process, and in the development of adipose tissues (Price and Fernandez-Hernando, 2016; Landrier et al., 2019). Knocking out DICER and the DGCR8 (DiGeorge syndrome critical region 8) protein in the miRNA biogenesis process prevents proper processing of adipose-specific miRNA resulting in the piling up of white adipose leading to metabolic damages (Mori et al., 2012; Kim et al., 2014; Mori et al., 2014). These observations justify our focus on analyzing the role of miRNA in adipogenesis.

Confusion in miRNA Nomenclature in Adipogenesis Studies

In the early miRNA literature in adipogenesis studies, the miRNA nomenclature used was ambiguous at 2 levels, causing some confusion:

- (i) Unspecified member(s) of the same miRNA gene family: In the genomes of the human and higher animal species, some miRNA genes are duplicated to form a gene family with members showing high degrees of sequence homology. The gene members are normally designated with a letter suffix, a, b or c. In early literature, miRNA family member designation was largely disregarded.
- (ii) Unspecified 5p or 3p species: As described above, a pre-miRNA may produce a 5p and/or 3p mature miRNA species that normally target different transcripts (Huang et al., 2014). Early miRNA literature often failed to clearly denote whether a 5p or 3p species was studied.

In this review, attempts have been made to clarify these 2 issues of confusion when citing and discussing the literature. On confirmation by interrogations of the miRbase (Griffiths-Jones et al., 2006) and TargetScan (Agarwal et al., 2015) databases, appropriate miRNA designations of family members and the 5p or 3p species

of the miRNA studied are recommended and used in this review.

MiRNA Prediction in Chicken Genomewide Studies

Increasing number of studies has linked miRNA with meat quality of domestic animals (Shen et al., 2016; Chen et al., 2017), supporting the role of miRNA in modulating adipogenesis in farm animals, including broiler chicken.

In the *Poultry Science* centennial review, Hicks and Liu (2021) have provided an excellent and comprehensive appraisal on the involvement of miRNA in modulating metabolic development and functions in chicken (Hicks and Liu, 2021). However, the miRNA species and the functions cited are mainly derived from predictions based on interrogation of the TargetScan database and bioinformatics analysis. This review is an extension of Hicks and Liu (2021) by focusing on the miRNA-signaling-PPAR γ axis; more importantly, only experimentally validated miRNA and signaling factors and the affected adipogenic gene expression are cited and discussed. It is also noted here that a number of genomewide studies on chicken miRNA and target genes in association of preadipocyte differentiation and adipogenesis have been reported (Huang et al., 2015; Chen et al., 2019b; Sun et al., 2019; Ma et al., 2020). Our analysis cross-references to these reviews as supportive evidence when relevant.

MIRNA TARGETING PPAR γ IN THE MODULATION OF ADIPOGENESIS

For identification of miRNA involved in adipogenesis, genomewide small RNA sequencing approaches have been used for several species. To date, three miRNA, miR-27a/b-3p, miR-130, and miR-128, have been identified and experimentally confirmed to target the PPAR γ mRNA in adipogenesis (Figure 2A). In chicken, gga-miR-200b-3p has been identified in a genomewide study predicted to target PPAR γ but no experimental validation data were presented (Chen et al., 2019b) and is noted but not further discussed here.

miR-27a/b-3p

The literature describing the miR-27 gene family in targeting PPAR γ in adipogenesis is rather confusing. In the genomes of higher vertebrates, including human and rodent, there are 2 independent miR-27a and miR-27b genes. Some reports used only the designation miR-27 (Lin et al., 2009; Jang et al., 2019), or miR-27a or miR-27b without specifying the 5p or 3p species (Karbiener et al., 2009; Kim et al., 2010; Gu et al., 2016; Deng et al., 2020). The one exception is the use of the miR-27a/b-3p designation in human cells; in that work, the data showed similar results with both miR-27a-3p and miR-27b-3p (Kulyte et al., 2019).

Bioinformatics analysis has, indeed, revealed that the 3p species of hsa-miR-27a and -27b are different only by a single nucleotide at the 3'-end of the miRNA, which

