RNA-MEDIATED PROCESSES IN EPIGENETICS; AN INTEGRATIVE VIEW IN THE MAINTENANCE OF HOMEOSTASIS

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RNA-MEDIATED PROCESSES IN EPIGENETICS; AN INTEGRATIVE VIEW IN THE MAINTENANCE OF HOMEOSTASIS

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EDITORIAL
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Editorial: RNA-Mediated Processes in Epigenetics; an Integrative View in the Maintenance of Homeostasis

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Editorial on the Research Topic

RNA-Mediated Processes in Epigenetics; an Integrative View in the Maintenance of Homeostasis

Epigenetics is a mechanism linking environmental factors to altered gene activity that are associated with environmental factors, and includes mechanisms such as DNA methylation, histone modifications and RNA-mediated processes. Epigenetic changes can hold the memory of the effects of environmental factors to which an individual is subjected throughout his life. These mechanisms are implicated in the selection of new phenotypes and proposed to reassess the theory of natural selection (Guerrero-Bosagna, 2017). Non-coding RiboNucleic Acids (small or long ncRNA) are involved in epigenetic regulation directly silencing or activating chromatin at specific loci or through their integral role in the machinery that drives DNA methylation. The Research Topic incorporates new data on the epigenetic effects of non-coding RNAs on generation F0, then presents the transgenerational influences of non-coding RNAs on the F1 and F2 generations.

The first group of papers explores the short-term epigenetic effects on the F0 generation detailing the molecular events related to the regulation by miRNAs and long ncRNAs.

In the bioprocessing of true miRNAs like miR-21, both the 3p and 5p molecules are expressed, each with a specific functional effect in various cells (Desvignes et al., 2015; Alles et al., 2019). Dai et al. describe the cell-type-specific functions of miR-21 in different cardiovascular diseases and its potential use in clinical therapy. The incidence of miRNA-21-5p or−3p is clearly shown opening an original perspective on the biological meaning of 3p and 5p importance in miRNA evolution (The 5p strand is present in the forward (5′-3′) position, while the 3p strand is located in the reverse position of the pre-miRNA hairpin). The paper is also rightfully pointing to the limitation of algorithms in the prediction of binding sites of miR-21 on PTEN, underlining the need for biological validation for predicted targets.

In their study of the intestine, Ruiz-Roso et al. use skillfully-designed experiments on Dicer-1 knock-out mouse and human organoids to investigate the expression of miRNA-regulated cholesterol and lipoprotein metabolism-related genes and proteins involved in the homeostatic regulatory machinery of the postprandial lipemia. In doing so, they are identifying potential novel therapeutic targets in lipid metabolism disorders. Outside the field of human diseases, Wang et al. provide a detailed insight into how miR-149-5p plays a crucial role in agronomic products derived from domestic mammals. The implication of a specific miRNA in the production of top-quality hair is not only of interest in the textile industry but also in the preservation of highly valuable domestic mammal races. Lin et al. discuss the properties of long ncRNAs as a biomarker of Tumor MicroEnvironment in bladder cancer and their links with miRNA. They emphasize cogently the importance of examining the levels of infiltrated immune cells in the tumor

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(T cells or myeloid cells). Indeed, the immune response is crucial in regard to proper cellular communication. Bosch et al. 's pioneering opinion paper has a similar focus. The TRL-7/8 toll-like receptors bind miRNAs and they are expressed on endosome membranes, suggesting that TLR-binding microRNAs transported via extracellular vesicles probably serve in stress responses.

The cellular microenvironment is also especially relevant to the growth of bone during tumorigenesis (Haider and Taipaleenmäki, 2018) and in the epigenetic regulation of skeletal impairment due to a high-fat diet (Tencerova et al., 2018). Penolazzi et al. have discussed the importance of joint homeostasis in skeletal development and are persuasive in their call for an international consortium on 3D models.

The second group of papers is exploring the long-term epigenetic effects on F1 and F2 generations.

In the literature, Harman et al. (2020) are proposing the first demonstration documenting the reprogramming of heritability in mammals to promote disease resilience in the next generation. A beneficial link of stress on the F1 generation connected with some miRNA pathway has been described on a mouse model of ophthalmic care. In nutrition, the microRNAs are now on the way to becoming new micronutrients according to Wang et al. (2018) who demonstrate the passage of microRNAs from cow's milk into consumer's plasma.

In this Research Topic, Ozkan et al. have cleverly designed a mouse model on breast milk siblings. The findings support the theory, for the first time, that the factors modifying the epigenetic mechanisms may be transmitted by breast milk and these epigenetic interactions may be transferred to offspring. These results are also suggesting hereditary epigenetic effects of cross-fostering on future generations and the impact of mother-infant dyad on epigenetic programming through miRNAs. Such works are calling for a future reappraisal of milk banking practices, but also on the conception of supplementation distributed during breast-feeding (Moro and Arslanoglu, 2020).

In addition to the stress of being breast-fed by an adoptive mother, there are others that can have deleterious consequences on adult life. Tavares et al. have identified an interesting relationship between Early Life Stress and the modulation of the serotonergic and dopaminergic systems, through post-transcriptional regulation by miRNAs. The use of miRNAs supplementation to prevent mid-term consequences of early weaning stress is under study with miR-320-3p (Tavares et al., 2020), a non-canonical miRNA with a non-described 5p form (Desvignes et al., 2015). Arzate-Mejía et al. provide a comprehensive overview of the effects of prolonged periods of social isolation with a focus on the molecular events leading to behavioral alterations (related to memory or cognition but also relevant for the modulation of mood and even of addictive behaviors). Important epigenetic modifiers such as the H3K9me2 histone methyltransferase G9a and histone deacetylases like HDAC-2 and−4, as well as regulatory ncRNAs like microRNAs, are also dysregulated, suggesting that social isolation could remodel chromatin and impact steady-state or stimulus-dependent transcriptional responses.

In conclusion, this eBook illustrates the complexity of epigenetic regulation through the valuable contributions of the authors, and is pioneering new avenues in the molecular regulation of homeostasis, diabetes, obesity and cardiovascular and psychiatric diseases related to environmental stress.

AUTHOR CONTRIBUTIONS

All authors have contributed and validated the editorial.

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Intestinal Lipid Metabolism Genes Regulated by miRNAs

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MicroRNAs (miRNAs) crucial roles in translation repression and post-transcriptional adjustments contribute to regulate intestinal lipid metabolism. Even though their actions in different metabolic tissues have been elucidated, their intestinal activity is yet unclear. We aimed to investigate intestinal miRNA-regulated lipid metabolism-related genes, by creating an intestinal-specific Dicer1 knockout (Int-Dicer1 KO) mouse model, with a depletion of microRNAs in enterocytes. The levels of 83 cholesterol and lipoprotein metabolism-related genes were assessed in the intestinal mucosa of Int-Dicer1 KO and Wild Type C57BL/6 (WT) littermates mice at baseline and 2 h after an oral lipid challenge. Among the 18 genes selected for further validation, *Hmgcs2*, *Acat1* and *Olr1* were found to be strong candidates to be modulated by miRNAs in enterocytes and intestinal organoids. Moreover, we report that intestinal miRNAs contribute to the regulation of intestinal epithelial differentiation. Twenty-nine common miRNAs found in the intestines were analyzed for their potential to target any of the three candidate genes found and validated by miRNA-transfection assays in Caco-2 cells. MiR-31-5p, miR-99b-5p, miR-200a-5p, miR-200b-5p and miR-425-5p are major regulators of these lipid metabolism-related genes. Our data provide new evidence on the potential of intestinal miRNAs as therapeutic targets in lipid metabolism-associated pathologies.

Keywords: Dicer1, lipid metabolism, small intestine, microRNA, organoids, *Hmgcs2*, *Acat1*, *Olr1*

INTRODUCTION

Lipid metabolism consists of anabolic and catabolic processes, in which the intestinal epithelium plays a very important role in maintaining systemic energy homeostasis (Zhou et al., 2020). Lipid absorption in the gastrointestinal tract is essentially carried out by the enterocytes, where digested lipids are packed into chylomicrons and secreted to circulation, through which they reach

distal tissues (Desmarchelier et al., 2019). The whole process involves considerable adjustments concerning morphological, transcriptional and posttranscriptional responses. Evidence shows that there is an increase in the uptake of fatty acids (FA) and cholesterol by the small intestine, which affects lipid metabolism gene and protein expression, in mice fed high-fat diets (HFD) (de Las Heras et al., 2017). Even though all cellular processes behind intestinal lipid metabolism are not fully deciphered, microRNAs (miRNAs), which are important posttranscriptional gene regulators (Sand, 2014; Lopez de Las Hazas et al., 2019), have been investigated in enterocytes (Gil-Zamorano et al., 2014). Indeed, miRNAs participate in processes of intestinal epithelial differentiation (Dalmasso et al., 2010) and barrier function (Ye et al., 2011). Moreover, miRNAs modulate the expression of certain lipids, cholesterol, and FA synthesis and metabolism-related genes (Gil-Zamorano et al., 2014), like 3-*Hydroxy-3-methylglutaryl coenzyme A synthase (Hmgcs)*, *Acetyl-CoA acetyltransferase (Acat)*, *fatty acid synthase (Fasn)*, *ATP-Binding Cassette Transporter A (Abca)*, *C-Protein reactive (Crp) or Scavenger receptor class B type 1* (Briand et al., 2016; Davalos-Salas et al., 2019). Yet, it is likely that many other miRNA-regulated genes involved in intestinal lipid metabolism remain unknown. Gain- and loss-of function studies showed that miRNA dysregulation may not be critical in normal tissues but can greatly affect the performance of cells and tissues undergoing stress conditions (Mendell and Olson, 2012). In this sense, the expression of miRNAs in the intestinal epithelium becomes dysregulated in several diseases, such as in various types of cancer (Ahmed et al., 2018), inflammatory bowel disease (Feng et al., 2019), necrotizing enterocolitis (Ng et al., 2015), and diabetes mellitus (Shan et al., 2016).

MiRNAs are small (19–25 nucleotides) non-coding RNA molecules, which modulate the activity of hundreds of genes and different pathways related to key biological processes in enterocytes, such as differentiation, proliferation and apoptosis (Bartel, 2009; Gadecka and Bielak-Zmijewska, 2019). Small intestine miRNAs are likely engaged in the regulation of processes such as energy homeostasis, lipid metabolism and HFD-induced weight increase (Briand et al., 2016; Mantilla-Escalante et al., 2019). MiRNAs generally repress the expression of target-genes in lipid metabolism-related pathways, such as insulin signaling, ketogenesis and homeostasis of cholesterol (Li et al., 2020; Wu et al., 2020). Nonetheless, the role of miRNAs concerning the regulation of lipid metabolism in the intestinal epithelium has not been fully investigated.

Proper miRNA production and function requires a complex machinery (Mendell and Olson, 2012; Sand, 2014). A vital element of the miRNA machinery is DICER1, a cytoplasmic RNase III type endonuclease necessary for the biosynthesis of miRNAs and small interfering RNAs (siRNAs). *Dicer1* depletion results in the build-up of miRNA precursors and in decreased levels of mature miRNAs (Huang et al., 2012; Robertson et al., 2018), which becomes useful when studying miRNAs functions.

Loss of *Dicer 1* in the intestinal epithelium has been previously established in intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mouse models, showing that depletion of microRNAs in enterocytes disturbs mouse intestinal crypts structure (McKenna et al., 2010), lipid metabolism (Mantilla-Escalante et al., 2019) and intestinal epithelial differentiation (McKenna et al., 2010). Here, the aim was to investigate the expression of miRNA-regulated cholesterol and lipoprotein metabolism-related genes and proteins involved in the homeostatic regulatory machinery of postprandial lipemia, and, in doing so, identifying potential novel therapeutic targets in lipid metabolism disorders.

RESULTS AND DISCUSSION

Lipid Metabolism Genes Modulated by the Loss of Intestinal *Dicer*1

The intestine is considered as the "gatekeeper" of intestinal lipid absorption (Krieger, 2001) and miRNAs are relevant players in lipid metabolism (Briand et al., 2016; Mantilla-Escalante et al., 2019), with peculiar roles under stress conditions (Small and Olson, 2011). Having these two concepts in mind, a mouse model lacking miRNAs in epithelial intestinal cells was created by backcrossing *Dicer1*$^{loxP/loxP}$ (The Jackson Laboratories, Bar Harbor, ME, United States) and *Villin-cre* mice (The Jackson Laboratories), showing a remarkable reduction of *Dicer1* gene expression in the small intestine and intestinal organoids compared to WT mice (**Figures 1A,B**). To search for genes directly modulated by miRNAs in response to a lipid challenge, Int-*Dicer1* KO mice were exposed to a single gavage of olive oil + cholesterol (lipid challenge) or water (controls), for 2 h (n = 5 per group). Compared to controls, *Dicer1* gene expression did not change after the lipid challenge. As expected, loss of *Dicer1* in cells expressing *Villin1* (*Vill1*) dramatically reduced the levels of DICER1 protein in the small intestine scraped mucosa from Int-*Dicer1* KO mice compared to WT (**Figure 1C**). Reduced DICER1 protein levels were accompanied by a considerable reduction of miR-192 (**Figure 1D**), one of the most expressed miRNA in the small intestines (Huang et al., 2012).

Next, the response of 83 genes directly related to cholesterol and lipid metabolism was evaluated by RT-qPCR (**Figure 1E**). After the lipid challenge, the expression of several genes (*Cyp51*, *Hmgcs1*, *Hmgcs2*, *insulin-induced gene (Insig)*, *LDL receptor (Ldlr)*, *Leptin (Lep)*, *nuclear receptor subfamily 1*, *group H*, *member 4 (Nr1h4)* and proprotein convertase subtilisin/kexin type 9 (*Pcsk9*)) was induced in WT and Int-*Dicer1* KO mice compared with controls. Moreover, the expression of *Abca1*, *Crp*, *low-density lipoprotein receptor-related protein-12 (Lrp12)*, *Lrp1b* and *Niemann-Pick C1 like 1 (Npc1l1)* was repressed, suggesting an appropriate response to dietary lipids. Besides causing detrimental effects on cardiovascular disease, fat-rich diets are linked to gastrointestinal transit disorders, although the mechanisms responsible for these actions are not entirely clear (Nezami et al., 2014). As many genes related to lipoprotein metabolism were differentially expressed in the Int-*Dicer1* KO mice, we next evaluated whether this had a physiological consequence in plasma lipid levels. We found that, after the lipid challenge, plasmatic postprandial triglycerides levels were reduced in Int-*Dicer1* KO mice compared to WT (**Figure 1F**). This effect was not observed at baseline levels.

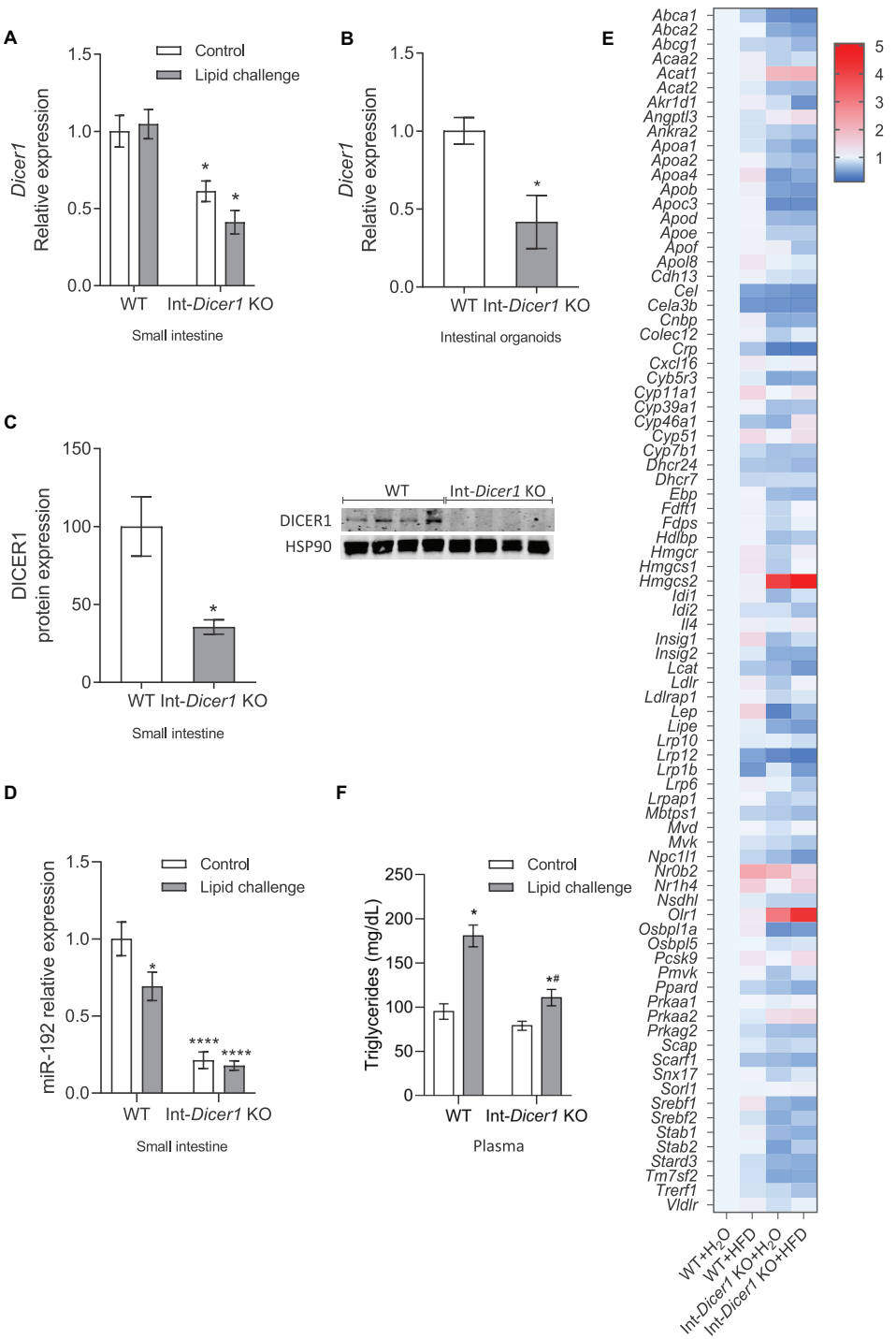


FIGURE 1 | (A) Effects of an oral high-fat dietary challenge (lipid challenge) on the gene expression of *Dicer1* in small intestine of Wild Type C57BL/6 (WT) and intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mice. *$p < 0.05$ compared to WT+H_2O mice ($n \geq 23$). **(B)** *Dicer1* gene expression in intestinal organoids isolated from WT and Int-*Dicer1* KO mice. *$p < 0.05$ compared to WT ($n = 6$). **(C)** DICER1 protein expression in small intestine scraped mucosa and intestinal organoids of WT and Int-*Dicer1* KO mice. *$p < 0.05$ compared to WT group ($n = 4$). **(D)** Intestinal levels of miR-192 in WT and Int-Dicer1 KO mice. *$p < 0.05$ compared to WT+H_2O mice ($n \geq 23$). **(E)** Effects of an oral lipid challenge on the expression of lipid and cholesterol metabolism-related genes, in small intestine from WT and Int-*Dicer1* KO mice. Upregulated genes are represented in red; downregulated genes are shown in blue. **(F)** Effect of an oral lipid challenge on plasma triglyceride levels in WT and Int-Dicer1 KO mice. *$p < 0.05$ compared to WT+H_2O, $^{\#}p < 0.05$ compared to WT+lipid challenge mice ($n \geq 23$). Data in panels **(A–D,F)** are means ± SEM. Comparison between groups by two-way ANOVA **(A,F)** or two-tailed unpaired *t*-tests **(B–D)**.

The hypothesis here was that, in the absence of *Dicer1*, genes whose expression is modulated by miRNAs would show upregulated levels or be differentially modulated in the absence of *Dicer1*. Thus, attention was set on genes whose expression increased in *Dicer1* KO mice compared to WT. Since their expression levels differed in Int-*Dicer1* KO mice compared to WT, we found 18 candidate genes of modulation by miRNAs, namely *Abca1*, *Abca2*, *apolipoprotein A4 (Apoa4)*, *Apoc3*, *Apob*, *Acat1*, *nuclear envelope-enriched activator of lipin (Cnep)*, *cytochrome b5 reductase 3 (Cyb5r3)*, *protein kinase AMP-activated* α *catalytic subunit (Prkaa)*, *orphan receptor small heterodimer partner (Nr0b2)*, *hormone-sensitive lipase (Lipe)*, *steroidogenic acute regulatory domain 3 (Stard3)*, *Hmgcs2*, *oxidized low-density lipoprotein receptor 1 (Olr1)* and a oxysterol-binding protein (*Osbpl1a)*. Although some of the above-mentioned genes, e.g., *Lipe* showed no changes or very low expression levels (**Supplementary Figure S1A**), 11 genes were more likely to be modulated by miRNAs, according to the validation assays performed in the small intestine of two different animal cohorts ($n \geq 23$ per group) (**Figure 2A**). Interestingly, among these genes, three were strong candidates for modulation by miRNAs in enterocytes and intestinal organoids, namely mitochondrial 3-Hydroxy-3-methylglutaryl coenzyme A synthase (*Hmgcs2*), acetyl-CoA acetyltransferase 1 (*Acat1*) and oxidized low-density lipoprotein receptor 1 (*Olr1*) (**Figure 2A**). These new potential miRNA-modulated genes have not been reported in previous studies and, together with other previously identified targets (Huang et al., 2012), provide additional evidence of the relevance of miRNAs in lipid metabolism.

Hmgcs2 and *ACAT1* Are Modulated by Intestinal miRNAs

Because the canonical function of miRNAs is to repress their target genes, focus was set on two candidate genes of modulation by miRNAs, i.e., *Hmcgs2* and *Acat1*, whose mRNA expression was increased in *Dicer1* KO mice. Here, *Hmgcs2* gene expression was seen to increase in small intestine and intestinal organoids of Int-*Dicer1* KO mice compared to WT, and to rise after the fat challenge (**Figures 2A,B**). Furthermore, HMCGS2 protein levels were also increased in the small intestine (**Figure 2C**) and in intestinal organoids (**Figure 2D**) of Int-*Dicer1* KO mice compared to WT. HMGCS2 catalyzes the first reaction of ketogenesis, or the formation of ketone bodies, in mitochondria of hepatocytes and gut epithelial cells, condensing acetyl-CoA with acetoacetyl-CoA to form methylglutaryl coenzyme A (HMG-CoA) (Puchalska and Crawford, 2017; Kim et al., 2019). The intestinal ability to oxidize fat and generate ketone bodies (a valuable energy resource in fasting cells) has been reported in many studies (Puchalska and Crawford, 2017). Fasting and intense lipolysis are, among other factors, ketogenesis inducers (Hegardt, 1999) and increased expression of HMGCS2 is related to increased ketogenesis in intestinal mucosa (Wang et al., 2017). Furthermore, ketone bodies are markers of mitochondrial dysfunction (Kennaway et al., 1984; Robinson et al., 1985), which can be intensified by high free cholesterol levels (Campbell and Chan, 2008). Here, mice received a single lipid challenge 2 h before sacrifice, rather than being fed a high-fat diet and this could result in a punctual increase in fat consumption at the expense of carbohydrates, promoting a situation of ketogenesis or the formation of ketone bodies. The most striking result we observed is that the absence of *Dicer1* in enterocytes leads to a significant increase in both gene and protein expression of HMGCS2, in small intestine and organoids. Therefore, it is conceivable that, in the small intestine, the physiological process of ketogenesis is highly regulated by miRNAs and that a deficiency in these molecules could affect it. Other genes involved in the metabolism of ketone bodies were also evaluated (i.e., *Bdh1* and *Hmgcl*, **Supplementary Figures S1A,B**, respectively). The induction of *Bdh1*in the intestines of Int-*Dicer1* KO mice compared to their WT littermates was statistically significant (**Supplementary Figure S1B**).

HMGCS2 contributes to the regulation of intestinal cell differentiation, modulates the balance of cell differentiation and proliferation patterns, which are associated with different intestinal pathologies (e.g., colorectal cancer, inflammatory bowel disease and necrotizing enterocolitis) (Wang et al., 2017). Furthermore, it participates in the regulation and maintenance of intestinal epithelial homeostasis (Hegardt, 1999; Kim et al., 2019). HMGCS2 expression was increased in differentiated sections of the intestinal mucosa and disturbed expression of HMGCS2 in intestinal cells impairs cell differentiation (Wang et al., 2017). In addition, intestinal cell differentiation is enhanced by ketogenesis, which also inhibits abnormal cell growth. Whether the overexpression of HMGCS2 is a consequence of a reduced differentiation of epithelial cells or responds to other mechanisms is unknown. Here, contrary to what happens in WT mice, HMGCS2 levels are affected by a fat challenge in Dicer1 KO mice (**Figure 2A**), and this dysregulation deserves further investigation.

On the other hand, ACAT1 acts in the final stage of ketone breakdown (ketolysis) in the processing of lipids and is also able to catalyze the reverse chemical reaction, promoting the first step of ketogenesis (Kano et al., 1991). Here, the increase in *Acat1* gene expression observed in small intestine and intestinal organoids from Int-*Dicer1* KO mice compared to WT mice (**Figures 2A,B**), could corroborate the notion that miRNAs in enterocytes participate in the modulation of the ketogenesis process. Nevertheless, when compared to WT mice, *Acat1* gene expression findings in enterocytes from Int-*Dicer1* KO mice were not accompanied by increased protein levels (**Figure 2C**).

Olr1 and Intestinal Epithelial Differentiation Are Modulated by Intestinal miRNAs

The loss of *Dicer1* in the small intestine causes changes in permeability and intestinal epithelial differentiation (McKenna et al., 2010). In our study, OLR1 gene and protein expressions were increased in small intestine samples from Int-*Dicer1* KO mice compared to WT mice (**Figures 2A,C**). Enhanced intestinal permeability or incorrect intestinal differentiation can induce an inflammatory response in intestinal microcirculation, which may be accompanied by a rise in the expression of *Olr1* (Al-Banna and Lehmann, 2013). OLR1 has a structure capable of

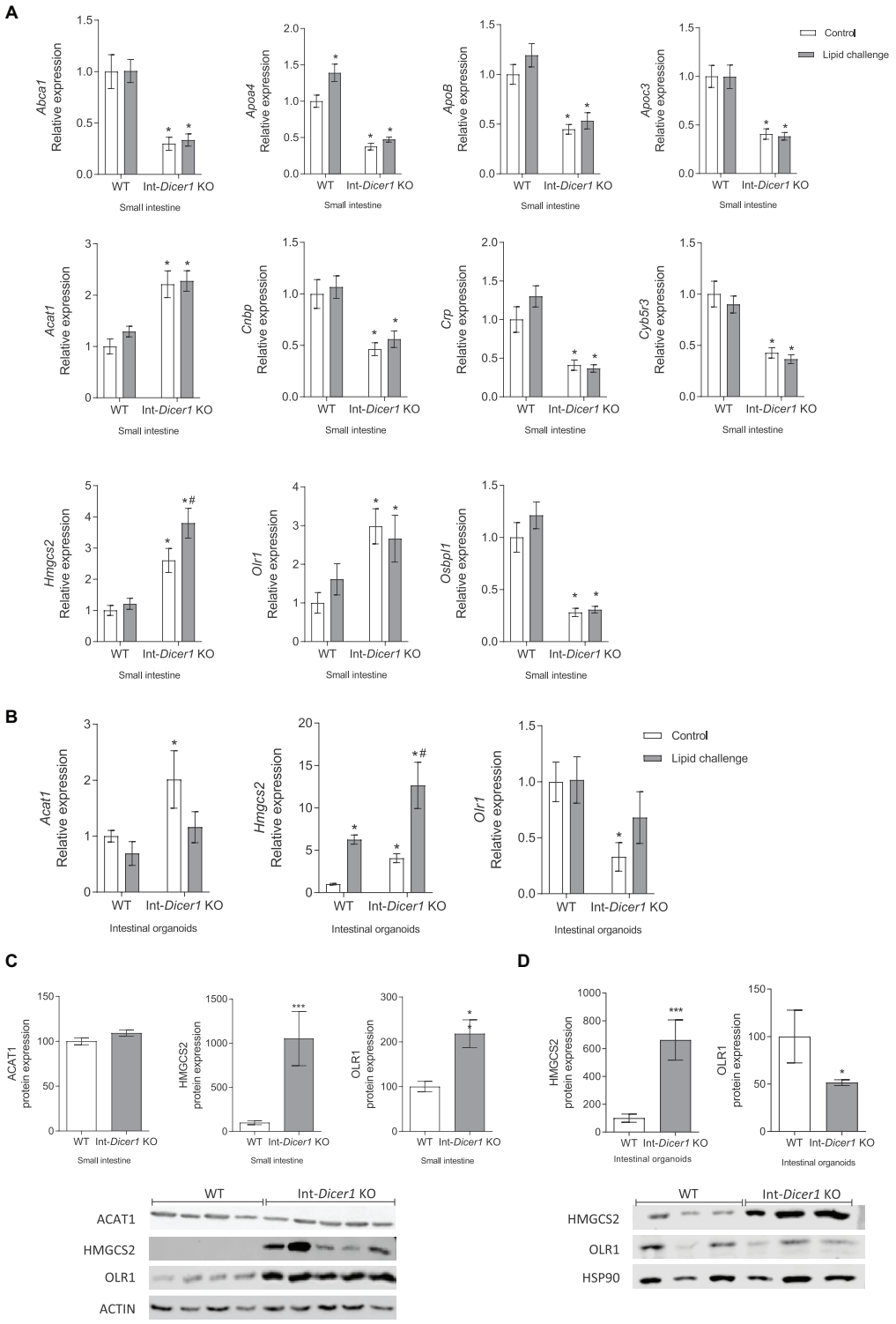


FIGURE 2 | (A) Effect of an oral high-fat dietary challenge (lipid challenge) on the expression of *Abca1*, *Apoa4*, *ApoB*, *Apoc3*, *Acat1*, *Cnbp*, *Crp*, *Cyb5r3*, *Hmgcs2*, *Olr1* and *Osbpl1*, in small intestine of Wild Type C57BL/6 (WT) and intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mice. $^{*}p < 0.05$ compared to WT+H_2O mice, $^{\#}p < 0.05$ compared to Int-*Dicer1* KO+H_2O ($n \geq 23$). **(B)** *Acat1*, *Hmgcs2* and *Olr1* gene expression in intestinal organoids isolated from WT and Int-*Dicer1* KO mice exposed to postprandial micelles of olive oil and cholesterol (PPM). $^{*}p < 0.05$ compared to control WT mice, $^{\#}p < 0.05$ compared to control Int-*Dicer1* KO group ($n = 9$). **(C)** ACAT1, HMGCS2 and OLR1 protein expression in small intestine of WT and Int-*Dicer1* KO mice. $^{**}p < 0.001$, $^{***}p < 0.0001$, compared to WT ($n = 9$). **(D)** HMGCS2 and OLR1 protein expression in intestinal organoids isolated from WT and Int-*Dicer1* KO mice. $^{*}p < 0.05$, $^{***}p < 0.0001$ compared to WT ($n = 3$). Data in all cases are means ± SEM. Comparison between groups by two-way ANOVA **(A,B)** or two-tailed unpaired *t*-tests **(C,D)**.

recognizing negatively charged substances, damaged cells, toxins, and bacteria (Wu et al., 2011; Al-Banna and Lehmann, 2013). Hence, OLR1 represents a therapeutic goal for modulating the inflammatory response in the intestinal microcirculation (Al-Banna and Lehmann, 2013). In agreement, and in contrast to what was observed in the intestinal tissue, OLR1 gene and protein expression in Int-Dicer1 KO mice was downregulated in intestinal organoids compared to WT (**Figures 2B,D**). To interpret these differences, we note that, although the intestinal organoids model approximates an *in vivo* situation better than single cell cultures. it lacks intestinal microbiota, lamina propria immune cells, and intestinal microcirculation (Angus et al., 2019).

We measured the levels of important proliferation/differentiation-related genes to assess if the aforementioned changes were associated with specific gene expression alterations. In small intestines, the expression of *Vil-1*, *Muc2*, *Chgb*, *Gata4*, *Alpi*, *Dpp4*, *Slc2a2* and *Lyz1* was significantly reduced in Int-*Dicer1* KO mice (**Figure 3A**). *Mucin 2* (*Muc2*) expression is frequently employed to identify variations in quantity functionality of intestinal goblet cells (Yu et al., 2016). *Chromogranin A (Chga)* is a marker of enteroendocrine cells (Andres et al., 2015). *Gata 4* is a marker of intestinal epithelial differentiation that is essential to preserve gut barrier function and mucosal integrity (Lepage et al., 2016). *Intestinal alkaline phosphatase (Alpi)* is a brush border enzyme that considerably diminishes the pro−inflammatory action of LPS (Parlato et al., 2018). *Dipeptidyl peptidase* (*Dpp4)* expression is increased in inflammatory bowel disease, atherosclerosis, obesity, and multiple sclerosis, implying its participation in the pathogenesis of inflammation (Zhou et al., 2019). *Solute carrier family 2 member 2 (Slc2a2)* is a β-cell glucose transporter necessary for standard glucose-stimulated insulin release (Novosadova et al., 2018). *Lysozyme 1* (*Lyz1)* is a marker of Paneth cells (Yu et al., 2016). Finally, *leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)* stands as a marker of intestinal stem cells (Yu et al., 2016). The changes seen here suggest there is a different intestinal epithelium state between both murine models and a different proliferative rate, gut barrier function and mucosal integrity in Int-*Dicer1* KO mice. These data suggest that the lack of miRNAs impacts on intestinal development and permeability by influencing intestinal stem cells and proliferating transit amplifying cells, which give rise to distinct cell types (absorptive, goblet, and enteroendocrine cells).

Furthermore, *Vil1*, *Lgr5* and *Chga* expression was increased in Int-*Dicer1* KO mice compared to WT (**Figure 3B**). Intestinal organoids were used as a model for assessing the importance of miRNAs in intestinal epithelial development. Intestinal organoids derived from a relatively pure population of intact intestinal Lgr5+ stem cell-containing crypts (Angus et al., 2019) present a morphology that closely resembles the ones obtained from the WT mice used here (**Figure 3C**). However, a deviation from the expected intestinal organoid structure was seen for organoids derived from Int-*Dicer1* KO mice. Int-*Dicer1* KO organoids showed an unstructured morphology with elongated organoids (**Figure 3D**), suggesting miRNAs play a relevant role in intestinal epithelial development.

miRNAs Candidates to Target Intestinal *Hmgcs2*, *Acat1* or *Orl1*

First, the possible miRNAs regulating these genes were bioinformatically analyzed as different miRNAs can target one gene. To do this, PITA and TargetScan were searched for miRNA-gene interactions. Twenty-six putative miRNAs capable of targeting *Hmgcs2*, *Acat1* and *Olr1* simultaneously, and 76 capable of targeting *Hmgcs2* and *Acat1* simultaneously were identified (**Figure 4A**). These results were compared with the highly enriched miRNAs expressed in intestinal epithelium (**Supplementary Figure S2**). The search was then centered in miRNAs that were both expressed in the small intestine and predicted by bioinformatic analysis, and 29 miRNAs were finally selected (**Figure 4B**).

Screening and validation *in vitro* lessens misleading estimations; thus, transfection with miRNA mimics for 24 or 48 h was performed in Caco-2 cells, and *Hmgcs2*, *Acat1* and *Olr1* gene expression levels were measured by RT-qPCR (**Figure 5**). A negative mimic miRNA with no known target served as a control. Many of these miRNA-target interactions were not validated (**Supplementary Figure S3**).

Validation Confirms Five Selected miRNAs

Our findings provide the first evidence that miR-425-5p negatively modulates the gene expression of *Hmgcs2* in Caco-2 cells (24 and 48 h) (**Figure 5A**). The miR-425 family (including miR-425-3p and miR-425-5p) participates in biological processes occurring in the gastrointestinal tract, among other tissues, and its abnormal expression is associated with the progression of several diseases (Lu et al., 2019). Interestingly, the increase in miR-425 levels is useful for fat production in muscle cells and adipocytes (Qi et al., 2019). According to the results shown here, miR-425-5p appears to be important in the regulation of lipid metabolism, ketogenesis, and intestinal differentiation in enterocytes.

Even though miR-31-5p, miR-99b-5p and miR-200a-5p exhibited significant *Acat1* gene expression downregulation capacity, 24 h after transfection, only the former exerted this effect also after 48 h (**Figure 5B**). MiR-31-5p, miR-99b-5p and miR-200b-5p downregulated *Olr1* levels in Caco-2 culture cells, at 24 and 48 h (**Figure 5C**). To determine whether the five above-mentioned miRNAs were modulated in the intestines of Int-*Dicer1* KO mice, we evaluated their expression by RT-qPCR (**Figure 5D**). We found that miR-425-5p, miR-31-5p, miR-200a-5p and miR-200b-5p were dramatically repressed in the small intestine of Int-*Dicer1* KO mice, which is in accordance with the depression seen for *Hmgcs2*, *Acat1* and *Olr1*. Related studies have found that levels of miR-31-5p were enhanced in inflamed mucosa from patients with ulcerative colitis and Chron's disease, and play a fundamental role in epithelial cell regeneration (Gupta et al., 2019; Tian et al., 2019). Mir-99b-5p is a marker of inflammation in tissues other than the intestine (Hildebrand et al., 2018) and miR-200b-5p was linked with the heightened expression of interleukin 8 (IL-8), CXCL2, IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6 in upper genital

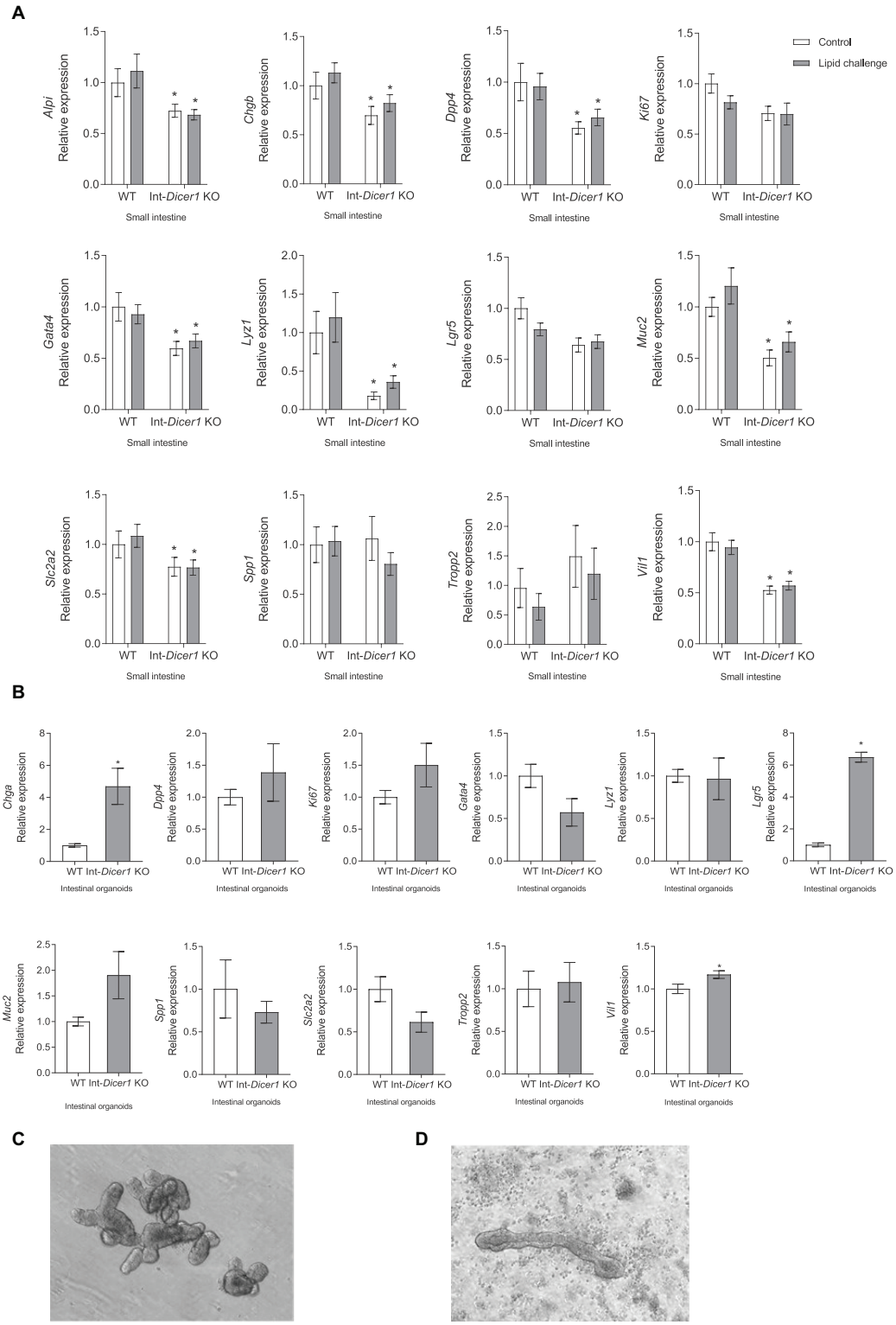
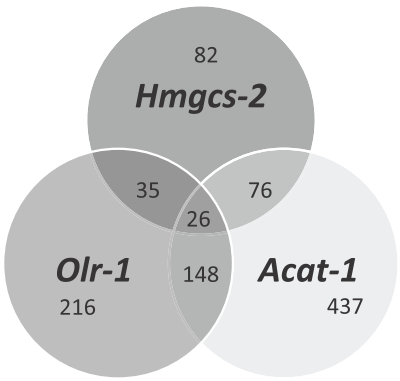


FIGURE 3 | (A) Effect of an oral high-fat dietary challenge (lipid challenge) on the expression of *Alpi*, *Chgb*, *Dpp4*, *Ki67*, *Gata4*, *Lyz1*, *Lgr5*, *Muc2*, *Slc2a2*, *Spp1*, *Tropp2* and *Vill1*, in small intestine of Wild Type C57BL/6 (WT) and intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mice. Data are means ± SEM. Comparison between groups by two-way ANOVA. *$p < 0.05$ compared to WT+H_2O mice ($n \geq 23$). **(B)** *Chga*, *Dpp4*, *Ki67*, *Gata4*, *Lyz1*, *Lgr5*, *Muc2*, *Slc2a2*, *Spp1*, *Tropp2* and *Vill1* gene expression in intestinal organoids isolated from WT and Int-*Dicer1* KO mice. Data are means ± SEM. Comparison between groups by two-tailed unpaired t-tests. *$p < 0.05$ compared to WT group ($n = 9$). **(C)** Light microscope visualization (10×) of mature intestinal organoids isolated from WT mice. **(D)** Light microscope visualization (10×) of mature intestinal organoids from Int-*Dicer1* KO mice.

A



B

miRNA	Mature sequence	Mirbase ID
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU	MI0000066
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	MI0000108
hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU	MI0000734
hsa-miR-107	AGCUUCUUUACAGUGUUGCCUUG	MI0000114
hsa-miR-1224-5p	GUGAGGACUCGGGAGGUGG	MI0003764
hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA	MI0000469
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	MI0000446
hsa-miR-132-5p	ACCGUGGCUUUCGAUUGUUACU	MI0000449
hsa-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCUG	MI0003129
hsa-miR-152-5p	AGGUUCUGUGAUACACUCCGACU	MI0000462
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	MI0000071
hsa-miR-182-5p	UUUGGCAAUGGUAGAACUCACACCG	MI0000272
hsa-miR-185-5p	UGGAGAGAAAGGCAGUUCCUGA	MI0000482
hsa-miR-18a-5p	UAAGGUGCAUCUAGUGCAGAUAG	MI0000072
hsa-miR-192-5p	CUGACCUAUGAAUUGACAGCC	MI0000234
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	MI0000489
hsa-miR-200a-5p	CAUCUUACCGGACAGUGCUGGA	MI0000737
hsa-miR-200b-5p	CAUCUUACUGGGCAGCAUUGGA	MI0000342
hsa-miR-200c-5p	CGUCUUACCCAGCAGUGUUUGG	MI0000650
hsa-miR-203a-5p	AGUGGUUCUUAACAGUUCAACAGUU	MI0000283
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	MI0000076
hsa-miR-215-5p	AUGACCUAUGAAUUGACAGAC	MI0000291
hsa-miR-27a-5p	AGGGCUUAGCUGCUUGUGAGCA	MI0000085
hsa-miR-31-5p	AGGCAAGAUGCUGGCAUAGCUG	MI0000089
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU	MI0001445
hsa-miR-425-5p	AAUGACACGAUCACUCCCGUUGA	MI0001448
hsa-miR-455-5p	UAUGUGCCUUUGGACUACAUCG	MI0003513
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	MI0000095
hsa-miR-99b-5p	CACCCGUAGAACCGACCUUGCG	MI0000746

FIGURE 4 | (A) PITA and TargetScan algorithms were run to predict 26 common miRNAs putatively regulating *Hmgcs-2*, *Acat-1 and Olr-1*. **(B)** miRNAs selected comparing the bioinformatic analysis with the list of 173 miRNAs expressed in intestinal epithelium.

tract disease (Yeruva et al., 2017). Further, increased intestinal permeability or incorrect intestinal differentiation can induce an inflammatory response in the intestinal microcirculation, which may be accompanied by a rise in the expression of *Olr1* (Al-Banna and Lehmann, 2013). Hence, results suggest that miR-31-5p, miR-99b-5p, and miR-200b-5p negatively modulate the gene expression of *Olr1* and could represent a novel therapeutic target against intestinal inflammatory processes.