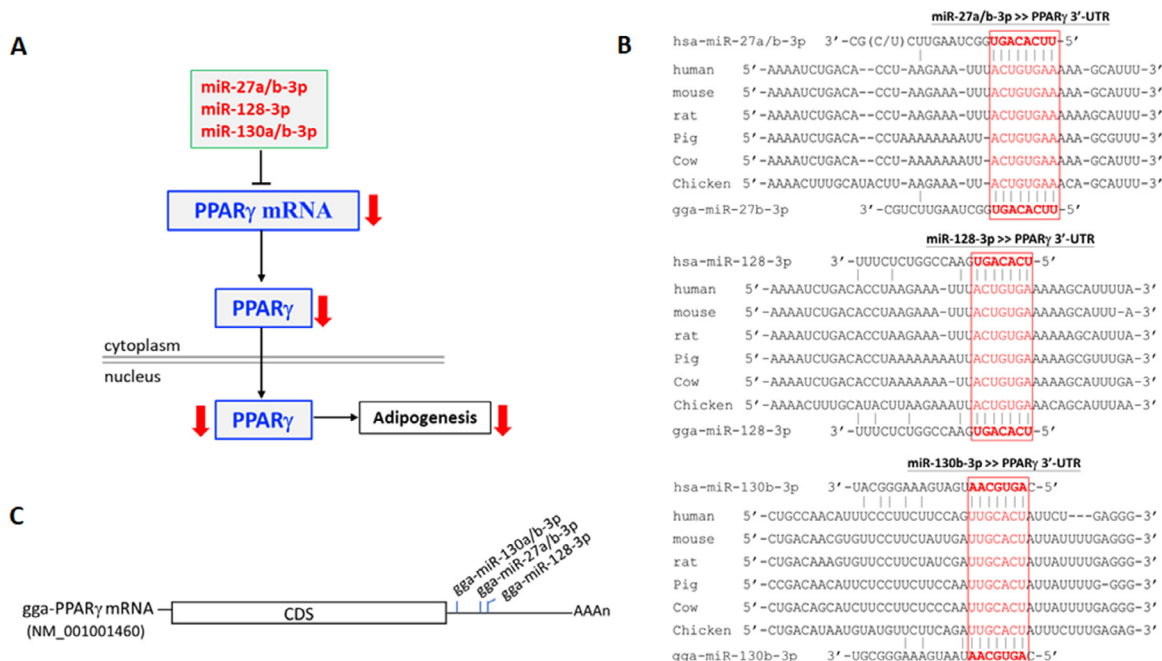


Figure 2. MiRNA regulation of PPAR γ expression and conservation of miRNA seed sequences in different species. (A) Experimentally validated miRNA regulation of PPAR γ expression. The three miRNA target the PPAR γ transcript to block PPAR γ translation to suppress adipogenesis. The downregulated effects of the miRNA on PPAR γ expression levels and adipogenesis are shown by downward-pointing thick-red arrows (the symbols are also used in other figures). (B) Bioinformatics analysis of the miRNAs that have been experimentally validated to target the PPAR γ transcript. The 3'-UTR sequences of the PPAR γ transcripts that embrace the miRNA seed sequences of human and five other animal species are shown; the seed sequences of the miRNAs are boxed and shown in red letters, as is also applied to other figures. (C) Mapping of the three putative chicken PPAR γ -targeting miRNA in the 3'-UTR of the chicken PPAR γ mRNA is shown; the map is derived from interrogation of the TargetScan database. CDS, coding sequence.

does not alter the miRNA seed sequence (Supplementary Table S1-A1). On the other hand, the 5p species of hsa-miR-27a and -27b are divergent by up to eight nucleotides in base substitutions and insertions/deletions (Supplementary Table S1-A1). Importantly, only the miR-27a/b-3p species, and not the -5p species, target the 3'-UTR of PPAR γ transcripts of various species (Figure 2B). It is, thus, highly likely that the unspecified miR-27 in the various previous studies was miR-27a-3p or -27b-3p. Hence, we propose to use the miR-27a/b-3p designation as in the work by Kultye et al. (2019) to collectively indicate the PPAR γ -targeting miR-27.

In chicken, there is only one gga-miR-27b gene based on interrogation of the miRbase database. The chicken gga-miR-27b-3p and the human hsa-miR-27b-3p are identical in sequence and that the seed sequence of miR-27a/b-3p is conserved in the 3'-UTR of the PPAR γ transcripts of human, rodents (mouse and rat), domestic animals (pig and cow), and poultry (chicken; Figure 2B, top panel). A putative gga-miR-27a-3p target site has also been mapped in the 3'-UTR of the chicken PPAR γ mRNA (Figure 2C). Such a high degree of cross-species conservation suggests important functionality, and allows projection of findings in human and other species to chicken. Involvement of gga-miR-27b-3p in adipogenesis has also been predicted in genomewide studies in chicken (Huang et al., 2015; Sun et al., 2019), consistent with our proposed projection.

MiR-27a/b-3p targeting of the PPAR γ transcript was confirmed in luciferase assays, as were negative regulation of adipogenesis and inhibition of adipocyte formation in human and rodent cells (Karbiener et al., 2009; Lin et al., 2009; Kim et al., 2010; Gu et al., 2016). Interestingly, miR-27a/b-3p concurrently downregulates C/EBP α (Karbiener et al., 2009; Lin et al., 2009) in the activation of PPAR γ -targeted adipogenic genes (see Section PPAR γ IS A GATEWAY TO ADIPOGENESIS).

In human orbital fibroblast cells, miR-27a/b-3p levels decreased on induction of adipogenic differentiation, an event coinciding with decline in the PPAR γ and C/EBP α levels (Jang et al., 2019). In steroid-induced rat bone marrow-derived mesenchymal stem cells, miR-27a/b-3p was shown to suppress adipogenesis but promoted osteogenesis by directly targeting PPAR γ and gremlin 1 (GREM1) (Gu et al., 2016). In sheep, miR-27a/b-3p downregulated PPAR γ leading to suppressed accumulation of triglyceride, and promoted preadipocyte proliferation (Deng et al., 2020).

miR-128-3p

Reports on miR-128-3p targeting PPAR γ and adipogenesis are more recent, and the authors have specified the 3p designation (Chen et al., 2018; Zhang et al., 2019). Our bioinformatics analysis has indicated that there are 2 gene copies of miR-128 in all species analyzed. Indeed, the miR-128-3p seed sequence is conserved in human and domestic animals, including

chicken (Figure 2B, middle panel, and Figure 2C; Supplementary Table S1-A2). The seed sequence of the putative gga-miR128-3p is mapped in the 3'-UTR of the chicken PPAR γ mRNA in close proximity with other PPAR γ -targeting gga-miRNA (Figure 2C).

Downregulation of miR-128-3p in adipogenesis was first reported in preadipocyte proliferation and differentiation of mouse 3T3-L1 cells (Chen et al., 2018). In another study that aimed to elucidate the functions of long noncoding RNA (lncRNA) in adipocyte differentiation, a lncRNA designated as IMFNCR (intramuscular fat-associated long non-coding RNA) was shown to promote intramuscular adipocyte differentiation by depleting miR-128-3p via molecular sponging (Zhang et al., 2019). miR-128-3p depletion was correlated with upregulation of PPAR γ expression and intramuscular adipocyte differentiation in chicken cells; miR-128-3p targeting of PPAR γ was unequivocally proven in luciferase assays (Zhang et al., 2019).

miR-130a/b-3p

In the literature, the designations of the human hsa-miR-130, porcine ssc-miR-130b, and bovine bta-miR-130a or bta-miR-130a/b are used without the 3p/5p specifications (Lee et al., 2011; Pan et al., 2013; Wei et al., 2017; Yang et al., 2017; Ma et al., 2018). Our bioinformatics analysis shows that there are two miR-130 gene copies in the human genome but 3 genes in the mouse and chicken genomes (Supplementary Table S1-A3). Our analysis also shows that the 3p species of miR-130 targets PPAR γ and the seed sequence of the miRNA is conserved in all species analyzed (Figure 2B, bottom panel). The consensus miR-130a/b-3p designation is used in this review.

By way of genomewide screening followed by experimental confirmation, miR-130a/b-3p was identified as a direct suppressor of PPAR protein expression, leading to triglyceride deposition and downregulated expression of adipogenic genes in human cells (Lee et al., 2011). Interestingly, miR-130a/b-3p targets not only the 3'-UTR but also the coding sequence of the PPAR γ transcript. Consistent with the human data, miR-130a/b-3p was subsequently shown to suppress fat deposition in pigs and bovine by downregulating expression of both PPAR γ and C/EBP α (Pan et al., 2013; Wei et al., 2017; Ma et al., 2018). In milk cattle, miR-130a/b-3p was also shown to lower fat contents in milk by targeting PPAR γ in the mammary gland (Yang et al., 2017). In an attempt to improve the quality of meat in pigs, microvehicles were used to deliver miR-130b-3p as a suppressant of fat deposition via blocking the PPAR γ -C/EBP α -adipogenesis connection (Pan et al., 2014).

In summary, database interrogation and bioinformatics analysis show that the seed sequences of the three PPAR γ -targeting miRNA are highly conserved in the 3'-UTR of the PPAR γ mRNA, as also in chicken (Figure 2B). In the chicken PPAR γ transcript, the putative binding sites of the 3 miRNA are clustered at the 5'-

end of the long 3'-UTR close to the coding sequence (Figure 2C). Such a genomic location has an advantage that if the 3'-UTR sequence is subjected to further post-transcriptional trimming by alternative splicing, the miRNA binding sites are less likely deleted, hence, maintaining the critical role of miRNA-mediated regulation of PPAR γ expression and adipogenesis.

MIRNA REGULATION OF SPECIFIC SIGNAL TRANSDUCTION PROTEINS IN MODULATING PPAR γ IN ADIPOGENESIS

As cellular environment changes, arising external stimuli need to be received by cells to elicit appropriate responses for survival. Signal transduction is a cellular process that responds to such external challenges. In the process, transmembrane protein receptors first bind with the external stimuli, such as growth factors, cytokines, or small peptide hormones, to trigger a cascade of intracellular biochemical events, collectively called signal transduction. At the end of the signaling is the transcriptional activation of genes, or other cellular responses, needed to appropriately deal with the external stimuli (Kramer, 2015).

This review focuses on 5 important signaling pathways chosen based on 2 criteria. First, each of the signaling pathway converges the biochemical effects onto PPAR γ to initiate the preadipocyte differentiation stage of adipogenesis. Second, one or more miRNA have been identified and experimentally validated to target a signaling protein in experimentally demonstrated effects on adipogenesis. Since there are abundant reviews on each of the signaling pathways, and are cited here, the pathways illustrated in this reviewed are simplified to highlight only the miRNA-signaling-PPAR γ connection, the

main theme of the review. Bioinformatics predictions of involvement of kinases and protein phosphorylation in signal transduction have previously been reported in chicken (Huang et al., 2015; Chen et al., 2019b; Sun et al., 2019; Ma et al., 2020), and are cited here where appropriate.

Wnt Signaling

The Wnt family proteins are glycoproteins that act as paracrine or autocrine factors to regulate cell fate and cell growth (Cadigan and Nusse, 1997; MacDonald et al., 2009; Kawakami et al., 2013). When a Wnt ligand binds to a transmembrane receptor of the Frizzled (Fzd) family, intracellular signaling is initiated (MacDonald and He, 2012). Before the initiation of the Wnt signaling, β -catenin is in a phosphorylated form, susceptible to destruction (Cadigan and Nusse, 1997; Kikuchi, 1999; Miller et al., 1999). On initiation of Wnt signaling, the lipoprotein receptor-related proteins 5/6 (LRP5/6) come into action as coreceptors: Fzd and LRP5/6 associate to form a heterodimer, freeing β -catenin from phosphorylation, and sparing its destruction (Wu et al., 2009; Kim et al., 2013; Nusse and Clevers, 2017). Alternatively, phosphorylation of LRP5/6 blocks β -catenin phosphorylation to stabilize β -catenin for nuclear transportation (Wu et al., 2009; Figure 3A). In the nucleus, β -catenin associates with members of the TCF (T-cell factor)/LEF (lymphoid enhancer-binding factor) transcription factor family to kickstart the canonical Wnt signaling pathway by modulating expression of downstream target genes, including PPAR γ and C/EBP α (Figure 3A; Cadigan and Nusse, 1997; Kikuchi, 1999; Miller et al., 1999).