CONCLUSION

Loss of Dicer1 in mouse intestinal epithelial cells unveiled three miRNA-regulated genes, i.e., *Hmgcs2*, *Acat1* and *Orl1*. Intestinal organoids isolated from these mice exhibited different morphological features from those of wild type mice. Although further validation is needed, we provide evidence that miR-425-5p, miR-31-5p, miR-99b-5p, miR-200a-5p and miR-200b-5p can function as major regulators of lipid metabolism-related genes in the intestine. Moreover, in view of the results, we hypothesize that miR-425-5p is involved in the expression level changes in ketogenesis-related genes observed after the dietary lipid challenge. Further insight on the role of miRNAs as modulators of the molecular actions in response to dietary cholesterol, lipid homeostasis and intestinal ketogenesis may reveal other potential therapeutic targets involved in the regulation of lipid and cholesterol metabolism, as well as ketogenesis, in the gut. In this sense, more *in vivo* research is needed before firm conclusions can be drawn in terms of therapeutic approaches to modulate intestinal miRNAs in health and disease.

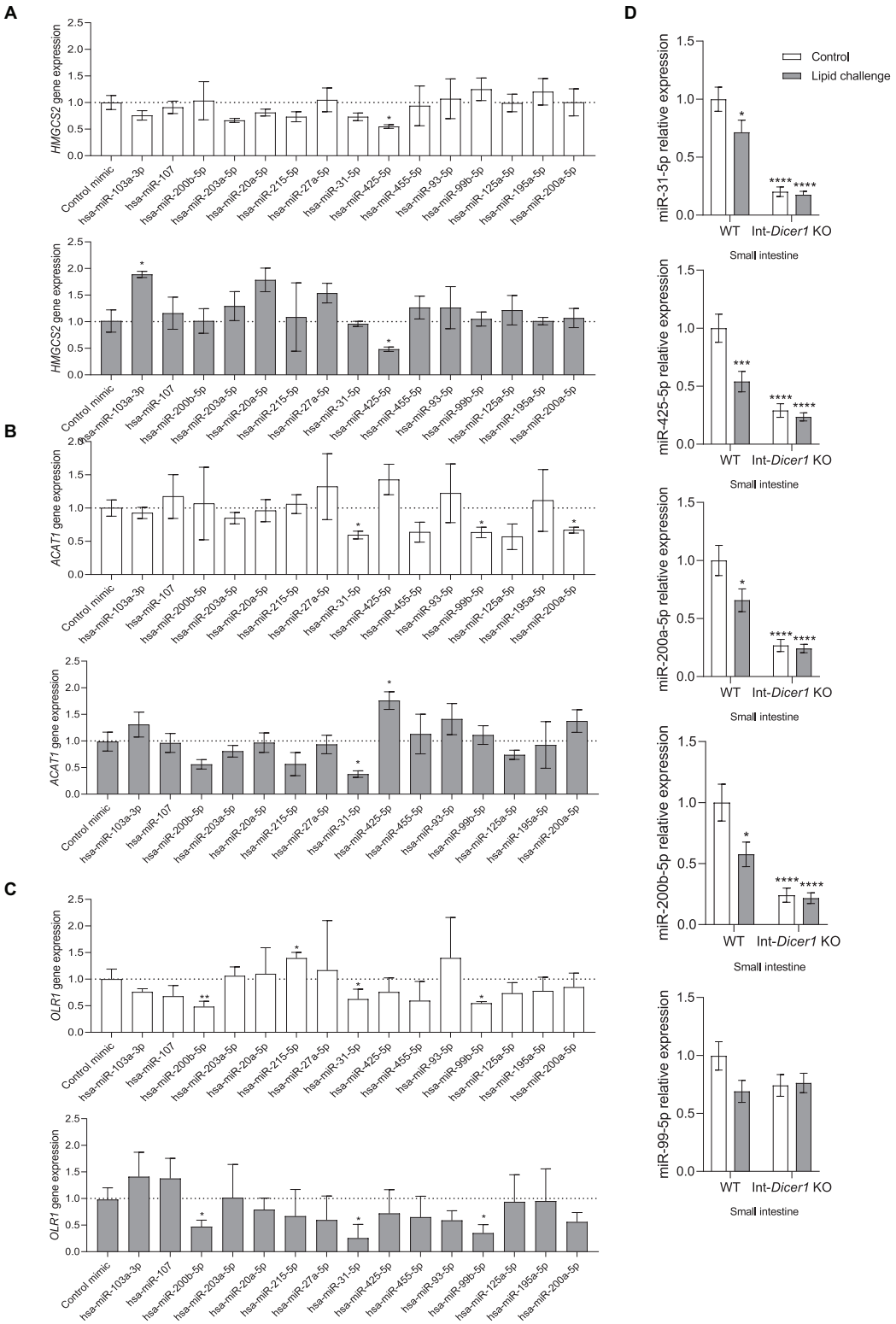


FIGURE 5 | Effects of several hsa-miRs on the expression levels of **(A)** *Hmgcs2*, **(B)** *Acat1* and **(C)** *Olr1*, in Caco-2 cells transfected for 24 or 48 h. Data are means ± SEM. Comparison between groups by two-tailed unpaired *t*-tests. $^{*}p < 0.05$ compared to control group. $^{**}p < 0.001$ compared to control group ($n = 4$). **(D)** Effects of an oral high-fat dietary challenge (lipid challenge) on the expression of selected miRNAs, in small intestine of Wild Type C57BL/6 (WT) and intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mice. Data are means ± SEM. Comparison between groups by two-way ANOVA. $^{*}p < 0.05$ compared to WT+H_2O mice ($n \geq 23$).

MATERIALS AND METHODS

Animals

Animal practices were performed in agreement with the guidelines of the European Communities Directive 86/609/EEC controlling animal research and was approved (Proex 281/15 and Proex 282/15) by the Animal Ethics Committee of the Ramón y Cajal Hospital (Madrid, Spain). Int-*Dicer1* KO and wild type (WT) littermates [fl/fl, tg(-)] C57BL/6J mice, 8–10 weeks old, were used in all experiments. Mice were housed in a standard animal facility and maintained in a temperature- ($25 \pm 2°C$) and light- (12 h light–dark cycles) controlled room. Food and water were available *ad libitum*.

Study Design

Male and female C57BL/6 WT littermates and Int-*Dicer1* KO mice were distributed into two experimental groups. Mice received (by oral gavage) either a high-fat dietary challenge (lipid challenge), consisting on the administration of 250 μL olive oil enriched with 40 mg of cholesterol, or water (controls). Two hours after administration, mice were anesthetized with ketamine/xylazine, sacrificed by exsanguination and perfused with phosphate-buffered saline (PBS).

Sample Collection

Blood samples were immediately collected in EDTA tubes and centrifuged at $1,500 \times g$, for 15 min, at 4°C, to obtain plasma. In addition, small intestine samples, including scraped mucosa, were collected and immediately frozen in liquid nitrogen. All samples were stored at −80°C.

Isolation of Mouse Small-Intestinal Crypts and Organoid Culture

Intestinal crypts were isolated from the small intestine of C57BL/6 WT [fl/fl, tg(-)] mice or littermates from intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mice, according to Stemcell's recommended protocol [Technical Bulletin: Intestinal Epithelial Organoid Culture with IntestiCult™ Organoid Growth Medium (Mouse)]. Intestinal crypts were plated in 24-well tissue culture plates (Corning, NY, United States) (200 crypts per well) and incubated at 37°C and 5% CO_2 until passage.

Caco-2 Culture Cells and Transfection

Caco-2 cells were acquired from the American Type Culture Collection (ATCC). Cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, CA, United States), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, United States) at 37°C in a humidified incubator with 5% CO_2.

All miRNAs were synthesized by Cultek Molecular Bioline (Madrid, Spain). Caco-2 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States), according to the manufacturer's recommendations, and 20 μM miRNAs diluted in Opti-MEM® (Invitrogen, Carlsbad, CA, United States). For conventional aqueous transfection, 4.5×10^4 cells per well were cultured in 24-well plates, for 24 h. Then, Lipofectamine 2000-miRNA complexes diluted (1:5) in culture medium were added. Medium was replaced 24 or 48 h after the transfection.

Intestinal Organoids Treatment

Organoids were incubated in 24-well tissue culture plates and treated with postprandial micelles (including olive oil and cholesterol, PPM) or DMEM (control group), for 24 h. Artificial micelles were prepared according to a previously described method (Briand et al., 2016). The final composition of this micelles was 0.6 mM oleic acid (OA), 0.2 mM L-α-lysophosphatidylcholine, 0.05 mM cholesterol, 0.2 mM 2-monooleylglycerol, 2 mM taurocholate. The emulsion was sonicated twice for 30 min. The micelle solution was incubated for 2 h at 37°C. Each compound was added to the micelle solution and incubated for a further 2 h at 37°C.

RNA Isolation and qRT-PCR

Total RNA was extracted from tissue samples with TRIzol reagent (Invitrogen, Carlsbad, CA, United States), according to the manufacturer's instructions. Total RNA was isolated using the miRNeasy Mini Kit (Qiagen). cDNA was synthesized using Taqman Reverse Transcription Reagents (Applied Biosystems). qRT-PCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosytems), using the miScript SYBR Green PCR kit (Qiagen). mRNA levels were normalized to those of the housekeeping gene *beta-Actin* and *Gapdh*. Relative expressions were calculated by the comparative threshold cycle method and presented as a relative expression ratio ($2\text{-}\Delta\Delta$ threshold cycle). For miRNA quantification, total RNA was reverse-transcribed using the miScript II Reverse Transcription Kit (Qiagen). Specific primers for each miRNA (miScript Primer Assay) were also obtained from Qiagen. MiRNA levels were normalized to that of the housekeeping RNU6 (U6). List of specific oligos for mRNA genes are described in **Supplementary Figure 4**.

Western Blotting

Proteins were separated on SDS-PAGE gels under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, United States). Membranes were blocked for 1 h with 5% (w/v) bovine serum albumin (BSA) as a blocking agent (Sigma, Madrid, Spain) in PBS with Tween 20 (PBST; 1% PBS, 0.1% Tween20; v/v) at room temperature. After washing, membranes were probed overnight, at 4°C, with appropriate primary antibodies: HMGCS2 1:1000 (D3U1A, Cell signaling technology), ACAT1 1:1000 (CSB-PA001134LA01HU, Cusabio technology), DICER1 1:2000 (A301-936A, Bethyl laboratories) and OLR1 (CSB-PA016331LA01HU, Cusabio technology). After washing, membranes were incubated for 1 h with peroxidase-conjugated rabbit or mouse anti-goat IgG secondary antibody (1:10,000). For detection, ECL Advance Western Blotting Detection kit (Amersham Bioscience, Amersham, United Kingdom) was used. Blots were probed with rabbit monoclonal anti-Actin antibody (1:10,000, Abcam, Cambridge, United Kingdom) or rabbit monoclonal anti-HSP90 antibody (1:10,000, Abcam, Cambridge, United Kingdom) as internal

control, to normalize between gels. Quantification was expressed as the percentage of relative protein expression (protein/Actin or HSP90) vs. control group.

Gene Expression Profiling of Small Intestine by RT2-PCR Array

Expression of 83 genes involved in lipid and cholesterol metabolism was analyzed by PCR array using a 384-well 96 × 4 Mouse Lipoprotein & Cholesterol Metabolism RT2 Profiler PCR Array Kit (Qiagen) and a 7900 HT Fast Real-Time PCR Software (Applied Biosystems). Gene expression was normalized to the mean of all house-keeping genes in the array.

Bioinformatic Analysis to Identify miRNAs-Gene Interactions

Two different algorithms were implemented simultaneously in order to identify miRNA-gene interactions: PITA and TargetScan. TargetScan (Friedman et al., 2009) uses the degree of sequence complementarity as the primary key parameter to identify miRNA-mRNA interactions. PITA (Kertesz et al., 2007) utilizes thermodynamics as the main criterion. A prediction was considered valid whenever it co-occurred in at least two algorithms.

Statistical Analysis

Data are shown as means ± standard error of the mean (SEM). Statistical analyses consisted of two-way analysis of variance (ANOVA) (genotype × lipid challenge), followed by Tukey's *post-hoc* tests, or two-tailed unpaired *t*-tests. A significance level of $p < 0.05$ was applied to all statistical analyses. GraphPad Prism 8 (version 8.3.0; Graph Pad Software Inc., San Diego, CA, United States) was used for all statistical analyses.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal research was approved (Proex 281/15 and Proex 282/15) by the Animal Ethics Committee of the Ramón y Cajal Hospital (Madrid, Spain).

AUTHOR CONTRIBUTIONS

AD: idea and coordination of work. MR-R: bioinformatic analysis, mathematical analysis, statistical treatment of the data and wrote the manuscript, prepared figures and illustrations. MR-R, JG-Z, MCL, OB, DS-L, MC, MJL, JT-C, and AD: performed experiments. JG-Z and AO: veterinary work. JT-C, FV, and AD: edited the manuscript. MR-R, AD, FV, OB, and JM: administrative support and discussion. All the authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00707/full#supplementary-material

FIGURE S1 | Effect of an oral high-fat dietary challenge (lipid challenge) on the expression of **(A)** *Abca2*, *Lipe*, *Nrob2*, and *Prkaa* (validation assays were not performed), **(B)** *Bdh1* and **(C)** *Hmgcl*, in small intestine of Wild Type C57BL/6 (WT) and intestinal-specific *Dicer1* knockout (Int-*Dicer1* KO) mice. Data are means ± SEM. Comparison between groups by two-way ANOVA **(A)** or two-tailed unpaired *t*-tests (B,C). $^*p < 0.05$ compared to WT+H_2O mice or WT ($n \geq 23$ per group).

FIGURE S2 | List of 173 miRNAs expressed in intestinal epithelium and their relative mean expression.

FIGURE S3 | Effect of selected miRNAs on relative gene expression of *Hmgcs-2*, *Acat-1* and *Olr-1* in Caco-2 cells after 24 and 48 h. Data are means relative to control.

FIGURE S4 | Sequences of quantitative RT-PCR Primers.

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MINI REVIEW
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Early Life Stress and the Onset of Obesity: Proof of MicroRNAs' Involvement Through Modulation of Serotonin and Dopamine Systems' Homeostasis

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Healthy persons hold a very complex system for controlling energy homeostasis. The system functions on the interconnected way between the nutritional, endocrine, neural, and epigenetic regulation, which includes the microRNAs (miRNAs). Currently, it is well accepted that experiences of early life stress (ELS) carry modification of the central control of feeding behavior, one of the factors controlling energy homeostasis. Recently, studies give us a clue on the modulation of eating behavior, which is one of the main factors associated with the development of obesity. This clue connected the neural control through the serotonin (5HT) and dopamine (DA) systems with the fine regulation of miRNAs. The first pieces of evidence highlight the presence of the miR-16 in the regulation of the serotonin transporter (SERT) as well as the receptors 1a (5HT1A) and 2a (5HT2A). On the other hand, miR-504 is related to the dopamine receptor D2 (DRD2). As our knowledge advance, we expected to discover other important pathways for the regulation of the energy homeostasis. As both neurotransmission systems and miRNAs seem to be sensible to ELS, the aim of this review is to bring new insight about the involvement of miRNAs with a central role in the control of eating behavior focusing on the influences of ELS and regulation of neurotransmission systems.

Keywords: miRNA, early life stress, obesity, serotonin, dopamine

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Abbreviations: 3′UTR, 3′Untranslated region; 5HT, 5-Hydroxytryptamine, serotonin system; 5HT1A, 5-Hydroxytryptamine receptor 1A; 5HT1B, 5-Hydroxytryptamine receptor 1B; 5HT2C, 5-Hydroxytryptamine receptor 2C; 5HT4, 5-Hydroxytryptamine receptor 4; 5HT6, 5-Hydroxytryptamine receptor 6; 5HT7, 5-Hydroxytryptamine receptor 7; AgRP, protein related to gene agouti; cAMP, cyclic adenosine monophosphate; CART, cocaine and amphetamine-related transcript; CpGs, methylated cytosines follow of guanine nucleotide sites; CUMS, chronic unpredictable mild-life stress; CUS, chronic unpredictable stress; DA, dopamine, dopamine system; DAT, solute carrier family 6, neurotransmitter transporter dopamine member 3, SLC6A3; Dicer, microRNA-processing ribonuclease III; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2; DRD3, dopamine receptor D3; DRD5, dopamine receptor D5; ELS, early life stress; EW, early weaning; has-mir-16, MI0000070, MI0000115; Let-7d, hsa-let-7d-5p, mmu-let-7d-5p, rno-let-7d-5p (MIMAT0000065, MIMAT0000383, MIMAT0000562); miR-103, has-mir-103a-1 MI0000109, has-mir-103a-2 MI0000108; miR-143-3p, hsa-miR-143-3p, mmu-miR-143-3p, rno-miR-143-3p (MIMAT0000435, MIMAT0000247, MIMAT0000849); miR-16-5p, hsa-miR-16-5p, mmu-miR-16-5p, rno-miR-16-5p (MIMAT0000069, MIMAT0000527, MIMAT0000785); miR-200a, rno-miR-200a-3p, mmu-miR-200a-3p, hsa-miR-200a-3p (MIMAT0000682, MIMAT0000519, MIMAT0000874); miR-96, hsa-miR-96-5p, mmu-miR-96-5p, rno-miR-96-5p (MIMAT0000095, MIMAT0000541, MIMAT0000818); miRNAs, microRNAs; mmu-miR-135, MI0000161, MI0000715; mPFC, medial pre-frontal cortex; mRNA, Messenger RNA; MS, maternal separation; NAcc, nucleus accumbens; NPY, neuropeptide Y; PLC, prelimbic cortex; PND, postnatal day; POMC, pro-opiomelanocortin; REST, repressor element-1 silencing transcription factor; RNAs, ribonucleic acids; SERT, solute carrier family 6 member 4 (SLC6A4/5HTT), serotonin transporter; TH, tyrosine hydroxylase.

INTRODUCTION

Almost over one-third of the world's population is overweight or obese (Chooi et al., 2019). This condition negatively affects the life quality, productivity, and costs with public health. One of the main aspects of this body weight regulation is feeding behavior (Remmers and Delemarre-van de Waal, 2011) which involves neural networks such as the serotonergic (5HT) and dopaminergic (DA) systems (Meguid et al., 2000). Several studies show that the disruption of those systems is strongly associated with increased food intake and/or preference for palatable food, which are important factors contributing for the onset of obesity (van Galen et al., 2018). Gene expression of both 5HT and DA systems can be influenced by miRNAs (Launay et al., 2011; Shi et al., 2014), and in this case, they would be the key regulatory molecules in the comprehension of the pathophysiology of the feeding behavior.

MiRNAs are small non-coding RNAs with an average length of approximately 22 nucleotides (Bartel, 2004). They regulate post-transcriptional gene expression by binding to the 3′UTR of mRNAs, some miRNAs also regulate the expression of another or several other miRNAs (Truscott et al., 2016), and even themselves (Zisoulis et al., 2012). Generally, miRNA specifically inhibit protein synthesis either by repressing translation or by inducing deadenylation and degradation of target mRNA (Bartel, 2004) but were also reported to activate translation (Huntzinger and Izaurralde, 2011). Each miRNA has the capacity to target hundreds of diverse transcripts, and a single messenger can be modulated by several miRNAs, this represents a highly coordinated system and fine-tuned regulation of protein expression (Krol et al., 2010; O'Carroll and Schaefer, 2013).

On the other hand, a healthy environment during the beginning of life is crucial for a proper development in mammals (Resnick et al., 1979; Morgane et al., 1993 2002). Maternal nutritional and emotional factors are critical during periconceptional and perinatal periods (Morgane et al., 1993; Chen and Baram, 2016). Early life stress (ELS) experiences can lead to long-term neurobehavioral complications. Both pre-clinical and clinical studies identify the influence of ELS on the development of several psychiatric disorders, including perturbation of feeding behavior, eating disorders and obesity (Chen and Baram, 2016; Entringer et al., 2016). Interestingly, the miRNAs are also sensible to ELS through several models, as showed on **Table 1**. In this context, this review brings a potential role of the miRNAs in the onset of obesity through modulation of 5HT and DA in response to ELS.

SEROTONIN: ROLE ON FEEDING BEHAVIOR, INFLUENCES OF ELS, AND MIRNA REGULATION

The 5HT system includes receptors, transporters and enzymes involved in the metabolism of serotonin (5-Hydroxytryptamine), and it regulates several functions in the organism as locomotors activity, body temperature, wake-sleep cycle, and feeding behavior (Lam et al., 2010; Olivier, 2015). Regarding the control of eating behavior, serotonin has a well-established anorectic role through promotion of satiety. In the arcuate nucleus of the hypothalamus, serotonin acts in different ways; it acts on 5HT1B, promoting inhibition of neurons that produce neuropeptide Y (NPY) and the cocaine and amphetamine-related transcript (CART), which are orexigenic. It also acts on 5HT2C, promoting activation of neurons that produce pro-opiomelanocortin (POMC) and the peptide related to the agouti gene (AgRP), which are anorexigenic, thus promoting satiety signaling (Heisler et al., 2006). In addition, recent studies also refer that serotonin has a role in the hedonic regulation of eating behavior. Receptors such as 5HT6 in areas of the mesocorticolimbic circuit have been associated with motivational feeding behavior (da Silva et al., 2017). The impairments of the homeostasis of the serotonergic system are associated with disorders of eating behavior, usually associated with increased food intake, either by homeostatic or hedonic changes. In particular, the serotonergic system appears extremely sensitive to environmental changes during the development of the organism, and several studies have shown that ELS impairs the function of the 5HT system (de Lima et al., 2020).

Models of ELS in animals are usually associated with deprivation of the mother–infant relationship, such as in maternal separation (MS) and early weaning (EW) models (Kikusui and Mori, 2009; Harrison and Baune, 2014). Previous studies from our laboratory show that the MS disrupts the 5HT system. In middle aged females, it increases the 5HT1B gene expression in the hypothalamus, associated with decreased food intake (de Souza et al., 2020a), and in adult males, we observed a decreased action of fluoxetine on food intake (de Souza et al., 2020b). In addition, MS promotes decreased 5HT concentration in hypothalamus and amygdala of young animals, associated with increased palatable food intake (de Lima et al., 2020). Together, these data suggest that MS alter the serotonergic system function, contributing to disorders of feeding behavior. On the other hand, we have been able to associate the EW with changes in gene expression of several components of the 5HT system in male and female rats, such as SERT, 5HT1B, and 5HT2C in hypothalamus and brainstem. Based on the patterns of expression in the brainstem and response to fenfluramine, we suggested a hypofunction of the serotonergic system in the EW animals (Tavares et al., 2019, 2020a,b). All these changes in the 5-HT system were accompanied by alterations on feeding behavior, which indicate that the 5HT system control of feeding behavior can be modulated by ELS, which can be directly linked to the onset of obesity.

Recently, studies have deepened about these compensatory changes and epigenetic modifications have been extensively investigated. In this respect, miRNAs have been shown to be important regulators y/o mediators of gene expression. In the case of depression, it is currently accepted that several miRNAs modulate the activity of the serotonergic system, but little is known about these regulators in the context of eating behavior. As far as we know, miR-16 is able to bind the SERT messenger

TABLE 1 | Influences of ELS on miRNA activity.

ELS model	Subjects	Region	Outcome	Authors
Pre-clinical studies				
MS	Rat	Hippocampus	Increased miR-16	Bai et al., 2012
MS	Mice	Cortical neurons	Impaired response of miR-212 to the learning process on a cocaine conditioned place preference test	Viola et al., 2016
MS + CUS	Rat	NAcc	Increased miR-504	Zhang et al., 2013
MS	Rat	mPFC	Increased REST4	Uchida et al., 2010
Morphine + Apnea + MS	Mice	Hippocampus	Decreased miR-204-5p, miR-455-3p, miR-448-5p, and miR-574-3p	McAdams et al., 2015
CUMS	Rat	Basolateral amygdala	Increased rno-miR-124a	Xu et al., 2017
Increased maternal care	Rat	Hypothalamus	increased rno-miR-488, rno-miR-144, and rno-miR-542-5p and decreased rno-miR-421 and rno-miR-376b-5p	Vogel Ciernia et al., 2018
Protein malnutrition	Mice	Hypothalamus	increased mmu-miR-187-3p, mmu-miR-369-3p and mmu-miR-132-3p	Berardino et al., 2019
Unpredictable maternal separation combined with maternal stress	Mice	Sperm	Changes in miRNA transmitted to F2 generation	Gapp et al., 2014
Prenatal stress	Rat	Hippocampus	Decreased hsa-miR-125b-1-3p	Cattane et al., 2019
Clinical studies				
Childhood maltreatment	both sexes	Leukocytes	Methylation changes in CpGs close to region coding miR-124-3	Prados et al., 2015
Childhood abuse	Men aged 45 years old	Whole blood	Methylation changes in promoter region of 39 miRNAs	Suderman et al., 2014
Child abuse	European adults of both sexes	Buccal mucosa cells	Association between the polymorphism rs3125 of 5HT2A and brooding. This region is predicted to be targeted by miR-1270, miR-1304, miR-202, miR-539 and miR-620	Eszlari et al., 2019
Childhood trauma	Adult of both sexes	Blood cells	Decreased hsa-miR-125b-1-3p	Cattane et al., 2019
Childhood trauma	Adult both sexes	Human hippocampus progenitor cells	Decreased hsa-miR-125b-1-3p	Cattane et al., 2019

ELS, early life stress; MS, maternal separation; CUS, chronic unpredictable stress; CUMS, chronic unpredictable mild-life stress; NAc, nucleus accumbens; mPFC, medial pre-frontal cortex; CpGs, methylated cytosines followed by guanine nucleotide sites.

(**Table 2**) and silence its expression in humans and animals (Baudry et al., 2010; Moya et al., 2013; Song et al., 2015; Shao et al., 2018). The relationship between miR-16 and SERT is even modulated by pharmacological antidepressant treatment and also alternative treatments as the electroacupuncture; besides different responses according to the affected brain area, these treatments improve the level of depressive behaviors, suggesting a highly specific regulation (Baudry et al., 2010; Zhao et al., 2019). SERT appears to be a key piece of regulation, as different miRNAs can modify its expression, as the mmu-miR-135 (Issler et al., 2014), rno-miR-18a-5p, rno-miR-34a-5p, rno-miR-135a-5p, rno-miR-195-5p, rno-miR-320-3p, rno-miR-674-3p, and rno-miR-872-5p (Zurawek et al., 2017). This relationship between miR-16 and SERT is interesting, since SERT activity is directly related to serotonergic signaling. SERT recaptures the remaining amount of serotonin from the synaptic clefts, and an increase in its activity may mean a decrease in serotonergic signaling. In depression, has been shown that decreased levels of miR-16 and elevated levels of SERT are associated with the pathology by promoting a reduction in serotonergic signaling. Drugs that block SERT activity and increase serotonin levels are used to treat this depressive behavior. Interestingly, the same drugs are used to treat obesity (Halford et al., 2012) as they also promote a reduction in food intake. This evidence gives a primary role to miR-16 that may also be a candidate to modulate SERT activity in the context of eating disorders.

In addition to SERT, miRNAs modulate the activity of other components of the serotonergic system (**Table 2**), such as 5HT1B, 5HT1A, 5HT4, 5HT2C, and 5HT7. The 5HT1B receptor is advised as a target of the miR-96 (Jensen et al., 2009). 5HT1A seems to be targeted by miR-16, miR-135 (Liu et al., 2017), and has-miR-26a-2 (Xie et al., 2019). The 5HT4 receptor acquire decreased expression in response to miR-103, has-miR-15b and a mix containing hsa-miR-103, has-miR-15b and hsa-miR-16 (Wohlfarth et al., 2017). In addition, miR-34 appears to bind the receptor 5HT2C (Andolina et al., 2016), hsa-miR-16 appears to reduce 5HT2A expression (Yang et al., 2017), and miR-29a decreases the expression of 5HT7 (Volpicelli et al., 2019). The impairment of the activity of these receptors is associated with disrupted food intake either by homeostatic or hedonic mechanisms. 5HT1A, 5HT1B, and 5HT2C are strongly associated with satiety signaling, and several studies report that their

TABLE 2 | Components of the serotonergic and dopaminergic systems and their regulatory-associated miRNAs.

Components	miRNAs	Authors
Serotonin or 5-Hydroxytryptamine (5HT) system		
SERT (SLC6A4/5HTT)	miR-16, miR-135, miR-18a-5p, miR-34a-5p, miR-135a-5p, miR-195-5p, miR-320-3p, miR-674-3p, and miR-872-5p.	Baudry et al., 2010; Launay et al., 2011; Moya et al., 2013; Issler et al., 2014; Song et al., 2015; Zurawek et al., 2017; Shao et al., 2018; Zhao et al., 2019
5HT1A	miR-16, miR-135, and miR-26a-2.	Liu et al., 2017; Xie et al., 2019
5HT1B	miR-96	Jensen et al., 2009
5HT2A	miR-16	Yang et al., 2017
5HT2C	miR-34	Andolina et al., 2016
5HT4	miR-103, miR-15b, and a mix containing miR-103, miR-15b, and miR-16	Wohlfarth et al., 2017
5HT7	miR-29a	Volpicelli et al., 2019
Dopamine (DA) system		
DRD1	miR-504, miR-105, miR-15a, miR-15b, miR-16 and miR-142-3p	Tobón et al., 2012, 2015; Zhang et al., 2013; Zhao et al., 2017; Wu et al., 2020
DRD2	miR-143, miR-200a, miR-504, has-miR-9 and miR-326	Zhang et al., 2013, 2015; Shi et al., 2014; Gangisetty et al., 2017; Wu et al., 2018; Mavrikaki et al., 2019; Wang et al., 2019
DRD3	let-7d	Bahi and Dreyer, 2018
DAT	miR-137 and miR-491	Jia et al., 2016

SERT, solute carrier family 6 member 4 (SLC6A4/5HTT), serotonin transporter; 5HT1A, 5-Hydroxytryptamine receptor 1A; 5HT1B, 5-Hydroxytryptamine receptor 1B; 5HT2C, 5-Hydroxytryptamine receptor 2C; 5HT4, 5-Hydroxytryptamine receptor 4; 5HT7, 5-Hydroxytryptamine receptor 7; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2; DRD3, dopamine receptor D3; DAT, dopamine transporter, solute carrier family 6 member 3 (SLC6A3).

disruption promotes increased food intake. On the other hand, 5HT4 is associated with hedonic modulation of food intake and obesity. Thus, the modulation of these receptors through miRNAs can also be associated with the onset of eating disorders leading to obesity.

DOPAMINE: ROLE ON FEEDING BEHAVIOR, INFLUENCES OF ELS, AND MIRNA REGULATION

The dopaminergic system, as well as the serotoninergic system, comprises a set of neurotransmitter, enzymes, receptors, and dopamine transporter (DAT). On the other hand, neurons that synthesize dopamine can be found in the brainstem and can be divided into three groups, which forms the Nigro Striatal system, the mesocorticolimbic system, and the mesocortical system (Ogawa and Watabe-Uchida, 2018). The principal role on feeding behavior is taken by the mesocorticolimbic system (Wise, 1989; Berridge and Kringelbach, 2008). Dopaminergic neurons are known to be involved in emotion-based behavior including motivation and reward (Phillips et al., 2008). Therefore, in the context of the feeding behavior, this system is mainly related to the hedonic component of feeding, but evidences also point out that dopamine is a key component on hypothalamic regulation of the homeostatic eating behavior (Meguid et al., 2000; Ikeda et al., 2018).

The DA system is sensible to ELS and its disruption is associated with several psychiatric disorders, such as eating disorders and obesity (Naef et al., 2015). Our previous study showed that DRD1 and DRD2 gene expression were increased in the brainstem of adult rats, accompanied by higher palatable food intake after MS (de Souza et al., 2018). The MS also modulates the DA system in other brain areas, such as PLC, NAcc, and striatum, changing the density of immunoreactive fibers of TH, and the mRNA expression of DRD2, DRD1, and DRD5 (Majcher-Maślanka et al., 2017). On the other hand, EW increases DRD1 mRNA expression in the hypothalamus and brainstem and DRD2 in the brainstem of middle-aged male rats (Tavares et al., 2020b). In all of these studies, disrupted patterns on feeding behavior are observed, indicating that alterations in the dopaminergic system can be one of the underlying mechanisms that lead to behavioral disorders.

Increased evidence points out that several components of the dopaminergic system are influenced by some miRNAs (**Table 2**). DRD1 appears to be regulated by miR-504 (Zhang et al., 2013), rno-miR-105 (Zhao et al., 2017), and for the cluster of hsa-miR-15a-5p, hsa-miR-15b-5p, and hsa-miR-16-5p, and mmu-miR-142-3p (Tobón et al., 2012, 2015). The expression of the DRD2 is modified by miR-143-3p (Wang et al., 2019), miR-200a (Wu et al., 2018), miR-504 (Zhang et al., 2013), hsa-miR-9, and hsa-miR-326 (Shi et al., 2014; Zhang et al., 2015; Gangisetty et al., 2017; Mavrikaki et al., 2019). Both receptors, DRD1 and DRD2, are associated with control of food intake, either homeostatic or hedonic, in several areas of the brain (Wise, 1989; Ikeda et al., 2018) which indicates that its modulation through miRNAs can modulate the food intake. In addition, overexpression of let-7d is negatively correlated with the expression of DRD3 in the hippocampus of mice (Bahi and Dreyer, 2018). The activity of the DRD3 is controversy in the context of food intake, but some evidences associate it with eating disorders and decreased food intake (Thomsen et al., 2017; González et al., 2019). The expression of DAT, the major controller of dopamine levels in the synaptic clefts, is post-transcriptionally regulated on cell culture of dopaminergic neurons by miR-137 and miR-491 (Jia et al., 2016). This transporter acts like SERT, reuptaking the dopamine from the synaptic cleft, so its function is extremely necessary to normal DA signalization, even in the context of eating behavior. On the other hand, the reduction of Dicer, a miRNA-processing ribonuclease III, in the ventral midbrain of DA neurons promotes changes in the miRNAs profile and altered the survival capacity of these dopaminergic neurons (Chmielarz et al., 2017). Together, these evidences extended the susceptibility of the DA system to the regulation of miRNAs, which can lead to modulation of eating behavior and may be associated with eating disorders.

PERSPECTIVES: ROLE OF THE MIRNAS ON THE ONSET OF OBESITY THROUGH 5HT AND DA SYSTEMS' DISRUPTION IN THE CONTEXT OF ELS

In addition to knowing that components of the 5HT and DA neurotransmission systems are susceptible to ELS, some evidence also shows that miRNAs have their expression and activity influenced by ELS, which is summarized in **Table 1**. Both, pre-clinical and clinical studies affirm that childhood trauma could be associated with the modulation of miRNA, as the case of the miR-16 and miR-504 which have their control of the serotonin and dopamine impaired by stress, with consequences such as depression, anhedonia, and body weight gain. However, more studies are needed to understand the full picture, specifically in the context of the control of the feeding behavior, which is directly involved in the development of obesity.

Conversely, both clinical and pre-clinical studies demonstrate that ELS is able to alter SERT activity (Wankerl et al., 2014; Van Der Knaap et al., 2015; Tavares et al., 2019, 2020a). Interestingly, differences in SERT activity are observed in obesity, both in humans and animals (Giannaccini et al., 2013; Borgers et al., 2014; Zha et al., 2017). For example, the density of SERT is reduced in obese humans (Giannaccini et al., 2013; Borgers et al., 2014) and increased in rats with abdominal obesity who were exposed to a diet rich in simple carbohydrates (Spadaro et al., 2015). In addition to being involved in the pathophysiology of obesity and being sensitive to ELS, several lines of evidence in the literature show that SERT is a target for miR-16 and propose an important role in regulating its activity (Baudry et al., 2010). On the other hand, animal studies demonstrate that the 5HT1A receptor is also modulated by ELS (Bravo et al., 2014; Razoux et al., 2017) and has increased density in the hippocampus and hypothalamus of rats chronically submitted to a Westernized diet (Yu et al., 2018). Interestingly, 5HT1A is also the target of miR-16, which has its expression modulated by ELS (Bai et al., 2012). The receptor 5HT2A is, as well, modulated by ELS in animals and humans (Rentesi et al., 2013; Parade et al., 2017) and involved with the pathophysiology of obesity (Rosmond et al., 2002; Huang et al., 2004). Interestingly, the 5HT2A is also targeted by the miR-16 (Yang et al., 2017). From these observations, we believe that miR-16 is an excellent candidate for moderating changes in SERT, 5HT1A, and 5HT2A due to ELS, in the context of the altered eating behavior.

Regarding the dopaminergic system, the DRD1 and DRD2 actively participate in the regulation of food intake, especially with regard to palatable foods, as these are related to the food reward system (Meguid et al., 2000; Berridge et al., 2009; Volkow et al., 2011). Changes in this reward system are linked to eating behavior disorders, with changes in the activity of DRD1 and DRD2 being observed in humans and animals (Guo et al., 2014; Rivera et al., 2015; Gaiser et al., 2016; de Souza et al., 2018; Romanova et al., 2018; Tavares et al., 2020b). In addition, both receptors are modulated by ELS (de Souza et al., 2018; Tavares et al., 2020b). Interestingly, we observed that miR-504 targets both DRD1 and DRD2, with their expression being altered by ELS (Zhang et al., 2015). Additionally, DRD1 has also been identified as a target for miR-16 (Wu et al., 2020). Thus, we believe that miR-504 and miR-16 modulate DRD1 and DRD2, in the context of eating disorders associated with ELS.

In summary, according to the evidence reported, we can infer that the serotonergic and dopaminergic systems undergo regulation of their activity through post-transcriptional modulation by miRNAs. Both systems participate in the physiological and pathological processes of eating behavior, which leads us to believe that miRNAs may be behind several changes in eating behavior as observed in several disorders such as obesity. Several studies point out that the genesis of these disorders is largely associated with experiences of stress early in life. Neonatal stress is already well described as a modulator of the serotonergic and dopaminergic systems associated with disorders of eating behavior, as well as a modulator of expression and activity of miRNAs. In addition, we know that miRNAs participate in the pathological processes of several psychiatric disorders. Thus, we establish here a relationship between neonatal stress and the modulation of the serotonergic and dopaminergic systems, through post-transcriptional regulation by miRNAs, as a possible pathophysiological mechanism behind eating behavior disorders. Future studies are needed to investigate this relationship and provide further support for the scientific community in the search for understanding and treatment of pathologies of eating behavior.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Epigenetic Programming Through Breast Milk and Its Impact on Milk-Siblings Mating

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Background: The epigenetic effects of transmission of certain regulatory molecules, such as miRNAs, through maternal milk on future generations, are still unknown and have not been fully understood yet. We hypothesized that breastfeeding regularly by adoptive-mother may cause transmission of miRNAs as epigenetic regulating factors to the infant, and the marriage of milk-siblings may cause various pathologies in the future generations.

Results: A cross-fostering model using a/a and A^{vy}/a mice had been established. F2 milk-sibling and F2 control groups were obtained from mating of milk-siblings or unrelated mice. Randomized selected animals in the both F2 groups were sacrificed for miRNA expression studies and the remainings were followed for phenotypic changes (coat color, obesity, hyperglycemia, liver pathology, and life span). The lifespan in the F2 milk-sibling group was shorter than the control group (387 vs 590 days, $p = 0.011$) and they were more obese during the aging period. Histopathological examination of liver tissues revealed abnormal findings in F2 milk-sibling group. In order to understand the epigenetic mechanisms leading to these phenotypic changes, we analyzed miRNA expression differences between offspring of milk-sibling and control matings and focused on the signaling pathways regulating lifespan and metabolism. Bioinformatic analysis demonstrated that differentially expressed miRNAs were associated with pathways regulating metabolism, survival, and cancer development such as the PI3K-Akt, ErbB, mTOR, and MAPK, insulin signaling pathways. We further analyzed the expression patterns of miR-186-5p, miR-141-3p, miR-345-5p, and miR-34c-5p and their candidate target genes Mapk8, Gsk3b, and Ppargc1a in ovarian and liver tissues.

Conclusion: Our findings support for the first time that the factors modifying the epigenetic mechanisms may be transmitted by breast milk and these epigenetic interactions may be transferred transgenerationally. Results also suggested hereditary epigenetic effects of cross-fostering on future generations and the impact of mother-infant dyad on epigenetic programming.

Keywords: breast milk, epigenetic regulatory mechanisms, miRNA, transgenerational inheritance, cross-fostering, life span

BACKGROUND

As an epigenetic regulator, breast milk provides growth factors, immune factors, microbiota, stem cells, and microRNAs (miRNAs) (Kosaka et al., 2010; Hassiotou et al., 2012; Alsaweed et al., 2016; Melnik and Gerd, 2017). Lactation-specific miRNAs are secreted as extracellular vesicles (exosomes) derived from mammary gland epithelial cells, reach the systemic circulation of the newborn infant, mediates cellular communication between the mother and her nursing infant and then, may exert gene regulatory functions in the infant (Kosaka et al., 2010; Baier et al., 2014; Arntz et al., 2015; Alsaweed et al., 2016). The epigenetic effects of transmission of certain regulatory molecules, such as miRNAs, through maternal milk on future generations, are still unknown and have not been fully understood yet.

Currently, there are more than 2000 miRNAs that have been discovered in humans and it is believed that they collectively regulate one third of the genes in the genome. For instance, human milk provides abundant amounts of miRNA-148a, miR-152, miR-29b, and miR-21, which all target DNA methyl transferases (DNMTs) that potentially affect whole genome DNA methylation patterns leading to genome-wide DNA hypomethylation, and thus modifies gene expression (Bodo and Melnik, 2017; Melnik and Gerd, 2017). Continued uptake of milk-derived exosomes that carry DNMTs targeting miRNAs may promote diabesity, allergy, neurodegenerative diseases, and cancer later in life (Melnik and Schmitz, 2017).

The milk of each mammal is unique for its own offspring. However, due to various problems caused by the baby or mother, babies can not be fed with their own mother's milk. In such cases, alternative breastfeeding practices allowing human milk sharing, such as cross—fostering or donor-milk banking are being considered (Arslanoglu et al., 2010; Palmquist and Doehler, 2016). In traditional cross-fostering, a wet nurse is a lactating woman who breastfeeds a child who is not her own; and individuals who are not biological siblings but breastfed by the same woman are defined as milk-siblings. To our knowledge, there is no study exist in the literature evaluating the issue of milk-siblingship and milk-sibling marriage on the basis of scientific evidence.

Until recently, transition of hereditary material only attributed to the Mendelian law. Therefore, it was impossible to understand how milk-sibling marriage could lead to heritable genetic transmission and increased risk of genetic diseases in future generations. Recent advances in epigenetic science have shown that there may be new and different perspectives. After the discovery of epigenetic regulatory factors, especially miRNAs, in breast milk, the milk-sibling hypothesis was first proposed in 2012 by our study group (Ozkan et al., 2012).

In this study, we hypothesized that breastfeeding regularly by adoptive -mother will cause transmission of some miRNAs as epigenetic regulating factors to the infant, and the marriage of milk-siblings may cause various pathologies in the future generations (Ozkan et al., 2012). Since it was impossible to create a model that will adapt our hypothesis to the human, an experimental model using a/a and A^{vy}/a mice on C57Bl6J background had been established to test this hypothesis. To our knowledge, this is the first study evaluating the issue of cross-fostering, and milk-sibling mating on the basis of scientific evidence.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Dokuz Eylul University Medical Faculty, Animal Care and Use Committee. All experiments were performed in accordance with relevant guidelines and regulations. A^{vy}/a and a/a mice on C57Bl6J background obtained from Missouri University, MMRRC laboratory via cryopreservation were used (RRID: MMRRC_000375-MU). All subjects were kept under standard conditions with a 12:12-h light-dark cycle and had free access to tap water and a standard pellet diet.