In adipogenesis, Wnt-1 was shown to suppress differentiation of preadipocytes by suppressing expression of

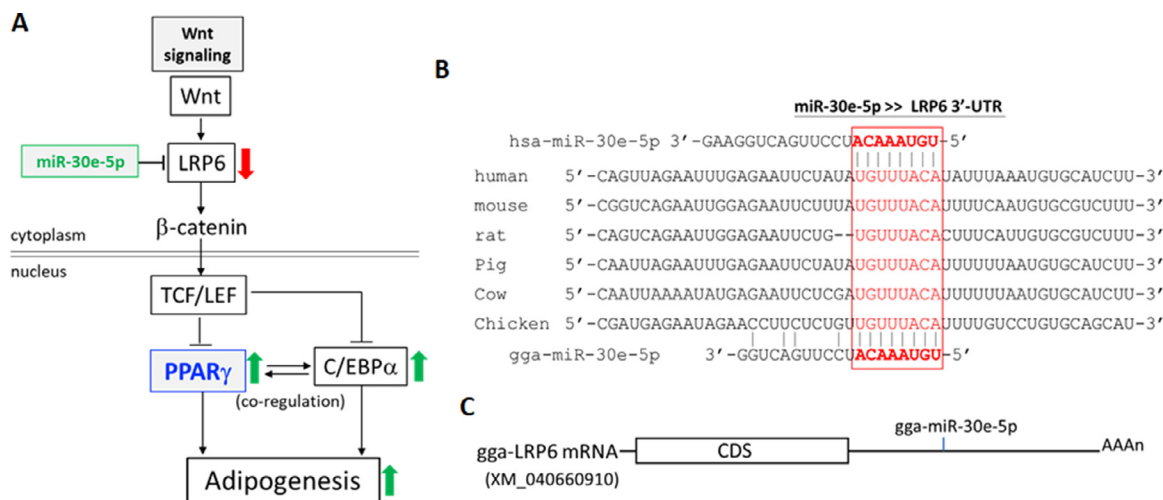


Figure 3. The canonical Wnt signaling pathway targeting PPAR γ in the adipogenesis process. (A) A simplified version of the Wnt pathway. Interactions of Wnt with a Wnt receptor and a co-receptor LRP6 induce intracellular signaling through β -catenin. β -catenin enters the nucleus to interact with TCF/LEF to form a transcriptional complex that suppresses expression of the PPAR γ and CEPB α , blocking adipogenesis. MiR-30e-5p targets the LRP6 transcript and suppresses LRP6 translation (thick red arrow), freeing β -catenin to enter the nucleus to associate with TCF/LEF to unblock the suppressed expression of PPAR γ and CEPB α , hence, promoting adipogenesis (upward-pointing thick green arrows; the symbols are also used in other figures). (B) The LRP6 mRNA and miR-30e-5p are highly conserved in different species, including chicken. The seed sequences of the miRNA-mRNA interactions are boxed. (C) Identification of the putative gga-miR-30e-5p seed sequences in the 3'-UTR of the chicken LRP6 transcript by bioinformatics analysis.

mRNA (Figure 4B). We propose the use of miR-15a/b-5p to represent miR-15 in FOXO1 targeting in adipogenesis, as in Dong et al. (2014). Bioinformatics analysis further shows that the FOXO1 mRNA is highly conserved, while the miR-15a/b-5p seed sequences are absolutely conserved in the species analyzed, including chicken (Figure 4B). The gga-miR-15a/b-5p seed sequence maps adjacent to the coding sequence in the 3'-UTR in the putative chicken FOXO1 mRNA (Figure 4C). In chicken genomewide analysis by others, the mir-16 family of gga-miR-15a, -15b, and -16 has been predicted to modulate FOXO1 expression (Sun et al., 2019; Hicks and Liu, 2021), consistent with our predicted involvement of gga-miR-15a/b-5p in adipogenesis in chicken. In essence, miR-15a/b-5p is a positive regulator of adipogenesis via FOXO1 in the PI3K/AKT signaling pathway.

MAPK/ERK Signaling

The mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated kinase (Akaike et al., 2004) are called by many names. At the top of the MAPK/ERK signaling pathway is MAP kinase kinase kinase 1, or MAP3K1, encoded by the gene MEK1; MAP3K1 phosphorylates MAP kinase kinase 1 and 2 (MAP2K1/2), gene products of MEK1/2, which, in turn, phosphorylate MAP kinase 3/1 (MAPK3/1), which is better known as extracellular signal-regulated kinase 1/2 (ERK1/2) (Figure 5A; reviewed in Wortzel and Seger, 2011). MAPK/ERK activation is important for initiating adipogenesis by promoting C/EBP β phosphorylation to transactivate C/EBP α (Prusty et al., 2002; Park et al., 2004). Likewise, MAPK/ERK activation also enhances C/EBP β phosphorylation and induces C/EBP α expression and adipogenesis in mouse fibroblasts (Park et al., 2004; Figure 5A).