Study Groups and Cross Fostering

Heterozygous viable yellow males (A^{vy}/a) were paired with non-mutant females (a/a; generation F0). Each mother had 6-9 pups (F1 generation) with a continuum of coat colors from all brown (Y0) to all yellow (Y5) with transitional mottled patterns (Y1–Y4). Cross fostering was performed by removing pups from one dam and transferring them to another lactating dam with pups of the same approximate age (Lohmiller and Swing, 2006). To establish a successful cross-fostering model and reduce the stress of dams and pups, previous published guidelines were followed (Fahrenkrog et al., 2004; Lohmiller and Swing, 2006). Three offspring (F1) from each mother were randomly selected, marked with intradermal staining, and transferred to the other dam at postnatal day (PND)1 (**Figure 1**). Pups were reared in these nests until weaning on PND28. At the end of the fourth week, the offspring were separated from the mother and started to be fed with a standard mice diet (Bilyem standard mouse pellet, Ankara, Turkey: 23% crude protein, 8% ash, 7% crude cellulose, 5% crude fat, and 2,900 kcal/kg). Beginning at

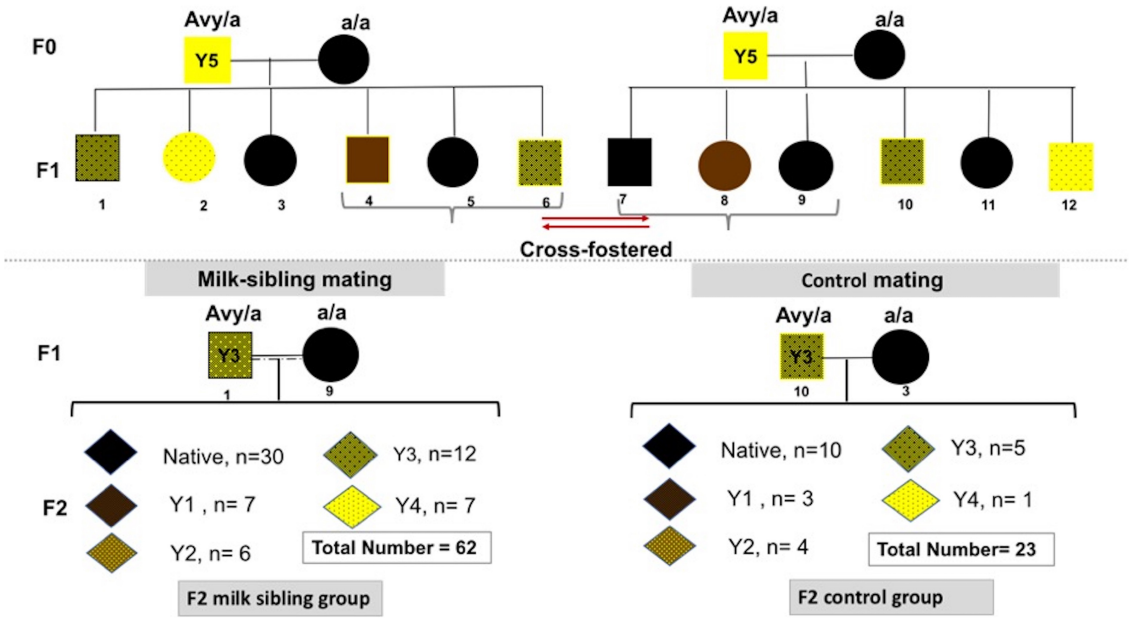

FIGURE 1 | Experimental scheme demonstrating cross-fostering. A^{vy}/a male and a/a female mice (F0) were mated. The numbers under the puppies are given as a representation to explain the model. Three offspring (F1) from each mother were randomly removed and transferred to the other dam at postnatal day 1. Beginning at the postnatal 6th week, F1 mice were mated with their cross-siblings or non-consanguineous mice to obtain F2 milk-sibling or F2 control groups. Starting from the F0 generation, the cross-fostering model was repeated 3 times and recurrent matings of certain animals in the F1 generation were performed to produce F2 control and F2 milk-sibling groups. Distribution of F2 offspring coat color in milk-sibling and control groups after repeated matings is given.

the postnatal 6th week, F1 mice were mated with their milk-siblings or unrelated mice to obtain the F2 milk-sibling group and F2 control group. Starting from the F0 generation, the cross-fostering model was repeated 3 times. Proper animals in the F1 generation were mated repeatedly to produce F2 control and F2 milk-sibling groups. Finally, a colony composed of 122 animals (F1 and F2 generation) was yielded.

Experimental Procedures in the Offspring

Since miRNAs in breast milk and tissues can vary in quantity and quality during different periods of breastfeeding and development, breast milk from mothers and tissues from developing puppies were obtained on PN10 to provide standardization. On PND10, when coat colors became clear, all of the animals were photographed and coat colors were classified from Y0 to Y5 by two researchers (Cooney et al., 2002). After coat color determination on PND10, 25 pups in the F2 milk-sibling group and 9 pups in the F2 control group were sacrificed.

Liver and gonad tissues of sacrificed animals were placed in liquid nitrogen in a protective container and stored at −80°C for real-time quantitative polymerase-chain-reaction (qPCR) studies. The remaining animals (37 pups in the F2 milk-sibling group and 14 pups in the F2 control group) were followed long term for phenotypic changes.

Follow Up of Phenotypic Characteristics

At follow-up, biweekly body weight measures, fasting blood glucose levels at 5th and 8th month, and changes in the animals' appearance or behavior were monitored closely. Whole blood glucose level was measured using a hand-held whole-blood glucose monitor (FreeStyle Optium Neo Blood Glucose and Ketone Monitoring System, sponsored by Abbott Diabetes Care) after 6 h of fasting period. Blood samples (5 μl or less) were taken from tail tip after needle punctuation. Sex and species specific reference ranges were used to define abnormal levels (Ayala et al., 2010; Benede-Ubieto et al., 2020). Lifespan of each animal were recorded individually. The autopsy of the animals resulting in spontaneous mortality was performed and macroscopically observed pathological changes were recorded. Additionally, three animals from each group were sacrificed at about 18 months and liver tissues evaluated by histological methods.

RNA Isolation

Total RNA was isolated from breast milk (F0 and F1 generations) and liver and gonad tissues (F2 generation) using TRIZOL (Roche, Germany; Trakunram et al., 2019). Concentration and purity of RNA for each sample were confirmed using a Nanodrop1000 (Thermo Fisher Scientific, MA, United States).

miRNA Expression Levels

cDNA was synthesized from the obtained RNA samples using a cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA samples were quantified using the Biomark Real Time PCR System for expression levels of 384 miRNAs (Tan et al., 2015).

The expression profiles of 384 miRNAs were determined using the following panels: miRNA serum plasma (MIMM-106Z, Qiagene, Germany), Neurological Development and disease (MIMM-107Z, Qiagene), Immunopathology (MIMM-104Z, Qiagene) and Cell Development and Differentiation (MIMM-103Z, Qiagene). ACTB and GAPDH were used as housekeeping genes. All samples were performed in duplicate and the values were normalized using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). All stages of PCR studies were performed at Erciyes University's Betul-Ziya Eren Genome and Stem Cell Center (GENKOK).

Reverse Transcription and Pre-amplification Reactions

Isolated RNA samples were reverse-transcribed into cDNA in 5 μl final reaction volumes using the TaqMan MicroRNA Reverse Transcription Kit (P/N: 4366596; Life Technologies, Foster City, CA, United States). All reactions were performed as specified in the manufacturer's protocol: 2 μl total RNA were added to 3 μl RT reaction mix (Megaplex RT Primers 10X dNTPs with 100 mM dTTP, 50 U/μl MultiScribe Reverse Transcriptase, 10X RT Buffer, 25 mM $MgCl_2$, 20 U/μl RNase Inhibitor, and nuclease free water). Reverse transcription was performed using a SensoQuest GmbH Thermal Cycler (Göttingen, Germany). Reaction conditions were 16°C for 120 s, 42°C for 60 s, and 50°C for 1 s repeated for 40 cycles. The final step was 85°C for 300 s and 4°C for at least 600 s until further processing or storage. cDNA samples were stored at −80°C until PCR analysis. Pre-amplification was performed following reverse transcription using the TaqMan SybrMaster Mix (P/N 4309155; Life Technologies). All reactions were performed as specified in the protocols of the manufacturer. For pre-amplification, 2 μl 1:5 diluted RT product was added to 3 μl PreAmp mix. miRNA TaqManPreAmp Thermal Protocol was performed using a SensoQuest GmbH Thermal Cycler (Göttingen) as follows: 95°C for 600 s, 55°C for 120 s, and 72°C for 120 s followed by 18 cycles of 95°C for 15 s, 60°C for 240 s, and 600 s at 99.9°C followed by a rest period at 4°C (Peng et al., 2019).

qPCR

Quantitative Real-Time PCR reactions (qPCR) were performed using the high-throughput BioMark Real-Time PCR system (Fluidigm, South San Francisco, CA, United States). Pre-amplified cDNA samples were diluted with low EDTA (0.1 mM) TE Buffer (1:5). TaqManSybr Master Mix (490 μl; P/N 4309155; Life Technologies) and GE Sample Loading Reagent (49 μl; P/N 85000746; Fluidigm, South San Francisco, CA, United States) were mixed and 3.85 μl was pipetted into a 96 well plate with 3.15 μl 1:10 diluted pre-amplified cDNA into each well and mixed. Then, 5 μl of this mixture and 4 μl 1:1 diluted assay mixture were pipetted into assay inlets of a 96.96 Dynamic Arrays (Fluidigm). The BioMark IFC controller HX (Fluidigm) was used to distribute the assay and sample mixes from the loading inlets into the 96.96 Dynamic array reaction chambers for qPCR by Fluidigm's Integrated Fluidic Circuit Technology. qPCR steps were performed using the BioMark System according to the following protocol: 50°C for 120 s, 70°C for 1.800 s, and 25°C for 600 s. Then UNG and Hot start protocol were performed at 50°C for 120 s and initial denaturation at 95°C for 600 s. Finally, PCR cycles followed with 30 cycles at 95°C for 15 s (denaturation) and 60°C for 60 s (annealing) (Kocyigit et al., 2017). We used a combination of two reliable housekeeping genes' [GAPDH (coding for glyceraldehyde 3-phospate dehydrogenase) and Beta-actin] expression for internal normalization after a preliminary test of the relative expression variance of these two internal controls across different sample types (Tan et al., 2015).

In silico Identification of miRNA Target Genes and Related Pathways

To identify potential target genes of the significantly differentially expressed miRNAs, we conducted an *in silico* analysis using DIANA Tools with mirPath v2.0, microT-CDS v5.0[1] that support all analyses for KEGG (Kyoto Encyclopedia of Genes and genomes, https://www.genome.jp/kegg/) molecular pathways, as well as multiple slices of Gene Ontology (GO, http://geneontology.org/) in Mus musculus (Kanehisa and Goto, 2000; Vlachos et al., 2012; Kanehisa et al., 2016). Analysis using DIANA were performed with the default parameters (p value threshold: 0.05, microT threshold: 0.8) (Vlachos et al., 2012). The web server identifies miRNAs targeting the selected pathway and ranks them according to their enrichment p values. We implemented functional enrichment analysis of miRNA target genes using annotation from the KEGG Pathway Database (Kanehisa and Goto, 2000; Kanehisa et al., 2016). Additionally, to functional annotation of miRNAs and miRNA combinations DIANA and KEGG as well as GO were used using all datasets or their subsets (genes union and pathways union parameters were selected). The biology process terms with $p < 0.05$ were considered statistically significant. Targeted Pathways clusters/heatmaps were generated from DIANA. By selecting Targeted Pathways Clusters/heatmap, miRpath flags all the significant pathways (with p values < 0.05) with 0 and the other pathways with 1. The miRD[2], microRNA.org-Targets and Expression, and miRbase: the microRNA database were also used to predicted miRNA-target interactions (http://www.microrna.org/microrna/home.do; http://mirbase.org/; http://mirdb.org/). Ensembl and miRbase were used to support miRNA nomenclature history (http://mirbase.org/; http://www.ensembl.org/index.html). The combination of validated and predicted miRNA-target interactions were used for further analyses with qPCR.

Determination of Gene Expression Levels

Gsk3b, Mapk8, and Ppargc1a genes were selected among miRNA target genes and expression levels were determined in all samples. To determine the expression levels of these genes, cDNA was

[1]http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index
[2]http://mirdb.org/

synthesized from the obtained RNA samples using a cDNA synthesis kit (Thermo Fisher Scientific). cDNA procedure was conducted according to the manufacturer's protocol. cDNA samples were quantified using the Roche 480 Real Time PCR System for expression levels of Gsk3b, Mapk8, and Ppargc1a genes (Kocyigit et al., 2017).

Gsk3b, Mapk8, and Ppargc1a expressions represented in **Figures 7**, **8** were normalized to Beta-Actin and GAPDH genes using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Expression changes in the F2 cross-fostering group compared to the F2 control group were calculated and shown as fold change in the graphs.

Histological Analysis

Liver tissues of three subjects from each group around the 18th month of age were fixed in formalin and processed. Formalin-fixed paraffin-embedded tissue sections were stained with hematoxylin and eosin for light microscopy. The sections were also stained with Prussian Blue and Masson trichrome to demonstrate tissue iron and fibrosis, respectively. A semi-quantitative scoring system was used to grade steatosis, hepatic nuclear degeneration, fibrosis, lymphoid aggregates and hemosiderin pigmentation (0 normal 1 mild, 2 moderate, and 3 severe histopathological changes) (Goodman, 2007; Liang et al., 2014).

Statistical Analysis

Data were collected using Fluidigm Real-Time PCR Analysis Software, the linear derivative baseline correction method, and the auto global Cq threshold method. System-given Cq values of 999 and values larger than 26 were considered non-speci?c and beyond detection limits and removed. Median limit of detection Cq values were calculated across all arrays to assign missing values. Data normalization was performed using the $2^{-\Delta\ \Delta\ Ct}$ method (Livak and Schmittgen, 2001). All of the analysis was performed through a web based software[3].

MicroRNAs fold change values were calculated using R 3.2.2 software (limma) and easy ROC packages (R Core Team, 2015). A p value < 0.05 was considered statistically significant. Benjamini-Hochberg error test was used to adjust p values by taking into account multiple tests (Vlachos and Hatzigeorgiou, 2017). The Student's t-test was used for comparisons between groups. A p value < 0.05 was considered statistically significant.

SPSS IBM 24 statistical package program was used for comparing phenotypic features of the F2 control and F2 milk-sibling groups. The normal distribution of the data was evaluated using a histogram, q-q graphs, and the Shapiro–Wilk test. Categorical data were evaluated using the chi-square test. Homogeneity of the variances tested by Levene's test. A resulting p-value > 0.05 means that variances are equal and then further parametric tests are suitable. If a resulting p value under 0.05, Mann–Whitney U test was performed.

[3]http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/

RESULTS

Coat color distribution was not significantly different between the two groups. Initial mean body weights were similar in both groups. However, the F2 milk-sibling group showed higher weight gain during the first two postnatal months than the F2 control group ($p < 0.05$). Although this difference was not significant during the intervening months, by the 24th month, the mean body weight was significantly higher in the F2 milk-sibling group ($p < 0.\ 05$). Mean fasting blood glucose levels at the 5th and 8th months were not significantly different between the two groups (**Table 1** and **Figure 2**). In the liver histological evaluation, the control group (having Y0, Y2, and Y3 coat colors) showed non-specific changes except for mild steatosis and hepatocytic regenerative changes. These non-specific changes were also seen in the F2 milk-sibling group (Y0, Y2, and Y4 coat colors). However, they also showed some additional pathological changes such as hemosiderin depositions, lymphoid aggregates, and fibrosis (**Figure 3**).

In order to understand the epigenetic mechanisms leading to these phenotypic changes, we performed miRNA expression analysis to identify differentially expressed miRNAs across milk-sibling and control groups. Comparisons of miRNA expression patterns were performed between the gonad and liver tissues of the F2 control and the F2 milk-sibling groups. Expression patterns of miRNAs demonstrating significant differences in paired comparisons were further analyzed using web-based bioinformatics tools to determine potential regulated targets and pathways (Vlachos et al., 2012). Based on our data showing that F2 milk-siblings had higher body weight and shorter life expectancy compared to control counterparts (**Table 1** and **Figure 2**), we focused on the signaling pathways regulating lifespan and metabolism (**Figures 4–6**). Thus, we concentrated on heat maps generated from F2-ovaries (**Figure 4**), F2-testes (**Figure 5**), and F2-livers (**Figure 6**). We observed the PI3K-Akt signaling pathway, ErbB signaling pathway, mTOR signaling pathway, MAPK signaling pathway, transcriptional misregulation in cancer, pathways in cancer, and insulin signaling pathway were most commonly affected in the milk-siblings group in comparison to controls. These signaling pathways are part of nutrient-sensing systems known to regulate reproductive and somatic aging as well as metabolic syndrome and its complications, including fatty liver disease, respiratory disease, diabetes, cardiovascular disease, osteoarticular disease, and cancer (Templeman and Murphy, 2018; Fadini et al., 2011).

Targeted pathway heatmaps show that miR-186-5p targets most of the signaling pathways playing a role in survival and metabolism. Target prediction analysis showed that miR-186-5p potentially targets Gsk3b (**Figure 7A**), which is involved in many pathways such as the PI3K-akt signaling pathway, ErbB signaling pathway, pathways in cancer, and insulin signaling pathway (**Figure 4A**). Gsk3b expression in F2-ovaries was analyzed using RT-qPCR to examine if there is a link between Gsk3b and miR-186-5p expression. miR-186-5p expression decreased in F2 milk-siblings, whereas Gsk3b increased compared to control counterparts (**Figure 7B**).

TABLE 1 | Phenotypic characteristics of the F2 generation.

	F2-cross-fostering *n* = 62	F2-control *n* = 23	*P*
Sex, female, n (%)	31 (50.0)	15 (65.2)	0.266*
Coat color			
Y0 (Native)	30 (48.3)	10 (43.5)	0.058*
Y1	7 (11.2)	3 (13.0)	
Y2	6 (9.7)	4 (17.4)	
Y3	12 (19.4)	5 (21.7)	
Y4	7 (11.2)	1 (4.3)	
Y5	0	0	
Long term follow up	*n* = 37	*n* = 14	
Blood glucose mg/dl (mean ± sd)			
5th month	110.5 ± 13.9	94.0 ± 23.2	0.062**
8th month	100.5 ± 13.9	90.0 ± 23.2	0.101**
Mean lifespan, days (mean ± sd)	387.9 ± 234.9	589.7 ± 208	0.011**

*Chi square test. **Student t test.*

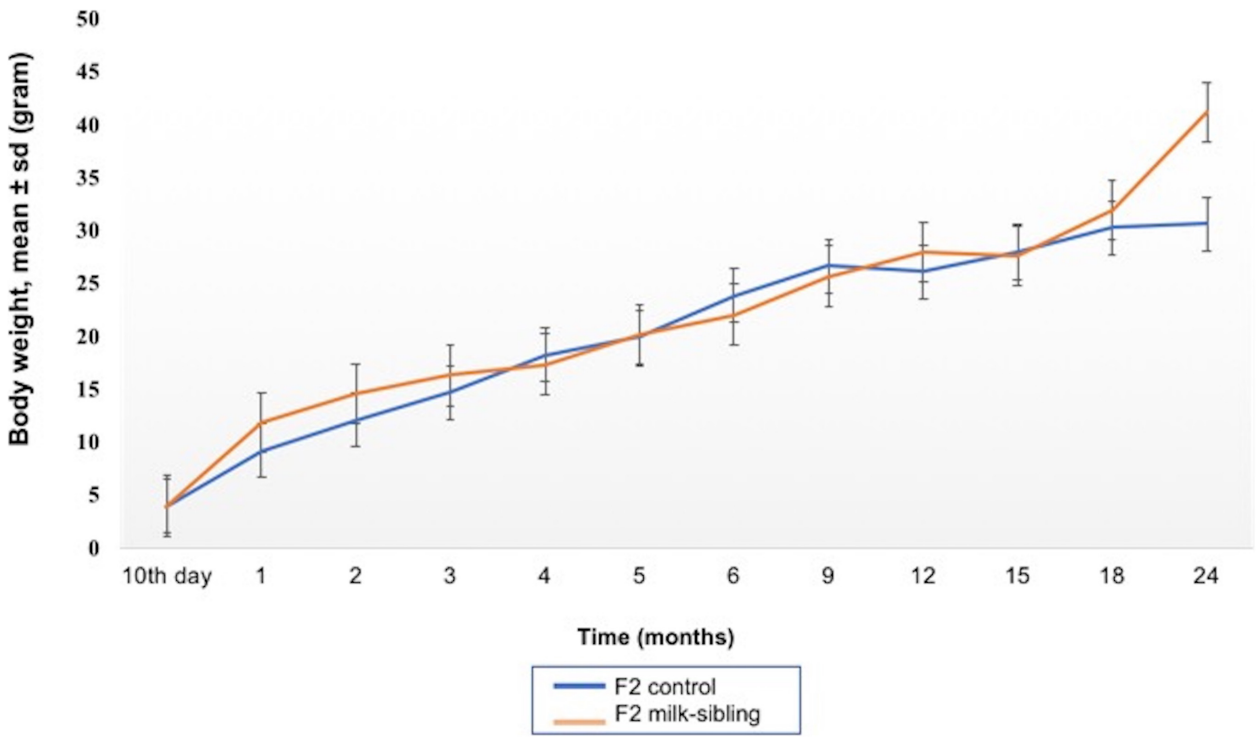

FIGURE 2 | Mean body weight follow up of the two groups. The difference between the groups at the 1st, 2nd, and 24th months were statistically significant (*p* values: 0.014, 0.029, and 0.044, respectively).

Following the same logic, we found that miR-141-3p targets most of the survival and metabolism related pathways, including the MAPK signaling pathway, mTOR signaling pathway, ErbB signaling pathway, and pathways in cancer (**Figure 4**). Target prediction analysis showed that miR-141-3p potentially targets Mapk8 (**Figure 7C**), which is involved in the MAPK signaling pathway, ErbB signaling pathway, and pathways in cancer When we analyzed Mapk8 expression in F2-ovaries using RT-qPCR, we observed that miR-141-3p expression increased in F2 milk-siblings, whereas Mapk8 expression decreased compared to the control group (**Figure 7D**).

Because metabolic homeostasis is dependent on proper liver function, we evaluated miRNAs and their potential targets' expressions in liver tissues. We detected only two miRNAs differentially expressed in milk-siblings in comparison to control groups in liver (miR-345-5p and miR-34c-5p; **Figure 6**). These miRNAs target the PI3K-Akt signaling pathway, transcriptional misregulation in cancer, pathways in cancer, and mTOR signaling pathway, which are among the target pathways significantly

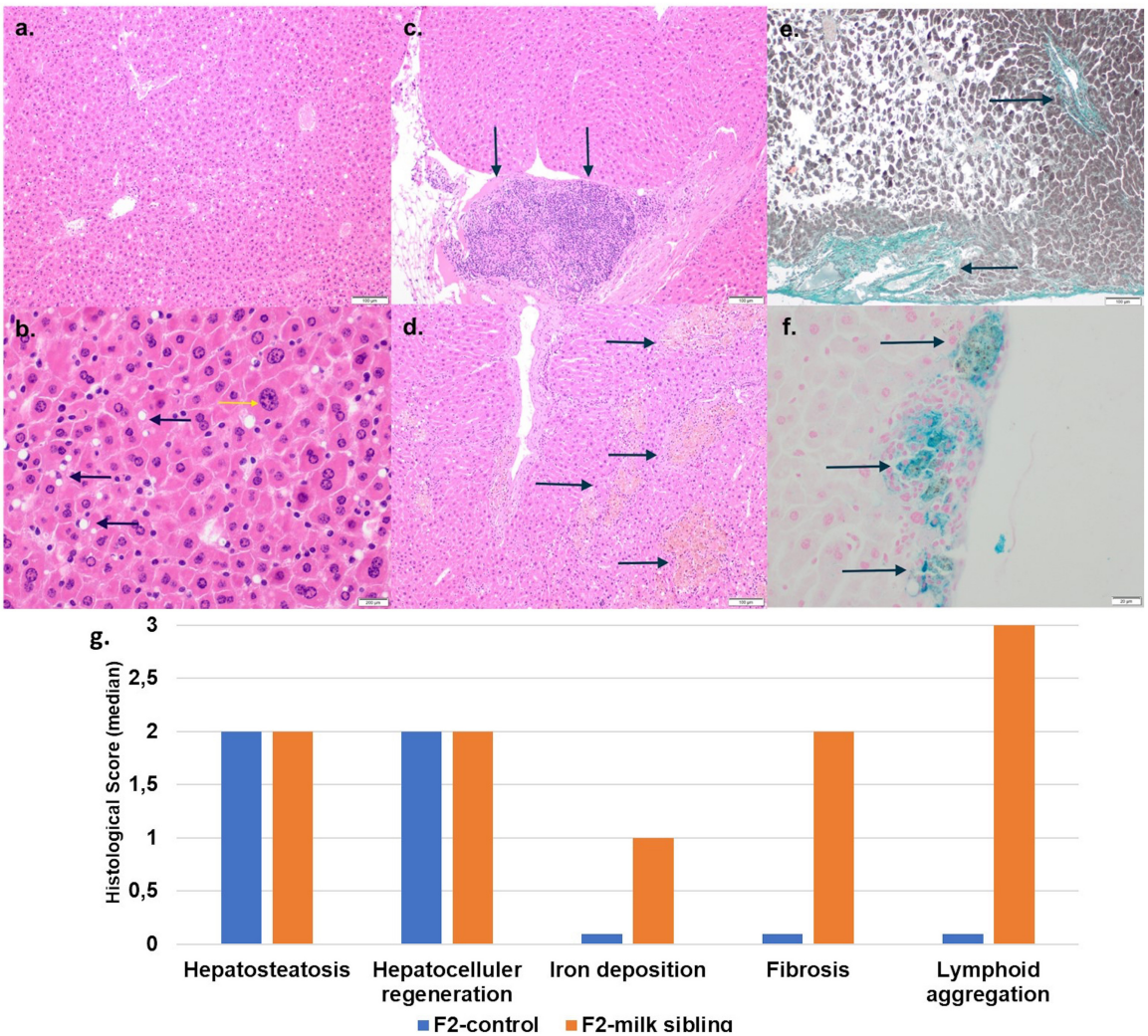

FIGURE 3 | Liver tissue histology. **(a)** F2 Control group: mild liver steatosis (H&E, x10 magnification). **(b)** F2 Control group: Mild liver steatosis (black arrows), hepatocytic regenerative changes, nucleomegaly, and coarse chromatin (yellow arrows; H&E, x10 magnification). **(c)** F2 Milk-sibling group: lymphoid aggregates (black arrows; H& E, x10 magnification) Focal. **(d)** F2 Milk-sibling group: Yellowish pigmented foci (black arrows; H& E, x10 magnification). **(e)** F2 Milk-sibling group: fibrosis around central ven (black arrows; Masson trichrome, x20 magnification). **(f)** F2 milk-sibling group: Hemosiderin containing nodules (black arrows; Prussian blue staining, x20 magnification). **(g)** Comparison of median liver histology scores across the F2-control and F2 milk-sibling groups sibling (statistical analysis could not be performed because of the small sample size).

affected in F2-gonad tissues. We investigated potential targets of mmu-miR-34c-5p and mmu-miR-345-5p using web-based bioinformatics tools (**Figure 8**). We identified a complementary site for mmu-miR-34c-5p in the 3'UTR of Gsk3b mRNA (**Figure 8A**). miRNA expression profiling revealed that mmu-miR-34c-5p expression increased in milk-siblings, whereas miR-345-5p expression decreased in milk-siblings in comparison to their control counterparts (**Figures 8B,D**). Expectedly, RT-qPCR analysis of Gsk3b, a predicted target of mmu-miR-34c-5p, decreased in milk-siblings and Ppargc1a, a predicted target for mmu-miR-345-5, increased in milk-siblings compared to their control counterparts (**Figures 8B,D**; for entire list and *p* values, see **Supplementary Data**).

DISCUSSION

The most striking result of study was that mating of milk-siblings resulted with various pathologies in offspring possibly because of the shared breast milk that can affect epigenetic regulation mechanisms. Results revealed that the life expectancy of the offspring obtained from milk-siblings mating was much shorter than the offspring from control matings. Offspring of milk-siblings were more obese during the aging period and histopathological examination of liver tissues revealed abnormal findings that were not shown in the offspring from control matings such as lymphoproliferative nodules, abnormal iron accumulation, and fibrosis.

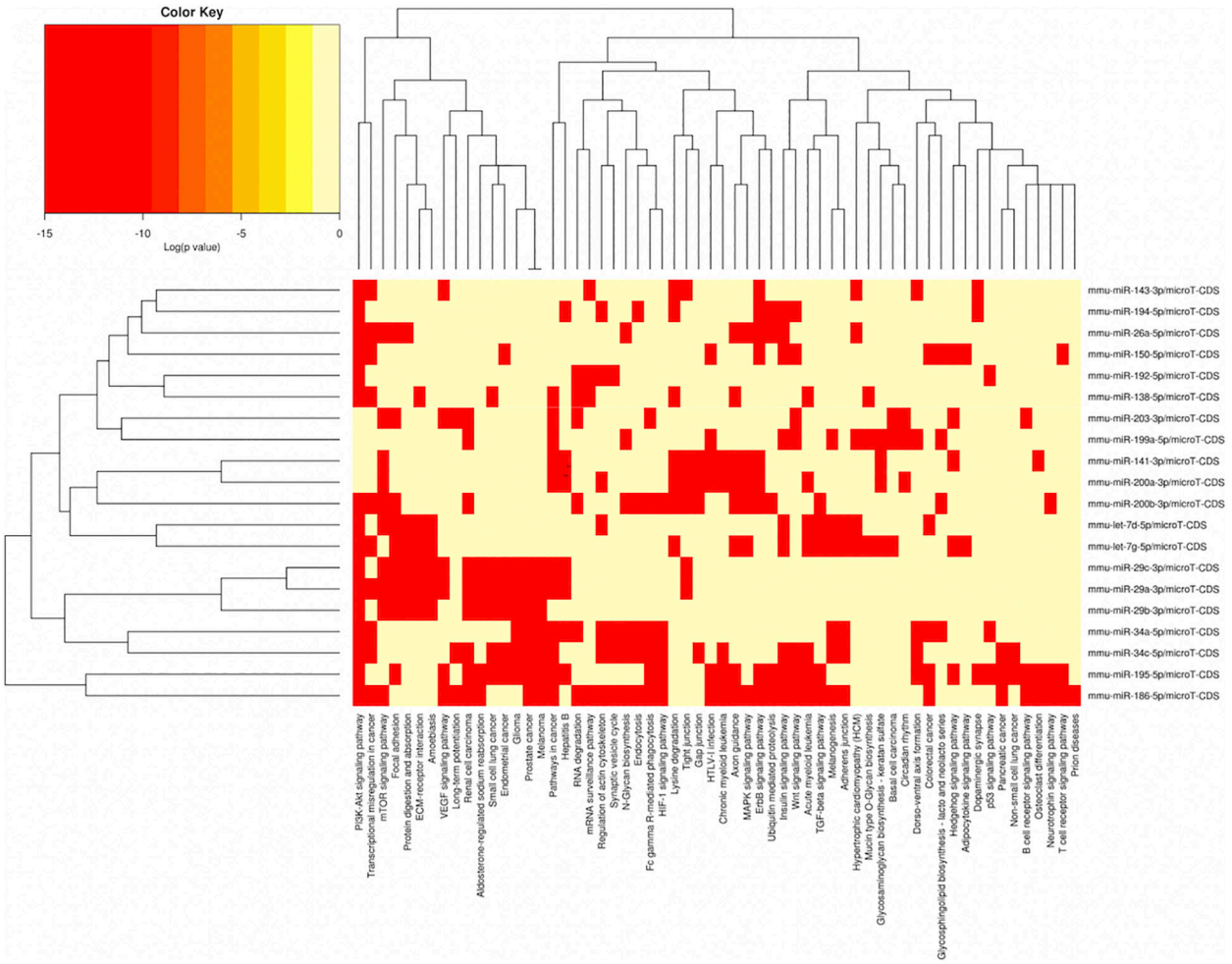

FIGURE 4 | miRNAs vs pathways heatmaps generated from DIANA miRPath. Targeted pathway heatmap shows differentially expressed miRNAs across F2 ovaries from milk-siblings and control groups. Darker colors represent lower significance values. Heatmaps on both axes represent hierarchical clustering results for miRNAs and Pathways, respectively. miRNA axis shows miRNAs clustered together by similar pathway targeting patterns. Similarly, an analogous clustering is shown on the pathway axis.

In our study, we performed miRNA microarray to profile mouse miRNAs in ovaries, testes, and livers and make expressional comparison between milk-siblings and control groups. The number of significantly differentially expressed miRNAs was higher in ovaries than testes than livers. Interestingly, these differentially expressed miRNAs mostly targets metabolism, survival and cancer associated pathways with very small *p*-values. Notably, these pathways strongly associated with the phenotypes we observed. Particularly, mTOR signaling, AMPK signaling, FOXO signaling, insulin signaling that are commonly targeted by miRNAs differentially expressed in milk-siblings are nutrient-sensing systems determine reproductive status and somatic tissue maintenance with age (Fadini et al., 2011; Templeman and Murphy, 2018). Accumulating evidences highlights the fact that longevity and metabolic signals interplay in a complex way in which lifespan appears to be strictly dependent on substrate and energy bioavailability. These pathways also determine the development of the metabolic syndrome and its complications affecting disparate organs and systems, such as fatty liver disease, respiratory disease, diabetes, cardiovascular disease, osteoarticular disease, and cancer (Fadini et al., 2011; Templeman and Murphy, 2018). Thus, it is not surprising that differentially expressed miRNAs mostly clustered into these pathways. For instance, in our study we found that while miR-186-5p downregulated in milk-siblings Gsk3b upregulated in milk-siblings compared to control groups. Downregulation of miR-186-5p and upregulation of GSK3B have been reported in ovarian carcinoma (Hilliard et al., 2011; Templeman and Murphy, 2018). Overexpression of GSK3B has been implicated in insulin resistance, polycystic ovary syndrome (PCOS), platinum-resistance in ovarian cancer (Cai et al., 2007; Goodarzi et al., 2007; Hilliard et al., 2011). In addition, we detected upregulation of miR-141-3p in milk-siblings and downregulation of its potential target MAPK8 in milk-siblings. Interestingly, miR-141-3p upregulation and MAPK8 (JNK1) signaling downregulation have been reported in platinum-based

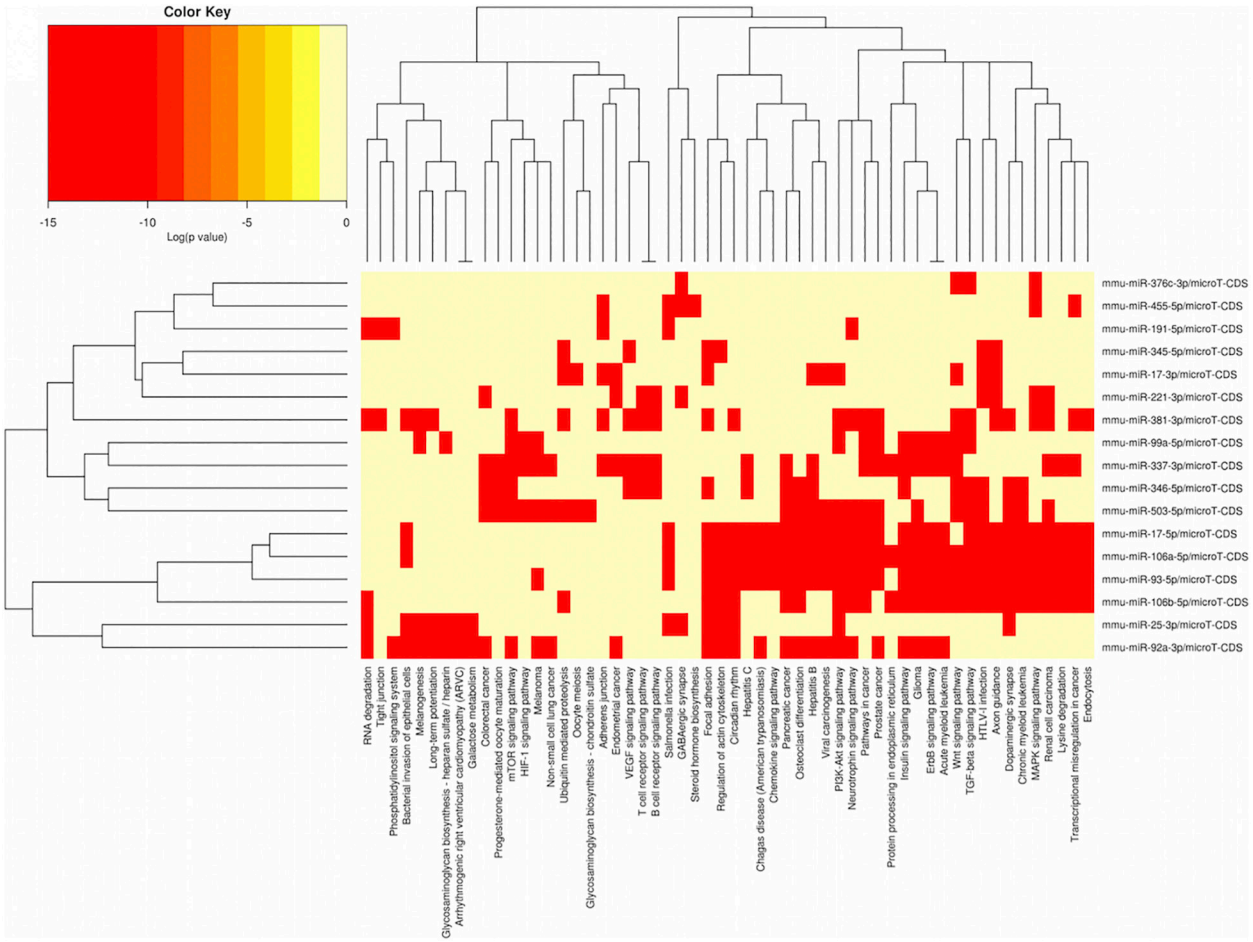

FIGURE 5 | miRNAs vs pathways heatmaps generated from DIANA miRPath. Targeted pathway heatmap shows differentially expressed miRNAs across F2 testes from milk-siblings and control groups. Darker colors represent lower significance values. Heatmaps on both axes represent hierarchical clustering results for miRNAs and Pathways, respectively. miRNA axis shows miRNAs clustered together by similar pathway targeting patterns. Similarly, an analogous clustering is shown on the pathway axis.

chemotherapy in ovarian cancer cell lines (Ying et al., 2015; Li et al., 2018). MAPK8 has been also implicated in the aging-related disease and overexpression of JNK1 in roundworms was reported to increase lifespan (Oh et al., 2005). Overall, even though our bioinformatics and gene expression studies support the data gathered from phenotypic observations, it is necessary to perform further analysis to conclude that these miRNAs and their potential targets specifically play roles in. We have not analyzed animals for insulin-resistance, PCOS or ovarian cancer cells; it would be worth studying the potential role of miR-186-5p/Gsk3b axis in ovarian tissues. Considering the fact that liver plays an essential role in metabolic homeostasis, we evaluated miRNAs and their potential targets expressions in liver tissues. We detected only two miRNAs statistically differentially expressed in milk siblings in comparison to control groups. miR-34c-5p showed a higher level in milk-siblings and accordingly its potential target Gsk3b was lower in the same tissues compared to control counterparts. It was shown that miR-34c-5p upregulated in hepatitis B-related acute-on-chronic liver failure (Ding et al., 2015). In addition, increased expression of miR-34c-5p was shown in hypoxic rat liver (Zhi et al., 2017). Accordingly, Gu et al. (2018) showed that Gsk3b signaling decreased under intermittent hypoxic conditions. These studies imply that increased expression of miR-34c-5p and decreased activity of Gsk3b signaling might be a response to cellular stress conditions. Furthermore, we found a decreased expression of miR-345-5p and an increase expression of its potential target gene Ppargc1a in milk-siblings. Interestingly, Chen et al., performed an integrated analysis of miRNA and gene expression profiles highlighted a functional miRNA-gene regulatory module associated with liver fibrosis and listed miR-345-5p among decreased miRNAs (Chen et al., 2017). Similarly, histological analysis showed fibrosis in milk-siblings' livers that have decreased expression of miR-345-5p. In addition, we observed fat accumulation in livers of milk-siblings. Liver fibrosis (Stal, 2015) and impaired PPARGC1A activity are associated with non-alcoholic fatty liver diseases (Aharoni-Simon et al., 2011). Taken together our results supports that miRNAs likely

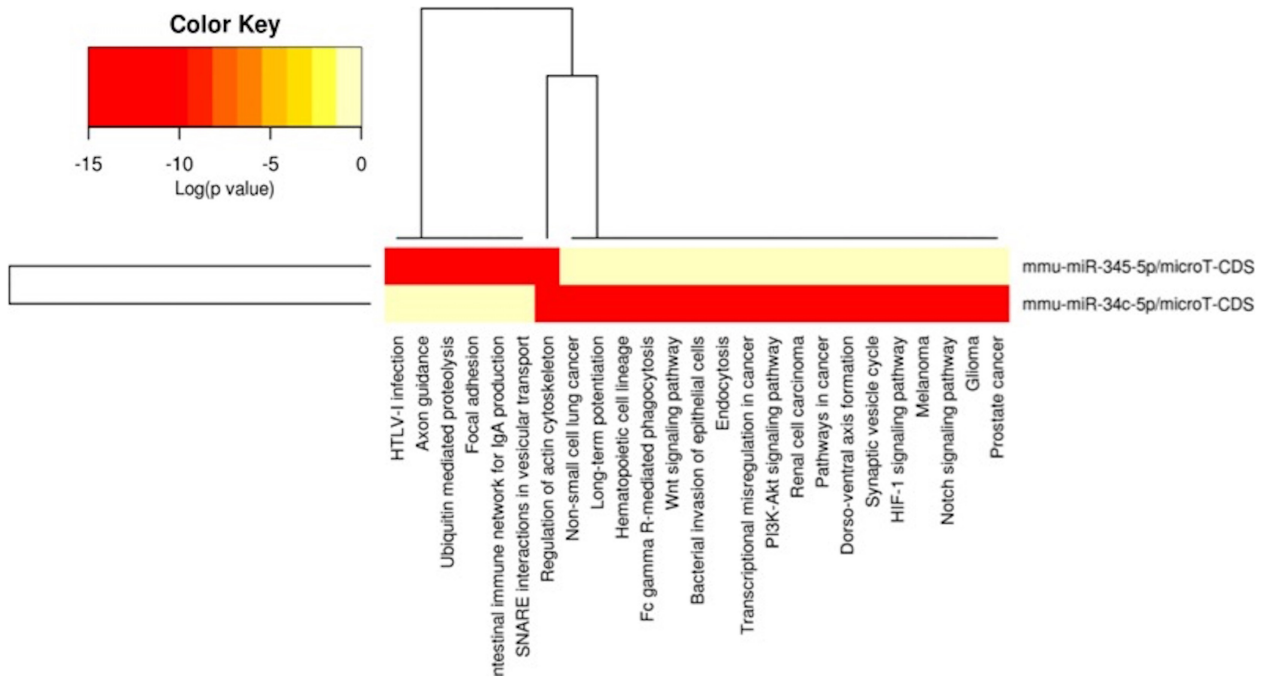

FIGURE 6 | miRNAs vs pathways heatmaps generated from DIANA miRPath. Targeted pathway heatmap shows differentially expressed miRNAs across F2 livers from female milk-siblings and control groups. Darker colors represent lower significance values. Heatmaps on both axes represent hierarchical clustering results for miRNAs and Pathways, respectively. miRNA axis shows miRNAs clustered together by similar pathway targeting patterns. Similarly, an analogous clustering is shown on the pathway axis.

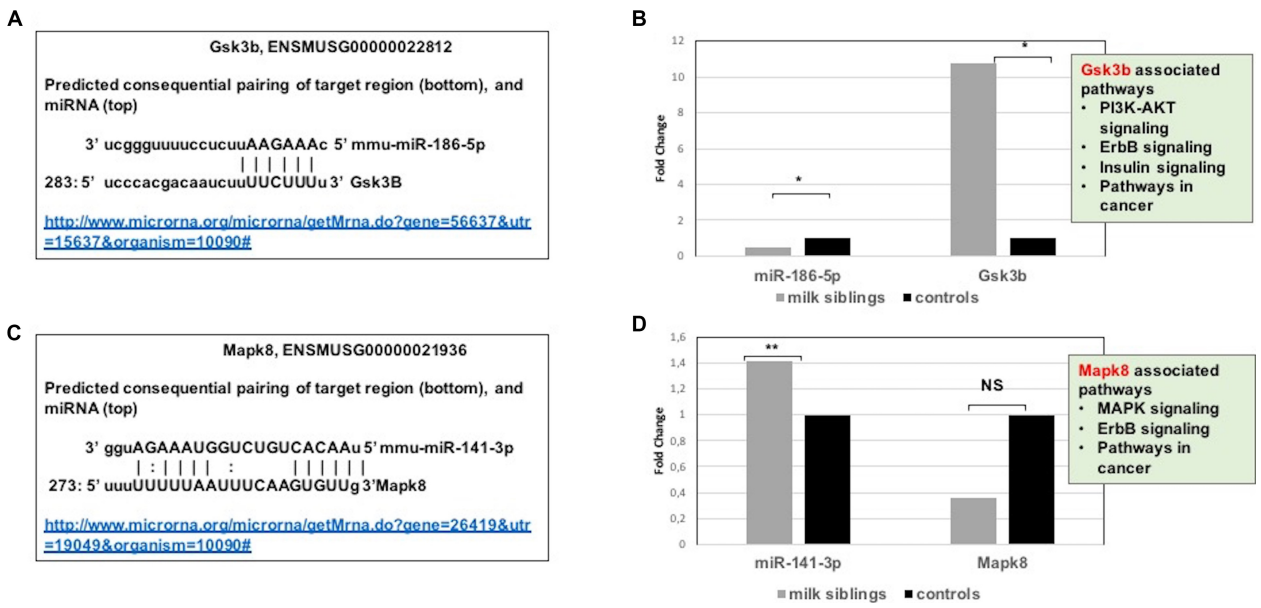

FIGURE 7 | (A) Schematic representation describing sequence alignment of mm-miR-186-5 with the 3′-UTR of Gsk3b (http://www.microrna.org/microrna/getMrna.do?gene=56637&utr=15637&organism=10090). **(B)** Graph shows corrected fold changes in miR-186-5p and Gsk3b expression levels (milk-siblings to control groups) in the ovary tissues. ACTH was used as a reference gene. Values from control groups were set at 1. (http://www.microrna.org/microrna/getMrna.do?gene=26419&utr=19049&organism=10090). **(C)** Schematic representation describing sequence alignment of mm-miR-141-3p with the 3′-UTR of Mapk8. **(D)** Graph shows corrected fold changes in mm-miR-141-3p and Mapk8 expression levels (milk-siblings to control groups). ACTH was used as a reference gene. Values from control groups were set at 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

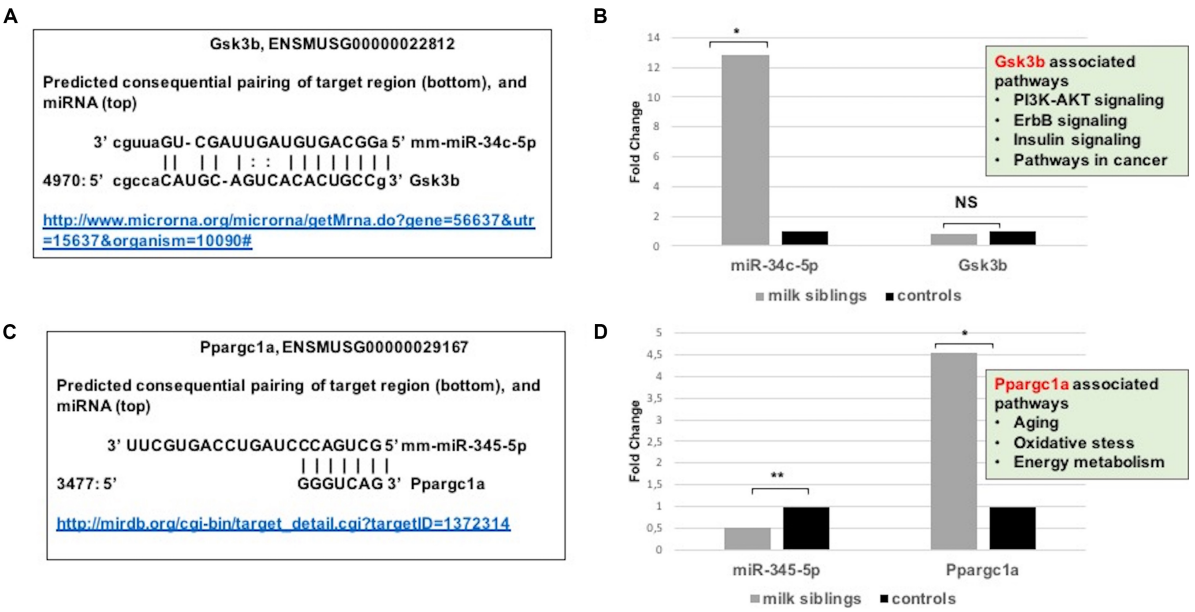

FIGURE 8 | (A) Schematic representation describing sequence alignment of mm-miR-34c-5p with the 3′-UTR of Gsk3b (http://www.microrna.org/microrna/getMrna.do?gene=56637&utr=15637&organism=10090). **(B)** Graph shows corrected fold changes in mmu-miR-34c-5p and Gsk3b expression levels (milk-siblings to control groups) in the liver tissues. ACTH was used as a reference gene. Values from control groups were set at 1 (http://mirdb.org/cgi-bin/target_detail.cgi?targetID~=~1372314). **(C)** Schematic representation describing sequence alignment of mm-miR-345-5p with the 3′-UTR of Ppargc1a. **(D)** Graph shows corrected fold changes in mm-miR-345-5p and Ppargc1a expression levels (milk-siblings to control groups). ACTH was used as a reference gene. Values from control groups were set at 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

mediate the phenotypes observed in survival-, obesity- related, transgenerational metabolic disturbances.

There might be a period in which offspring are susceptible to breast milk induced epigenetic changes. The period that begins with conception and covers the first 2 years of life is suggested as the most active period in terms of epigenetic regulation, especially in terms of DNA imprinting. Therefore, this period is referred to as "1000 day period" (Shenderov and Midtvedt, 2014; Walker, 2016; Indrio et al., 2017; Linner and Almgren, 2020). Growing amount of evidence supports that epigenetic programming affected by early nutrition may result in adult disease in the long run (Shenderov and Midtvedt, 2014; Indrio et al., 2017). Considering that, there may be a sensitivity period to epigenetic factors in breast milk all suckling pups were weaned on the same PND in this study.

Epigenetic developmental plasticity allows an organism to adapt to environmental signals, especially during fetal and early life (Shenderov and Midtvedt, 2014). The cross fostering may have disrupted the epigenetic developmental plasticity starting during fetal life and continuing during the early postnatal life. Furthermore, the milk of each mother is specific to her own baby, and the maternal-fetal interaction during fetal life may affect the composition of breastfeeding (Twigger et al., 2015) and the baby's tolerance to the biological mothers' milk. Therefore, epigenetic differences in F2 milk-sibling group may have been aroused not only due to mating of milk-siblings, but also due to the disruption of epigenetic developmental plasticity due to lack of breast milk of their own and breastfeeding by foster-mother.

Since the genetic structures and environmental conditions of the cross-sibling and control groups were the same in this model, breast milk was supposed to be the only responsible factor for existing epigenetic changes. Because of the similarity of the genetic backgrounds and environmental exposures of the two groups, posttranscriptional epigenetic mechanisms, especially through miRNAs, were investigated instead of DNA methylation patterns or histone modifications. Small transcoded RNAs (microRNA, pi-RNA, etc.) play an important role in transgenerational epigenetic inheritance. Sperm not only transfers DNA to ovum, but also transfers different kinds of RNAs including miRNAs and pi-RNAs. These transferred RNAs play an important role in embryo development by influencing various mRNA expressions (Smythies et al., 2014). Future experiments analyzing the milk in each generation could be performed in order to establish the precise participation of miRNAs.

When the results of this study are interpreted, the following conclusions may be drawn: i.e., substances that may affect epigenetic regulation mechanisms may be transferred to F1 generation via breastfeeding, ii. epigenetic interactions or epimutations occurring in F1 generation can be transferred to the next generation. It is not clear whether this kind of inheritance occurs in monoepigenic or polyepigenetic as in the other genetic diseases. The results of this study can not claim exactly the mechanisms by which breast milk affects meiotic epigenetic inheritance and it is not exactly known yet, how it has been carried over generations. If the results of this study are supported by further studies, it may be questionable

that milk-sibling marriages cause hereditary diseases as in the consanguineous marriages.

There are several limitations of this study. Although bioinformatics approaches were used to predict functionality of miRNAs, experimental validation is necessary to show miRNA: gene interactions. Secondly, in the F1 generation, cross fostering may have stressed animals and may have affected subsequent metabolic responses in the F2 generation. However, to test the effect of milk-siblingship on subsequent generations, this breeding scheme allowed us to keep the genetic background of the animals comparable in the two F2 groups. Furthermore, consequences of disruption of maternal-fetal dyads by cross fostering should also be considered when interpreting the results as discussed above. Small sample size was another important limitation of the study. Further experiments are needed to the differentiate the epigenetic effects of milk-sibling and cross fostering on future generations.

CONCLUSION

The results of this study support that epigenetic regulation mechanisms may be transmitted to the baby through breast milk and these epigenetic interactions or epimutations may be transferred to the next generation transgenerationally. The current results indicate that milk-sibling mating may cause various diseases in offspring. Results may also indicate the heritable epigenetic effects of cross-fostering on future generations. Similar results are not verified in humans. Nevertheless, with these remarkable findings, it is time to reconsider what we know about this issue.

CODE AVAILABILITY

DIANA Tools with mirPath v2.0, microT-CDS v5.0 (see text footnote[1]), KEGG (Kyoto Encyclopedia of Genes and genomes, https://www.genome.jp/kegg/) molecular pathways, and multiple slices of Gene Ontology (GO, http://geneontology.org/) in Mus musculus were used for bioinformatics analysis (Kanehisa and Goto, 2000; Vlachos et al., 2012; Kanehisa et al., 2016).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Dokuz Eylul University Multidisciplinary Animal Laboratory Ethical Committee.

AUTHOR CONTRIBUTIONS

HO revealed the main hypothesis. HO, FT, and YÖ designed the study. FT and PA performed most of the experiments. ST performed the PCR studies and wrote some part of the manuscript. PK performed the data and bioinformatics analysis and also contributed to writing. EÖ performed the histological analysis. ET contributed to PCR studies. OY, ND, and AK contributed to the experimental assistance. YÖ provided conceptual advice. FT and PK wrote the manuscript with comments from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.569232/full#supplementary-material

Supplementary Additional File 1 | Comparison of F2 female gonads (milk sibling vs control).

Supplementary Additional File 2 | Comparison of F2 livers (milk sibling vs control). Comparison of F2 livers.

Supplementary Additional File 3 | Comparison of F2 male gonad (milk sibling vs control).

Supplementary Additional File 4 | miRNA expression-Comparison of F2 female gonads (milk sibling vs control).

Supplementary Additional File 5 | miRNA expression comparison of F2 male gonads (milk sibling vs control).

Supplementary Additional File 6 | miRNA expression Comparison of F2 livers (milk sibling vs control).

Supplementary Additional File 7 | RT-mRNA expression a. Comparison of F2 female gonads (milk sibling vs control) b. Comparison of F2 livers (milk sibling vs control).

Supplementary Additional File 8 | Representation of the biological process that Mapk8 involved in. The data are obtained from Ensembl (http://www.ensembl.org/index.html).

Supplementary Additional File 9 | Representation of the biological process that and Ppargc1a involved in. The data are obtained from Ensembl (http://www.ensembl.org/index.html).

Supplementary Additional File 10 | Representation of the biological process that Gsk3b involved in. The data are obtained from Ensembl (http://www.ensembl.org/index.html).

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OPINION
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The Adequacy of Experimental Models and Understanding the Role of Non-coding RNA in Joint Homeostasis and Disease

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INTRODUCTION

RNA-mediated processes by non-coding RNA (ncRNAs) namely microRNAs, long ncRNAs and circular RNAs, as all epigenetic mechanisms are particularly sensitive to the effects of tissue microenvironment and environmental factors (Chisolm and Weinmann, 2018; Ning et al., 2019; Pagiatakis et al., 2019). In recent years research has focused on the development of smart cell culture *in vitro* systems one step closer to natural conditions, paying particular attention to the cellular microenvironment and cell culture conditions. 3D cell culture and co-culture systems based on cultivating a single cell population or different cell populations combined together, have found growing interest as useful tools to better understand cell biology and to offer more physiological relevant results by tightly controlling experimental parameters (Dhaliwal, 2015; Duval et al., 2017; Mirbagheri et al., 2019). The development of these *in vitro* models is a promising approach even if the limited availability of human tissue from which to obtain the cells have to take into account. Such an approach by using tissue specimens of human origin can allow the realization of suitable 3D *in vitro* models overcoming the limits of traditional 2D monolayer cell cultures, or expensive animal models that often cannot accurately recapitulate human etiopathogenesis and are not suited to develop novel drugs. It is widely recognized that in a 3D environment cells tend to be more subjected to morphological and functional changes differently to those grown in simplistic cellular monolayer. Another important issue is the methods matter regarding the employment of cell culture conditions that have to take into account the physiological parameters such as oxygen concentration, chemical and biophysical components.

As a whole, new 3D technologies recapitulating the essential aspect of the dynamic *in vivo* environment can meet the need of achieving stringent information to design adequate prevention strategies and to develop more effective therapeutics, including those based on RNA. Epigenetic investigation in all areas cannot underestimate this aspect. It is in fact important to take into consideration that ncRNAs display more tissue-specific expression patterns than protein-coding genes, and cell differentiation is particularly affected by fine tuning of ncRNAs level (Fatica and Bozzoni, 2014; Jiang et al., 2016; Ramón Y Cajal et al., 2019). Therefore, it follows that changes in the ncRNAs expression and functions, as well as their cellular delivery through extracellular vesicles (Di Liegro et al., 2017) may be particularly influenced by experimental conditions and methodological approach of carrying out the experiments.

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This general premise is particularly appropriate for research aimed at studying joint homeostasis and skeletal pathologies, an area in which a great effort is being made to compare the effectiveness of different experimental models.

Bone and cartilage cells together with all other cells localized in the different types of skeletal joints are extremely sensitive to the effects of interstitial pH, oxygen levels, glucose concentration, mechanical stimuli, calcium content, and paracrine signaling.

Many skeletal degenerating conditions may be seen as the failure of soluble factors and epigenetic regulation to establish the correct microenvironment in bone and cartilage (van Meurs et al., 2019). Emerging evidence showed that ncRNAs, in particular miRNAs, are deregulated in many bone and joint diseases (Zhou et al., 2017; Wang et al., 2019; Wu et al., 2019; Zhu et al., 2019; Li et al., 2020; Song et al., 2020). A better understanding of RNA-mediated processes in epigenetics will help to deepen how bone and cartilage are damaged, providing novel repair strategies to restore joint function, as well as new insight on the pathogenesis of acquired and inherited bone or joint diseases (Houard et al., 2013; Sun et al., 2019; van Meurs et al., 2019).

However, artifacts arising from not satisfactory experimental models and/or contaminants in culture medium can produce incorrect information, weakening the biomedical relevance (Tosar et al., 2017).

Here we would like to express our opinion on (i) the importance of the adequacy of experimental models to understand the specific role of ncRNA, in joint/skeletal homeostasis, and (ii) the careful surveillance for warning signs of contamination and possible artifacts.

The final aim is to keep high attention and to stimulate discussion on the need to adopt effective tools to design appropriate human-oriented joint disease research focused on understanding the mechanisms of regulation of gene expression to develop specific RNA-based therapeutics.

THE JOINT OF SKELETAL SYSTEM: THE CHALLENGE OF DEVELOPING APPROPRIATE EXPERIMENTAL MODELS

Re-creating *in vitro* skeletal tissue microenvironment is a challenge that is engaging many researchers who are looking for the molecular mechanisms underlying tissue homeostasis maintenance for preclinical drug testing purposes. This challenge is especially hard for what concerns the joint of the skeletal system, due to its intrinsic biology. Currently, various strategies recapitulating the complex and dynamic three-dimensional (3D) environment experienced by cells *in vivo* are proposed by several research groups (Baker and Chen, 2012; Cassotta et al., 2020). These include the development of:

- various 3D—engineered systems based on co-cultures (Owen and Reilly, 2018),
- biomimetic materials resembling the natural extracellular environment combined with cells at different stage of maturation (Park et al., 2018),
- drug-releasing scaffolds (Zeng et al., 2019),
- bioreactors for dynamic culture conditions (Bicho et al., 2018),
- organ-on-a chip and integrated microfluidic culture platforms (Dai et al., 2019).

Methods and models listed above can offer great benefits especially when combined with a careful control of chemical and physical parameters (Bader et al., 2011; Baker, 2016). This means exposing the cellular system to:

- physiological oxygen concentration, by using specific cell culture solutions to create hypoxic environment. Primary cultures of chondrocytes or osteoblasts are typically carried out at normoxia (21% oxygen), while these cells are *in vivo* exposed to an oxygen gradient of 1–8%;
- mechanical stimuli, maintaining, for example, the cartilage integrity through the process of mechanotransduction obtained with suitable devices;
- refined and more physiologically relevant culture media, including serum.

The optimization and a wider use of these methods is essential. However, most of the data in the literature are still based on the use of cellular monolayers and conventional culture conditions. Yet it is well-known that, for example, isolated chondrocytes may change their phenotype after expansion in 2D monolayer cell culture (Caron et al., 2012).

Here we would like to draw attention to the need to create guidelines, in the near future, for the development of experimental models suitable for producing stringent data on joint homeostasis and new therapeutics development for joint degeneration. In particular, the common effort should be directed to identify experimental strategies and standardize reagents and protocols to optimize the results.

These reflections have the aim of ruling out misleading approaches, allowing the attribution of specific roles to molecular regulators, such as those supporting the RNA-mediated processes (Raman et al., 2018; Razmara et al., 2019; Zhang et al., 2019).

JOINT DEGENERATION AND ncRNAs

Joint degeneration includes over 100 different types of arthritis conditions affecting usually the hips, knees, spine column, hands and feet (Xu and Li, 2020). The most widespread degenerative joint disease is the osteoarthritis (OA) which is one of the leading causes of disability in the middle-aged and elderly (Glyn-Jones et al., 2015). To date there is no cure for these disorders. The prevalence of this pathology is expected to increase in the coming years because the risk factors that favor it are inherent in today's society (aging, overweight and obesity, sedentary lifestyle, or uncontrolled sports practice) (Hoy et al., 2014; O'Neill et al., 2018; Zhang et al., 2019).

Joint degeneration process involves cartilage, bone and synovium (Xu and Li, 2020). It is only partially understood, and many aspects of the complex signaling between the joint forming cells and mechanisms driving cell fate decision inside the degenerated microenvironment have yet to be fully elucidated. In order to develop new therapeutics fighting joint degeneration, most studies are focusing on intra-articular injection of nucleic

acid-based drugs including ncRNAs (Kawanishi et al., 2014; Wang et al., 2017; Cheng et al., 2018; Rai and Pham, 2018). Also in this case, improved *in vitro* experimental models are required to aid the identification of key regenerative ncRNAs that, once injected in a damaged joint, could potentially enhance endogenous repair and slow down the progression of joint tissues degeneration in all types of arthritis including after traumatic injury.

Abundant examples concerning the description of the expression and role of specific ncRNAs in the homeostasis of bone and cartilage tissues are present in the literature. It is difficult to choose the most significant and for this reason, please refer to recent reviews that gather the most important evidences (Endisha et al., 2018; Razmara et al., 2019; Li et al., 2020). Many of the papers cited in these reviews investigated the mechanism by which a ncRNA acts, by evaluating the variation in the expression of specific differentiation markers and the functionality of the cells following the silencing or overexpression of the ncRNA. In most cases, such an approach leads to conclusions about regulatory molecular circuits supported by specific epigenetic RNA-mediated processes. Most of the conclusions are certainly valid for the model used, but need to be confirmed in more sophisticated systems respecting biological complexity, and in view of a pre-clinical utility. In many cases a limited reproducibility was observed in studies aimed at elucidating the association of epigenetic signals with bone or joint phenotypes.

THE RISKS THAT CAN BE TAKEN USING INAPPROPRIATE EXPERIMENTAL MODELS

There are highly variable or unreliable conclusions about the role of ncRNAs in the maintenance of joint homeostasis or in contributing to the onset and progression of a joint disease. Literature often provides data that may have been influenced by the choice of partial and not fully representative experimental conditions considering the biochemical and structural complexity of the joint.

The main critical issues that we have identified are related to (1) the employment of unsuitable experimental models; (2) the occurrence of unperceived artifacts; (3) the subsequent impossibility to identify applications for personalized medicine.

Here are just a few examples. Recent studies focusing on lncRNA HOTAIR (HOX transcript antisense intergenic RNA) in cartilage and synovium suggest that it is involved in the regulation of the pathogenesis of OA and synovial inflammation, as HOTAIR silencing could restore collagen II and aggrecan expression (Chen et al., 2020). The analysis on extracellular matrix (ECM) degradation of human chondrocytes revealed that other lncRNAs such as H19, Nespas (a "sponge" targeting mir-291a-3p,−23a-3p,−24-3p,−196a-5p, and let-7a-5p), lncRNA-MSR (targeting miRNA-152) and lncRNA-CIR (targeting miR-27) display a similar role (Sun et al., 2019). In experiments like these, was the influence of physiological cues including mechanical loading or oxygen concentration considered to preserve the native epigenetic profile?

Deregulation of circSEMA4B, circRNA_104670, circ-4099, circ-GRB10, circVMA21 modulates the phenotype of nucleus pulposus cells inside the intervertebral disc (IVD), through sponging their target miRNAs and repressing (or derepressing) the corresponding downstream mRNAs (Li et al., 2020). With current evidence, is treatment of IVD degeneration through restoring the expression of downregulated circRNA or silencing of the aberrantly upregulated circRNAs feasible? To understand the difficulty of answering this question just think that the best way to achieve NP cell-specific delivery of ncRNA-based therapeutics remains undefined.

Regarding the risk of artifacts, it has been shown, for example, that the application of RNA-seq or "single-cell"—OMIC to profile ncRNAs from human cartilage biopsy and isolated chondrocytes suffer the influence of handling procedure and the choice of sample to include in the study (van Meurs et al., 2019). Careful handling and processing of cells is in fact critical to preserve the native epigenetic and expression profile. For instance, different results were obtained from live tissue donors or post-mortem, freshly isolated human chondrocytes or de-differentiated fibroblast like cells cultured in presence of chondrogenic inducers (Ajeekigbe et al., 2019).

We believe that efforts to develop and optimize particularly promising approaches such as those listed below should increase. For example, osteochondral plugs (from bovine, equine, and human specimens) which represent promising *ex-vivo* models for the study of joint diseases (Cope et al., 2019) are able to provide a reliable throughput model for proof of concept and mechanistic studies, applicable also to epigenetic analysis. Attractive alternative has been reached from multicompartmental modular bioreactors, microfluidic-based chip technology that developed "joint-on-a-chip," and high-throughput single cell technologies to measure the epigenomic together genomic, transcriptomic or proteomic state of individul cells at high resolution (Groen et al., 2017; Hwang et al., 2017).

Most researchers rightly work with models that are easy to handle that allow to repeat the experiment numerous times to demonstrate the statistical significance of data: there is no doubt about the correctness of the method. However, in our opinion, it is important that studies relying on a large number of different tests have to be accompanied by "proof of concept" experiments by using methods we here mentioned to pave the way for the development of effective drugs.

In this regard, another aspect not to be overlooked is the added value that can derive from considering the data obtained from each single cell source and therefore from each individual donor/patient. Also with regard to epigenetics, research is heading toward an evolving concept based on a patient-oriented research. Research on RNA-mediated processes by specific ncRNAs in the joint homeostasis and the impact on potential development of new drugs requires also an analysis of the heterogeneity of different kinds of patients. It is important to mention that the best therapeutic decisions for the average patient are not necessarily the best decisions for an individual patient.

CONCLUSIONS AND FUTURE PERSPECTIVES

The applicability of results from *in vitro* studies to *in vivo* situations, especially as regards the molecules involved in regulatory mechanisms, is directly dependent on the degree of similarity between the *in vitro* experimental condition and the *in vivo* environment. We believe a major effort and investment of time in this direction by the scientific community is necessary. The effort should be toward the improvement and the use of technology which allows cells from a specific donor to grow and behave *in vitro* in a manner that more closely represents that experienced by their native counterparts. This approach will likely have a significant impact on the understanding the real role of critical regulators of tissue homeostasis such as ncRNAs, and on improving drug discovery.

This objective can be achieved through different types of initiatives that connect the scientists who deal with joint homeostasis and disease, such as: (1) the creation of an international research Consortium dedicated to support the development and optimization of 3D cell culture models, (2) specific workshops for promoting the development of guidelines in order to minimize controversies on mechanisms of disease and potential therapeutic targets, (3) the creation of a blog managed by a joint scientific organization that promotes debate and where it is possible to meet the experts.

Certainly the biggest challenge is to convince those scientists to move from their already well-established 2D, and often successfully funded, cellular models. Therefore, we think that a critical point is represented by adequate funding policy that takes these issues into account and makes *ad hoc* funds available for studying and developing more relevant experimental models.

AUTHOR CONTRIBUTIONS

LP contributed the idea and the draft. EL contributed the overall structure and corrected the text. RP wrote the paper and conceived the study. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Long-Term Impact of Social Isolation and Molecular Underpinnings

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Prolonged periods of social isolation can have detrimental effects on the physiology and behavior of exposed individuals in humans and animal models. This involves complex molecular mechanisms across tissues in the body which remain partly identified. This review discusses the biology of social isolation and describes the acute and lasting effects of prolonged periods of social isolation with a focus on the molecular events leading to behavioral alterations. We highlight the role of epigenetic mechanisms and non-coding RNA in the control of gene expression as a response to social isolation, and the consequences for behavior. Considering the use of strict quarantine during epidemics, like currently with COVID-19, we provide a cautionary tale on the indiscriminate implementation of such form of social isolation and its potential damaging and lasting effects in mental health.

Keywords: non-coding RNAs, microRNA, long non-coding (lnc) RNAs, epigenetics, social isolation, behavior, COVID-19

INTRODUCTION

Social behavior is a major life component of many organisms. Proper behavior in response to environmental conditions and signals is critical for development, reproduction, and survival (Chen and Hong, 2018). In mammals, social behavior is exquisitely regulated by brain mechanisms that depend on the control of gene expression during development and in response to life experiences (Cole et al., 2007; Zayed and Robinson, 2012; Chen and Hong, 2018). Accumulating evidence suggests that chromatin-based processes and molecular mechanisms including DNA methylation, non-coding RNA (ncRNA) and transcription factors play critical roles in the control of gene regulatory networks that establish and modulate social behavior (Yao et al., 2016; Hwang et al., 2017; Bludau et al., 2019; Seebacher and Krause, 2019; Nord and West, 2020). However today, how the modulation of gene expression can shape behavioral responses to experiences, such as social isolation, during early postnatal development and in adult life is poorly understood (Hilakivi et al., 1989; Weiss et al., 2004; Zelikowsky et al., 2018). Particularly, when social interactions are perturbed by social isolation, a special condition during periods of pandemics like the one we are currently going through, this can directly impact mental health and have consequences throughout life.

This review provides a comprehensive overview of the effects of prolonged periods of social isolation on the body and describes the known molecular events leading to behavioral alterations.

We review the current evidence linking social isolation with changes in gene expression in the brain, and the effects on regulators of genome activity such as epigenetic modifiers, ncRNA and transcription factors. Direct functional evidence supporting the role of miRNAs and long ncRNAs (lncRNAs) as modulators of social behavior and their link to behavioral abnormalities observed during and after prolonged social isolation are discussed. Finally, we reflect on the effects that prolonged social isolation, such as observed during strict quarantine in epidemics, can have on mental health and discuss interventions that may help to ameliorate their burden.

Effects of Social Isolation in Humans

In humans, chronic social isolation can have detrimental health effects (House et al., 1988) (summarized in **Table 1**). Social isolation is associated with increased blood pressure, C-reactive protein, and fibrinogen levels (Shankar et al., 2011). It is also associated with an increased risk to be inactive (Shankar et al., 2011; Schrempft et al., 2019), have motor decline (Buchman et al., 2010) and impaired cognitive functions (Shankar et al., 2013). Loneliness or living alone has been linked with poorer immediate and delayed recall (Shankar et al., 2013) and dementia (Holwerda et al., 2014), as well as higher odds of mental health problems (Coyle and Dugan, 2012). Social isolation can as well result in health-risk behaviors, smoking (Shankar et al., 2011), and reduced self-related physical health (Cornwell and Waite, 2009; Coyle and Dugan, 2012). Therefore, social isolation affects physiology, cognition, and behavior in humans.

Effects of Social Isolation in Animal Models

In rodents, social isolation has multiple effects on physiology and behavior (summarized in **Table 2**). Chronic social isolation (at least 2 weeks) results in complex behavioral responses characterized by increased aggressive behavior toward a submissive intruder, enhanced reactivity to footshock, and freezing to threatening ultrasonic stimulus (Zelikowsky et al., 2018). It also reduces time spent in the center of the arena during open field test (OFT) and increases the propensity to jump off an elevated plus maze (EPM) test (Zelikowsky et al., 2018). Chronically-isolated rodents spend less time interacting with a novel individual, but more time closer to a predator (Zelikowsky et al., 2018). They also have higher anxiety, depression, and anhedonia-like behaviors (Wallace et al., 2009), indicating that chronic social isolation alters behavioral responses in multiple ways.

Prolonged social isolation also affects different aspects of physiology. It can impair neurogenesis in the olfactory bulb (OB), the ventral hippocampus (VH), and the dentate gyrus (DG), and lead to reduced volume of some of these structures and the prefrontal cortex (Lu et al., 2003; Day-Wilson et al., 2006; Guarnieri et al., 2020). The loss of medial prefrontal cortex volume, but not its total number of neurons, resembles that observed in individuals with schizophrenia (Day-Wilson et al., 2006). Social isolation also affects the activity of the hypothalamic-pituitary-adrenal (HPA) axis, which controls the reaction to stress. In prairie voles, chronic isolation differentially affects the expression of the corticotropin-releasing factor receptor 2 (*CRF2)* between the hippocampus and the hypothalamus (Pournajafi-Nazarloo et al., 2011), two brain regions with major roles in regulating stress responses.

Notably, social isolation can promote tumor progression in animal models (Williams et al., 2009; Volden et al., 2013), and correlats with increased expression of key metabolic genes, upregulated lipid synthesis, and glucose metabolism in pre-malignant mammary gland (Williams et al., 2009) and mammary adipocytes (Volden et al., 2013).

Molecular Underpinnings of Social Isolation

Social Isolation and Loneliness Can Be Influenced by Genetic Variation

Loneliness is a social state strongly associated with mortality that is influenced by genetic variation (Gao et al., 2017; Day et al., 2018). A genome-wide association study (GWAS) including almost half a million participants from the UK Biobank study revealed the existence of genetic variants associated with loneliness and regular participation in social activities (Day et al., 2018). A total of 15 genomic loci were significantly associated with loneliness. Interestingly, the association was stronger in regions close to genes expressed preferentially in the brain where they are enriched for epigenetic modifications (Day et al., 2018), suggesting that loneliness can be influenced by genetic variants affecting the activity of regulatory elements in the brain. Interestingly, the expression of 8 genes was linked to susceptibility to loneliness: *GPX1, C1QTNF4, C17orf58, MTCH2, BPTF, RP11-159N11.4, CRHR1-IT1,* and *PLEKHM1*. The case of *BPTF* is of interest as it encodes the Bromodomain PHD finger transcription factor (BPTF) which is the largest subunit of the nucleosome remodeling factor (NURF), a major regulator of chromatin structure and gene expression (Barak et al., 2003; Stankiewicz et al., 2017). *BPTF* is highly expressed in the fetal brain and the brain of patients with neurodegenerative conditions, such as Alzheimer's disease (Bowser et al., 1995). Mutations in *BPTF* have been found in patients with intellectual disability, speech delay, and microcephaly, while genetic inactivation of BPTF in Zebrafish leads to neurodevelopmental phenotypes (Stankiewicz et al., 2017). Therefore, genetic variation affecting the expression of *BPTF* could influence neurodevelopment and social states such as loneliness. Overall, results derived from GWAS suggest that in addition to life experiences, a specific genetic composition could influence social isolation and social interaction. However, whether these genetic associations truly influence brain development and function remains to be determined. Modeling genetic variants identified in humans using murine models and CRISPR-Cas9 editing (Zhu et al., 2019; Sandoval et al., 2020) could prove valuable to decipher the functionality of genetic variants associated with loneliness. Furthermore, it would be of great interest to increase the population diversity of GWAS to provide a comprehensive catalog of genetic variations influencing social behavior across human populations.

TABLE 1 | Physiological and mental health effects of decreased social interactions in humans.

Exposure	Age	Participants (*N*)	Effects of exposure	References
Effects of non-enforced loneliness and social isolation				
Loneliness Social isolation Old age	50+ Mean: 66.9	8,688	-Social isolation was positively associated with blood pressure, C-reactive protein, and fibrinogen levels. -Social isolation and loneliness were associated with higher risk of being inactive, smoking, as well as reporting multiple health-risk behaviors.	Shankar et al., 2011
Social disconnectedness Perceived isolation Old age	57–85	2910	-The correlation between social disconnectedness and perceived isolation is only weak to moderate in strength (r = 0.25, $p < 0.001$). -Results indicate that social disconnectedness and perceived isolation are independently associated with lower levels of self-rated physical health.	Cornwell and Waite, 2009
Social isolation Loneliness Old age	65–84	4004	-The mortality hazard ratio for feelings of loneliness was 1.30 [95% confidence interval (CI) 1.04–1.63] in men and 1.04 (95% CI 0.90–1.24) in women. -No higher risk of mortality was found for social isolation.	Holwerda et al., 2012
Feelings of loneliness (FoL) Living alone Old age	Elderly people mean age: 76.5	3620 community-dwelling elderly people	-Living alone and FoL were both independent predictors of death after 22 years of follow-up (hazard ratio, 1.14; 95% CI, 1.05–1.23; p = 0.001) and (hazard ratio, 1.20; 95% CI, 1.08–1.33; p =0.001), respectively. -No significant interaction was found between feelings of loneliness and living alone (β = 0.08; relative risk = 0.85; 1.40; p = 0.48).	Tabue Teguo et al., 2016
Feelings of loneliness Social isolation	Older persons	2173 non-demented community-living older persons	-Factors positively associated with developing dementia: living alone (p = 0.001), no longer being married (p = 0.001), feelings of loneliness (p = 0.000) and receiving social support (p =0.000). -Social isolation was not associated with a higher dementia risk in multivariate analysis.	Holwerda et al., 2014
Loneliness Old age	Older people Average age: 79.67	985 persons without dementia ∼25% male	-The level of loneliness at baseline was associated with the rate of motor decline (Estimate, −0.016; *SE:* 0.006, p = 0.005). -When terms for both feeling alone (loneliness) and being alone were considered together in a single model, both were relatively independent predictors of motor decline.	Buchman et al., 2010
Social isolation Loneliness Old age	Older adults	11,825	-Loneliness and social isolation were not highly correlated with one another (r = 0.201, p = 0.000). -Loneliness was associated with higher odds of having a mental health problem (OR: 1.17; CI: [1.13, 1.21], p = 0.000). -Isolation was associated with higher odds of reporting one's health as being fair/poor (OR: 1.39; CI: [1.21, 1.59], p = 0.000).	Coyle and Dugan, 2012
Social isolation Loneliness	Mean age at baseline: 65.6 years	6034	-Baseline isolation was associated with decreases in all cognitive function measures at follow-up (β = −0.05 to −0.03, $p < 0.001$). -Loneliness was associated with poorer immediate recall (β = −0.05, $p < 0.001$) and delayed recall (β = −0.03, p = 0.02). -Interaction between educational level and both isolation (p = 0.02) and loneliness (p = 0.01) for delayed recall, such that isolation and loneliness were associated with poorer recall only among those with low levels of education.	Shankar et al., 2013
Social isolation Loneliness Old age	Aged 50–81 years (mean 66.01)	267 community-based men (n = 136) and women (n = 131)	-Total 24 h activity counts were lower in isolated compared with non-isolated respondents (β = −0.130, p = 0.028). -Loneliness was not associated with physical activity or sedentary behavior.	Schrempft et al., 2019
Transition to living alone, Old age	65+	4587	-Living consistently alone did confer increased odds for caseness. -Living alone in later life is not in itself a strong risk factor for psychological distress. -Greater risk of caseness for women, risk increases with age. -A likelihood ratio test confirms that the key interaction between time and living arrangements adds significant explanatory value to the model ($p < 0.001$).	Stone et al., 2013
Solitude	Mean age: 21	44 female college students	-Cortisol levels were significantly higher when individuals were alone. -Trait affectivity moderated the association between solitude and cortisol.	Matias et al., 2011
Effects of enforced social isolation				
30 days isolation	(Age mean: 36.3 ± 7.2) (Age mean: 31.8 ± 8.7)	16 isolated participants 17 non-isolated	30 days of isolation do not have a significant impact on brain activity, neurotrophic factors, cognition, or mood, even though stress levels were significantly increased during isolation.	Weber et al., 2019
Quarantine	64% were 26–45 years of age	129 quarantined persons	-Symptoms of post-traumatic stress disorder (PTSD) and depression were observed in 28.9% and 31.2% of responders. (median duration of quarantine: 10 days). -Longer quarantine was associated with an increased prevalence of PTSD symptoms. -Acquaintance with or direct exposure to someone with a diagnosis of SARS was also associated with PTSD and depressive symptoms.	Hull, 2005

(Continued)

TABLE 1 | continued

Effects of non-enforced loneliness and social isolation

Exposure	Age	Participants (*N*)	Effects of exposure	References
Stress related to epidemic	Mean age: ∼39	Randomly selected employees (n = 549) of a hospital in Beijing	-About 10% of the respondents had experienced high levels of post-traumatic stress (PTS) symptoms since the SARS outbreak. -Respondents who had been quarantined, or worked in high-risk locations such as SARS wards, or had friends or close relatives who contracted SARS, were 2 to 3 times more likely to have high PTS symptom levels, than those without this exposure. -Altruistic acceptance of work-related risks was negatively related to PTS levels.	Wu et al., 2009
SARS quarantine	Mean age: 49	1057	-Self-reported compliance with all required quarantine measures was low (15.8 ± 2.3%), although significantly higher when the rationale for quarantine was understood (p = 0.018). -Health-care workers (HCW) experienced greater psychological distress, including symptoms of PTSD ($p < 0.001$). -Increasing perceived difficulty with compliance, HCW, longer quarantine and compliance with quarantine requirements were significant contributors to higher IES-R scores.	Reynolds et al., 2008
9 days SARS quarantine	Mean age: 39	338 hospital staff	-Quarantine was detected as a relevant factor leading to acute stress disorder (5%) -Feeling stigmatized and rejected in the neighborhood (20%) -Considered resignation (9%)	Bai et al., 2004
2 weeks after contact with MERS patients	Mean age: 44	1656	-During the isolation period, 7.6% of participants had anxiety symptoms, 16.6% had feelings of anger. -After 4–6 weeks, 3% of participants had anxiety symptoms, 6.4% had feelings of anger. -Risk factors: inadequate supplies, social networking activities, history of psychiatric illnesses, financial loss.	Jeong et al., 2016
SARS quarantine	Mean age: 39	903	-Most residents of the first officially recognized site of community outbreak were affected by stigma. -Forms: being shunned, insulted, marginalized, rejected. -Stigma was associated with psychical distress.	Lee et al., 2005
Hospital staff SARS quarantine	Mean age: ∼40	549 hospital employees, 104 quarantined	-Increased odds of having depression 3 years later: being single, having been quarantined, exposure to other traumatic events before SARS, perceived SARS-related risk level. -Decreased odds: altruistic acceptance of risk.	Liu et al., 2012b
SARS quarantine	Mean age: 44	333 nurses	-Lower levels of avoidance behavior, emotional exhaustion, anger, and burnout: high levels of vigor, organizational support, trust in equipment, low levels of contact with SARS patients, time spent in quarantine.	Marjanovic et al., 2007
10 days SARS quarantine	ND*	99 Health care workers 19 patients with SARS	-Patients with SARS reported fear, loneliness, boredom, anger, and worries about family members, anxiety, insomnia, uncertainty, and stigmatization. -Staff: fear of contagion and infecting family, uncertainty, and stigmatization.	Maunder et al., 2003
City isolation because of SARS	ND*	187	-26.2% of participants had psychological disorders. -Prediction factors: income reduction (odds ratio: 25.0), gender, range of activities, eating restrictions, restrictions in going out, disinfection of clothing, infection control.	Mihashi et al., 2009
Ebola quarantine	ND*	432 (focus group) and 30 (interviews)	-High level of social insecurity. -Stress because of forced cremation of death for poor people. -Quarantine raised condemnation, strengthened stigmatization, created socio-economic distress.	Pellecchia et al., 2015
10 days SARS quarantine	Mean age: 43	10 health-care workers	Experienced stigma, fear, frustration.	Robertson et al., 2004
Equine influenza quarantine	Mean age: ∼40	2760 horse owners	34% reported high psychological distress (12% in the general population).	Taylor et al., 2008
H1N1 quarantine	Mean age: 20	419 undergraduates	No significant differences between quarantined and non-quarantined group.	Wang et al., 2017
MERS quarantine	ND*	6231	1221 people placed in quarantine experienced psychological and emotional difficulties, 350 required continuing services.	Yoon et al., 2016

ND = No Data.*

TABLE 2 | Effects of social isolation (SI) in animal models.

Exposure	Organism	Duration of SI	Effects of exposure	References
Social isolation, running, adjusting corticosterone levels	Sprague-Dawley rats:adult, male	12 days	High corticosterone levels in response to stress after social isolation cause running to decrease neurogenesis.	Stranahan et al., 2006
Social isolation Antidepressants (fluoxetine, desipramine) Enriched environment	Swiss mice, adult, male/female	1 week	-Decreased neurogenesis in the olfactory bulb and ventral hippocampus, reduced norepinephrine in OB, and decreased NE and serotonin in the dorsal hippocampus. -Many effects can be prevented by fluoxetine and desipramine.	Guarnieri et al., 2020
Social environments	Male Sprague-Dawley rats (young)	4 or 8 weeks	-Decreased newborn neurons in the dentate gyrus and reduced long-term potentiation in the hippocampus.	Lu et al., 2003
Social isolation	Male Lister Hooded rats; 28 days old	8 weeks	-Volume loss of medial prefrontal cortex, but no loss in neuron number- > loss of volume of the neuropil.	Day-Wilson et al., 2006
Social isolation	Mice: male, 9 weeks old	4 weeks	-Alteration of neuroplasticity related genes.	Ieraci et al., 2016
Exposure to chronic stress (social deprivation)	Mice: male, 3-month-old (C57BL/6)	3 weeks	-Increased HPA axis reactivity and reduced BDNF levels.	Berry et al., 2012
Chronic social isolation stress (CSIS) Acute stress	Rats	21 days of chronic social isolation	Changes in redox-status associated with decreased Hsp70i protein expression enabled NF-jB translocation into the nucleus, causing increased cytosolic nNOS and iNOS protein expression- > oxidative stress.	Zlatković and Filipović, 2013
Social isolation	Rats	30 days	The decrease in neuroactive steroids could be due to a decrease in activity of the HPA axis or the peripheral benzodiazepine receptor response.	Serra et al., 2004
Social isolation	Prairie voles, female/male, adult (2 months old)	4 weeks	Reduction in hypothalamic CRH-R2 and increase in hippocampal CRH-R2 expression	Pournajafi-Nazarloo et al., 2011
Loss of bonded partner, monogamous rodent	Prairie voles	4 days separation from partner	Long-term intracerebroventricular infusion of a non-selective corticotropin-releasing factor (CRF) receptor antagonist.	Bosch et al., 2009
Chronic social isolation	Male Wistar rats	21 days	Suppressed proplastic response and promoted proapoptotic signaling in prefrontal cortex, mediated by unbalance in glucocorticoid receptor and NFkB Transcription factors.	Djordjevic et al., 2010
Social isolation Intrahippocampal interleukin-1 receptor antagonist	Adult male Sprague-Dawley rats	6 h after contextual fear conditioning	Hippocampal-dependent memory impairments induced by elevated levels of brain IL-1 could occur via an IL-1 -induced downregulation in hippocampal BDNF.	Barrientos et al., 2003
Social isolation	Sprague-Dawley rats, 2 months old, male	8 weeks	Reduction on BDNF protein concentrations in the hippocampus.	Scaccianoce et al., 2006
Social isolation, oxytocin administration	Prairie voles: female, adult (60–90 days old)	4 weeks	Oxytocin can prevent effects of social isolation.	Grippo et al., 2009
Social isolation in experiment 1	Prairie voles: female/male, adult (60–90 days old)	4 weeks	Elevated plasma oxytocin and oxytocin immunoreactive cell density in females.	Grippo et al., 2007
Social isolation	Male C57BL/6 mice	3 months	Changes in methylation in the midbrain	Siuda et al., 2014
Individual housing	Male Wistar rats	12 weeks	Sympathetic nervous system: immunocompetent tissues are depleted of catecholamine, this leads to an impairment of immune response.	Gavrilovic et al., 2010
Social isolation	Male Wistar rats, 45 days old at start	12 weeks	Increased Neuropeptide Y in caudate putamen, more explorative rats.	Thorsell et al., 2006
Social isolation stress	Mice	2 weeks	-Upregulation of the neuropeptide tachykinin 2 (Tac2)/neurokinin B (NkB). -Nk3R antagonist prevented the effects of SI.	Zelikowsky et al., 2018
Social isolation Breast cancer	Mouse model of "triple-negative" breast cancer	12 weeks	Increase in mammary tumor growth and metabolic gene expression.	Volden et al., 2013
Social isolation	Female C3 (1)/SV40 T-antigen mice	9.5 weeks	Significantly larger mammary gland tumors burden and increased expression of key metabolic genes.	Williams et al., 2009
Social isolation during adolescence	Male Wistar rats	3 weeks	-Social isolation in adulthood: reduced systolic arterial pressure and increased diastolic arterial pressure. -Most changes caused in adolescence can be reversed by later group housing, except for body weight and baroreflex sensitivity.	Cruz et al., 2016
Social isolation	Prairie voles	4 weeks	Beneficial effects of an enriched environment on depression- and anxiety-relevant behaviors.	Grippo et al., 2014

Social Isolation Induces Changes in Gene Expression

Conversely to the observation that loneliness is influenced by genetic makeup, social experiences can themselves alter gene transcription and have consequences for behavioral responses. In particular, social isolation can modulate gene expression across tissues in many species, from *Drosophila* to mammals (Wallace et al., 2009; Zelikowsky et al., 2018; Agrawal et al., 2020). In *Drosophila*, adult male flies exposed to social isolation for 4 days show robust changes in the expression of 90 genes mostly related to immune response (Agrawal et al., 2020). This is consistent with findings that social isolation modulates immune responses and induces inflammation (Powell et al., 2013; Cole et al., 2015), a condition also associated with depressive-like behaviors in animal models and depression in humans (Ma et al., 2020). The brain-specific neuropeptide *Drosulfakinin* (*Dsk*) was shown to be upregulated in the head of socially isolated males. It was proposed to act as a brake for aggressiveness induced by social isolation as *Dsk* knockdown increases aggressive behaviors of isolated male flies (Agrawal et al., 2020). Notably, its mammalian homolog cholecystokinin (CCK) regulates aggression and anxiety and has been implicated in panic disorder (Zwanzger et al., 2012; Katsouni et al., 2013). CCK transcription can also be modulated by other stressors such as maternal separation (Weidner et al., 2019).