Activation of MAPK/ERK signaling in the initial phase of adipogenesis significantly enhances the transactivation of the C/EBP α minimal promoter and the expression of both C/EBP α and PPAR γ (Prusty et al., 2002). Later in adipogenesis, a second MAPK/ERK activation blocks adipogenesis (Bost et al., 2005; Rodriguez et al., 2006) to prevent PPAR γ promoter binding (Hu et al., 1996). Likewise, MAPK/ERK mediates phosphorylation and inactivation of RXR, hindering PPAR γ -RXR dimerization (see Figure 1B; Solomon et al., 1999).

MiR-375-3p is recruited to shutdown MAPK/ERK signaling to block PPAR γ phosphorylation for preadipocyte differentiation to proceed (Figure 5A; also see below; Ling et al., 2011). In mouse 3T3-L1-derived adipocytes, miR-375-3p levels were first shown to be upregulated during differentiation, suppressing ERK2 expression and blocking the phosphorylation of C/EBP β and PPAR γ (Ling et al., 2011). In later studies, the true target of miR-375-3p was identified to be the MAP3K1 transcript (Salem et al., 2017; Figure 5A). Hence, miR-375-3p is also a positive regulator of adipogenesis via targeting the MAPK/ERK signaling (Figure 5A).

Database interrogation has indicated the existence of highly conserved miR-375-3p in the animals analyzed (Supplementary Table S1-D). The 3'-UTR of the human MAP3K1 mRNA that harbors the miR-375-3p seed sequence is highly conserved (Figure 5B), and the gga-miR-375-3p target site is mapped at the 3'-end of the putative chicken MAP3K1 mRNA (Figure 5C). The analysis suggests that the miR-375-3p-MAPK/ERK signaling connection is indispensable in chicken and other species, as supported by chicken genomewide analysis (Chen et al., 2019b).

TGF- β Signaling

Transforming growth factor β (TGF- β) is a ubiquitous growth factor detected in adipose tissues, preadipocytes,

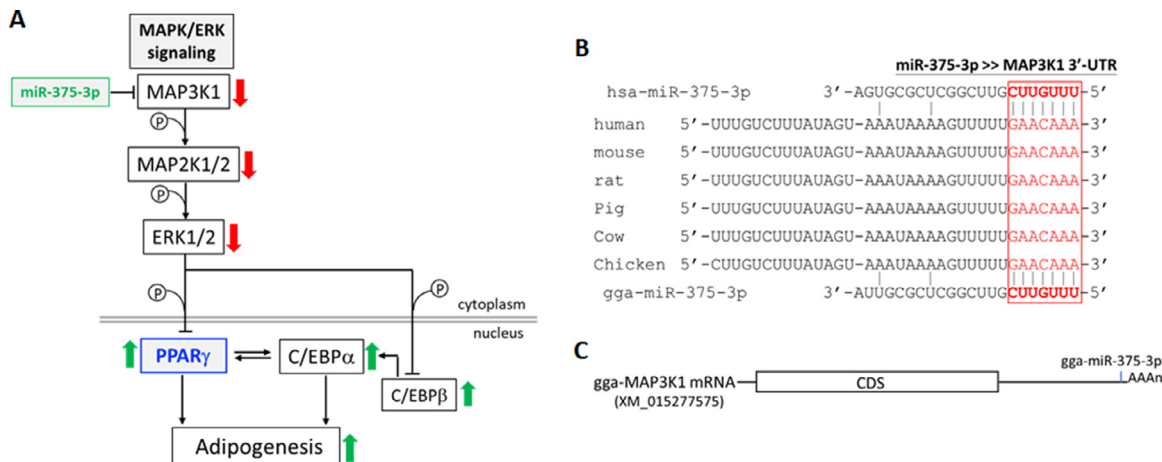


Figure 5. MAPK/ERK signaling in adipogenesis. (A) The MAPK/ERK signaling and PPAR γ -C/EBP α regulation. Signaling begins at MAP3K1 phosphorylation (P) of MAP2K1/2, which, in turn, phosphorylates ERK1/2. The activated ERK kinases further phosphorylate C/EBP β , impacting on C/EBP α expression and coregulation with PPAR γ leading to suppressed adipogenesis. MAP3K1 is negatively modulated by miR-375-3p (thick red arrow) to reverse the chain of phosphorylation steps resulting in positive modulation of PPAR γ expression and adipogenesis (thick green arrows). (B) Bioinformatics analysis shows that the 3'-UTR sequences of the MAP3K1 transcripts are highly conserved, whereas the seed sequence (boxed) of hsa-miR-375-3p is absolutely conserved in the MAP3K1 mRNAs of all the species analyzed, including chicken. (C) The chicken gga-miR-375-3p is identified in the TargetScan database and is mapped in the 3'-UTR of the putative chicken MAP3K1 mRNA.

and adipocytes in culture (Sparks et al., 1993; Bortell et al., 1994; Samad et al., 1997; Rahimi et al., 1998). TGF- β signaling is, in general, initiated with the binding of a TGF- β ligand to the transmembrane serine-threonine kinase receptor type II (**TGFBR2**), which then phosphorylates and activates **TGFBR1**, the type 1 receptor (Heldin et al., 1997). Subsequently, the activated TGFBR1 phosphorylates the Mothers Against Decapentaplegic homolog 2 and 3 proteins (**SMAD2** and **SMAD3**) in the cytoplasm, inducing conformational changes to enable the formation of a SMAD2/3 complex for translocation into the nucleus (Itoh et al., 2000; Massague and Wotton, 2000). In the nucleus, the SMAD2/3 protein complex interacts with other transcription factors to bind to specific DNA sequences to upregulate expression of targeted genes (Derynck and Zhang, 1996; Heldin et al., 1997).