In rodents, chronic social isolation stress can trigger widespread changes in the transcription of protein-coding and non-coding genes (Karelina et al., 2009; Wallace et al., 2009; Liu et al., 2012a; Jin et al., 2016; Kumari et al., 2016; Verma et al., 2016, 2018; Zelikowsky et al., 2018; Mavrikaki et al., 2019; Chang et al., 2020). In adult mice, social isolation for 8 weeks induces transcriptional changes in the myelin genes *Mbop* and *Mobp* in oligodendrocytes of the prefrontal cortex (PFC) (Liu et al., 2012a). Two weeks of social isolation induces a gradual transcription of *Tact2* gene in the brain and peripheral endocrine tissues such as testis (Zelikowsky et al., 2018). *Tact2* codes for the neuropeptide neurokinin B (NkB), necessary for behavioral responses observed in mice subjected to chronic social isolation (Zelikowsky et al., 2018). In rats, prolonged social isolation for 6–12 weeks induces changes in gene expression in the cortex and the nucleus accumbens shell (NAcSh), a brain region important for responses to emotional stimuli (Wallace et al., 2009; Kumari et al., 2016). In cortex, post-weaning social isolation increases the expression of the brain-derived neurotrophic factor (BDNF), the cAMP response element binding protein (CREB-1), and the histone acetyltransferase CREB-1 binding protein (CBP), but reduces the transcription of the histone deacetylase-2 (HDAC2). In the NAcSH, adult chronic social isolation also upregulates many genes coding for K^+ channels and major regulatory proteins such as the activating transcription factor-2 (ATF2), Janus kinase and genes coding for epigenetic factors such as the histone deacetylase-4 (HDAC4) (Wallace et al., 2009). This suggests that chronic social isolation can potentially rewire gene regulatory networks by altering the amount of activity-dependent transcription factors and chromatin-modifying proteins.

Social isolation in rodents can also affect the expression of non-coding RNAs like miRNAs (Kumari et al., 2016; Verma et al., 2018; Mavrikaki et al., 2019; Antony et al., 2020, p. 181; Chang et al., 2020). Prolonged isolation of postnatal rats resulted in differential miRNAs expression in the anterodorsal bed nucleus of the stria terminalis (adBNS), a region involved in anxiety responses (Mavrikaki et al., 2019). A total of 12 miRNAs were differentially regulated in both socially-isolated males and females, with the majority being downregulated, e.g., miR-181c, miR-143, miR-29a, miR-434, and miR-22 (Mavrikaki et al., 2019). Interestingly, the level of miR-29a was also altered in other tissues such as the oral cavity (Yang et al., 2013), suggesting systemic responses to social isolation. miR-181c expression was also downregulated in the brain of isolated mice after stroke (Verma et al., 2018; Antony et al., 2020) while the levels of miR-181a are affected in blood of adult humans with a history of childhood trauma (Mavrikaki et al., 2019), suggesting that miR-181 family members could be a common target of stress responses in mammals.

Changes in miRNAs expression after social isolation can vary depending on sex (Kumari et al., 2016; Mavrikaki et al., 2019). For example, chronic social isolation upregulates miR-132, a direct target of CREB-1, and downregulates miR-134 in the cortex of female rats (Kumari et al., 2016). In female adBNS, twice more miRNAs were affected than in their male counterparts (Mavrikaki et al., 2019), and this correlated with an anxiety behavior (Kumari et al., 2016; Mavrikaki et al., 2019). These findings suggest that chronic social isolation can differentially modulate behavior and transcriptional programs depending on sex. While in females, target genes of miRNAs altered by social isolation are involved in drug addiction and MAPK signaling suggesting effects on reward pathways, in males, target genes are involved in GABAergic synapses thus affect inhibitory neurons (Mavrikaki et al., 2019). Consistently, social isolation increases the propensity to self-administer drugs and to develop addictive behaviors (Green et al., 2010). Overall, different lines of research strongly support that social isolation can alter transcriptional programs in the brain affecting both protein-coding and non-coding genes.

Transcription Factors and Epigenetic Mechanisms Modulate Behavioral Responses to Social Isolation

The mechanisms linking social isolation with changes in gene expression likely involve different molecular cascades with one of the major consequences being perturbed activity of transcription factors (Wallace et al., 2009; Kumari et al., 2016). In the rodent brain, the activity of the transcription factor CREB is diminished in NAcSh of rats exposed to chronic social isolation (Wallace et al., 2009). CREB has been associated with differential expression of a subset of genes, like those coding for K + channels, in the NAcSh of socially isolated rats. Notably, CREB overexpression is sufficient to revert the anxiety-like behavior observed in isolated individuals but not the anhedonia-like phenotype (Wallace et al., 2009). This suggests that CREB is a major player in the regulation of emotional hyper-reactivity in NAcSH and that additional molecular pathways likely regulate other behavioral abnormalities observed in socially-isolated animals. A major

question regarding the role of CREB in social isolation is the molecular nature of its reduced activity during prolonged social isolation. To date, it is unknown whether transcriptional or post-transcriptional mechanisms operating in the NAcSh are responsible for its reduced regulatory activity during chronic social isolation.

Classical epigenetic mechanisms for the control of gene expression are also implicated in the effects of prolonged social isolation (Weaver et al., 2004; Murgatroyd et al., 2009; Gapp et al., 2014; Siuda et al., 2014; Wang et al., 2017). Intermittent social isolation in early postnatal life in rodents, such as induced by maternal separation, can modulate DNA methylation and histone post-translational modifications at regulatory elements of genes involved in stress reactivity including the glucocorticoid receptor (GR) gene (*Nr3c1*) (Weaver et al., 2004) and the mineralocorticoid receptor (MR) gene (*Nr3c2*) (Gapp et al., 2014). This has been associated with a rewiring of stress responses and behavioral adaptation. Chronic social isolation during the juvenile period can also alter the epigenome. Pups at postnatal day (PND) 21 subjected to social isolation for 2 months show a global increase in the level of the repressive histone post-translational modification H3K9me2 in neurons, an effect correlated with increased transcription of the H3K9me2 histone methyltransferase (HMT) G9a and GLP in the hippocampus (Wang et al., 2017). In adult male mice, chronic social isolation for 3 months induced a significant global increase in DNA methylation, H3K4 di, and trimethylation as well as a trend toward an increase in the global levels for H3K9ac, in the midbrain (Siuda et al., 2014). In all cases, the increase in epigenetic modifications was associated with an increase in the catalytic processes leading to such epigenetic modifications. For example, H3K4 HMT activity was significantly enhanced in the midbrain of socially isolated male mice, which could suggest increased transcription of genes coding for H3K4 HMT (Siuda et al., 2014). Prolonged social isolation also increased the transcription of genes coding for HDACs such as *Hdac1* and *Hdac3* which correlated with decreased CpG methylation at their promoter regions (Siuda et al., 2014), supporting the hypothesis that chronic isolation can perturb gene regulatory networks by altering epigenetic modifiers. In contrast, the transcription of the gene coding for the serotonin transporter *Slc6a4* was markedly reduced by social isolation and this correlated with increased DNA methylation at its promoter region (Siuda et al., 2014).

Non-coding RNAs Are Major Regulators of Social Behavior

Non-coding RNAs such as miRNAs and lncRNAs are major regulators of gene expression across the animal kingdom (Jonas and Izaurralde, 2015; Engreitz et al., 2016; Kim et al., 2016; Li and Fu, 2019). Although different lines of evidence suggest that ncRNAs are transcriptionally altered in the brain of rodents after social isolation, direct and functional evidence on their contribution to behavioral and physiological consequences of prolonged social isolation is still sparse (Verma et al., 2018; Antony et al., 2020; Chang et al., 2020). However miRNAs and lncRNAs were proven to modulate social behaviors which are also altered as the result of prolonged social isolation (Haramati et al., 2011; Dias et al., 2014; Issler et al., 2014; Jin et al., 2016; Zhu et al., 2017; Cheng et al., 2018; Lackinger et al., 2019; Labonté et al., 2020; Ma et al., 2020).

MiRNAs

Different miRNAs have been documented to modulate aggressive-, anxiety-, and depression-like behaviors as responses to prolonged social isolation. For example, miR-206 is responsible for the stress-induced aggressive behavior of socially isolated mice via direct targeting of *BDNF* mRNA in the ventral hippocampus (Chang et al., 2020). MiR-34c is downregulated in the brain of socially isolated female rats (Mavrikaki et al., 2019) and it is responsive to chronic stress in the adult central nucleus of the amygdala (CeA) where it has been shown to have an anxiolytic effect when overexpressed (Haramati et al., 2011). Since prolonged social isolation is a form of chronic stress and anxiety a behavioral response of socially isolated female rodents (Kumari et al., 2016; Mavrikaki et al., 2019), miR-34c could be a modulator of anxiolytic responses due to prolonged social isolation.

MiR-135 can modulate serotonin functions by targeting the serotonin transporter *Slc6a4* (Issler et al., 2014), which is downregulated in the midbrain of socially isolated adult mice (Siuda et al., 2014). Consistently, deletion of miR-135 gene in serotonergic neurons results in anxiety- and depression-like behaviors while miR-135 overexpression induces resilience to the behavioral effects of chronic social stress (Issler et al., 2014). The miRNA cluster miR-17-92 is of particular interest as it can also regulate anxiety- and depression-like behaviors by targeting transcripts of the glucocorticoid receptor (GR) pathway in the adult brain (Jin et al., 2016). Deletion of the miRNA cluster in neural progenitors in the adult brain resulted in mice displaying anxiety-, depression-, and anhedonia-like behaviors while miR-17-92 cluster overexpression had anxiolytic and antidepressant-like effects (Jin et al., 2016). Notably, anxiety-, depression-, and anhedonia-like behaviors are all behavioral manifestations of adult rodents exposed to chronic social isolation (Wallace et al., 2009; Zelikowsky et al., 2018) which suggest that chronic pervasive stress can modulate the expression of miRNAs and in such way, impact behavior. In support of this, chronic stress results in reduced expression of the miR-17-92 cluster while the overexpression of the miR-17-92 cluster was anxiolytic and protected against the deleterious effect of chronic stress on neurogenesis (Jin et al., 2016). MiR-137 is another important modulator of social behavior. Heterozygous mice for miR-137 show impaired social behaviors, such as reduced social preference toward other mice, as well as impaired response to social novelty (Cheng et al., 2018), all behavioral manifestations of prolonged social isolation in rodents.

The miR-379-410 cluster is the best-characterized group of miRNAs with a demonstrated role in fine-tuning social behavior in mammals (Lackinger et al., 2019). It is specifically expanded in placental animals and contains 38 miRNAs with documented

roles in neuronal processes. Constitutive removal of the entire cluster results in hyper-social behavior characterized by increased ultrasonic vocalizations both during early and juvenile postnatal life, exaggerated reciprocal social interactions, and increased social approach behavior, suggesting that such miRNAs as a group can function to buffer social behavior in mammals (Lackinger et al., 2019). Knockout mice also had reduced repetitive behaviors and attenuated anxiety-related behaviors. Molecularly, the loss of the miR-379-410 cluster leads to a major up-regulation of the transcript levels for more than 3,000 genes in neurons, consistent with the role of miRNAs in suppressing gene expression. Interestingly, some of the up-regulated genes code for glutamate receptor components which was linked with increased neuronal excitability in the hippocampus and hyper-social behavior (Lackinger et al., 2019). Therefore, the miR-379-410 cluster is a genomic regulatory hub for the fine-tuning of social behavior in mammals. Whether members of the cluster are implicated in behavioral abnormalities due to prolonged social isolation remains to be determined.

While it is clear that miRNAs are transcriptionally dysregulated by social isolation and some of them directly modulate behaviors characteristic of chronically isolated animals, manipulating specific miRNAs *in vivo* has recently emerged as a promising therapeutic approach to ameliorate the negative effects of social isolation on behavior and physiology (Verma et al., 2018; Antony et al., 2020; Chang et al., 2020). For example, inhibition of miR-206 in the hippocampus of socially isolated mice or intranasal administration of an antagonist of miR-206 eliminates stress-provoked attacks via BDNF upregulation (Chang et al., 2020). Also, social isolation can negatively influence stroke recovery in humans and rodents and this has been associated with the dysregulation of miRNAs, such as miR-181c and miR-141, in a mouse model of stroke (Verma et al., 2018; Antony et al., 2020). Mice that were socially isolated post-stroke showed a gradual decrease in the levels of miR-181c in the ipsilateral cortex as compared with group-housed mice also subjected to stroke. Remarkably, the systemic upregulation of miR-181c using a miRNA mimic significantly increased miR-181c levels in the brain and improved survival rate after stroke in isolated mice. This also partially rescued locomotor effects and ameliorated anxiety. Molecularly, the re-establishment of miR-181c levels reduced glial activation in isolated mice (Antony et al., 2020), a remarkable finding as glia activation after stroke has been related to increased inflammation and poorer prognosis (Xu et al., 2020). This data suggest that social isolation could compromise neuroinflammatory responses in the brain after stroke. In support of this, social isolation after stroke impairs the transcriptional upregulation of interleukin-6 (IL-6*)* in the brain (Karelina et al., 2009). While IL-6 is a cytokine involved in the induction of inflammatory responses, IL-6 induction in the brain is neuroprotective (Loddick et al., 1998). Importantly, the systemic inhibition of miR-141c, which is upregulated in the brain of socially isolated mice after stroke, resulted in the transcriptional upregulation of *IL-6* (Verma et al., 2018). Thus, miRNAs act as major modulators of inflammatory responses via regulation of pro-inflammatory genes in the context of social isolation after stroke.

LncRNAs

LncRNAs can also affect social behavior in mice through different mechanisms. The antisense lncRNA of synapsin II (AtLAS) is differentially expressed in the mPFC between dominant and subordinate mice (Ma et al., 2020) and its downregulation in excitatory neurons of the mPFC is sufficient to establish social dominance in grouped mice. Since chronically isolated mice have altered behaviors toward other individuals, such as increased aggression but also blunted response to social novelty (Zelikowsky et al., 2018), it is possible that lncRNAs are important modulators of behavioral responses due to chronic social isolation in mammals. Consistently, aggressive behaviors have also been associated with changes in lncRNAs expression (Punzi et al., 2019; Labonté et al., 2020). The monoamine oxidase A (MAOA) associated ncRNA (MAALIN) is a lncRNA located in the 3′ intergenic region separating the tail-to-tail oriented MAO genes A and B. In humans, the promoter of MAALIN is hypomethylated in neurons of the DG from suicidal subjects with a history of impulsive aggressive disorders and this correlates with lower expression of *MAOA* gene, which has been implicated in aggressive disorders both in humans and animals (Labonté et al., 2020). Overexpression of MAALIN in the hippocampus of aggressive mice induces a discrete downregulation of *MAOA* and results in a trend for increased duration of attacks toward other mice, suggesting that MAALIN could affect aggressive behavior (Labonté et al., 2020), a stereotypic behavioral response for male mice exposed to chronic social isolation.

Neuropeptides Are Major Drivers of Behavioral Responses to Social Isolation

Neuropeptides are major modulators of the behavioral effects observed during extended periods of social isolation across the animal kingdom. In *Drosophila*, the neuropeptides drosulfakinin and tachykinin modulate aggressive behavior in isolated male flies (Asahina et al., 2014; Agrawal et al., 2020). In mice, the expression of the neuropeptide NkB is sufficient and necessary for the behavioral abnormalities observed in socially isolated mice (Zelikowsky et al., 2018). Interestingly, NkB acts regionally in different brain areas to modulate specific behavioral responses due to chronic social isolation stress (Zelikowsky et al., 2018). Therefore, neuropeptides in conjunction with activity-dependent transcription factors, epigenetic modifiers, and ncRNAs are major modulators of behavioral and physiological responses to social isolation.

Behavioral Implications of Quarantine During Epidemics: A Cautionary Tale

In the past centuries, the timely implementation of isolation and quarantine of human populations has shown to be an effective public health intervention to stop the spread of viruses, such as the Ebola virus, MERS-CoV, SARS-Cov, and more recently, SARS-CoV2, the causal agent of COVID-19 (Hull, 2005; Pellecchia et al., 2015; Yoon et al., 2016; Prem et al., 2020). Countries all over the world have applied this strategy, resulting in mandatory or voluntary confinement for several months for more than a third of the world's population up to this point.

Various countries pursued different approaches to prevent and reduce the spread of the virus. The first and strictest type of quarantine was enforced in Wuhan, China, the origin of the coronavirus outbreak (Prem et al., 2020). In some areas of the city, residents were completely forbidden to leave their home. Authorities went from door to door for health checks and forced the ill into isolation (Wuhan's coronavirus outbreak: life inside the quarantine). Italy was the second country to enforce quarantine, then most European countries followed with different level of restrictions. Some countries pursued a more relaxed approach, such as Sweden where confinement was not mandatory, resulting in differences on the overall infectious and death rate due to COVID-19 (Habib, 2020).

While isolation refers to the separation of infected people from those who are healthy, quarantine separates and restricts the movement of people who might be infected but are not yet symptomatic. Physical distancing reduces the frequency and closeness of social contact between people. Although quarantine has been successful in slowing down the spread of the virus, poor implementation can cause additional problems in the exposed people (Pellecchia et al., 2015; Brooks et al., 2020; Buttell and Ferreira, 2020). The current quarantine due to COVID-19 has increased domestic violence, fear of people losing their jobs, reduced physical activity, altered sleep, and increased anxiety (KANTAR, 2020; How the Pandemic Could Be Messing With Your Sleep; Agren et al., 2020; Bouillon-Minois et al., 2020; Economic Commission for Latin America and the Caribbean, 2020; Mahase, 2020; Mazza et al., 2020; Spinelli et al., 2020; Thomas et al., 2020). These effects can even be more pronounced in people in developing countries where most of the population lives under the poverty line, including nations in Africa, Asia, and Latin America. Residents of such countries are in a tremendous hazard on suffering from lasting effects of forced confinement as they can not fulfill even their most basic need. They experience the quarantine as a major physical and psychological stressor for extended periods of time (Madhav et al., 2017; Yatham et al., 2018; Agren et al., 2020; Economic Commission for Latin America and the Caribbean, 2020).

More than 50 years of research in animal models and humans have conclusively shown the detrimental effects of chronic stress on health, highlighting the necessity for more empathic interventions to protect or reduce the sequelae of confinement on mental health and well-being of the population. Simple yet effective strategies could be implemented to reduce social isolation and perceived loneliness among older people, which are a sector of the population at risk to experience the detrimental effects of social isolation (Gardiner et al., 2018). Animal interventions, like animal-assisted therapy or having a pet has been shown to alleviate loneliness in the elderly (Shankar et al., 2011; Krause-Parello, 2012). The use of electronic devices, specifically computer and internet in older adults has also been found to decrease loneliness (Heo et al., 2015). In this regard, the use of a mobile phone for sociability has been associated with decreased loneliness, particularly when used in the context of face-to-face interactions (Wang et al., 2018). Therefore, the knowledge gained by previous research on the biological effects of social isolation on behavior has been an important driving factor for the realization that quarantine can have long-lasting effects on the population.

CONCLUSION

Prolonged social isolation has detrimental effects on humans and animals. In humans, chronic social isolation perturbs physical and mental health and we are just starting to uncover the molecular mechanisms driving behavioral effects associated with social withdrawal. Evidence derived from different animal models strongly suggests that social isolation can induce transcriptional changes in different brain areas fundamental for memory and cognition and also relevant for the modulation of mood and even addictive behaviors. Some of the affected genes are major transcriptional regulators such as the AP-1 transcription factors and CREB, both mediators of transcriptional responses due to neuronal activation in mammals (Yap and Greenberg, 2018). Furthermore, important epigenetic modifiers such as the H3K9me2 histone methyltransferase G9a and histone deacetylases like HDAC-2 and -4, as well as regulatory ncRNAs like miRNAs are also dysregulated, suggesting that social isolation could remodel chromatin and impact steady-state or stimulus-dependent transcriptional responses. While current findings suggest such a possibility, direct causal evidence linking the potential mediators, e.g., transcription factors and epigenetic modulators, with the establishment and maintenance of behavioral and physiological abnormalities associated with social isolation, is still sparse. A major missing information also is the identification of signaling pathways responsible for transcriptional events observed in the brain of socially isolated animals such as CREB activation or transcriptional downregulation of HDACs. Also, the molecular events leading to specific regulation of a subset of miRNAs that modulate important signaling molecules such as BNDF and IL-6 during social isolation are not known.

Furthermore, although available evidence suggests that GR signaling is implicated in the response to acute social isolation in mice (Kamal et al., 2014), whether it contributes to transcriptional effects observed during chronic social isolation is unknown. Based on the available evidence, we envision that chronic social isolation induces remodeling of chromatin structure and organization as a consequence of exposure to chronic stress. Such modification could affect not just brain cells but also other tissues, persistently modifying regulatory programs which in turn change behavior and physiology.

From a public health perspective, major attention should be paid to the physiological and psychological consequences of social withdrawal on the general population. Given that loneliness in humans has been documented to be linked to all-cause increased mortality and with an effect on mortality comparable to smoking, it is fundamental to gain better knowledge of the molecular mechanisms that promote the behavioral and physiological effects of isolation with the

long-term goal to develop new pharmacological and non-pharmacological interventions. While in most cases, social isolation has detrimental effects on the exposed individual in humans and animals, it is possible that some individuals show some resilience. It may be linked to better coping strategies, a isolation habituated state due to a lifestyle based on loneliness, or a natural lower sensitivity to such social stress.

Finally, current actions to mitigate the pandemic of COVID-19 is a call to revisit and implement the best possible public health interventions to protect people against infectious diseases without affecting their physical and mental health. The imposed regulations by governments around the world may have consequences that people do not anticipate and may reverberate for years and possibly decades. Given that the emergence and spread of viruses that infect humans are and will be a constant thread for humankind, a more thoughtful strategy is needed to reduce social interaction while taking into consideration the extraordinary impact that social interactions can have in life.

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AJ, ZL, and VS wrote a draft of the review, and RGA-M and IMM finished it.

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Epigenetic Mechanisms in Immune Disease: The Significance of Toll-Like Receptor-Binding Extracellular Vesicle-Encapsulated microRNA

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WHY WHAT IS OUT THERE MAKES US SICK?

Immune and inflammatory diseases arise from a complex combination of genetic and environmental factors (David et al., 2018; Surace and Hedrich, 2019). MicroRNA are a class of non-coding single-stranded RNA molecules of 19–23 nucleotides in length. In response to environmental triggers, microRNA mediate epigenetic cell fate decisions critical in immune homeostasis by driving cellular activation, polarization, and immunological memory cell development (Mehta and Baltimore, 2016; Curtale et al., 2019). Pattern recognition receptors (PRR) recognize conserved molecular components of pathogens and respond by secreting reactive oxygen species and cytokines that alert the immune system about infection (Medzhitov et al., 1997). They can also interact with various endogenous ligands i.e., lipids, glycans, proteins, and nucleic acids, when released under sterile conditions of cellular stress, tissue injury, and transplantation. As activators of PRR-signaling, endogenous ligands initiate immune cell recruitment and tissue repair. However, sustained PRR-signaling may result in an exacerbated inflammatory response, which can have lethal effects or lead to autoimmunity (reviewed in Yu et al., 2010). In addition to their well-documented canonical function regulating gene expression through RNA interference in the cytoplasm (Bartel, 2004), specific GU-rich microRNA sequences can activate pro-inflammatory signaling pathways by direct interaction with the ribonucleic-acid binding Toll-like receptor 7/8 (TLR-7/8) of innate immunity located in cellular endosomes (Heil et al., 2004). Extracellular vesicles are a heterogeneous population of membrane vesicles naturally secreted by living cells that facilitate intercellular exchanges (Valadi et al., 2007; Raposo and Stoorvogel, 2013). Exported inside extracellular vesicles, Toll-like receptor-binding microRNA released by cells from injured or stressed tissues can reach the endosomal compartment and propagate inflammatory signals in distant recipient cells (**Figure 1**). The contributions of a dozen of TLR-7/8-binding microRNA (let-7b/c, miR-7a, miR-21, miR-29a/b, miR-34a, miR-122, miR-133a, miR-142, miR-145, miR-146a, miR-208a, and miR-210) to inflammation have been described to date in settings of cancer, sepsis, neurological, autoimmune, and graft-vs.-host diseases (Fabbri et al., 2012; Lehmann et al., 2012; He et al., 2014; Park et al., 2014; Salama et al., 2014; Liu et al., 2015; Yelamanchili et al., 2015; Kim et al., 2016; Coleman et al., 2017; Feng et al., 2017; Ranganathan et al., 2017; Young et al., 2017; Salvi et al., 2018; Xu et al., 2018; Wang et al., 2019). Using confocal microscopy co-localization, co-precipitation, and TLR inhibitors, these studies demonstrate direct binding of these microRNA to TLR-7 in mouse and TLR-8 in human, independently of RNA interference. Furthermore, transgenic

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TLR-7$^{-/-}$ mice are protected against the degenerative and inflammation-related effects of TLR-binding microRNA (Fabbri et al., 2012; Lehmann et al., 2012; Yelamanchili et al., 2015; Liang et al., 2019). Since their discovery in 2012, the significance of microRNA as endogenous ligands of innate immunity in health and disease is still a matter of debate (Chen et al., 2013; Fabbri et al., 2013; He, X. et al., 2014; Bayraktar et al., 2019). As part of the dynamic continuum of the endocytic intercellular communication pathway, TLR-binding microRNA transported via extracellular vesicles likely serve both adaptive and maladaptive stress responses in cells expressing TLR-7/8.

MICRORNA TLR-BINDING ACTIVITY: AN EXTRACELLULAR VESICLE PHENOMENON?

So far, unconventional TLR-binding activity has been observed solely for extracellular microRNA and, out of 14 studies, 11 ascertain transfer in association with extracellular vesicles. The effects of danger-associated molecular patterns depend on their detection, a truism applicable to TLR-7-binding microRNA: they can act as such if and only if they reach the endosomal compartment. Encapsulation within extracellular vesicles constitutes a means for microRNA to enter the endocytic pathway where they may directly engage TLR-7/8 signaling (Mulcahy et al., 2014). In contrast, for RNA-interference activity, internalized microRNA have to escape from the endosome (Montecalvo et al., 2012), a rate-limiting step identified in the delivery of therapeutic short interference RNA (Johannes and Lucchino, 2018) and viral infection (Staring et al., 2018). It is conceivable that TLR-binding microRNA are conducive to exerting RNA interference-mediated effects in donor cells and TLR-binding effects or combinations of both after transfer via extracellular vesicles in recipient immune cells, i.e., major sites of TLR-7/8 expression (Lin et al., 2020; Sun et al., 2020).

The relative proportion of free and particulate microRNA in biofluids still raises controversy, which is in part linked to technical pitfalls in the proper assessment of RNA concentrations in extracellular vesicles and biofluids (Arroyo et al., 2011; Turchinovich et al., 2011; Gallo et al., 2012; Crossland et al., 2016; Jeppesen et al., 2019). While free soluble RNA are short-lived due to high physiological levels of ribonuclease activity, microRNA chaperone protein complexes, or extracellular vesicle microRNA have sufficiently low clearance to support autocrine and paracrine signaling loops (Mitchell et al., 2008). Interaction of extracellular vesicles with patrolling immune cells can further transmit local signals of inflammation to the level of the organism. Useful on one hand for systemic coordination, this transmission can prove detrimental in the case of self-sustaining inflammatory responses. Indeed, let-7b for example, whose production can be enhanced by NF-κb activation (Wang et al., 2012) is also a potent TLR-ligand and thus may enhance its own synthesis, a mechanism perpetuating the vicious circle of inflammation in rheumatoid arthritis (Kim et al., 2016). We have demonstrated previously that liposome-encapsulated miR-21 can induce enhanced extracellular secretion in hematopoietic cells through TLR-7/8 signaling (Chang, 2010; Yang et al., 2015; Young et al., 2017). Similarly, the activation of the type 1 interferon/NF-κb pathway has been shown to induce let-7e, miR-21 and miR-146a expression by a positive amplification loop (Chang, 2010; Yang et al., 2015).

QUANTITY MATTERS

If the body produces endogenous ligands of innate immunity, then how does this influence immune homeostasis? Fabbri and colleagues suggested that it is "the type and amount of information that cells exchange that ultimately affect cancer phenotype" (Fabbri et al., 2013). Indeed, biological active cargo is exported within extracellular vesicles sometimes at higher concentrations than in the donor cells and enhanced vesicle release has been broadly associated with inflammation and degeneration in pathological settings (Valadi et al., 2007; Zomer et al., 2015; Robbins et al., 2016; Young et al., 2017; Giri et al., 2020). As detoxifying "garbage bags" (Vidal, 2019), the enhanced extracellular vesicle outflow is presumably beneficial for the donor cell by permitting material clearance, but might entail deleterious consequences for the organism as a whole. The largely overlapping data reported in biomarker studies have built consensus indicating that measurable changes in circulating microRNA do not directly mirror changes in the diseased tissue, but are indicative of a secondary non-specific inflammatory response (Chen et al., 2008; Witwer, 2015). Presumably not by coincidence, TLR-binding microRNA overexpression is recurrently observed in pathological settings for miR-21, miR-7 and members of the let-7 and miR-29 families opening the way for subsequent polyvalent stimulation of the immune system. Although seemingly a critical factor, the quantitative requirements to modulate functional cellular responses are not well-understood. The biological activity of extracellular vesicle-encapsulated microRNA in recipient cells was first demonstrated in microRNA overexpression reporter experiments *in vitro* or after the transfer of concentrated suspensions of purified vesicles (Kosaka et al., 2010; Montecalvo et al., 2012). However, the number of copies measured per vesicles of a given endogenous microRNA is very low, even for abundant microRNA in extracellular vesicles, which raises questions about the physiological relevance of cell-to-cell microRNA-based communication (Williams et al., 2013; Chevillet et al., 2014). For RNA interference–mediated effects, a threshold of 1,000 copies of microRNA has to be reached in the recipient cells to trigger measurable effects (Brown et al., 2007), which represents the successful delivery of an estimated $\approx 10^5$ extracellular vesicles (Igaz, 2015). While these concentrations seem realistic for extracellular vesicles released from broadly distributed tissues such as blood, fat, or muscle (Sender et al., 2016), this seems unlikely for less abundant cell types. In contrast, in the attoliter (10^{-18} L) volume of the endosome, a single RNA molecule equates to a 3 μM concentration, which given the micromolar-affinity of the TLR-7 receptor for guanosine-uracil oligomers, might more easily elicit an immune response (Crozat and Beutler, 2004; Zhang et al., 2016). If present,

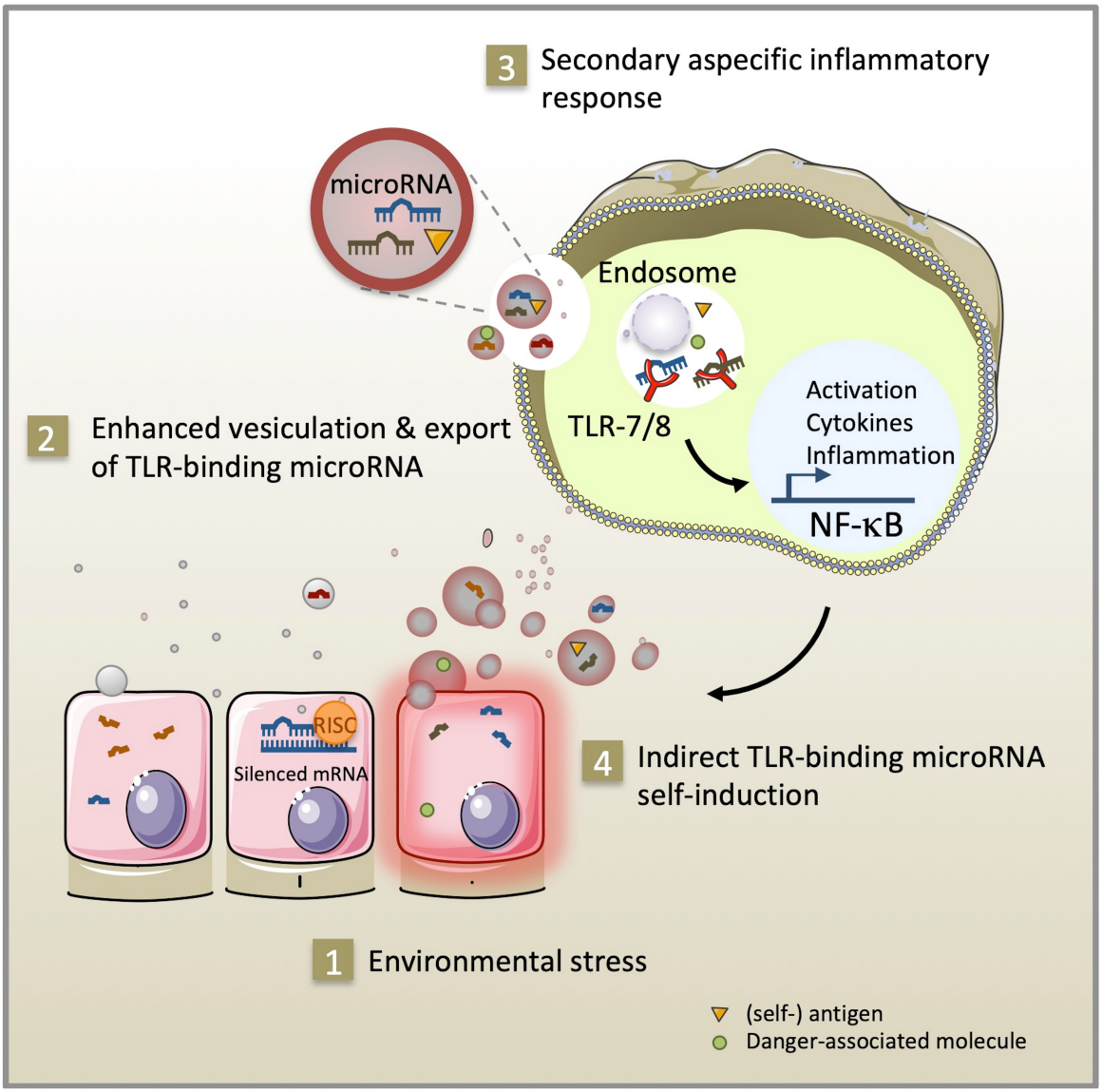

FIGURE 1 | As part of the intercellular endocytic communication pathway, TLR-binding microRNA transmitted via extracellular vesicles serve adaptive and maladaptive stress responses. Environmental stress (1) promotes secretion of extracellular vesicles and microRNA, (self-) antigen and danger-associated molecule release (2). After uptake by innate immune cells, specific GU-rich extracellular vesicle-encapsulated microRNA sequences can stimulate TLR-7/8 signaling in the endosome of recipient cells. Subsequent activation of the NF-κB pathway exacerbates inflammation through cytokine secretion, expression of co-stimulatory molecules (3) and self-induction of TLR-binding microRNA expression and extracellular vesicle secretion (4).

distinct TLR-binding microRNA sequences could synergistically activate TLR-7/8.

ARE ALL EXTRACELLULAR VESICLES EQUAL TLR-7/8 STIMULATORS?

The absolute quantity and diversity of microRNA exported is highest in large apoptotic bodies and shedding microvesicles. Yet, the majority of evidence on TLR-binding microRNA activity has focused on small ∼100 nm vesicles. Similarly, among extracellular vesicles released by serum-starved endothelial cells, only small exosome-like vesicles display immunogenic properties involving the activation of innate PRR by a specific repertoire of non-coding self-RNA (Hardy et al., 2019). In addition to being a consequence of exosome-focused research predominating the field in the past decade, this phenomenon may be explained by evidence of preferential sorting of GU-rich RNA and TLR-binding microRNA into small vesicles in situations of stress (Kouwaki et al., 2016; Fleshner and Crane, 2017; Hardy et al., 2019; Giri et al., 2020; Mensà et al., 2020). Differences in the mechanism and cellular targeting

of extracellular vesicle uptake could further influence the impact of TLR-ligand microRNA on recipient cells. Studies on synthetic RNA-containing particles provide evidence that nanometric particles are selectively internalized by plasmacytoid dendritic cells leading to the production of large amounts of interferon-α whereas micrometric particles preferentially induce tumor necrosis factor-α secretion from monocytes (Rettig et al., 2010). The authors infer that, in addition to surface protein expression, nano- or micro-particle size discrimination *per se* allows the immune defense to adapt to viral or bacterial/fungal infection, respectively. In line with this hypothesis, small extracellular vesicles from systemic lupus erythematosus patients and apoptotic lymphoblasts readily stimulate interferon release form plasmacytoid dendritic cells via TLR-signaling (Schiller et al., 2012; Salvi et al., 2018).

In contrast, extracellular vesicles derived from healthy tissue are essentially immune-silent (reviewed in Fleshner and Crane, 2017). For apoptotic bodies, the intrinsic tolerogenic properties rely on the expression of "find" and "eat-me" signals like phosphatidyl-serine that promote the production of anti-inflammatory mediators like the cytokine transforming growth factor-β and the prostaglandin E2 (Fadok et al., 1998; Pujol-Autonell et al., 2013). Equivalent signals may be absent, weak, or masked in microvesicles and exosomes in pathological settings. Indeed, encapsulated inside extracellular vesicles, microRNA are delivered as a bundle, along with many other immune active molecules i.e., lipids (Sagini et al., 2018), cytokines (Fitzgerald et al., 2018), prostaglandins (Lacy et al., 2019), auto-antigens, ATP or danger-associated molecules (Chalmin et al., 2010; Fleshner and Crane, 2017), which have been shown to concentrate in small extracellular vesicles in acute stress responses (Beninson et al., 2014). Evidence from kidney transplant recipients suggests that small exosome-like vesicles released from stressed or injured tissues create a permissive environment promoting the production of autoantibodies against formerly cryptic antigens (Dieudé et al., 2015; Cardinal et al., 2017). In concert with extracellular vesicle-independent co-stimulants, these factors may further shape the outcome of immune responses that rely on the combination of several activation signals.

CONCLUSIONS

As part of the oldest arm of the immune system, TLR developed 1,350 million years ago to adapt to environmental changes by controlling the activation and differentiation of immune cells by epigenetic mechanisms (Nie et al., 2018). Recent drastic alterations in our environment have been linked to an imbalance in immunity and the spread of inflammatory diseases. As catalyst of inflammation, the physiological significance of extracellular vesicle-encapsulated microRNA binding to TLR-7/8 has probably been over-looked. Further experimental evidence is needed to establish the dominant endogenous activator(s) of the inflammatory response. In particular, we lack (i) studies correlating TLR-binding microRNA expression to disease activity (ii) side-by-side comparisons of the dichotomous function of a given microRNA in its soluble form or encapsulated within specific subpopulations of extracellular vesicles, and (iii) evaluation of extracellular vesicle self-antigen modulation of (auto-) immune responses. The use of animal models should be valuable to further explore thresholds of physiological consequences of TLR-7/8 microRNA activation and systemic interactions in an integrated fashion, *in vivo*. Ultimately, new medication antagonizing TLR-binding microRNA may present an opportunity to prevent excessive inflammatory responses.

AUTHOR CONTRIBUTIONS

All authors conceptualized, wrote, edited, and approved the manuscript. SB designed the figure.

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miR-149-5p Regulates Goat Hair Follicle Stem Cell Proliferation and Apoptosis by Targeting the CMTM3/AR Axis During Superior-Quality Brush Hair Formation

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The Yangtze River Delta white goat is a unique goat species that can produce superior quality brush hair. CKLF-like MARVEL transmembrane domain-containing 3 (CMTM3), which influences the transcriptional activity of androgen receptor (AR), was identified as a candidate gene related to superior-quality brush hair formation. CMTM3 is generally expressed at low levels, but miR-149-5p is highly expressed in the skin tissues of these goats. The mechanism by which CMTM3 regulates the proliferation and apoptosis of goat hair follicle stem cells has not been elucidated. Here, RT-qPCR, western blotting, 5-ethynyl-2′-deoxyuridine (EdU), cell cycle, apoptosis, and dual-luciferase assays were used to investigate the role and regulatory mechanism of CMTM3 and miR-149-5p. Functional studies showed that CMTM3 overexpression inhibited proliferation and induced apoptosis in cultured hair follicle stem cells, whereas silencing CMTM3 markedly facilitated cell proliferation and deterred apoptosis in cultured hair follicle stem cells. Then, using bioinformatic predictions and the aforementioned assays, including dual-luciferase assays, RT-qPCR, and western blotting, we confirmed that miR-149-5p targets CMTM3 and preliminarily investigated the interaction between CMTM3 and AR in goat hair follicle stem cells. Furthermore, miR-149-5p overexpression significantly accelerated the proliferation and attenuated the apoptosis of hair follicle stem cells. Conversely, miR-149-5p inhibition suppressed the proliferation and induced the apoptosis of hair follicle stem cells. These results reveal a miR-149-5p-related regulatory framework for the miR-149-5p/CMTM3/AR axis during superior quality brush hair formation, in which CMTM3 plays a negative role.

Keywords: hair follicle stem cells, miR-149-5p, CMTM3, AR, proliferation, apoptosis, superior-quality brush hair formation, Yangtze River Delta white goat

INTRODUCTION

The Yangtze River Delta white goat, also known as the Haimen goat, is the exclusive goat breed in the world that can produce superior-quality brush hair. This hair is the finest raw material used for making Chinese calligraphy brushes; this breed has also been praised for this unique characteristic, which has resulted in it receiving a complimentary name: the brush hair goat (National livestock and Poultry Genetic Resources Committee, 2011). Brush hair is usually separated into three categories: Type I, inferior-quality hair; Type II, normal-quality hair; and Type III, superior-quality hair (Li and Huang, 2005). Our previous research revealed that the formation of Type III superior-quality brush hair is stimulated by androgen secretion and cold stress, which then activates and modifies the synthesis of certain proteins that participate in the formation stage of hair growth (such as fibrinogen) (Li et al., 2013; Yang et al., 2015). In previous studies, we screened and identified CKLF-like MARVEL transmembrane domain-containing 3 (CMTM3), which modulates the transcriptional activity of androgen receptor (AR), as a putative candidate gene associated with the superior-quality hair trait in Yangtze River Delta white goats (Ji et al., 2018). Upon further investigation with bioinformatic predictions and analyses, CMTM3 was identified as a target gene of miR-149-5p. The chemokine-like factor (CKLF) superfamily is a family of proteins that connects classical chemokines and transmembrane-4 superfamily factors (Han et al., 2001). The CMTM family consists of nine genes, including CKLF and CMTM1-8, and each member plays various roles in multiple biological processes (Gao et al., 2015; Zhang et al., 2016). CMTM3, also known as CKLFSF3, is closely related to AR, has a regulatory role in the male reproductive system, and is characterized by its specific leucine zipper domain and "LXXLL" (where L represents leucine and X represents any amino acid) motifs (Zhong et al., 2006).

MicroRNAs (miRNAs) are short (approximately 22 nt) non-coding RNA molecules that negatively regulate gene expression via posttranscriptional mechanisms, such as inducing target mRNA degradation or repressing translation (Wang et al., 2010). To date, many miRNAs have been identified in the skin tissues of cashmere goats (Zhang et al., 2007; Liu et al., 2012), and some other miRNAs were revealed to play critical roles in coat color genetics. Previously, several studies have reported that miRNAs, including the miR-202 (Qu et al., 2017), miR-181a (Frucht et al., 2011), and miR-let7 (Ma et al., 2018) families, are differentially expressed because their key regulators are involved in skin tissues with different hair colors and skin melanin formation in mice, goats, and sheep. Some miRNAs in skin tissue, such as miR-21 (Ahmed et al., 2011), miR-31 (Mardaryev et al., 2010), miR-214 (Ahmed et al., 2014; Du et al., 2018), miR-218-5p (Zhao et al., 2019a), and miR-320-3p (Zhao et al., 2019a), are essential for the regulation of skin and hair follicle development and regeneration. For example, miR-21 can regulate mouse hair follicle development via the BMP signaling pathway (Ahmed et al., 2011), miR-214 has been shown to suppress human hair follicle stem cell proliferation and differentiation by downregulating EZH2 with Wnt/β-catenin signaling (Du et al., 2018), and overexpression of miR-218-5p in skin fibroblast cells promotes proliferation and represses apoptosis by targeting SFPR2 (Zhao et al., 2019b). miR-149-5p and miR-149-3p comprise the miR-149 family and originate from the miR-149 precursor (based on miRbase v22.1). Recent studies have shown that miR-149-5p is a tumor-related miRNA that can play an important role in regulating cell migration by targeting GIT1 in medullary thyroid carcinoma (Ye and Chen, 2019); in addition, miR-149-5p has been confirmed as an independent prognostic indicator of clear cell renal cell carcinoma (Xie et al., 2018). Furthermore, miR-149-5p could be sponged by lncRNA SNHG8, which results in the promotion of hepatocellular carcinoma tumorigenesis and metastasis (Dong et al., 2018). However, little is known about the function of miR-149-5p in Yangtze River Delta white goats and its regulation in goat hair follicle stem cells during superior-quality hair formation.

According to our previous high-throughput sequencing results of Yangtze River Delta white goat skin tissues, CMTM3 was significantly differentially expressed between superior-quality brush hair goats and normal-quality brush hair goats (Li et al., 2013; Yang et al., 2015; Ji et al., 2018). In addition, the levels of methylated CMTM3 were notably higher while AR expression was significantly higher in superior-quality brush hair goat skin tissues than in normal-quality brush hair goat skins; this was accompanied by higher androgen levels, which are advantageous for the superior-quality brush hair trait (Wang et al., 2018). In the present study, we explored the role of CMTM3 and miR-149-5p in goat hair follicle stem cell function during the formation of superior-quality brush hair. By constructing an overexpression vector and using shRNAs to overexpress or silence endogenous CMTM3, we demonstrated that CMTM3 serves as a negative regulator of goat hair follicle stem cell proliferation and plays a positive role in apoptosis. Furthermore, we confirmed that miR-149-5p could directly target the 3′-UTR of CMTM3 mRNA, which resulted in the upregulation of AR expression. Additionally, we found that miR-149-5p serves as a positive regulator of goat hair follicle stem cells by inhibiting CMTM3, which then accelerates hair follicle stem cell proliferation and mitigates apoptosis. In total, our studies provide numerous supports for the role of miR-149-5p in regulating goat hair follicle stem cell proliferation and apoptosis and reveal a miRNA-related regulatory mechanism involving miR-149-5p, CMTM3, and AR during superior-quality brush hair formation.

Abbreviations: ANOVA, analysis of variance; AR, androgen receptor; BMP signaling pathway, bone morphogenetic proteins signaling pathway; CDS, coding sequence; CKLF, chemokine-like factor; CMTM, CKLF-like marvel transmembrane domain-containing; CMTM3, CKLF-like marvel transmembrane domain-containing 3; DSG1, desmoglein 1; DMEM, Dulbecco's modified eagle's medium; EGFP, enhanced green fluorescent protein; EZH2, enhancer of zeste homolog 2; FBS, fetal bovine serum; FOXM1, forkhead box m1; GIT1, G protein-coupled receptor kinase interacting ArfGAP 1; HDAC4, histone deacetylase 4; HEK293T, human embryonic kidney 293T cells; IGF-I, insulin-like growth factors 1; IGF-IR, insulin-like growth factors 1 receptor; KATAPs, mammalian keratin-associated proteins; lncRNA SNHG8, long non-coding small nucleolar RNA host gene 8; MAPK, mitogen-activated protein kinase; RIPA, radio immunoprecipitation assay; PMSF, phenylmethanesulfonyl fluoride; SFPR2, the secreted frizzled-related protein 2; shRNA, short hairpin RNA; Sirt1, sirtuin1.

MATERIALS AND METHODS

Animal Tissue Sample Collection

Yangtze River Delta white goats, also known as brush hair goats, were obtained from the Haimen State Goat Farm (Haimen City, Jiangsu Province, China). The skin tissues from the cervical spine were collected from three normal-quality brush hair goats and three superior-quality brush hair goats (aged 4–5 months, half sibling rams). Skin tissues were immediately frozen in liquid nitrogen after harvesting. The experimental procedures used in this study were approved by the Animals Care and Use Committee of Yangzhou University.

Expression Profiling and miRNA Prediction

Total RNA was extracted from Yangtze River Delta white goat skin tissues using TRIzol (Takara, Tokyo, Japan) and reverse-transcribed to cDNA using the PrimeScript RT reagent kit (Takara, Tokyo, Japan), which was then immediately used to perform the RT-qPCR assay of skin tissues from normal-quality and superior-quality brush hair goats. All primers used in this study were designed with Primer 5.0 software (Premier Biosoft, CA, United States) and the NCBI Primer-BLAST online website.[1] The primers for the CMTM3 and AR genes are listed in **Table 1**. The GAPDH (for gene detection) gene was used as an internal control.

miRecords[2] and TargetScan[3] software were employed to predict miRNAs (that could target *CMTM3*). miR-149-5p was selected based on prediction, and the specific primers for miR-149-5p used for RT-qPCR of skin tissues from normal-quality and superior-quality brush hair goats are listed in **Table 1**. 18S-rRNA (for miR-149-5p) was used as an internal control (Zhu and Altman, 2005; Kozera and Rapacz, 2013).

Plasmid Construction and RNAi

The stem-loop sequence (precursor) of miR-149-5p from miRbase Release 22.1[4] and the CDS and 3′-UTR of goat *CMTM3* from NCBI[5] were generated and amplified from the Yangtze River Delta white goat genomes. Then, the miR-149-5p precursor sequence was cloned into the *Hin*dIII and *Xba*I sites of the overexpression vector pcDNA3.1(+) to overexpress miR-149-5p (pcDNA3.1[+]-miR-149-5p). The CDS of goat *CMTM3* was cloned into the *Not*I and *Hin*dIII sites of the pDC316-mCMV-EGFP vector to overexpress CMTM3 (CMTM3-OE). Finally, the 3′-UTR of goat *CMTM3* was cloned into the luciferase reporter vector psiCHECK-2 (Promega, Madison, WI, United States) using the *Xho*I and *Not*I restriction sites. The mutant *CMTM3*

[1]https://www.ncbi.nlm.nih.gov/tools/primer-blast/index

[2]http://c1.accurascience.com/miRecords

[3]http://www.targetscan.org/-vert_72

[4]http://www.mirbase.org

[5]https://www.ncbi.nlm.nih.gov/

TABLE 1 | Primer information for miRNA and mRNA quantitative reverse transcription.

Gene	Primer name	Primer sequence (5′ to 3′)	Length
miR-149-5p	Stem-loop RT-miR-149-5p[1]	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACGGGAGTGA	
	miR-149-5p Stem-loop-F	TCTGGCTCCGTGTCTTC	
	miR-149-5p Stem-loop-R	GTGCAGGGTCCGAGGT	
18S-rRNA ID:493779	18S-rRNA-F	GTGGTGTTGAGGAAAGCAGACA	79 bp
	18S-rRNA-R	TGATCACACGTTCCACCTCATC	
PCNA ID:102172276	PCNA-F	ATCAGCTCAAGTGGCGTGAA	213 bp
	PCNA-R	TGCCAAGGTGTCCGCATTAT	
CDK1 ID:10086361	CDK1-F	AGATTTTGGCCTTGCCAGAG	103 bp
	CDK1-R	AGCTGACCCCAGCAATACTT	
CCND2 ID:102180657	CCND2-F	GGGCAAGTTGAAATGGAA	173 bp
	CCND2-R	TCATCGACGGCGGGTAC	
CMTM3 ID:102174055	CMTM3-F	CCTCTGCTTCCTCTTTGCTGATG	129 bp
	CMTM3-R	ACGGCTGTGATGGAGATGGC	
AR ID:100860827	AR-F	CCATCTCTTCCAAGGACAGTTACC	115 bp
	AR-R	TGCTCCAATGCCTCCACACC	
Bcl2 ID:100861254	Bcl2-F	ATGTGTGTGGAGAGCGTCAA	187 bp
	Bcl2-R	CCTTCAGAGACAGCCAGGAG	
Caspase3 ID:102177031	Caspase3-F	AGGCAGACTTCTTGTACGCA	170 bp
	Caspase3-R	TTCTGTCGCTACCTTTCGGT	
Caspase9 ID:102174681	Caspase9-F	GGGGACTTCTGGTGGTTAGT	118 bp
	Caspase9-R	GAGTCAGGAGGGAGAAAGCTG	
GAPDH ID:100860872	GAPDH-F	AGGTCGGAGTGAACGGATTC	259 bp
	GAPDH-R	CCAGCATCACCCCACTTGAT	

[1]*Stem-loop RT-miR-149-5p was applied for the reverse transcription of miR-149-5p.*

3′-UTR luciferase reporter vector was obtained by changing the miR-149-5p binding site from GAGCCAG to GTCGGTG. The primers used for plasmid construction are shown in **Table 2**. ShRNAs (CMTM3-sh1, CMTM3-sh2, and CMTM3-sh3) targeting goat *CMTM3* and a shRNA scramble (sh-NC) were purchased from GenePharma (GenePharma, Suzhou, China); the sequences are shown in **Table 3**.

TABLE 3 | Sequence information for RNA oligonucleotides.

Name	Sequence name	Sequence information (5′ to 3′)
miR-149-5p	miR-149-5p mimics	UCUGGCUCCGUGUCUUCACUCCC (sense)
		GAGUGAAGACACGGAGCCAGAUU (antisense)
	miR-149-5p NC	UUCUCCGAACGUGUCACGUTT (sense)
		ACGUGACACGUUCGGAGAATT (antisense)
	miR-149-5p inhibitors	GGGAGUGAAGACACGGAGCCAGA
	miR-149-5p inhibitor NC	CAGUACUUUUGUGUAGUACAA
CMTM3	CMTM3-NC	UUCUCCGAACGUGUCACGUTT (sense)
		ACGUGACACGUUCGGAGAATT (antisense)
	CMTM3-sh1	GGCCAAATTCCTCAAACAAGA (sense)
		TCTTGTTTGAGGAATTTGGCC (antisense)
	CMTM3-sh2	GCAGAAGAAGAGAATTCCGAC (sense)
		GTCGGAATTCTCTTCTTCTGC (antisense)
	CMTM3-sh2	GCTAGGCACTTTGTCAATAAT (sense)
		ATTATTGACAAAGTGCCTAGC (antisense)
	sh-NC	GGACAGTCAGAGTGTTACAGC (sense)
		GCTGTAACACTCTGACTGTCC (antisense)

Cell Culture and Transfection

Hair follicle stem cells from Yangtze River Delta white goats were isolated from newborn ram lamb neck skin and cultured, as described in our previous study (Wang et al., 2019). The procedures are briefly described below: (1) Skin tissues were washed with 0.9% normal saline followed by 75% ethanol with 1% penicillin-streptomycin (Invitrogen, CA, United States) three times. (2) Tissues were then rinsed with phosphate buffered saline (PBS, Solarbio, Beijing, China) three times and cut into small pieces (approximately 1 mm^3). (3) Digestion was performed with 0.25% Trypsin-EDTA (Gibco, New York, NY, United States) at 37°C for 1.5 h. (4) After digestion, hair follicles were picked and harvested by means of a stereomicroscope (Leica, Wetzlar, Germany). (5) The harvested hair follicles were digested with 0.25% Trypsin-EDTA again at 37°C for 30 min. (6) The digested follicles were placed in DMEM-F12 (Gibco, New York, NY, United States) supplemented with 20% FBS (Gibco, New York, NY, United States) and 2% penicillin-streptomycin and were ground in a homogenizer. (7) Finally, the mixed medium was filtered through a 200-mesh cell strainer (Corning, New York, NY, United States) and cultured in 60-mm culture plates (Corning, New York, NY, United States) at 37°C. The morphological images of hair follicle stem cells and measurements of integrity are presented in **Supplementary Figure S1**. Hair follicle stem cells and HEK293T cells were separately cultured in 6-well plates (Corning, New York, NY, United States) with growth medium (GM) comprising DMEM-F12 supplemented with 20% FBS and 2% penicillin-streptomycin and incubated at 37°C in an atmosphere containing 5% CO_2.

The effects of miR-149-5p on hair follicle stem cell proliferation and apoptosis were investigated by transfecting hair follicle stem cells with pcDNA3.1(+)-miR-149-5p (**Table 2**) and negative control (NC), miR-149-5p mimics (Mimics), single-stranded negative control (Anti-NC), and 2′-O-methylated oligonucleotides against miR-149-5p (Inhibitors) purchased from Gene Pharma (Suzhou, China) (**Table 3**) using Lipofectamine 3000 (Invitrogen, CA, United States) following the manufacturer's instructions. The effects of CMTM3 on hair follicle stem cell proliferation and apoptosis were determined via transfection with CMTM3-OE (**Table 2**), CMTM3-NC, sh-NC, CMTM3-sh1, CMTM3-sh2, and CMTM3-sh3 (**Table 3**). Transfection was performed when the stem cells grew to ∼70–80% confluence. After transfection, the cells were incubated and cultured in Opti-MEM (Gibco, New York, NY, United States) for 6 h, after which the medium was replaced with fresh GM for 96 h. Then, the stem cells were collected at 24-h intervals for further experiments. All hair follicle stem cell cultures were performed at least in triplicate.

Cell Proliferation Assay

First, hair follicle stem cells were seeded at a density of 5×10^5 cells/well in 6-well plates with GM. When the stem cells grew to

TABLE 2 | Primers used to construct the plasmids.

Gene	Primer name	Primer sequence (5′ to 3′)
pcDNA3.1(+) -miR-149-5p	Pre-miR-149-5p-F	*CCCAAGCTT*TGGGAAGAGAATTGCATCCGT
	Pre-miR-149-5p-R	*GCTCTAG*AAGGACACACAGGAAGCCCT
Wild-type CMTM3	Wild-CMTM3-F	CCGCTCGAGGGCATTTCCTGTGACCCAA
	Wild-CMTM3-R	ATAAGAATGCGGCCGCGGACCACGCTGTGCTGATA
Mutant-CMTM3	Mutant-CMTM3-F	CCGCTCGAGTTGTGAATGTCGGTGAGTTCT GGACCCA
	Mutant-CMTM3-R	ATAAGAATGCGGCCGCGGACCACGCTGTGCTGATA
CMTM3-OE	CMTM3-OE-F CMTM3-OE-R	ATAAGAATGCGGCCGCATGTGGCCCCCAGACC CGGAGCC
		*CCCAAGCTT*TCTGCCTTGTCAGCTGTGGTCTC

HindIII and XbaI restriction sites are italicized; XhoI and NotI restriction sites are underlined.

∼70–80% confluence, NC, miR-149-5p mimics, anti-NC, miR-149-5p inhibitors, or pcDNA3.1(+)-miR-149-5p was separately transfected. After 6 h, the transfection medium was replaced with fresh GM for 96 h. The mRNA and protein levels of PCNA, CDK1, and CCND2 were detected by RT-qPCR and western blotting, respectively, at 24-h intervals to analyze hair follicle stem cell proliferation. Second, hair follicle stem cells were seeded at a density of 1×10^5 cells/well in a 24-well plate (Corning, New York, NY, United States) and transfected with miR-149-5p oligonucleotides (**Table 3**) or pcDNA3.1(+)-miR-149-5p. In brief, stem cells were incubated for 2 h with serum-free medium containing 50 μM 5-ethynyl-2′-deoxyuridine (EdU) reagent from an EdU cell proliferation kit (RiboBio, Guangzhou, China) prior to immunostaining. Six independent replicate experiments were performed for each group. Fluorescent images were collected using a Leica fluorescence microscope (Leica, Wetzlar, Germany), and the imaging parameters were identical in all fluorescence microscopy images.

Assessment of Apoptosis

First, hair follicle stem cells were cultured at a density of 5×10^5 cells/well in 6-well plates with GM. After reaching ∼70–80% confluence, the stem cells were transfected with miR-149-5p oligonucleotides (**Table 3**) or pcDNA3.1(+)-miR-149-5p. After 6 h, the transfection medium was replaced with fresh GM for 96 h. The mRNA and protein levels of Bcl2, Caspase3, and Caspase9 were detected by RT-qPCR and western blotting, respectively, at 24-h intervals to analyze hair follicle stem cell apoptosis. Second, an Annexin V-FITC/propidium iodide (PI) staining assay was used to assess the apoptosis of hair follicle stem cells. After transfection with the abovementioned oligonucleotides or plasmids and culture for 48 h, cells subjected to different treatments were washed at least three times with 1 ml of 1 × PBS (pH = 7.4), digested and collected by trypsin, washed once more with 1 ml of 1 × PBS, and resuspended in 1 ml of 1 × binding buffer (Solarbio, Beijing, China). Afterward, the cells were treated with 5 μl of Annexin V-FITC and 10 μl of PI (Solarbio, Beijing, China) and incubated in the dark at room temperature for 10 min. Finally, the cells were analyzed using flow cytometry (FACSAria SORP, BD BioSciences, NJ, United States).

Stem Cell RNA Isolation, Reverse Transcription PCR (RT-PCR), and Real-Time Quantitative PCR (RT-q-PCR)

Total RNA was extracted from hair follicle stem cells cultured *in vitro* using a TRIzol kit (Takara, Tokyo, Japan). For gene quantification, 1 μl of total RNA (1000 ng/μl) was reverse-transcribed into cDNA using the PrimeScript RT kit (Takara, Tokyo, Japan) and then quantified on an ABI 7500/7500-Fast Real-Time PCR System (Applied Biosystems, CA, United States) with TB Green II Master Mix Reagent Kit (Takara, Tokyo, Japan). For miR-149-5p quantification, 1 μl of total RNA (1000 ng/μl) and a miR-149-5p stem-loop primer or a pair of miR-149-5p-specific primers (**Table 1**) were used for miR-149-5p RT-PCR and RT-qPCR, respectively. GAPDH (for gene detection) and 18S-rRNA (for miR-149-5p) were selected as internal normalization controls. The reaction conditions were as follows: 95°C for 30 s (initial denaturation), 40 cycles of 95°C for 10 s (denaturation) and then 60°C for 1 min (annealing), and an elevated optimum temperature for 5 min (final extension). The relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Arocho et al., 2006; Adnan et al., 2011).

Western Blotting

Total cellular protein was extracted from each treatment group using RIPA lysis buffer (Solarbio, Beijing, China) supplemented with 1% PMSF (Solarbio, Beijing, China). Cell protein fractions were prepared and collected by centrifugation (13 000 × g, 4°C, 5 min) and then quantified using a BCA protein assay kit (Solarbio, Beijing, China). For detection, 20 μg of cellular proteins was separated via SDS-polyacrylamide gel electrophoresis with 8% or 10% gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Darmstadt, Germany), which were then blocked with 5% skim milk (Sangon Biotech, Shanghai, China) for 2 h at room temperature. Subsequently, the blocked PVDF membranes were incubated overnight at 4°C with primary antibodies against PCNA (MW: 29 kDa, Abcam, Cambridge, United Kingdom, 1:1000 dilution), CDK1 (MW: 34 kDa, Abcam, Cambridge, United Kingdom, 1:1000 dilution), CCND2 (MW: 33 kDa, Abcam, Cambridge, United Kingdom, 1:1000 dilution), Bcl2 (MW: 26 kDa, Proteintech, Rosemont, IL, United States, 1:1000 dilution), Caspase3 (MW: 32 kDa, Proteintech, Rosemont, IL, United States, 1:1000 dilution), Caspase9 (MW: 46 kDa, Proteintech, Rosemont, IL, United States, 1:1000 dilution), AR (MW: 68 kDa, Abcam, Cambridge, United Kingdom, 1:1000 dilution), CMTM3 (MW: 20 kDa, Bioss, Beijing, China, 1:1000 dilution) and β-actin (MW: 42 kDa, Abcam, Cambridge, United Kingdom, 1:500 dilution). Then, the membranes were washed with 1 × Tris-buffered saline buffer supplemented with Tween 20 (TBST) (Solarbio, Beijing, China) before they were incubated with horseradish peroxidase-conjugated secondary antibodies, including goat-specific anti-rabbit IgG and rabbit-specific anti-goat IgG (Bioworld, Nanjing, China, 1:5000 dilution), for 2 h. Protein bands were visualized using Super-enhanced ECL Reagent (Biosharp, Hefei, China) and then analyzed on a FluorChem FC3 system (Protein-Simple, CA, United States). Finally, the band intensities on the images were analyzed using ImageJ software.

Cell Cycle Assay

A cell cycle PI staining assay was performed to determine the effects of miR-149-5p oligonucleotides, pcDNA3.1(+)-miR-149-5p, CMTM3-OE, and CMTM3-shRNAs on the different phases of the cell cycle in hair follicle stem cells. After cells were transfected for 48 h, they were collected in 6-well plates and centrifuged at 1500 rpm/min for 5 min. The supernatant was discarded after centrifugation, and the pelleted cells were washed once with 1 ml of precooled 1 × PBS. Afterward, cells were incubated with 1 ml of precooled 70% ethyl-alcohol at 4°C for 12 h. Subsequently, the cells were resuspended in 500 μl of PI staining buffer (Beyotime, Shanghai, China) and incubated at 37°C for 30 min in the dark. Then, the cell suspensions were subjected

to flow cytometry analysis (FACSAria SORP, BD BioSciences, NJ, United States). ModFit LT^{TM} software (Verity Software House, Topsham, ME, United States) was used to create and analyze the cell cycle histograms.

Dual-Luciferase Assay

HEK293T cells were cultured in 24-well Corning plates in DMEM-F12 supplemented with 20% FBS and 2% penicillin-streptomycin. Transfection was performed when cells grew to ∼70–80% confluence. The miR-149-5p oligos comprising miR-149-5p NC, miR-149-5p mimics, miR-149-5p anti-NC, and miR-149-5p inhibitors were cotransfected with psiCHECK-2 goat *CMTM3*-3′-UTR (wild-type *CMTM3*-3′-UTR) or psiCHECK-2 goat CMTM3-mut-3′-UTR (mutant-*CMTM3*-3′-UTR) into HEK293T cells with Lipofectamine 3000. Additionally, pcDNA3.1(+)-miR-149-5p was cotransfected with wild-type *CMTM3*-3′-UTR or mutant *CMTM3*-3′-UTR into HEK293T cells with Lipofectamine 3000. Forty-eight hours after transfection, a Dual-Luciferase Reporter Assay kit (TransGen, Beijing, China) was used to quantify the relative luciferase activity in each well according to the manufacturer's protocols. Firefly and Renilla luciferase activities were assessed on a BioTek Synergy 2 Multimode Microplate Reader (BioTek, VT, United States). The firefly luciferase activity was normalized to the Renilla luciferase activity.

Statistical Analysis

All data produced in this study are shown as the mean ± standard error of the mean (SEM) and are based on at least three or six independent biological replicates for each assay. One-way ANOVA was performed in SPSS v24 software (IBM, Armonk, NY, United States) to analyze miR-149-5p, CMTM3, and AR expression levels in hair follicle stem cells at 24 h intervals. Independent-samples *t*-tests were performed in SPSS v24 and Origin 7.5 software (OriginLab, MA, United States) to analyze and compare two different treatment groups (such as normal-quality vs. superior-quality; NC vs. Mimics; Anti-NC vs. Inhibitors; CMTM3-NC vs. CMTM3-OE; etc.). *P*-values < 0.05 were considered to be significant. $^{*}P < 0.05$ and $^{**}P < 0.01$.

RESULTS

miR-149-5p, CMTM3, and AR Expression in Yangtze River Delta White Goat Skin Tissue and Goat Hair Follicle Stem Cells

RT-qPCR analysis of miR-149-5p, CMTM3, and AR mRNA levels was performed on skin tissues from three normal-quality brush hair goats and three superior-quality brush hair goats to investigate differences in expression and explore the potential function of miR-149-5p and CMTM3. Difference analysis showed that miR-149-5p expression was higher in skin tissues from superior-quality brush hair goats than in those from normal-quality brush hair goats ($P < 0.05$) (**Figure 1A**). By contrast, CMTM3 expression was lower in superior-quality brush hair goats than that in normal-quality brush hair goats, but this difference was not significant ($P > 0.05$) (**Figure 1B**). AR expression was also higher in superior-quality brush hair goat skin samples than that in normal-quality brush hair goat skin samples ($P < 0.05$) (**Figure 1C**) because superior-quality brush hair is only formed in ram goats (aged 4–5 months), and this expression trend is consistent with the RNA-seq results of AR expression in goat hair follicle stem cells after CMTM3 interference (unpublished data). We also investigated miR-149-5p, CMTM3, and AR expression levels in untreated hair follicle stem cells in GM at 24 h, 48 h, 72 h, and 96 h. We observed that miR-149-5p expression levels were increased in untreated hair follicle stem cells at 24 h, 48 h, 72 h, and 96 h (at 48 h, $P > 0.05$; at 72 h and 96 h, $P < 0.05$) (**Figure 1D**); the mRNA level of CMTM3 was significantly decreased in untreated hair follicle stem cells at 24 h, 48 h, 72 h, and 96 h (at 48 h, $P < 0.05$; at 72 h and 96 h, $P < 0.01$) (**Figure 1E**); and the mRNA level of AR was increased over time in untreated hair follicle stem cells, but this change was not significant at 24 h, 48 h, 72 h, and 96 h (at 48 h, 72 h, and 96 h, $P > 0.05$) (**Figure 1F**). These results suggested that miR-149-5p and CMTM3 play antagonistic roles in hair follicle stem cells, and we hypothesized that the miR-149-5p/CMTM3/AR axis regulates the process of superior-quality brush hair formation. Thus, we further investigated miR-149-5p by assessing the individual effects of synthetic miR-149-5p mimics, miR-149-5p inhibitors, and a constructed overexpression vector (pcDNA3.1[+]-miR-149-5p plasmid) on hair follicle stem cells. The delivery of miR-149-5p duplexes was remarkably effective, increasing or reducing miR-149-5p levels by > 30-fold, respectively, in hair follicle stem cells ($P < 0.01$) (**Supplementary Figures S2A,B**).

CMTM3 Inhibits Proliferation and Promotes Apoptosis of Goat Hair Follicle Stem Cells

We first identified the role of CMTM3 during hair follicle stem cell proliferation and apoptosis by overexpressing or silencing endogenous CMTM3 in these cells using the pDC316-mCMV-EGFP-CMTM3 vector (CMTM3-OE) or shRNA targeting CMTM3, respectively. CMTM3 mRNA expression was notably increased in the CMTM3-OE group and extremely reduced in the CMTM3-sh1 group ($P < 0.01$) (**Figure 2A**). Fluorescent images of EGFP in hair follicle stem cells indicated that CMTM3-OE and CMTM3-sh1 were successfully transfected into the stem cells (**Figure 2B**). These results showed that CMTM3 expression (at the mRNA level) was effectively overexpressed or suppressed by CMTM3-OE and CMTM3-sh1, respectively, in hair follicle stem cells.

Next, cell cycle and Annexin V-FITC/PI staining assays were performed to investigate the role of CMTM3 during the proliferation and apoptosis of hair follicle stem cells. Cell cycle analysis revealed that CMTM3 overexpression via transfection with CMTM3-OE decreased the number of hair follicle stem cells at S-phase (from 13.10% down to 12.38%, $P > 0.05$) and increased the proportion of cells in G0/G1-phase (from 80.00% up to 82.55%, $P > 0.05$). In contrast, knocking down CMTM3 via transfection with CMTM3-sh1 significantly increased the number of hair follicle stem cells at the S-phase (from 12.08% up

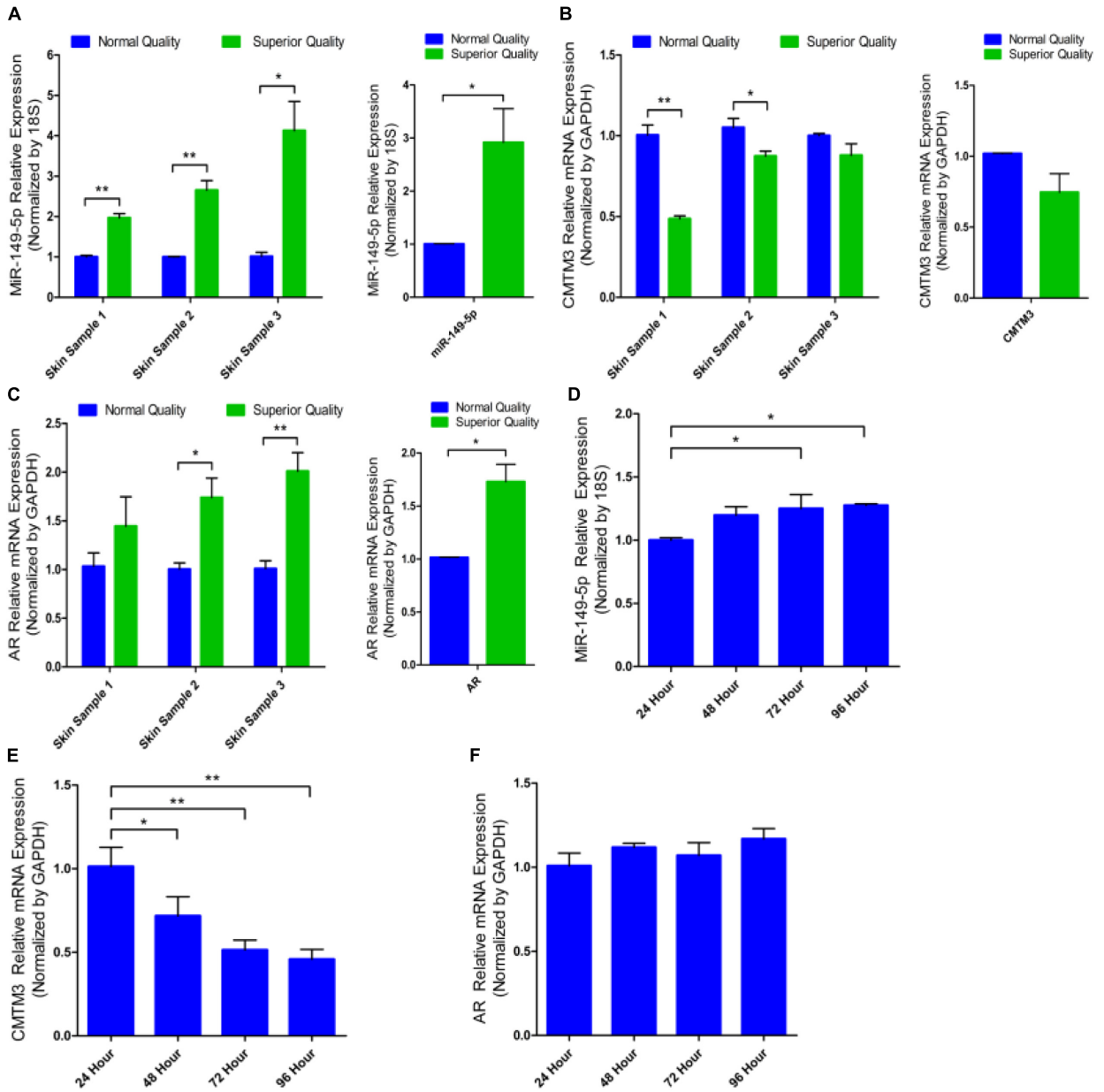

FIGURE 1 | miR-149-5p, CMTM3, and AR expression in skin tissues and cultured hair follicle stem cells from brush hair goats. The differences in miR-149-5p **(A)**, CMTM3 gene **(B)**, and AR gene **(C)** expression in skin tissues between three normal-quality brush hair goats and three superior-quality brush hair goats. Relative expression of miR-149-5p **(D)**, CMTM3 **(E)**, and AR **(F)** at 24 h, 48 h, 72 h, and 96 h were examined in GM by RT-qPCR of hair follicle stem cells. The results from each group are shown as the mean ± SEM of three independent replicates. One-way ANOVA and independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

to 16.30%, $P < 0.01$) and notably decreased the proportion of the cells in G0/G1-phase (from 82.99% down to 76.93%, $P < 0.05$) (**Figures 2C,D**). The Annexin V-FITC/PI staining assay showed that CMTM3 overexpression clearly accelerated apoptosis in hair follicle stem cells and strongly increased the apoptotic cell proportion ($P < 0.01$) (**Figure 2E**), while decreased CMTM3 expression protected hair follicle stem cells from apoptosis and profoundly decreased the proportion of apoptotic cells ($P < 0.01$) (**Figure 2F**). These results showed that CMTM3 overexpression functions in a manner consistent with that of miR-149-5p inhibition on hair follicle stem cell proliferation and apoptosis. Conversely, the effect of CMTM3 inhibition on hair follicle stem cell proliferation and apoptosis was consistent with that of miR-149-5p overexpression. Moreover, RT-qPCR and western blotting were used to assess AR mRNA and protein expression levels, respectively, in hair follicle stem cells with CMTM3 overexpression or knockdown. The results showed that AR mRNA and protein expression levels were markedly decreased in

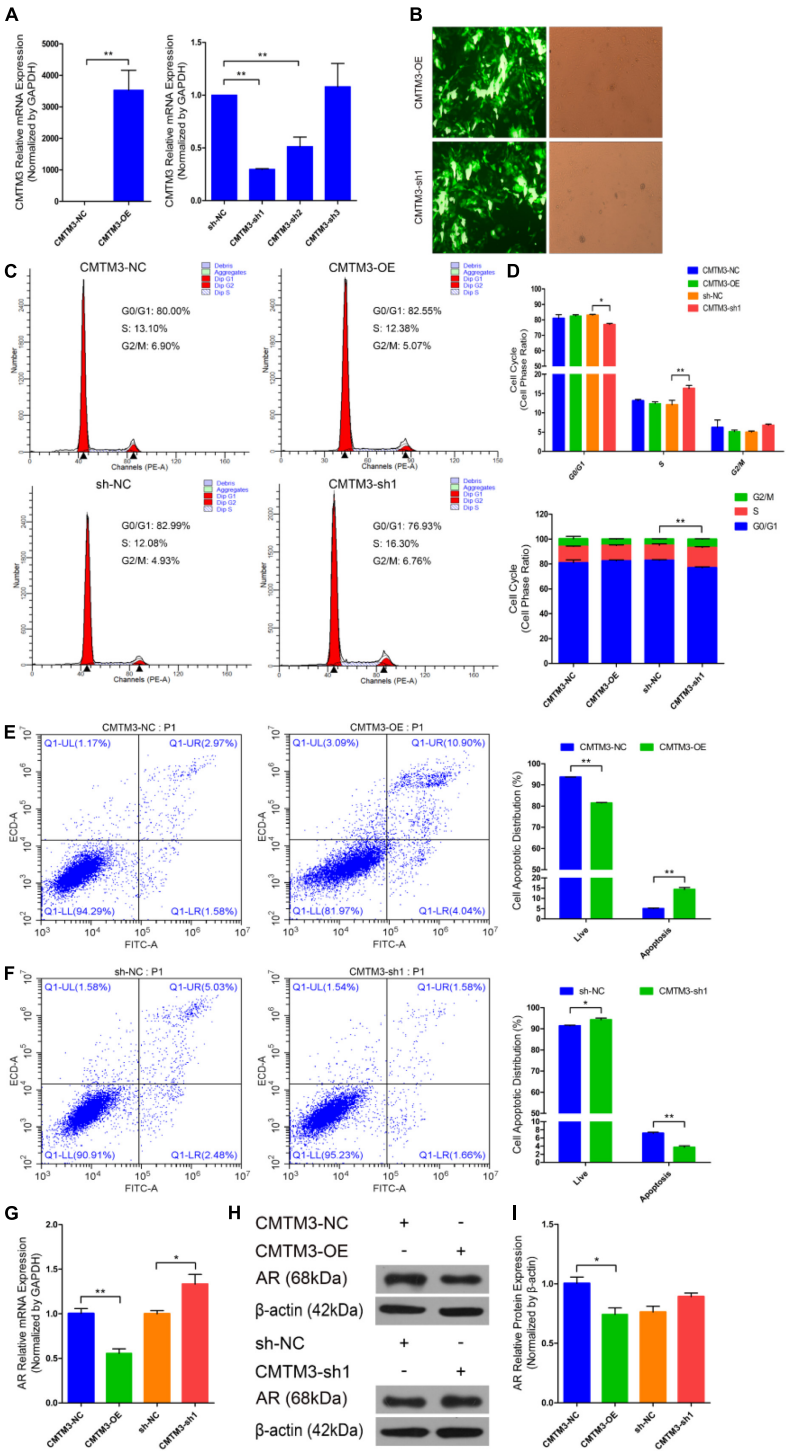

FIGURE 2 | Role of CMTM3 in goat hair follicle stem cell proliferation and apoptosis. **(A)** CMTM3 expression was measured 24 h after transfection with CMTM3-NC, CMTM3-OE, sh-NC, CMTM3-sh1, CMTM3-sh2, and CMTM3-sh3 in GM. **(B)** Images of EGFP in hair follicle stem cells at 24 h after transfection with CMTM3-OE and CMTM3-sh1 in GM. Hair follicle stem cells were transfected with CMTM3-NC, CMTM3-OE, sh-NC, and CMTM3-sh1 in GM, and cell phases were analyzed 24 h after transfection by flow cytometry **(C)** and counted **(D)**. **(E)** Hair follicle stem cells were transfected with CMTM3-NC or CMTM3-OE in GM, and cell apoptosis was analyzed 48 h after transfection by Annexin V-FITC/PI binding followed by flow cytometry. **(F)** Hair follicle stem cells were transfected with sh-NC or CMTM3-sh1 in GM, and cell apoptosis was analyzed 48 h after transfection by Annexin V-FITC/PI binding followed by flow cytometry. **(G)** AR expression was measured after transfection with CMTM3-NC, CMTM3-OE, sh-NC, and CMTM3-sh1 in GM. **(H,I)** AR (1:1000 dilution) protein expression was examined after transfection with CMTM3-NC, CMTM3-OE, sh-NC, and CMTM3-sh1 in GM. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

hair follicle stem cells transfected with CMTM3-OE ($P < 0.05$) but enhanced ($P < 0.05$ and $P > 0.05$, respectively) in cells transfected with CMTM3-sh1 (**Figures 2G–I**). Taken together, these results indicated that overexpressing endogenous CMTM3 repressed hair follicle stem cell proliferation and induced apoptosis, whereas silencing endogenous CMTM3 expression accelerated hair follicle stem cell proliferation and inhibited apoptosis. In addition, AR expression was regulated by CMTM3 overexpression or inhibition in hair follicle stem cells.

miR-149-5p Directly Targets the 3′-UTR of Goat CMTM3 and Upregulates AR Expression

We explored the mechanisms of the effects of CMTM3 and miR-149-5p using bioinformatic databases (*TargetScan;*[6] *miRecords;*[7] *David*[8]); CMTM3 (the candidate gene involved in the growth and formation of superior-quality brush hair) was selected among the potential target genes of miR-149-5p. We found that the 3′-UTR of goat CMTM3 mRNA contained a highly conserved binding site capable of complementing the miR-149-5p seed sequence in the databases (**Figure 3A**).