Following the initial report that TGF- β inhibited adipogenesis (Roelen and Dijke, 2003), later reports showed in human subcutaneous preadipocytes that TGF- β , acting through SMAD3, but not SMAD2, resulted in down-regulated PPAR γ and C/EBP α mRNA levels (Figure 6A; Chen et al., 1999; Choy et al., 2000; Choy and Derynck, 2003; Wei et al., 2010). The phosphorylation-activated SMAD3 interacts strongly with C/EBP α to repress C/EBP α functions and PPAR γ -C/EBP α coregulation in adipogenesis (see Section PPAR γ IS A GATEWAY TO ADIPOGENESIS) (Choy and Derynck, 2003; Roelen and Dijke, 2003; Maeda et al., 2004).

In miRNA regulation, miR-140-5p has been shown to target and suppress TGFBR1 gene expression, consequently inhibiting SMAD3 phosphorylation leading to upregulation of expression of PPAR γ and C/EBP α and promoting adipogenesis (Figure 6A; Zhang et al., 2015). Hence, miR-140-5p is a positive regulator of adipogenesis. Interestingly, C/EBP α loops back to transactivate the miR-140-5p promoter to further upregulate miR-140-5p expression in a feedforward circuit to further

block TGFBR1 expression to maintain high C/EBP α protein levels (Figure 6A; Zhang et al., 2015).

On bioinformatics analysis, the seed sequence of hsa-miR-140-5p is found to be absolutely conserved in the species analyzed, including chicken (Figure 6B; Supplementary Table S1-E). The gga-miR-140-5p seed sequence is mapped in the middle segment of the relatively short 3'-UTR of the putative chicken TGFBR1 mRNA (Figure 6C). It is proposed here that gga-miR-140-5p does play a similar regulatory role in targeting TGFBR1 in modulating adipogenesis in chicken as in other species. Surprisingly, none of the genomewide studies has predicted involvement of gga-miR-140-5p and TGF- β signaling in adipogenesis in chicken (Huang et al., 2015; Chen et al., 2019b; Sun et al., 2019; Ma et al., 2020).

ERK5 Signaling

Preadipocyte differentiation also involves successive phosphorylation steps in the extracellular signal-regulated kinase 5 (**ERK5**) signaling pathway (Figure 7A; Akaike et al., 2004), independent of the MAPK/ERK1/2 phosphorylation, described under section "MAPK/ERK signaling" and Figure 5A. ERK5 is first activated by phosphorylation by mitogen-activated protein kinase kinase 5 (**MAP2K5**) (Figure 7A, route (i); Zhou et al., 1995), which, in turn, phosphorylates the PPAR γ protein to block adipogenesis (Hu et al., 1996; Akaike et al., 2004; Chen et al., 2014).

To alleviate the deregulatory effects of ERK5 signaling, miR-143-3p targets both the MAP2K5 and ERK5 transcripts, suppressing ERK5 phosphorylation of PPAR γ in a positive regulatory mode with respect to adipogenesis (Figure 7A, route (ii)). Using rat adipose-derived stem cells, it was shown that miR-143-3p inhibited differentiation by targeting both MAP2K5 and ERK5 (Chen et al., 2014). Furthermore, when a high level of the PPAR γ protein is reached, PPAR γ loops

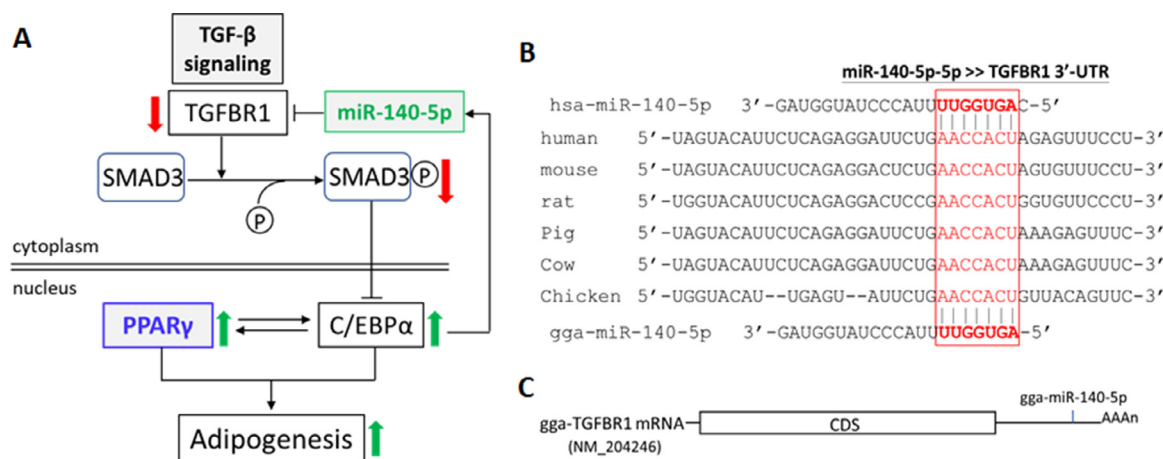


Figure 6. TGF- β signaling targeting PPAR γ in adipogenesis. (A) Modulation of PPAR γ and C/EBP α expression via TGF- β /SMAD signaling and miR-140-5p. Through TGFBR1, TGF- β signaling induces SMAD3 phosphorylation, which, in turn, suppresses the C/EBP α levels, affecting the PPAR γ -C/EBP α coregulation of adipogenesis. MiR-140-5p targets TGFBR1 to positively regulate C/EBP α and adipogenesis (thick green arrows), and C/EBP α may loop back to upregulate miR-140-5p expression to further enhance adipogenesis. (B) Bioinformatics analysis of miR-140-5p targeting TGFBR1. The seed sequence (boxed) of miR-140-5p is highly conserved in the species analyzed, including chicken. (C) Mapping of the miR-140-5p seed sequence in the 3'-UTR of the chicken TGFBR1 mRNA.