In the preliminary experiments, we observed a notable reduction in luciferase activity in only the pcDNA3.1(+)-miR-149-5p treatment in the pre-dual-luciferase assay ($P < 0.05$), and luciferase activity was non-significantly decreased in the pre-dual-luciferase assay in the pcDNA3.1(+)-miR-365-3p, pcDNA3.1(+)-miR-23a-3p, and pcDNA3.1(+)-miR-23b-3p treatments compared with the control ($P > 0.05$) (**Supplementary Figure S3**). We further verified whether CMTM3 is a precise target gene of miR-149-5p by introducing NC, miR-149-5p mimics, anti-NC, miR-149-5p inhibitors, a pcDNA3.1(+) null-plasmid, and pcDNA3.1(+)-miR-149-5p into HEK-293T cells and hair follicle stem cells cultured in GM. We constructed psiCHECK-2 double-luciferase reporters that included a separate fragment of the wild-type or mutant goat *CMTM3* 3′-UTR (**Figure 3A**). The wild-type- or mutant-*CMTM3*-3′-UTR plasmids were cotransfected with miR-149-5p oligos, pcDNA3.1(+) null-plasmid, or pcDNA3.1(+)-miR-149-5p into HEK-293T cells. The results showed that compared with the NC and wild-type *CMTM3*-3′-UTR plasmid cotransfection group, the miR-149-5p mimics and wild-type *CMTM3*-3′-UTR plasmid cotransfection group showed significantly reduced luciferase activity, whereas no noticeable reduction in luciferase activity was observed with the miR-149-5p mimics and mutant-*CMTM3*-3′-UTR plasmid cotransfection group compared with the NC and mutant-*CMTM3*-3′-UTR plasmid cotransfection group ($P < 0.01$) (**Figure 3B**). Additionally, no noticeable reduction in luciferase activity was observed in cells cotransfected with the wild-type- or mutant-*CMTM3*-3′-UTR plasmid and either anti-NC or miR-149-5p inhibitors ($P > 0.05$) (**Figure 3C**). Similarly, a clear reduction in luciferase activity was obtained in the pcDNA3.1(+)-miR-149-5p and wild-type *CMTM3*-3′-UTR plasmid cotransfection groups, which is consistent with the miR-149-5p mimics-treated groups ($P < 0.05$) (**Figure 3D**). These results preliminarily confirmed that miR-149-5p directly targets the 3′-UTR of goat *CMTM3*. Furthermore, CMTM3 mRNA and protein expression was measured to verify the relationship between CMTM3 and miR-149-5p, and AR mRNA and protein expression was preliminarily assessed to investigate the correlation between CMTM3 and AR in hair follicle stem cells. The results indicated that miR-149-5p overexpression repressed CMTM3 expression (at the mRNA and protein levels) at 24 h, 48 h, 72 h, and 96 h in cells cultured in GM alone but that this difference did not reach a significant level at some time points (such as CMTM3 mRNA expression at 48 h, $P > 0.05$) and was accompanied by the upregulation of AR mRNA and protein expression, which was not significant at some time points (such as AR mRNA expression at 24 h and 48 h, $P > 0.05$). Conversely, miR-149-5p inhibition increased CMTM3 mRNA (**Figure 3E**) and protein expression (**Figures 3G–I**) and downregulated AR mRNA (**Figure 3F**) and protein expression (**Figures 3G–I**); however, no level of significance was reached at some time points (such as CMTM3 mRNA expression at 24 h and 48 h, AR mRNA expression at 48 h to 96 h, $P > 0.05$). Taken together, these results indicated that miR-149-5p inhibits goat CMTM3 mRNA and protein expression in hair follicle stem cells by directly targeting the CMTM3 mRNA 3′-UTR region. The inhibition of CMTM3 was accompanied by the upregulation of AR mRNA and protein expression.

miR-149-5p Positively Regulates Goat Hair Follicle Stem Cell Proliferation

We further examined the function of miR-149-5p in hair follicle stem cell proliferation by introducing NC, miR-149-5p mimics, anti-NC, or miR-149-5p inhibitors into stem cells. Hair follicle stem cells were transfected with miR-149-5p mimics or inhibitors and incubated for 96 h in GM. Stem cells were collected at 24-h intervals for RT-qPCR and western blotting against PCNA, CDK1, and CCND2, which are indicators of proliferation. miR-149-5p overexpression enhanced the mRNA expression of proliferation indicators (PCNA, CDK1, and CCND2) during hair follicle stem cell proliferation, but this change was not significant at some time points (such as PCNA mRNA expression in the mimics-treated group at 48 h, $P > 0.05$). By contrast, miR-149-5p inhibition downregulated PCNA, CDK1, and CCND2 mRNA levels during hair follicle stem cell proliferation, but this change did not reach a significant level in some periods (such as PCNA and CCND2 mRNA expression in the inhibitors-treated group at 24 h, $P > 0.05$) (**Figures 4A–C**). Similarly, PCNA, CDK1, and CCND2 protein levels were increased during hair follicle stem cell proliferation after miR-149-5p overexpression, whereas miR-149-5p inhibition decreased the protein expression of these proliferation indicators; however, this change was not significant at some time points (such as PCNA protein expression in the mimics-treated and inhibitors-treated groups at 48 h, $P > 0.05$). This decrease was accompanied by a decrease in the mRNA expression of proliferation indicators (PCNA, CDK1, and CCND2) during hair follicle stem cell proliferation (**Figures 4D–G**). Second,

[6]http://www.targetscan.org/vert_72

[7]http://c1.accurascience.com/miReco-rds

[8]https://david.ncifcrf.gov/

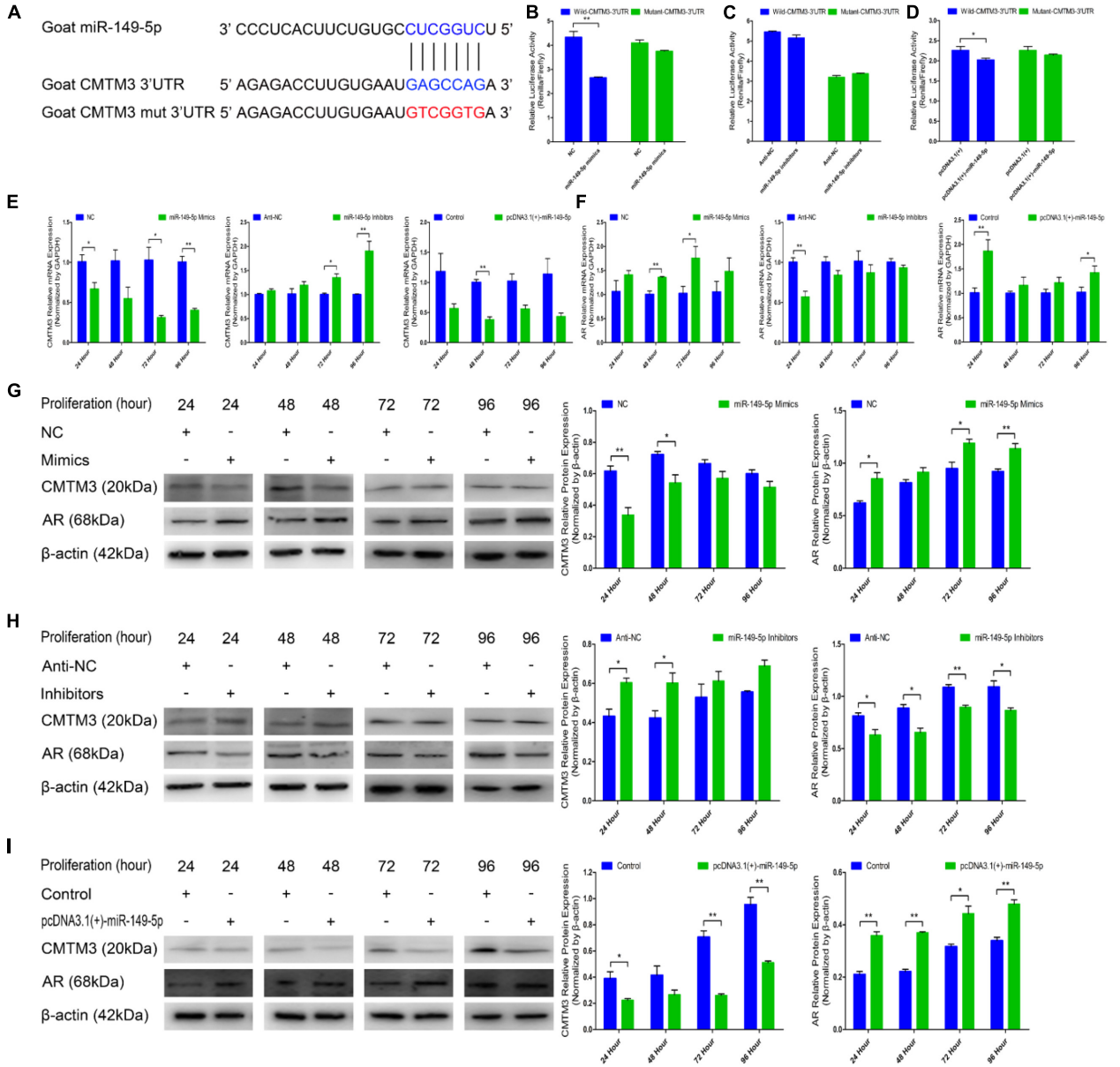

FIGURE 3 | miR-149-5p suppresses CMTM3 expression and accelerates AR expression by directly targeting the 3′-UTR of CMTM3. **(A)** The predicted binding site (blue) and mutated site (red) of miR-149-5p in the 3′-UTR of goat CMTM3. **(B–D)** Dual-luciferase activity assay of the wild-type or mutant 3′-UTR of CMTM3. NC or miR-149-5p mimics were cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells **(B)**. Anti-NC or miR-149-5p inhibitors were cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells **(C)**. pcDNA3.1(+) or pcDNA3.1(+)-miR-149-5p was cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells **(D)**. **(E)** CMTM3 mRNA expression during hair follicle stem cell proliferation after transfection with NC, miR-149-5p mimics, anti-NC, miR-149-5p inhibitors or pcDNA3.1(+)-miR-149-5p as evidenced by RT-qPCR; total RNA was harvested at 24 h, 48 h, 72 h, and 96 h after transfection. **(F)** AR mRNA expression during hair follicle stem cell proliferation after transfection with NC, miR-149-5p mimics, anti-NC, miR-149-5p inhibitors or pcDNA3.1(+)-miR-149-5p was evidenced by RT-qPCR; total RNA was harvested at 24 h, 48 h, 72 h, and 96 h after transfection. **(G–I)** CMTM3 (1:1000 dilution) and AR (1:1000 dilution) protein expression levels were examined after transfection with NC, miR-149-5p mimics **(G)**, anti-NC, miR-149-5p inhibitors **(H)**, and pcDNA3.1(+)-149-5p **(I)** in GM at 24 h, 48 h, 72 h, and 96 h. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

we examined the role of miR-149-5p in hair follicle stem cell proliferation with an EdU cell proliferation assay. We found that miR-149-5p overexpression (miR-149-5p mimics-treated group compared with NC counterparts) significantly increased the proportion of EdU-positive stem cells, but the proportion was notably decreased after miR-149-5p inhibition (miR-149-5p inhibitors treatments compared with anti-NC controls; $P < 0.01$) (**Figures 5A–C**). Moreover, the cell cycle assay further confirmed

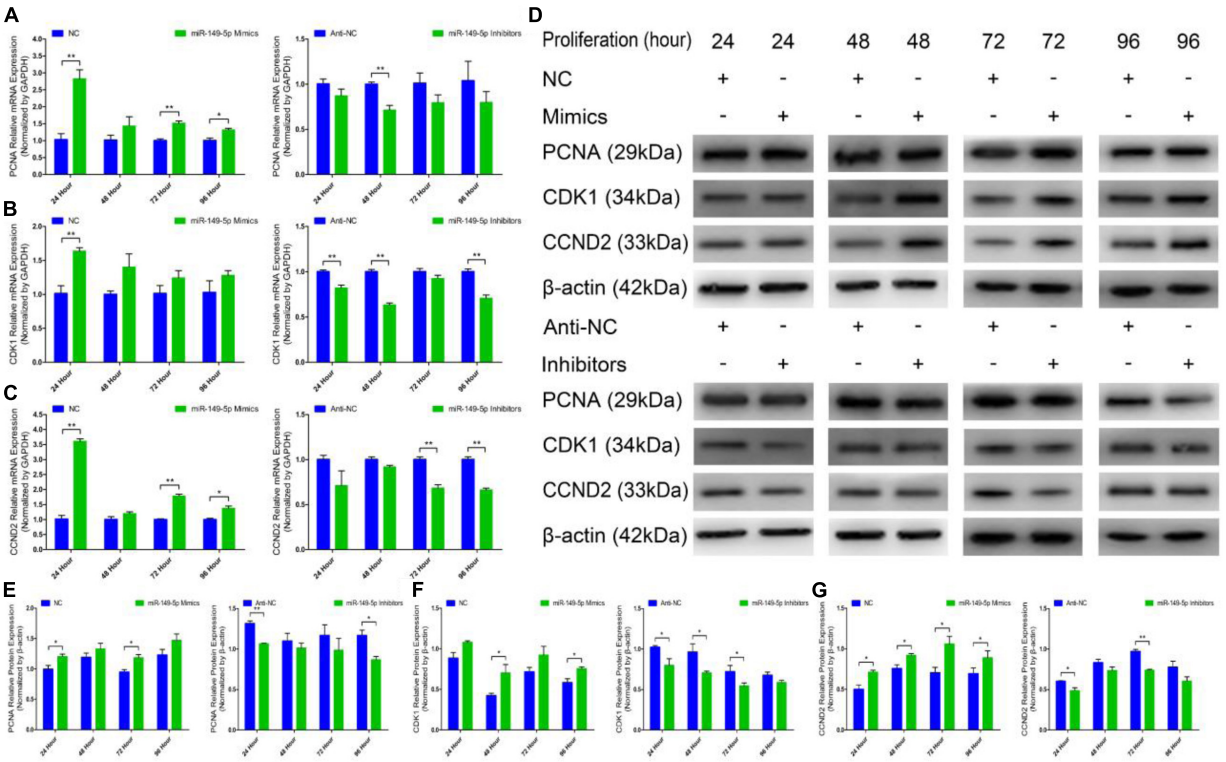

FIGURE 4 | Expression of proliferation marker genes and regulation of miR-149-5p during the proliferation of goat hair follicle stem cells. PCNA **(A)**, CDK1 **(B)**, and CCND2 **(C)** mRNA expression at 24 h, 48 h, 72 h, and 96 h after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM as determined by RT-qPCR. **(D)** Protein expression of PCNA (1:1000 dilution), CDK1 (1:1000 dilution), and CCND2 (1:1000 dilution) was measured at 24 h, 48 h, 72 h, and 96 h after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM. β-actin (1:500 dilution) was used as an internal control. **(E)** Relative PCNA protein expression after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM at 24 h, 48 h, 72 h, and 96 h. **(F)** Relative CDK1 protein expression after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM at 24 h, 48 h, 72 h, and 96 h. **(G)** Relative CCND2 protein expression after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM at 24 h, 48 h, 72 h, and 96 h. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

that miR-149-5p mimics promoted hair follicle stem cell proliferation, increased the number of hair follicle stem cells at S-phase (from 9.67% up to 12.36%, $P < 0.05$), and decreased the proportion of cells in G0/G1-phase (from 84.16% down to 82.53%, $P > 0.05$). Conversely, the results showed that after transfection with miR-149-5p inhibitors, the number of hair follicle stem cells in S-phase was reduced (from 14.06% down to 12.10%, $P < 0.05$), and the proportion of cells in G0/G1-phase was increased (from 79.32% down to 80.83%, $P > 0.05$) (**Figures 5D–F**). We also used a histogram to present the percentage of cells in each stage of the cell cycle, and only the difference in the percentage of cells in S-phase was significant ($P < 0.05$) after different treatments (**Figure 5G**). Taken together, these results indicated that miR-149-5p facilitates goat hair follicle stem cell proliferation.

miR-149-5p Suppresses Goat Hair Follicle Stem Cell Apoptosis

Next, we examined the role of miR-149-5p in hair follicle stem cell apoptosis. We found that Bcl2 (an antiapoptotic gene) mRNA expression was increased in miR-149-5p mimics-treated cells compared with NC-treated cells at 24 h, 48 h, 72 h, and 96 h but was decreased in the miR-149-5p inhibitors-treated group at 24 h, 48 h, 72 h, and 96 h; however, these decreases were not significant at some time points (similar to Bcl2 mRNA expression in mimics-treated group at 24 h, 48 h, and 72 h, $P > 0.05$) (**Figure 6A**). The mRNA levels of Caspase3 and Caspase9, two marker-genes of apoptosis, were decreased in miR-149-5p mimics-treated cells compared with NC-treated cells at 24 h, 48 h, 72 h, and 96 h but increased in miR-149-5p inhibitors treated cells at 24 h, 48 h, 72 h, and 96 h; however, these changes were not significant at some time points (such as Caspase3 mRNA expression in inhibitors-treated group at 24 h and 96 h, $P > 0.05$) (**Figures 6B,C**). Similarly, Bcl2 protein levels were increased in the miR-149-5p mimics-treated group compared with the NC-treated group at 24 h, 48 h, 72 h, and 96 h and decreased in the miR-149-5p inhibitors-treated group at 24 h, 48 h, 72 h, and 96 h, but these differences were not significant at some time points (similar to the pattern of Bcl2 protein expression in the mimics-treated group at 24 h, $P > 0.05$) (**Figures 6D,E**). In contrast, Caspase3 and Caspase9 protein levels

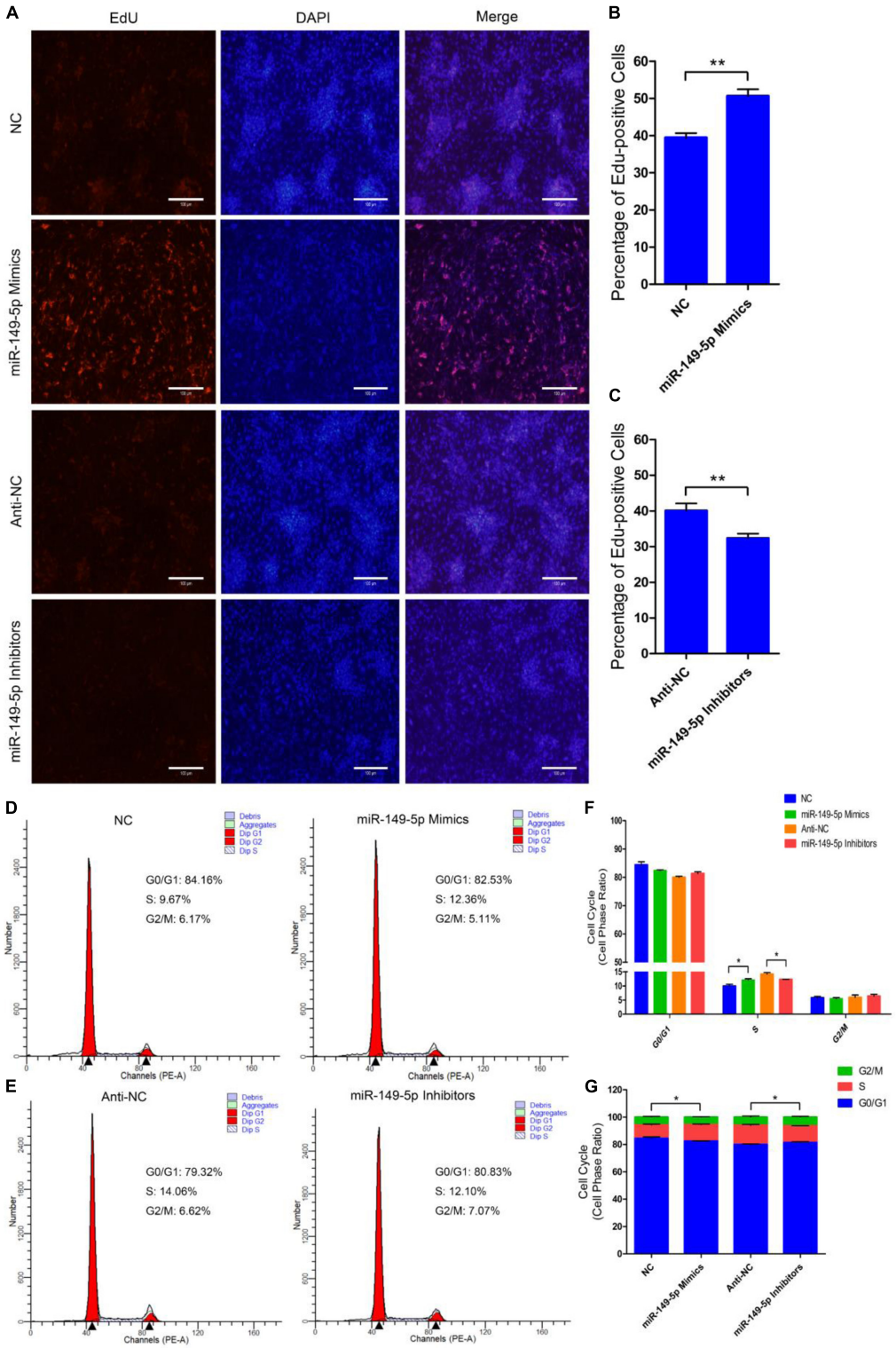

FIGURE 5 | miR-149-5p accelerates goat hair follicle stem cell proliferation. **(A)** Representative images of the EdU assay of hair follicle stem cells at 24 h after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM. Bars, 100 μm. **(B,C)** Quantification of EdU-positive cells ($n = 6$). The ratio of EdU-positive cells was calculated as (EdU-positive cells/Hoechst stained cells) × 100%. **(D–G)** Hair follicle stem cells were transfected with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM, and cell cycle phases were analyzed at 48 h after transfection by flow cytometry **(D,E)** and counted **(F,G)**. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

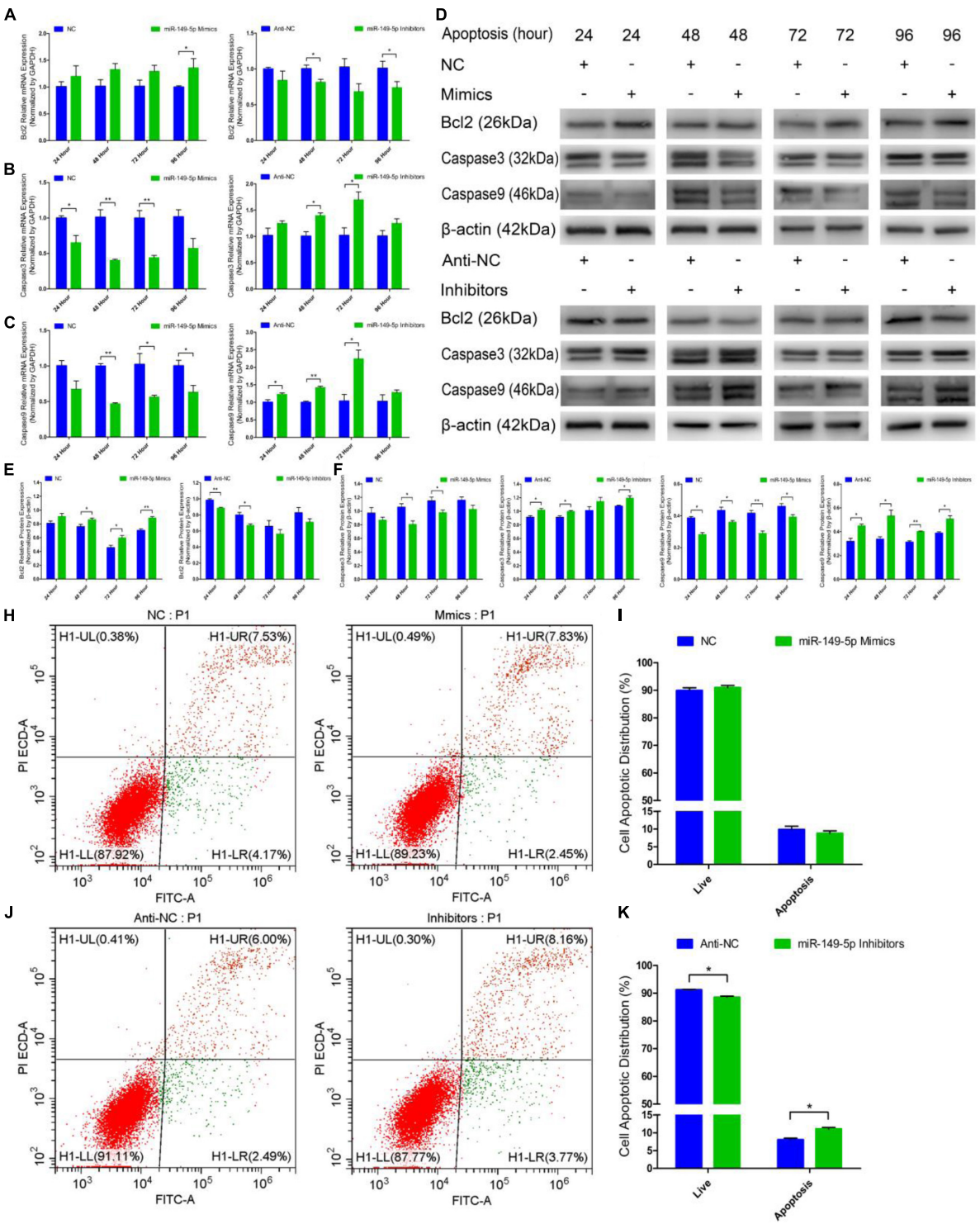

FIGURE 6 | miR-149-5p suppresses goat hair follicle stem cell apoptosis. **(A)** mRNA expression of antiapoptotic gene (Bcl2) at 24 h, 48 h, 72 h, and 96 h after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM as determined by RT-qPCR. **(B,C)** mRNA expression of apoptosis marker genes (Caspase3 and Caspase9) at 24 h, 48 h, 72 h, and 96 h after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM as determined by RT-qPCR. **(D)** Protein expression of Bcl2 (1:1000 dilution), Caspase3 (1:1000 dilution), and Caspase9 (1:1000 dilution) was measured at 24 h, 48 h, 72 h, and 96 h after transfection with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM. β-actin (1:500 dilution) was used as an internal control. **(E)** Relative Bcl2 protein expression with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM at 24 h, 48 h, 72 h, and 96 h. **(F)** Relative Caspase3 protein expression with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM at 24 h, 48 h, 72 h, and 96 h. **(G)** Relative Caspase9 protein expression

(Continued)

FIGURE 6 | Continued
with NC, miR-149-5p mimics, anti-NC, and miR-149-5p inhibitors in GM at 24 h, 48 h, 72 h, and 96 h. **(H,I)** Hair follicle stem cells were transfected with NC or miR-149-5p mimics in GM, and cell apoptosis was analyzed 48 h after transfection by Annexin V-FITC/PI binding followed by flow cytometry. **(J,K)** Hair follicle stem cells were transfected with anti-NC and miR-149-5p inhibitors in GM, and cell apoptosis was analyzed 48 h after transfection by Annexin V-FITC/PI binding followed by flow cytometry. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

were reduced in the miR-149-5p mimics-treated group compared with the NC-treated group at 24 h, 48 h, 72 h, and 96 h and increased in the miR-149-5p inhibitors-treated group at 24 h, 48 h, 72 h, and 96 h; however, the differences did not reach a significant level at some time points (such as Caspase3 protein expression in the mimics-treated group at 24 h and 96 h, $P > 0.05$) (**Figures 6D,F,G**). The Annexin V-FITC/PI staining assay showed that miR-149-5p overexpression protected hair follicle stem cells from apoptosis and decreased the proportion of apoptotic cells ($P > 0.05$) (**Figures 6H,I**), while miR-149-5p inhibition induced apoptosis in hair follicle stem cells and increased the proportion of apoptotic cells ($P < 0.05$) (**Figures 6J,K**). Together, these results demonstrated that miR-149-5p suppresses goat hair follicle stem cell apoptosis.

Ectopic miR-149-5p Expression Accelerates Goat Hair Follicle Stem Cell Proliferation and Inhibits Apoptosis

We constructed a miR-149-5p overexpression vector (pcDNA3.1[+]-miR-149-5p plasmid) to investigate the role of the miR-149-5p precursor in hair follicle stem cell proliferation and apoptosis. pcDNA3.1(+)-miR-149-5p was transfected into hair follicle stem cells to overexpress miR-149-5p, and the cells showed appreciably upregulated expression compared with that in the blank control or null-plasmid (pcDNA3.1[+])-treated groups, as detected by RT-qPCR (**Supplementary Figure S2B**). Next, pcDNA3.1(+)-miR-149-5p was transfected into hair follicle stem cells in GM, and the cells were collected at 24-h intervals. PCNA, CDK1, and CCND2 mRNA (**Figure 7A**) and protein (**Figures 7B,C**) levels were measured to determine the effect of miR-149-5p on hair follicle stem cell proliferation.

Compared with control cells, cells with miR-149-5p overexpression exhibited elevated levels of PCNA, CDK1, and CCND2 at 24 h, 48 h, 72 h, and 96 h, but these increases were not significant at some time points (such as PCNA mRNA expression in the overexpression vector treated group at 24 h, $P > 0.05$). Second, an EdU cell proliferation assay revealed that pcDNA3.1(+)-miR-149-5p clearly increased the proportion of EdU-positive stem cells ($P < 0.01$) (**Figure 7D**). Furthermore, the cell cycle assay confirmed that pcDNA3.1(+)-miR-149-5p promoted hair follicle stem cell proliferation, which was accompanied by a marked increase in the number of hair follicle stem cells in S-phase (from 10.94% up to 16.08%, $P < 0.01$) and a significant decrease in the proportion of cells in G0/G1-phase (from 84.88% down to 77.19%, $P < 0.01$) (**Figures 7E,F**).

pcDNA3.1(+)-miR-149-5p was also transfected into hair follicle stem cells in GM to assess the role of the miR-149-5p precursor in hair follicle stem cell apoptosis. The mRNA and protein expression levels of Bcl2, Caspase3, and Caspase9 were observed after transfection with the pcDNA3.1(+)-miR-149-5p plasmid in GM for 24 h, 48 h, 72 h, and 96 h. Compared with the control, the miR-149-5p precursor upregulated Bcl2 expression and downregulated Caspase3 and Caspase9 expression at 24 h, 48 h, 72 h, and 96 h in hair follicle stem cells, but these changes were not significant at some time points (such as Bcl2 mRNA expression in the overexpression vector treated group at 72 h, $P > 0.05$) (**Figures 7G–I**). Moreover, the Annexin V-FITC/PI staining assay showed that pcDNA3.1(+)-miR-149-5p protected hair follicle stem cells from apoptosis and decreased the proportion of apoptotic cells ($P > 0.05$) (**Figures 7J,K**). Taken together, these results revealed that the overexpression of miR-149-5p was able to promote hair follicle stem cell proliferation and inhibit stem cell apoptosis, which was consistent with the role of miR-149-5p mimics in both processes.

DISCUSSION

CMTM3, a member of the CMTM family, has been shown to play an important role in the development and progression of tumors (Zhong et al., 2006; Su et al., 2014) and to function as a regulator of AR transcriptional activity (Wang et al., 2008, 2018). Interestingly, our study confirmed that CMTM3 is a target gene of miR-149-5p by bioinformatic prediction and selection, RT-qPCR, western blotting, and dual-luciferase reporter gene assays. Androgen secretion could stimulate the formation of Type III superior-quality brush hair, and androgen can play its physiological function only after binding to AR. Hence, AR also plays a critical role in superior-quality brush hair traits. Androgens have also been identified to upregulate IGF-I expression and function as an important regulator of hair follicle growth (Philpott et al., 1994). In addition to these findings, we reported in a previous study that CMTM3 gene methylation promotes AR activity and upregulates the androgen hormone levels, which then results in the formation of superior-quality brush hair (Wang et al., 2018). In this work, CMTM3 expression was lower in the skin tissue of superior-quality brush hair goats than in those of the normal-quality brush hair goat but was not significantly different after analyzing three independent samples; furthermore, CMTM3 expression was decreased in cultured hair follicle stem cells in GM. In contrast, AR expression was higher in the skin tissues of superior-quality brush hair goats and increased in cultured hair follicle stem cells in GM. In addition, miR-149-5p expression was markedly higher in superior-quality brush hair goats than in normal-quality brush hair goats and was also upregulated in cultured hair follicle stem cells. These results suggested that miR-149-5p is involved in the regulation of hair follicle development and the formation of superior-quality brush

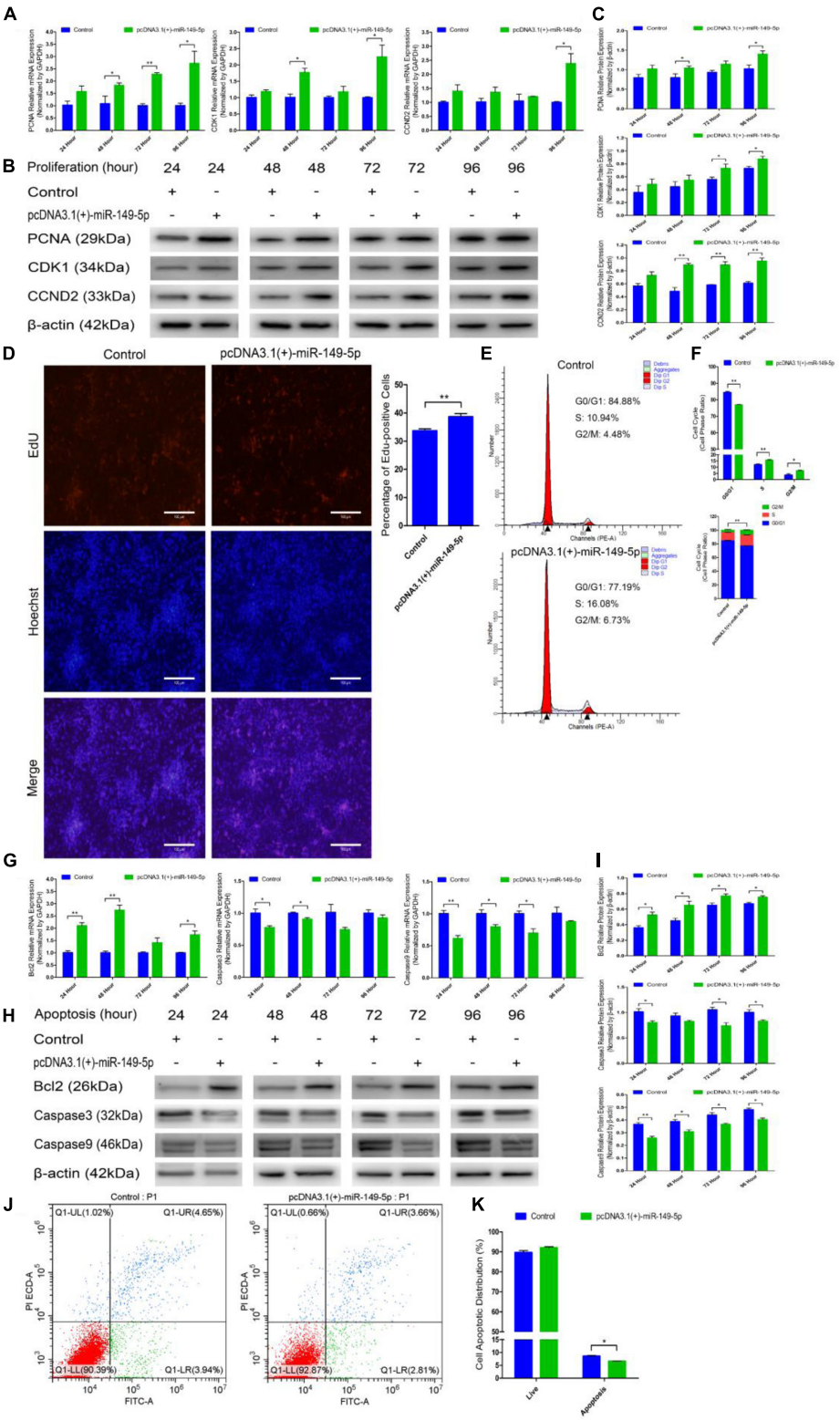

FIGURE 7 | Ectopic miR-149-5p expression facilitates proliferation and inhibits apoptosis in goat hair follicle stem cells. **(A)** PCNA, CDK1, and CCND2 mRNA expression at 24 h, 48 h, 72 h, and 96 h after transfection with pcDNA3.1(+)-miR-149-5p in GM as determined by RT-qPCR. **(B,C)** Protein level of PCNA (1:1000 dilution), CDK1 (1:1000 dilution), and CCND2 (1:1000 dilution) were measured following overexpression of miR-149-5p with pcDNA3.1(+)-miR-149-5p in GM at 24 h, 48 h, 72 h, and 96 h. β-actin (1:500 dilution) was used as an internal control. **(D)** Representative images of the EdU assay of hair follicle stem cells at 24 h after

(Continued)

FIGURE 7 | Continued
transfection with pcDNA3.1(+)-miR-149-5p in GM. Bars, 100 μm. Hair follicle stem cells were transfected with pcDNA3.1(+)-miR-149-5p in GM, and cell phases were analyzed at 24 h after transfection by flow cytometry **(E)** and counted **(F)**. **(G)** mRNA expression of antiapoptotic gene (Bcl2) and apoptosis marker genes (Caspase3 and Caspase9) at 24 h, 48 h, 72 h, and 96 h after transfection with pcDNA3.1(+)-miR-149-5p in GM as determined by RT-qPCR. **(H,I)** Bcl2 (1:1000 dilution), Caspase3 (1:1000 dilution), and Caspase9 (1:1000 dilution) protein levels were measured following overexpression of miR-149-5p with pcDNA3.1(+)-miR-149-5p in GM at 24 h, 48 h, 72 h, and 96 h. β-actin (1:500 dilution) was used as an internal control. **(J,K)** Hair follicle stem cells were transfected with pcDNA3.1(+)-miR-149-5p in GM, and cell apoptosis was analyzed 48 h after transfection by Annexin V-FITC/PI binding followed by flow cytometry. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

hair. The formation of superior-quality brush hair is complex and involves hair follicle growth and development, which are driven by many biological and physiological processes (Stenn and Paus, 2001; Botchkarev and Sharov, 2004; Chen et al., 2012; Kretzschmar and Clevers, 2017). Therefore, based on these findings and bioinformatic analysis, we speculated that miR-149-5p influences CMTM3 expression, thereby increasing the expression of AR during the formation of superior-quality brush hair, which is similar to other miRNA/mRNA regulatory axes in skin and hair development (Botchkareva, 2012; Cai et al., 2013).

RT-qPCR and western blotting assays showed that AR mRNA and protein expression levels were increased after miR-149-5p overexpression and decreased when miR-149-5p was inhibited, suggesting that the miR-149-5p–CMTM3–AR axis is a critical regulator of hair follicle stem cell proliferation, apoptosis, and superior-quality brush hair traits. Moreover, we used the pDC316-mCMV-EGFP-CMTM3 vector (CMTM3-OE) and shRNA mediated CMTM3 experiments to investigate the effects of overexpressing or knocking down CMTM3, respectively, on the proliferation and apoptosis of hair follicle stem cells. We found that overexpressing CMTM3 decreased the number of hair follicle stem cells in S-phase and increased the number of cells in G0/G1-phase. However, knocking down CMTM3 conspicuously increased the proportion of cells in S-phase and decreased the proportion of cells in G0/G1-phase, which indicated that CMTM3 mainly plays an antiproliferative role during S-phase in goat hair follicle stem cell proliferation and functions contrary to miR-149-5p. These results were consistent with the regulatory role of CMTM3 in hepatocellular carcinoma cells (Li and Zhang, 2017) but in contrast to its function in gastric cancer cells (Lu et al., 2018). Annexin V-FITC/PI staining assays also showed that compared with the NC or sh-NC control, CMTM3 overexpression induced hair follicle stem cell apoptosis, whereas CMTM3 knockdown protected stem cells from apoptosis. RT-qPCR and western blotting assays showed that AR expression was decreased at both the mRNA and protein levels after CMTM3 overexpression, which is similar to the effect of miR-149-5p inhibitors on hair follicle stem cells. Conversely, CMTM3 knockdown increased the expression of AR, which is similar to the role of miR-149-5p mimics in stem cells. These results revealed that CMTM3 plays a negative role in hair follicle stem cell proliferation and apoptosis by influencing the expression levels of AR.

miR-149-5p is a member of the goat miR-149 family. Recent work has shown that miR-149-5p overexpression can activate Sirt1 activity and consequently protect the brain from resveratrol-induced ischemia by targeting p53, which offers a novel therapeutic approach during acute ischemic stroke (Teertam et al., 2020). The miR-149-5p precursor mitigates cell migration and invasion in renal cell carcinoma by targeting FOXM1 (Okato et al., 2017). However, miR-149-5p overexpression inhibits vascular smooth muscle cell proliferation, invasion, and migration by interacting with HDAC4 (Zhang et al., 2019), and it can function as a negative regulator of melanoma cell proliferation and cell survival and promote apoptosis by targeting LRIG2 (Chen et al., 2017), suggesting that miR-149-5p functions as a therapeutic molecule against melanoma. These studies have highlighted the different functions of miR-149-5p in cardiovascular disease and cancers. Our research indicated that miR-149-5p expression was upregulated in the skin tissues of superior-quality brush hair goats and in cultured hair follicle stem cells, similar to the expression trend of miR-128-3p in skin samples from angora rabbits (Zhao et al., 2019b). miR-149-5p overexpression promotes the expression of functional genes linked to proliferation (PCNA, CDK1, and CCND2) and represses the expression of apoptotic genes (Caspase3 and Caspase9) while upregulating the expression of the antiapoptotic gene Bcl2. By contrast, miR-149-5p inhibition suppresses the expression of functional genes related to proliferation (PCNA, CDK1, and CCND2) and antiapoptotic Bcl2 at the mRNA and protein level but accelerates the expression of proapoptotic genes (Caspase3 and Caspase9), which preliminarily illustrates that miR-149-5p upregulation can promote goat hair follicle stem cell proliferation and inhibit apoptosis. However, the mRNA levels of PCNA, CDK1, and CCND2 rapidly increased during the proliferation of hair follicle stem cells at 24 h after transfection with miR-149-5p mimics but decreased at 48 h, 72 h, and 96 h compared with the levels at 24 h. This phenomenon may be caused by the transient high expression of miR-149-5p after transfection with miR-149-5p mimics. In contrast to this phenomenon, the mRNA levels of Bcl2, Caspase3, and Caspase9 neither increased nor decreased quickly during the apoptosis of hair follicle stem cells at 24 h after miR-149-5p mimics treatment; however, Caspase3 and Caspase9 expression significantly decreased at 48 h, 72 h, and 96 h after miR-149-5p mimics treatment. These results could be explained by the fact that goat hair follicle stem cells are primarily engaged in proliferation for self-renewal purposes and show little apoptosis activity, and this trend could be maintained by miR-149-5p overexpression but weakened by miR-149-5p inhibition (Vasyliev et al., 2017; Lu et al., 2019). Additionally, EdU and cell cycle assays showed that

miR-149-5p overexpression increased the proportion of EdU-positive cells, increased the number of hair follicle stem cells in S-phase, and decreased the proportion of cells in G0/G1-phase, whereas miR-149-5p inhibition decreased the proportion of EdU-positive cells, reduced the number of hair follicle stem cells in S-phase, and increased the proportion of the cells in G0/G1-phase. Interestingly, the trend of increased or decreased cells in G0/G1-phase was not significant in mimics-treated and inhibitors-treated groups. This result may be because miR-149-5p mainly plays a role in promoting proliferation at S-phase in goat hair follicle stem cell proliferation. In contrast to miR-149-5p, miR-134 could mediate S-phase arrest in human hepatocellular carcinoma cells (Ahn et al., 2019). Furthermore, Annexin V-FITC/PI staining assays showed that miR-149-5p overexpression protected hair follicle stem cells from apoptosis (decreased percentage of cells in early apoptosis), and miR-149-5p inhibition induced stem cell apoptosis (increased the rates of early and late apoptosis). These results indicate that miR-149-5p accelerates hair follicle stem cell proliferation and represses apoptosis, which is consistent with the role of miR-149-5p in pancreatic beta cells (Ruan et al., 2019). Interestingly, in contrast to our results, in renal cell carcinoma, the use of synthetic mimics to overexpress miR-149-5p can suppress cancer cell proliferation and migration but promote cancer cell apoptosis, with the apoptotic rate increasing from 1.89% (NC treatment) to 17.15% (miR-149-5p mimics treatment) (Jin et al., 2016). These findings indicate that the same miRNA plays diverse regulatory roles in different mammalian cells and tissues.

In the above results, the differences in some of the assays were not significant and only indicated increasing or decreasing trends, and we preliminarily characterized the relationship between CMTM3 and AR by using RT-qPCR and western blotting but not any other assays (such as coimmunoprecipitation assay) to further confirm the association between CMTM3 and AR. Therefore, elucidating the underlying mechanisms of superior-quality brush hair formation warrants further research.

CONCLUSION

Our study determined that CMTM3 overexpression represses hair follicle stem cell proliferation and induces apoptosis; in contrast, CMTM3 knockdown accelerates hair follicle stem cell proliferation and protects stem cells from apoptosis. Moreover, AR expression was decreased after CMTM3 overexpression, which is consistent with the role of miR-149-5p inhibitors in hair follicle stem cells and was increased after CMTM3 knockdown, which is consistent with miR-149-5p mimics in stem cells.

In this study, we also showed that miR-149-5p is important for the formation of superior-quality brush hair traits and can promote goat hair follicle stem cell proliferation and suppress hair follicle stem cell apoptosis by inhibiting CMTM3 expression via a posttranscriptional mechanism. These results reveal a regulatory mechanism involving miR-149-5p, CMTM3, and AR, in which miR-149-5p controls goat hair follicle stem cell proliferation and apoptosis via the suppression of *CMTM3* and the upregulation of *AR*, and this mechanism further regulates the formation of superior-quality brush hair traits in Yangtze River Delta white goats.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Yangzhou University.

AUTHOR CONTRIBUTIONS

JW and YL: conceptualization. JW, YF, and JM: methodology. JW and JQ: software. LZ: validation. JW, JQ, YF, and JM: formal analysis. JW, CC, and HH: investigation. YL: resources. YW and DJ: data curation. YL: supervision and validation. JW: writing – original draft preparation. YL: writing – review and editing. All authors have read and agreed to the published version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.529757/full#supplementary-material

Supplementary Figure 1 | Morphological images and integrity checks in hair follicle stem cells. **(A,B)** Morphological images of hair follicle stem cells in GM harvested with a microscope. **(C)** Images of the integrity analysis of hair follicle stem cells with immunocytochemical staining (this result can be found in our published research paper in Gene Journal in 2019, Gene 698, 19–26).

Supplementary Figure 2 | Efficiency of miR-149-5p oligos and its overexpression vector in goat hair follicle stem cells. **(A)** miR-149-5p expression 48 h after transfection with negative control (NC), miR-149-5p mimics (Mimics), single-stranded negative control (Anti-NC), and 2′-O-methylated oligonucleotides against miR-149-5p (Inhibitors) as determined by RT-qPCR. **(B)** miR-149-5p expression 48 h after transfection with pcDNA3.1(+) plasmid and pcDNA3.1(+)-miR-149-5p as determined by RT-qPCR. The results from each

group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. $^{*}P < 0.05$, and $^{**}P < 0.01$.

Supplementary Figure 3 | Results of the preliminary dual-luciferase assay. **(A)** pcDNA3.1(+) or pcDNA3.1(+)-miR-365-3p was cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells. **(B)** pcDNA3.1(+) or pcDNA3.1(+)-miR-23a-3p was cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells. **(C)** pcDNA3.1(+) or pcDNA3.1(+)-miR-23b-3p was cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells. **(D)** pcDNA3.1(+) or pcDNA3.1(+)-miR-149-5p was cotransfected with wild-type or mutant CMTM3 3′-UTR luciferase reporters in HEK293T cells. The results from each group are shown as the mean ± SEM of three independent replicates. Independent-samples *t*-tests were used for statistical analysis. Asterisks indicate significant differences. No asterisk means $P > 0.05$, $^{*}P < 0.05$.