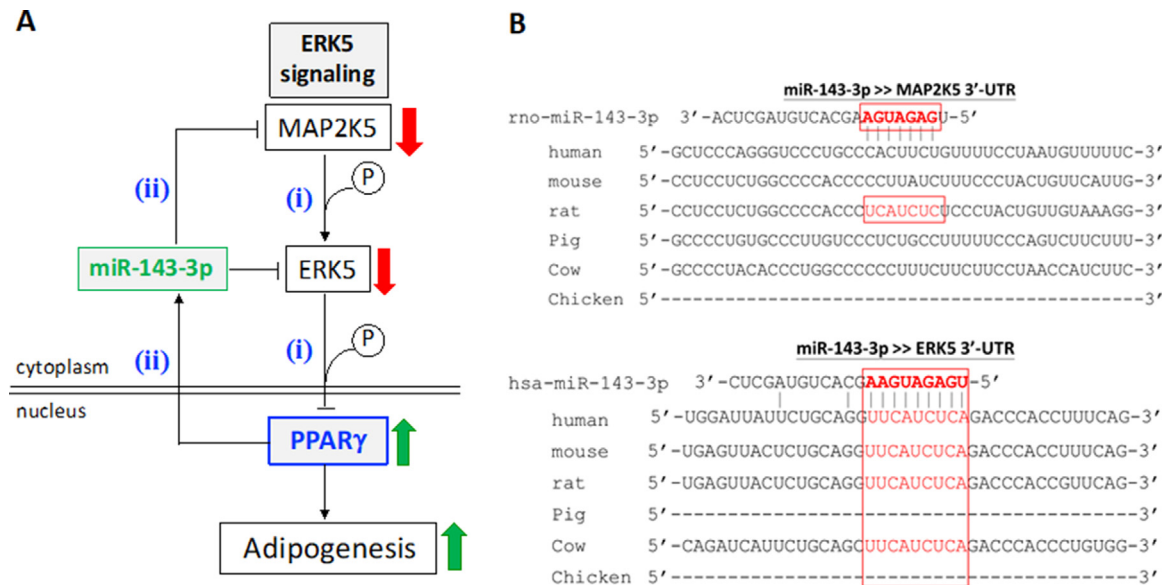


Figure 7. ERK5 signaling in adipogenesis. (A) A two-pronged regulatory mechanism involving ERK5: (i) phosphorylation (P) of MAP2K5 and ERK5 leads to downregulated PPAR γ expression; (ii) repression of ERK5 expression by miR-143-3p relieves PPAR γ suppression, and PPAR γ loops back to further activate miR-143-3p expression to upregulate PPAR γ expression. Effects of miR-143-3p are shown by thick arrows. (B) Bioinformatics analysis shows divergent and partial conservation of the miR-143-3p seed sequence in the 3'-UTR of MAP2K5 and ERK5 mRNA, respectively in different animal species. Dashed lines indicate the absences of the miRNA sequence in the pig and chicken transcripts.

back to bind to the promoter of the miR-143-3p gene to further upregulate miR-143-3p expression (Bae et al., 2017), leading to further suppression of MAP2K5 and ERK5 transcription, blocking PPAR γ phosphorylation and promoting adipogenesis (Figure 7A, thick green and red arrows; Chen et al., 2014).

Database search reveals that miR-143-3p is absolutely conserved in the species analyzed (Supplementary Table S1-F). However, the 3'-UTR sequences of MAP2K5 transcripts of different species are not conserved (Figure 7B, top panel), and the seed sequence of rat rno-miR-143-3p shown in the work of Chen et al. (2014) is not found in the 3'-UTR segments of other five species analyzed (Chen et al., 2014); chicken MAP2K5 mRNA even lacks the 3'-UTR segment harboring the seed sequence (Figure 7B, top panel). On the other hand, analysis suggests that the miR-143-3p seed sequence is found in ERK5 mRNA in rat, human, mouse and cow, but not in pig and chicken (Figure 7B, bottom panel). Taken together, miR-143-3p does not seem to participate in ERK5 signaling in the adipogenesis process in chicken. ERK5 and miR-143-3p are included here as a reminder of the fallacy of cross-species database interrogation and bioinformatics analysis without experimental validation.

OTHER ADIPOGENESIS-RELATED SIGNALING PATHWAYS

There are other signaling pathways that regulate adipogenesis through PPAR γ that are noteworthy but are not included in this review because they do not directly impact the preadipocyte differentiation step and that

there is no experimentally proven involvement of miRNA in the pathway.

Bone Morphogenetic Protein Signaling

As pointed out in section "miR-27a/b-3p", miR-27a/b-3p targets PPAR γ and gremlin 1 (GREM1) (Gu et al., 2016) to suppress preadipocyte differentiation. BMP4, a member of the bone morphogenetic protein family, is also a member of the TGF- β (section "TGF- β signaling") superfamily. In relation to BMP signaling, it suffices to note here that GREM1 is a known inhibitor of BMP4 (Gustafson et al., 2015). BMP4 signaling, acting via ZNF423, regulates mainly the adipogenic commitment step of adipogenesis (Bowers et al., 2006; Bowers and Lane, 2007; Gupta et al., 2010); also reviewed by Hammarstedt et al. (2018).

Fibroblast Growth Factor Signaling

There are 18 secreted FGF proteins that interact with 4 FGF receptors (FGFRs) (reviewed by Ornitz and Itoh, 2015). On activation, FGF signaling is linked to numerous other signaling pathways, one of which is the PI3-AKT pathway involved in FOXO1 phosphorylation (Section "PI3K/AKT (FOXO1) Signaling", Figure 4). In goat intramuscular preadipocytes, FGF21 knock-down promotes both expression of PPAR γ and C/EBP α , negatively regulating adipogenic differentiation (Xu et al., 2021). In goat cells, miR-26b-5p was upregulated during preadipocyte differentiation, and that the miRNA targeted and suppressed FGF21 expression (Ma et al., 2021). However, database interrogation did not identify a chicken FGF21 transcript (data not

Table 1. Signaling proteins that converge on the PPAR γ gateway and miRNA targeting in adipogenesis.

Figure	Signaling pathway	Proteins	miRNA: confirmed target ^a	Sequence conservation (human vs. other species) ^b		References
				mRNA 3'UTR	miRNA seed sequence	
3	Wnt signaling	Wnt LRP6 β -catenin TCF/LEF PPAR γ	miR-30e-5p: LRP6	LRP6 : highly conserved	miR-30e-5p : absolutely conserved	Wang et al., 2013; Chen et al., 2016
4	AKT signaling	PI3K/AKT FOXO1 PPAR γ	miR-15a/b-5p: FOXO1	FOXO1 : highly conserved	miR-15a/b-5p : absolutely conserved	Dong et al., 2014
5	MAP3K1/ERK signaling	MAP3K1 MAP2K1/2 ERK1/2 C/EBP α / β PPAR γ	miR-375-3p: MAP3K1	MAP3K1 : highly conserved	miR-375-3p : absolutely conserved	Ling et al., 2011; Salem et al., 2017
6	TGF- β signaling	TGFBR1 SMAD3 C/EBP α / β PPAR γ	miR-140-5p: TGFBR1; TGFBR1: miR-140-5p (loop back)	TGFBR1 : highly conserved in all species except chicken	miR-140-5p : absolutely conserved in all species	Zhang et al., 2015
7	ERK5 signaling	MAP2K5 ERK5 PPAR γ	rno-miR-143-3p: MAP2K5 miR-143-3p: ERK5; PPAR γ ; miR-143-3p (loop back)	MAP2K5 : absent in chicken; conserved in other species ERK5 : absent in pig & chicken; conserved in other species	rno-miR-143-3p : found only in rat mRNA; absent in all other species miR-143-3p : absent in pig & chicken; absolutely conserved in other species	Chen et al., 2014; Esau et al., 2004; Bae et al., 2017

^aProposed miRNA designations are used as described in the text.

^bBased on database interrogation and bioinformatics analysis; the species analyzed are human, mouse, rat, pig, cow, and chicken.

shown). Amongst other FGF members, FGF-8b has been shown in rat adipose-derived stem cells to determine the progenitor cell fate (Otsuka et al., 2021). FGF-10, acting through Krüppel-like factors, was also shown to upregulate C/EBP α , affecting PPAR γ functions in adipogenesis (Section "PPAR γ IS A GATEWAY TO ADIPOGENESIS", Figure 1B; Xu et al., 2018).

Notch Signaling

Notch signaling controls critical functions in multiple tissues through the binding of Notch ligands to Notch receptors, one of which is Notch 1; kinases are not involved in Notch signaling (Siebel and Lendahl, 2017; Shen et al., 2021). Notch1 has been shown to be both a positive and a negative regulator of preadipocyte differentiation in different systems under different experimental conditions (Shan et al., 2017). Hence, further works are needed to clarify the regulatory role of Notch1 signaling in adipogenesis. However, there has been a report that shows that on differentiation of mouse 3T3-L1 cells, miR-139-5p is upregulated, an event linked to PPAR γ downregulation and transition of preadipocytes from clonal expansion to terminal differentiation (Mi et al., 2015). In the study, the miR-139-5p regulatory effect was experimentally shown to target Notch1 and IRS1 in the Notch and IRS1/PI3K/Akt insulin signaling pathways. The seed sequence of miR-139-5p is conserved in mammals, and a gga-miR-139-5p transcript has also been reported in the GenBank database (GenBank: AM691526.1). However, gga-139-5p does not align with the chicken Notch1

mRNA sequence (data not shown) suggesting no role for this miRNA in chicken Notch1 signaling and adipogenesis.

CONCLUSIONS

In the preadipocyte differentiation step in the adipogenesis process, PPAR γ is a gateway protein that modulates expression of other downstream adipogenic genes. In this review, we show by examples how effects of interactions between signaling factors and the targeting miRNA are channeled towards PPAR γ (see summary in Table 1). We also demonstrate here that database interrogation and bioinformatics analysis may allow us to project to chicken what has been reported for other animal species (Table 1), pending experimental validation but shortening the research route to similar findings in chicken. Such bioinformatics projections may be useful for further functional appraisal in chicken of the many predicted metabolic miRNA derived from TargetScan database interrogations (Hicks and Liu, 2021), and other predicted miRNA-target mRNA pairings reported for chicken in genomewide analyses (Huang et al., 2015; Chen et al., 2019b; Sun et al., 2019; Ma et al., 2020).

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

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