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Impact of Long Non-coding RNAs Associated With Microenvironment on Survival for Bladder Cancer Patients

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Background: Cumulative evidence from several tumor studies, including bladder cancer, emphasizes the importance of the tumor microenvironment (TME) in tumorigenesis, development, and metastasis, which can be regulated by long non-coding RNAs (lncRNAs). This study aims to identify bladder cancer (BC) microenvironment–associated lncRNAs for their prognostic value predicting the survival of BC patients.

Methods: The data of BC patients regarding lncRNA expression and corresponding clinical characteristics were obtained from The Cancer Genome Atlas (TCGA). The Cox regression analysis and the least absolute shrinkage and selection operator (LASSO) regression analysis were performed to screen lncRNAs following the calculation of the immune score for each sample. For the screened lncRNAs, a risk score model was constructed to predict the survival, and 3- and 5-year overall survival (OS) rates were assessed using a nomogram. The calibration curve and concordance index (C-index) validated the performance of the nomogram. Finally, to explore the potential function related to the screened lncRNAs, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed.

Results: The multivariate Cox regression analysis screened five TME-associated lncRNAs regarded as independent factors influencing the tumor progression. The corresponding risk score model was established as follows: (−0.15816 AC064805.1) + (0.10015 AC084033.3) + (−0.17977 AC092112.1) + (−0.05673AC103691.1) + (0.17789 AL391704.1) + (−0.16258 LINC00892). The C-index for the nomogram was 0.63 (95% CI: 0.625–0.635). Also, the calibration curve verified the predictive effectiveness by showing a good concordance between the nomogram prediction and the actual observation. GO and KEGG analysis demonstrated that six TME-associated lncRNAs were most likely linked to tumor metastasis and progression.

Conclusion: The present study determined six lncRNAs as independent immuno-biomarkers in the TME, constructed a nomogram to predict their prognostic value, and investigated the potential biological processes to understand their regulatory roles in the progression of BC.

Keywords: bladder cancer, long non-coding RNA, immune score, microenvironment, nomogram, prognosis

INTRODUCTION

Due to poor prognosis and high recurrence, bladder cancer (BC) is considered a significant threat to male health (Cumberbatch et al., 2018). Concerning the mortality rates, BC ranks 13th, affecting up to 165,000 cases worldwide (Cumberbatch et al., 2018). For histological typing, staging, and stopping recurrence, transurethral resection of the bladder (TURB) is the most preferred method in the management of non-muscle-invasive bladder cancer (NMIBC) (Babjuk et al., 2017). Also, during disease management combined with chemotherapy or immunotherapy, close follow-up after patient discharge can effectively reduce the risk of tumor recurrence and progression to muscle-invasive bladder cancer (MIBC) (Babjuk et al., 2017). In some patients, an early radical cystectomy could be beneficial in the case of non-metastatic MIBC, but the corresponding comprehensive treatment scheme must be applied to improve the prognosis in a case-specific manner (Alfred et al., 2017). However, regardless of vigorous intervention measures, the final treatment outcome of MIBC is mostly unfavorable. Recently, antitumor fibroblast growth factor receptor (FGFR) targeting agents have shown promising results in clinical trials (Nogova et al., 2017). Apart from the traditional treatment strategy, novel U.S. FDA-approved immune checkpoint inhibitors are now being suggested as a first-line and metastatic treatment for BC (Rouanne et al., 2016). Similarly, identifying the key biomarkers linked to the tumor regulatory network could facilitate early diagnosis and timely targeted treatment to reduce the risk of recurrence, progression, and mortality in BC.

Long non-coding RNAs (lncRNAs) are transcribed from the non-coding regions that are ∼75% of the genome exposed to frequent genomic mutations (Djebali et al., 2012). Overcoming the experimental challenges, high-throughput sequencing technologies and functional tracking permit a profound understanding of alternation in the structure and function of lncRNAs caused by persistent genomic mutations. These changes disrupt the intracellular equilibrium of the regulatory network, giving rise to various cellular activities, including tumor cell transformation (Huarte, 2015). Prostate cancer–associated 3 (PCA3) (Bussemakers et al., 1999) and prostate cancer gene expression marker 1 (PCGEM1) (Srikantan et al., 2000) were the first discovered cancer-associated lncRNAs. PCA3 facilitates prostate cancer progression and tumor cell proliferation via regulation of the miR-218-5p/HMGB1 pathway (Zhang et al., 2019) and is also considered to be a molecular diagnostic biomarker in clinical practice (Hessels et al., 2003). PCGEM1, by activating transcription, regulates the expression of androgen receptor 3, which leads to castrate-resistant prostate cancer (Yang et al., 2013; Zhang et al., 2016). Likewise, the intracellular regulatory roles of several lncRNAs among other tumors have also been examined. For instance, MALAT1 in lung cancer (Gutschner et al., 2013), H19 in colorectal cancer (Ren et al., 2018), HULC in liver cancer (Xin et al., 2018), UCA1 in bladder cancer (Xue et al., 2017), and PVT1 in renal cancer (Ren et al., 2019) are the known diagnostic biomarkers in respective cancers and are under investigation for the targeted treatment.

In various tumors, the infiltrating immune cells are heterogeneous in nature. Therefore, the clinical outcomes and prognosis are closely linked to the level and types of immune cells in the local tumor site (Fridman et al., 2012). It is reported that lncRNAs can regulate the immune response by interacting with genomes, chromatin, RNA, and proteins and have also been linked to the differentiation and activation of immune cells, such as T cells and myeloid cells (Gomez et al., 2013; Hu et al., 2013; Wang et al., 2014). Therefore, to find specific immuno-biomarkers, it is important to examine the role of immune cells in tumor development and infiltration from the perspective of lncRNA-mediated regulation.

Recently, several studies screened for lncRNAs to develop relevant models for the prognosis of corresponding tumors (Cai et al., 2019; Miao et al., 2019). Furthermore, the prognostic value can be assessed based on the components involved in the progression of tumors, such as immune genes and immune cells. However, there are rare studies on tumor microenvironment (TME)-associated lncRNAs that may indirectly regulate tumorigenesis and progression of BC. Here, we screened six TME-associated lncRNAs, evaluated their prognostic value, and investigated potential function. These findings pave the way to look for novel immuno-biomarkers for diagnosis and immunotherapy to decrease recurrence and drug resistance in BC.

MATERIALS AND METHODS

Data Acquisition Relevant to BC

The Cancer Genome Atlas (TCGA)[1] database was used to retrieve the lncRNA expression profiles related to BC patients (n = 433) and clinical characteristics (n = 412) that included age, gender, stage, grade, and TNM staging. Also, the immune scores of the corresponding samples (n = 408) were obtained from the Estimation of STromal and Immune cells in Malignant Tumor tissues using Expression data (ESTIMATE).[2] Data lacking missing or unknown values were excluded.

[1]https://portal.gdc.cancer.gov/

[2]https://bioinformatics.mdanderson.org/estimate/disease.html

Screening of TME-Associated lncRNAs for Prognosis

Using the lncRNA expression profiles and the corresponding immune score, the BC samples were distinguished into either the high or low immune score group based on the median immune score. The sample sizes of the groups were comparable; 206 cases belonged to the high immune score group and 208 cases formed the low immune score group. The "limma" R software package (version 3.6.2) was utilized to screen the differentially expressed lncRNAs (DElncRNAs) with the inclusion criteria of log2-fold change >1 and false discovery rate (FDR) <0.05. The univariate Cox regression analysis, the least absolute shrinkage and selection operator (LASSO) regression analysis, and the multivariate Cox regression analysis were performed to identify the independent TME-associated lncRNAs. These models were used to analyze the influence of multiple independent variables on a dependent variable and were a major method for screening independent factors. Kaplan–Meier analysis and log-rank tests were employed to determine the survival-related lncRNAs. Then, the risk score model for lncRNAs was built to estimate survival risk for each patient as follows:

$$\text{Risk score} = \sum_{i=1}^{N} (\mathrm{E_i} * \beta_i)$$

Here, N, E_i, and β_i denote the number of selected lncRNAs, the expression level of the ith lncRNA, and the ith lncRNA coefficient, respectively. Receiver operating characteristic (ROC) analysis was used to estimate the sensitivity and specificity of the prognosis-related lncRNAs. $P < 0.05$ was considered as statistical significance.

Constructing a Predictive Survival Model

Combined with the clinical characteristics, a nomogram consisting of age, gender, stage, immune score, and risk score was established to predict the 3- and 5-year overall survival (OS) probability for BC patients. The nomogram was validated using the calibration curve and the concordance index (C-index).

Assessing the Immune Cell–Specific Expression and the Potential Function of the Six lncRNAs

To further examine the expression of the selected lncRNAs in specific immune cells, the promoters and enhancers targeting the selected lncRNAs were obtained using the Human Gene database.[3] Then, functional enrichment analysis was performed to assess the biological process and functional pathway related to these six lncRNAs along with analysis of coexpressed mRNAs. Pearson correlation coefficient analysis was utilized to find the correlation between the lncRNAs and the protein-coding genes. Using the threshold Pearson correlation coefficient >0.10 and $p < 0.01$, a total of 2,764 protein-coding genes were selected for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis in the Metascape project[4] (Zhou et al., 2019). The GO interaction networks and KEGG pathways were visualized using the Cytoscape software (Shannon et al., 2003).

RESULTS

DElncRNAs

After excluding the missing and unknown data, the remaining relevant data related to clinical characteristics used in this study are presented in **Supplementary Table 1**. Out of 10,933 lncRNAs, a total of 627 DElncRNAs are shown in **Figure 1A**. Combined with the immune score, the top 10 DElncRNAs with a high immune score and the other top 10 DElncRNAs with a low immune score are presented in **Figure 1B**.

LncRNAs as the Independent Prognostic Factors Based on the Risk Score Model

The results of the univariate Cox regression analysis of prognosis-associated independent lncRNAs ($p < 0.05$) are exhibited in **Supplementary Table 2**. Then, to avoid overfitting in the final model, LASSO regression analysis was performed on the results obtained from the univariate Cox regression analysis as shown in **Figures 2A,B**. Finally, six lncRNAs were identified for the subsequent multivariate Cox regression analysis. These are displayed using a forest plot in **Figure 2C**. The results demonstrate that AC064805.1 (HR: 0.854; 95% CI: 0.748–0.975; $P = 0.0194$), AC092112.1 (HR: 0.835; 95% CI: 0.737–0.947; $P = 0.0049$), and LINC00892 (HR: 0.850; 95% CI: 0.758–0.953; $P = 0.0054$) with prolonged OS probability were regarded as independent positive prognostic factors. On the contrary, AC084033.3 (HR: 1.105; 95% CI: 1.004–1.217; $P = 0.0406$) and AL391704.1 (HR: 1.195; 95% CI: 1.074–1.329; $P = 0.0011$) with the higher mortality were identified as independent negative prognostic factors. Based on the risk score and multivariate Cox regression analysis coefficients, the predicted OS for the six lncRNAs is as follows: (−0.15816 AC064805.1) + (0.10015 AC084033.3) + (−0.17977 AC092112.1) + (−0.05673AC103691.1) + (0.17789 AL391704.1) + (−0.16258 LINC00892). Using the median risk score, the BC samples were separated into either a high- or a low-risk score group as exhibited in **Figure 2D**.

Correlation of the Five lncRNAs With Overall Survival

Depending on the median expression level, the individual expression level of the five lncRNAs ranged from high to low expression levels. Kaplan–Meier analysis was used to find the correlation between the five lncRNAs and the overall survival rates (**Figures 3A–E**). We found that the high expression of AC064805.1 and LINC00892 were related to prolonged OS; however, high expression of AC084033.3 was associated with an unfavorable OS rate. The survival analysis of the risk scores is displayed jointly in **Figure 3F**. A high-risk score denotes an increased risk of mortality.

[3] https://www.genecards.org/

[4] http://metascape.org

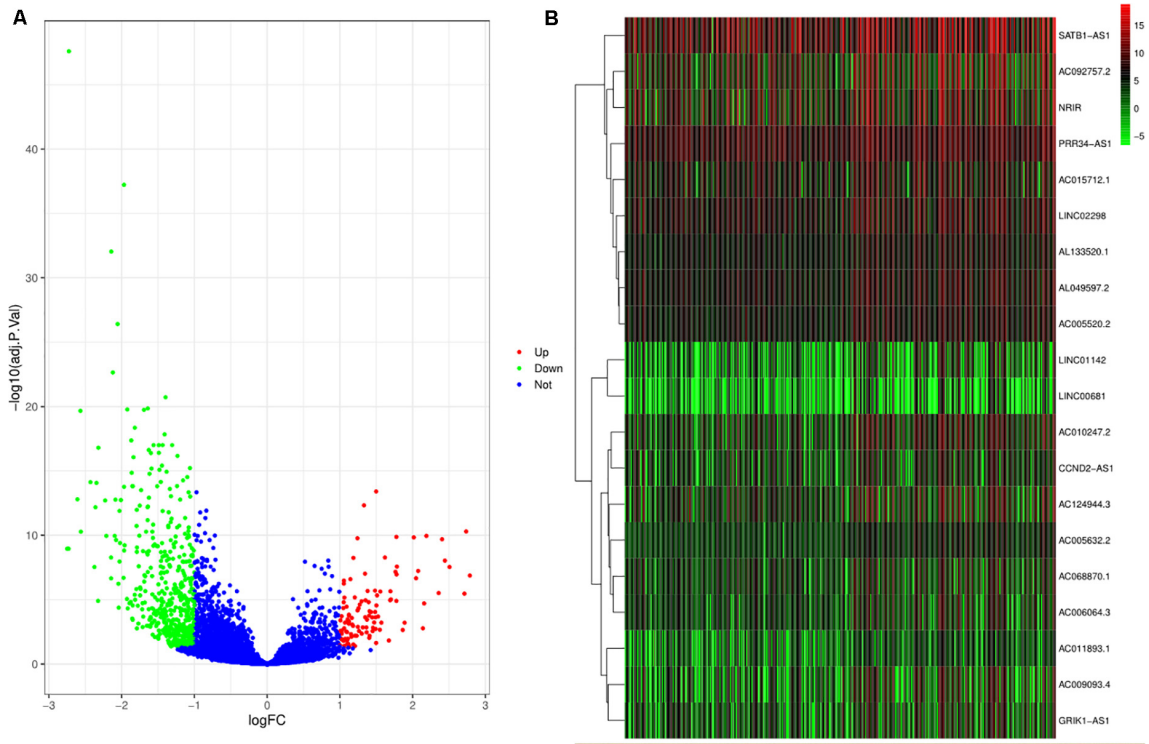

FIGURE 1 | Differentially expressed lncRNAs (DElncRNAs) and lncRNAs with immune score. **(A)** The volcano plot depicts the expression level of 10933 lncRNAs. The green, red, and blue dots imply downregulated and upregulated lncRNAs and no differential expression, respectively. **(B)** The heat plot shows lncRNAs with immune score. The top half of the heat plot refers to the top 10 expressing lncRNAs with a high immune score. Likewise, the bottom half of the heat plot refers to the top 10 expressing lncRNAs with a low immune score.

Prediction of OS Rates Using a Nomogram

Based on the risk score model, the predicted 3- and 5-year OS rates of the six lncRNAs were subjected to ROC analysis as demonstrated in **Figure 4A**. The area under the ROC curve for the predicted 3- and 5-year OS was 0.70 and 0.71, respectively, emphasizing the effectiveness of the model in predicting the OS rates. Next, the risk score; the immune score; and the clinical characteristics such as age, gender, and stage were incorporated to construct a nomogram to predict the 3- and 5-year OS for BC patients as displayed in **Figure 4B**. The nomogram was validated using the concordance index (C-index) and the calibration curve. The C-index was 0.63 (95% CI: 0.625–0.635). Also, the calibration curve revealed good concordance between the prediction and the actual observation, indicating the predictive effectiveness of the nomogram as illustrated in **Figures 5A,B**.

Expression of Selected lncRNAs in Specific Immune Cells and Investigation of the Potential Function of the Six lncRNAs

The results from the database about data analysis related to specific immune cells demonstrated that some promoters and enhancers could specifically target the genes to selectively transcribe the lncRNAs enhancing their expression as presented in **Supplementary Table 3**. Then, a total of 2,764 protein-coding genes coexpressing with the six lncRNAs were subjected to the GO and KEGG analysis using the Metascape platform. From GO functional annotation, we retrieved the top 20 significantly enriched GO terms, which are shown in **Figure 6A**. The six lncRNAs were found to be associated with several biological processes, including GO:0005912∼adherens junction, GO:0050839∼cell adhesion molecule binding, GO:1901361∼organic cyclic compound catabolic process, etc. The interaction network of the top 20 enriched GO terms were visualized as shown in **Figure 6B**. Also, KEGG pathway analysis demonstrated that the genes coexpressing with the lncRNAs were majorly enriched in hsa04520: Adherens junction, hsa04310: Wnt signaling pathway, M00141: C1-unit interconversion, eukaryotes, etc. The top 20 KEGG pathways are shown in **Figure 6C**. GO and KEGG analysis demonstrated that six TME associated lncRNAs were potentially related to tumor metastasis and progression. Correspondingly, the interaction network for the top 20 KEGG pathway is displayed in **Figure 6D**.

DISCUSSION

Over the recent decade, a vast majority of tumor studies focused on the influence of biological processes and relevant pathways

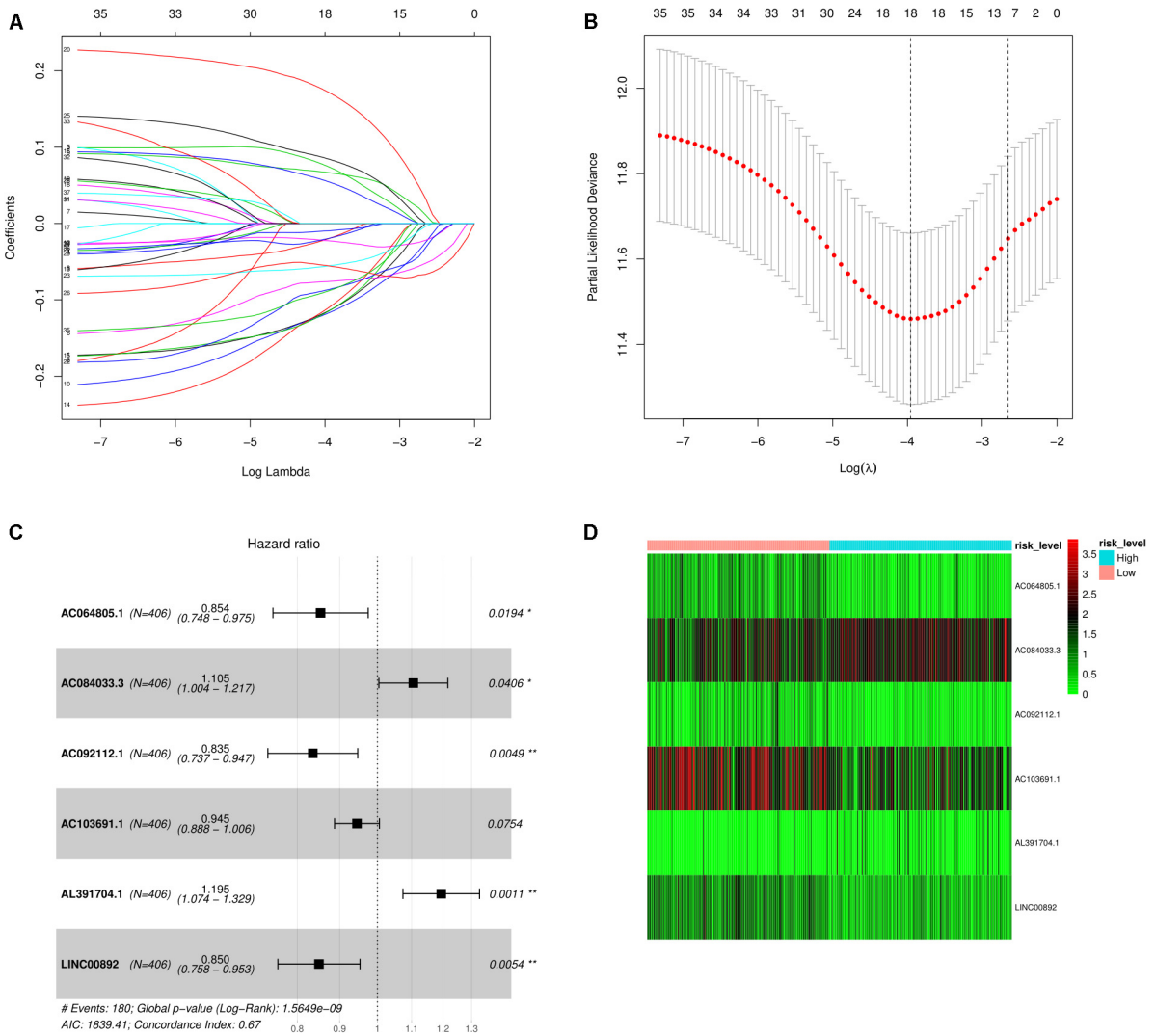

FIGURE 2 | Screening of lncRNAs as independent prognostic factors. **(A)** The LASSO regression model was utilized to validate the parameter selection adjustment. **(B)** The distribution of LASSO coefficient profiles for lncRNAs as prognostic factors. **(C)** Based on the multivariate Cox regression analysis, the forest plot shows lncRNAs with independent prognostic value. **(D)** The heat plot depicts the low- and high-risk expression profiles of the six lncRNAs in BC samples.

related to oncogenes, tumor suppressor genes, and their products to understand tumor development, progression, and metastasis. However, considerable tumors, such as BC are heterogenic in nature with a high mortality rate. This has compelled researchers to focus on the impact of the TME, especially the associated lncRNAs affecting the OS of tumor patients (Fridman et al., 2012; Huarte, 2015; Bhan et al., 2017). The status and expression levels of diverse infiltrating immune cells affect the biological processes of tumors, leading to distinct clinical outcomes (Fridman et al., 2012). The lncRNAs are known to affect the state of infiltrating immune cells by regulating the related biological processes or pathways. The lncRNAs CCAT1 and NIFK-AS1 alter the polarization of M2 macrophages by regulating the expression of miRNA to modulate the infiltration of macrophage subtypes in TME and expedite tumor progression and invasion (Zhou et al., 2018; Liu et al., 2019). These studies suggest that lncRNAs can be utilized as biomarkers of macrophage polarization and potential drug targets for immunotherapy in the corresponding tumors. The lncRNAs are also known to modulate the biological processes related to T cells, such as activation, development, and differentiation (Heward and Lindsay, 2014). Upregulated lnc-Tim3 and lnc-sox5 destroy the balance of the TME by decreasing the infiltration of antitumor CD8 + T cells aiding tumor progression (Wu et al., 2017; Ji et al., 2018). lnc-EGFR via regulating AP-1/NF-AT1/Foxp3 signaling pathway and lncRNA SNHG1 by regulating miR-448/IDO affect the differentiation and growth of immunosuppressive regulatory T cells (Tregs) and enable immune escape for tumor (Jiang et al., 2017; Pei et al., 2018). Similarly, the dendritic cell differentiation is modulated by lnc-DC via activation of transcription factor STAT3, weakening the antitumor response (Wang et al., 2014). Concisely, all these findings indicate that lncRNAs play an

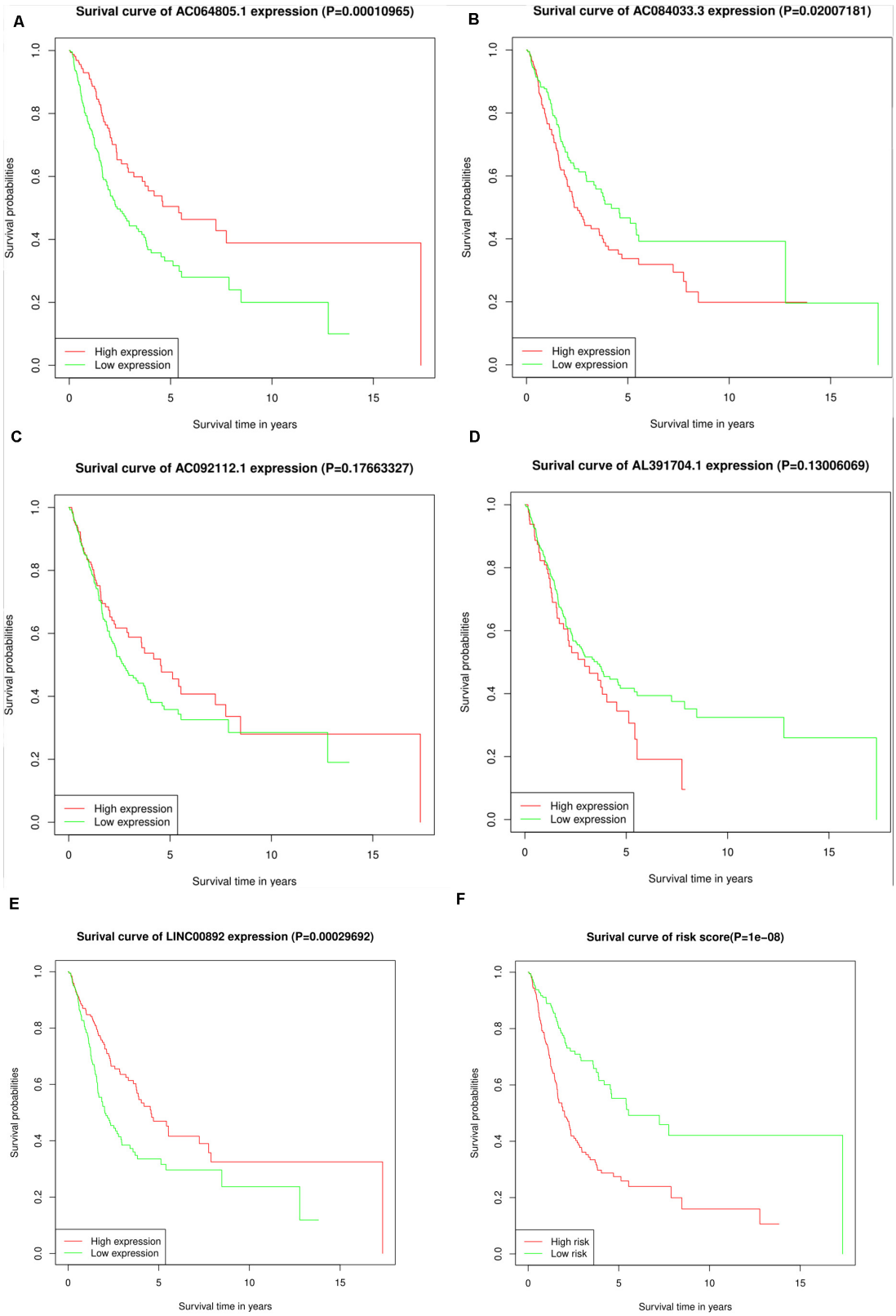

FIGURE 3 | KM survival analysis for the five lncRNAs and the risk score. **(A–F)** Kaplan–Meier curves of OS for AC064805.1, AC084033.3, AC092112.1, AL391704.1, LINC00892, and risk score, respectively.

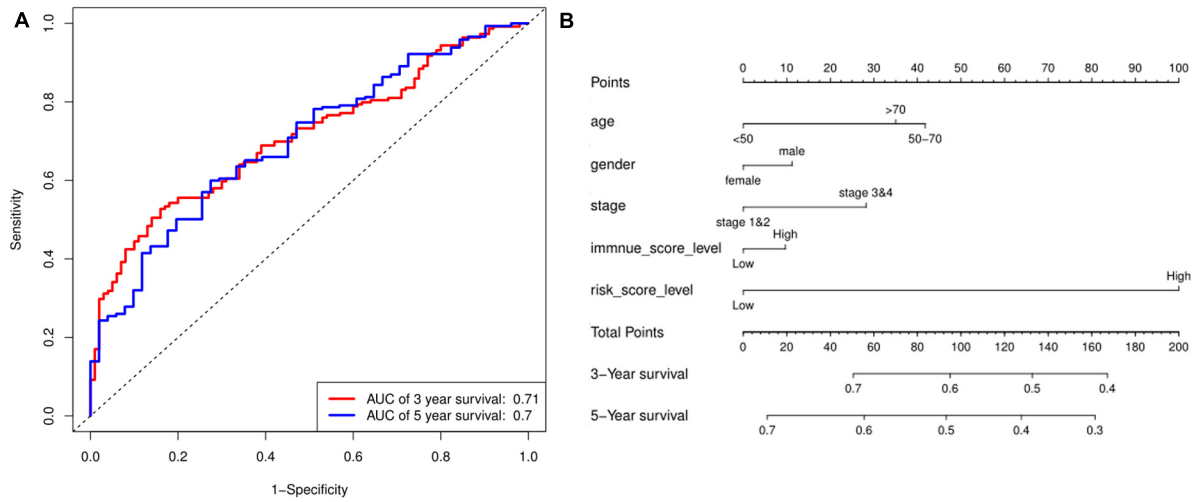

FIGURE 4 | Prediction for OS. **(A)** ROC curve shows the sensitivity and specificity of the risk score model for predicting 3- and 5-year OS probability. **(B)** Each variable was scored according to the patient's status, and the total score was obtained by adding each variable score. The vertical line represents the corresponding 3- and 5-year OS probability prediction.

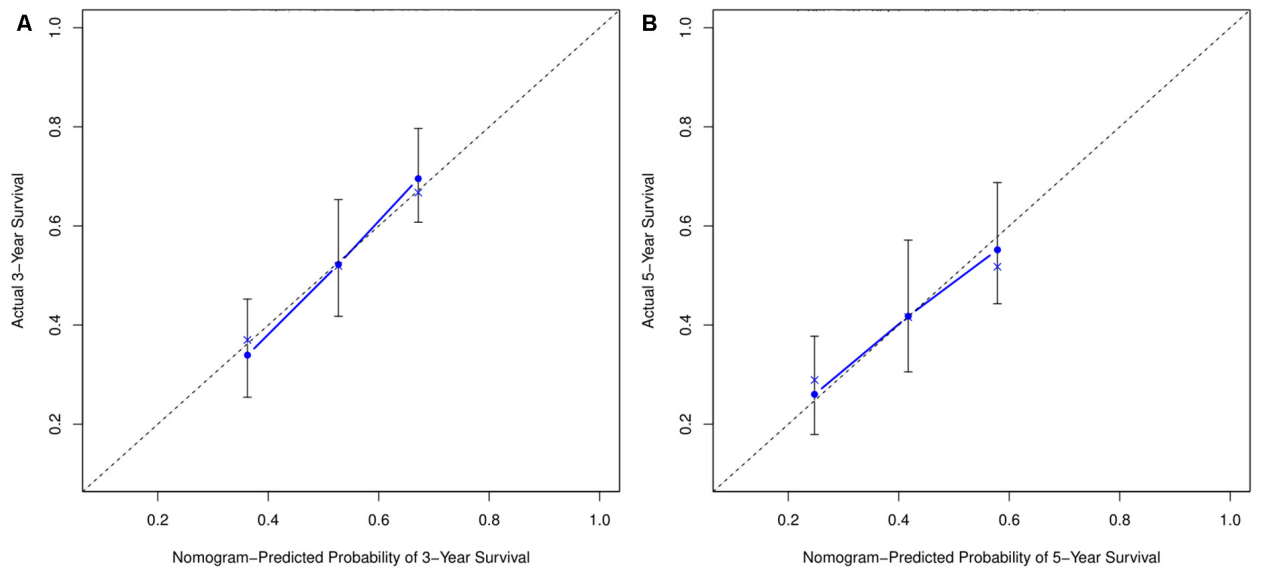

FIGURE 5 | Calibration curve for nomogram prediction. **(A,B)** 3- and 5-year OS prediction performed by the nomogram. A small gap between the blue solid and black dotted lines demonstrates good performance of the nomogram.

essential role in regulating the activities of immune cells in TME, further emphasizing the relationship between tumor heterogeneity and immune cell infiltration in a variety of tumors. Notably, tumor exosome–derived lncRNAs have been shown to enable specific communications between tumor cells and immune cells. For example, tumor cell–derived lncRNA TUC339 activates macrophages to modulate the macrophage cytokine production, phagocytosis, M1/M2 polarization, and cell proliferation (Li X. et al., 2018). It further strengthens the notion that lncRNAs may influence the infiltration of immune cells to regulate their functions, which are closely related to tumor growth, progression, and metastasis. Therefore, lncRNAs can indeed serve as novel immuno-biomarkers in the corresponding tumors.

Because several lncRNAs are involved in tumor growth, progression, and metastasis, a risk score model was established to comprehensively consider their role in prognosis. The utility of LASSO regression analysis identified six TME-associated lncRNAs, and the multivariate Cox regression analysis recognized five lncRNAs as potential immuno-biomarkers and therapeutic targets. The ROC score for the six lncRNAs for 3- and 5-year OS was predicted to be 0.70 and 0.71, respectively.

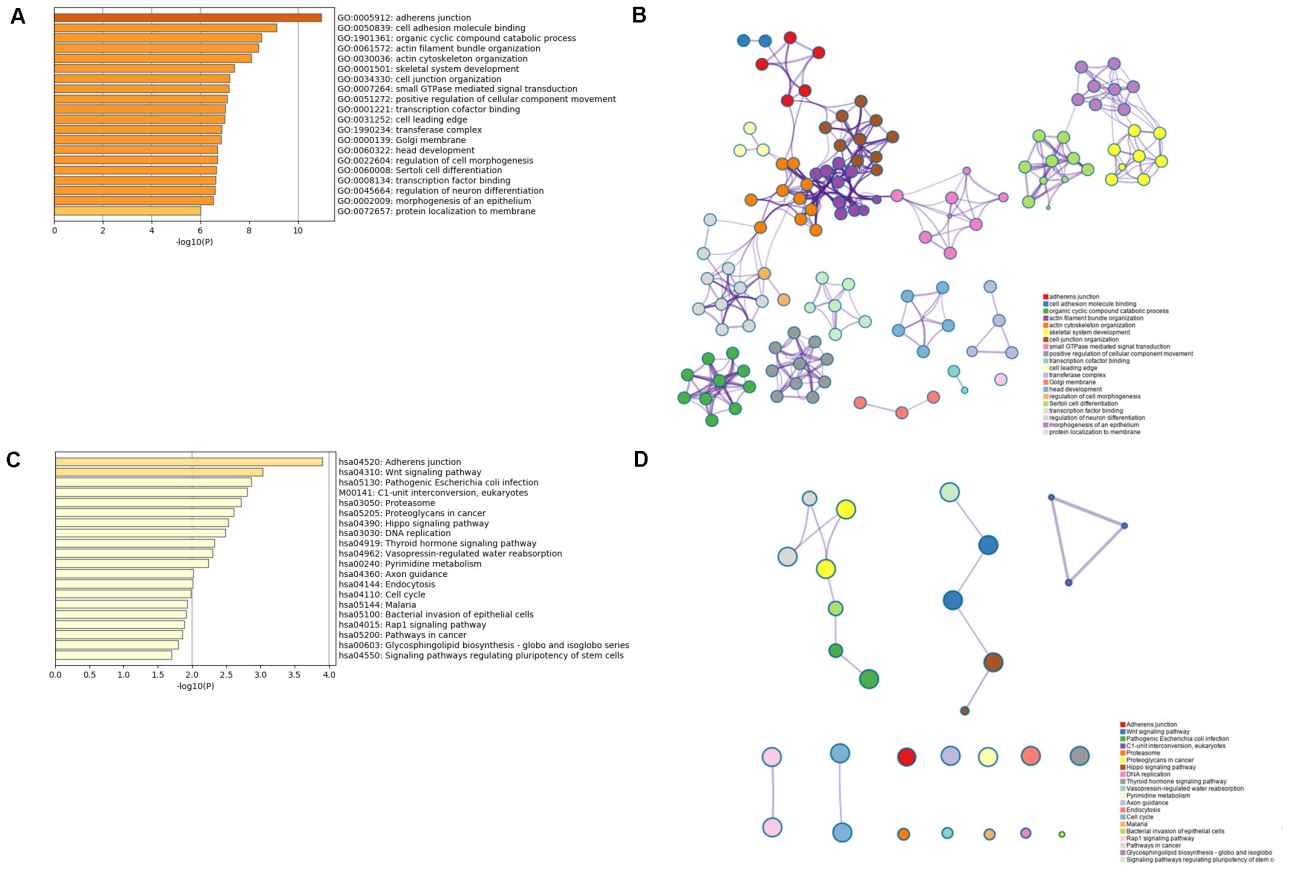

FIGURE 6 | GO and KEGG analysis for the six lncRNAs coexpressed with mRNAs. **(A,B)** The bar plot and interaction network plot for the top 20 enrichment of GO analysis. **(C,D)** The bar plot and interaction network plot for the top 20 enrichment of KEGG analysis.

This model can also be utilized to evaluate the prognostic values of the multiple lncRNAs. Finally, a nomogram was developed to attain the individual prognosis information based on patient-specific conditions. These results could help clinicians in the early prognosis of the disease and to timely intervene reducing the mortality rate in BC patients.

Almost 75% of the genome can produce lncRNAs, which are now being explored as tumor hallmarks (Djebali et al., 2012). They exist in TME and may also influence other tumor hallmarks, including the immune system. Cancer cell–derived lncRNA H19 targets endothelial cells to promote angiogenesis by modulating the production and release of VEGF, contributing to tumor growth (Conigliaro et al., 2015). Exosomal lncRNA PTENP1 inhibits tumor progression by regulating PTEN expression via binding to microRNA-17 (Zheng et al., 2018). lncRNA FAL1 binds to miR-1236 to promote tumor cell proliferation and metastasis (Li B. et al., 2018). In addition, lncRNAs are also associated with antitumor drug resistance. For instance, sunitinib resistance is the result of altered expression of AXL and c-MET caused by lncARSR binding to miR-34/miR-449 (Qu et al., 2016). In this study, mRNA coexpression analysis was carried out to investigate the potential function of the six lncRNAs. The enrichment analysis was conducted using GO and KEGG analysis. The results suggest that these lncRNAs could be involved in critical cellular processes, such as tumor metastasis, catabolism, cell cycle, DNA replication, and so on.

Nonetheless, there are limitations to our study. First, the TCGA database is limited to constructing a risk score model and nomogram. Therefore, additional databases should be utilized to validate the six lncRNA signatures and the nomogram performance. Second, only limited clinical characteristics were included in the nomogram to uphold the accuracy of the prediction. Third, because the several lncRNAs have not been fully validated yet, the selected lncRNAs lack laboratory evidence. Using the database, we could only infer about the expression of the six lncRNAs in specific immune cells. Fourth, the associated biological processes and pathways need to be validated experimentally to investigate the molecular mechanism revealing the characteristics of the TME-associated lncRNAs.

CONCLUSION

In a nutshell, our study screened six BC microenvironment–associated lncRNAs, identified independent risk factors

influencing OS, and constructed models to predict the prognostic value. Also, functional enrichment analysis revealed that the six lncRNAs were most likely related to BC metastasis and progression. However, further experiments are required to investigate the associated pathways.

DATA AVAILABILITY STATEMENT

The data used and analyzed during the current study are available from TCGA (https://portal.gdc.cancer.gov/).

AUTHOR CONTRIBUTIONS

GLi, SW, and GLin: conception and design. GLin, BG, YW, and TL: data collection, data analysis and interpretation. GLin and GLi: manuscript writing. All authors: final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.567200/full#supplementary-material

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REVIEW
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The Cell Type–Specific Functions of miR-21 in Cardiovascular Diseases

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Cardiovascular diseases are one of the prime reasons for disability and death worldwide. Diseases and conditions, such as hypoxia, pressure overload, infection, and hyperglycemia, might initiate cardiac remodeling and dysfunction by inducing hypertrophy or apoptosis in cardiomyocytes and by promoting proliferation in cardiac fibroblasts. In the vascular system, injuries decrease the endothelial nitric oxide levels and affect the phenotype of vascular smooth muscle cells. Understanding the underlying mechanisms will be helpful for the development of a precise therapeutic approach. Various microRNAs are involved in mediating multiple pathological and physiological processes in the heart. A cardiac enriched microRNA, miR-21, which is essential for cardiac homeostasis, has been demonstrated to act as a cell–cell messenger with diverse functions. This review describes the cell type–specific functions of miR-21 in different cardiovascular diseases and its prospects in clinical therapy.

Keywords: miR-21, cardiovascular diseases, cardiomyocytes, fibroblast, endothelial

INTRODUCTION

According to the 2013 global burden of disease study (GBD), 17.3 million individuals died due to cardiovascular diseases (CVDs) worldwide. It causes twice as many deaths as cancer, contributing to 31.5% of total deaths and 45% of deaths due to non-infectious disease (Townsend et al., 2016). The unmanageable factors of CVDs comprise family history, sex, and age, and the manageable factors comprise cigarette smoking, dyslipidemia, diabetes mellitus, sedentary lifestyle, unhealthy diet, and stress. The most common types of CVDs are acute myocardial infarction (AMI), arrhythmias, vascular diseases, cardiomyopathy, and heart failure (HF) (Olson, 2014). With the development of certain effective drugs and devices, the incidence rates of CVDs were controlled to a certain extent. However, no significant improvements in overall outcomes have been observed, which necessitates further insights into molecular and pathological features of the diseased vessel and heart along with innovative therapeutic strategies (MacKenna et al., 2000; Bruneau, 2008). Lately, microRNAs (miRNAs), a kind of conserved small non-coding RNA (ncRNAs), have garnered interest as critical regulators of CVDs.

The biogenesis of miRNAs is a multistep process. Briefly, they are first transcribed by RNA polymerase II into primary-miRNAs (pri-miRNAs). The pri-miRNA hairpin is then excised in the nucleus by complexes that contain the RNase III enzyme Drosha and the RNA-binding protein

DiGeorge syndrome critical region 8 (DGCR8). Drosha recognizes the junction at the base of the hairpin (the junction formed by double-stranded RNA–single-stranded RNA), and the two DGCR8 proteins bind to the stem and ensure proper cleavage. The pre-miRNA hairpins are composed of ∼70 nucleotides, whose ends are characterized by a 2′ nucleotide overhang of the 3′ end, a 5′ phosphate, and a 3′ hydroxyl at the 3′ end, which are recognized by Exportin 5 (XPO5) and transferred into the cytoplasm. In the cytoplasm, the RNase III enzyme Dicer binds to pre-miRNAs by identifying the structures comprising the 3′ overhang, 5′ phosphate, and the loop and cuts the pre-miRNAs at a length that is species-specific and produces a mature miRNA duplex with another classical 2-nucleotide overhang of the 3′ end.

One strand of the mature miRNA duplex (also called the guide strand) binds to Argonaute protein-containing RISC, and the other strand (also called the passenger strand) is degraded. The strand with less stable 5′ pairing ends is prioritized (Gebert and MacRae, 2019; Trabucchi and Mategot, 2020). miRNAs bind to the 3′ untranslated region (UTR) of target mRNAs and mediate their regulation at the post-transcriptional level by inhibiting translation or initiating mRNA decay (Latronico and Condorelli, 2009). Recent studies have reported that, other than gene silencing, miRNAs could stimulate transcription and translation by binding to the promoter region, 5′ UTR, or the open reading frame in target genes (Iwakawa and Tomari, 2013; Li et al., 2019). In 1993, the first animal miRNA was identified that regulates the development in *Caenorhabditis elegans* (Rosalind and Ambrost, 1993). Later, miRNAs attracted the attention of researchers (Cordes and Srivastava, 2009). A single miRNA often regulates the function of multiple mRNAs, and each mRNA can also be altered by different miRNAs, participating in precise adjustments in a complex web of cellular interactions (Leite-Moreira et al., 2013). Moreover, the two mature miRNAs originating from the different arms of a single pri-miRNA typically act on varied mRNA targets. miRNAs may contribute to cardiac homeostasis via diverse cell types, such as cardiomyocytes (CMs), cardiac fibroblasts (CFs), endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and immune cells (Cordes and Srivastava, 2009; Small et al., 2010; Small and Olson, 2011; Barwari et al., 2016).

The functions of miR-21 in the cardiovascular system have been extensively investigated but are still shrouded in controversy (Krichevsky and Gabriely, 2009). Although the inhibition of miR-21 in CFs via the systemic delivery of antagomirs demonstrated a significant benefit during HF (Thum et al., 2008), miR-21 knockout or knockdown of systemic expression of miR-21 with a locked nucleic acid–modified (LNA-modified) anti-miR oligonucleotide did not convert the pathological processes of HF (Patrick et al., 2010). Meanwhile, accumulating evidence suggests that the overexpression of miR-21 in the CMs demonstrates a protective role in cardiac function (Cheng et al., 2009b). This divergence might be a result of the distinct regulatory mechanisms of miR-21 in different cellular subtypes (Mishra et al., 2009) (**Figure 1**). Elucidating the cell type–specific functions and precise targets of miR-21 is important for potential clinical application. We have summarized the cell-specific functions and discussed the therapeutic prospects of miR-21 in CVDs in this review.

BIOLOGY AND FUNCTION OF miR-21

For a long time, the human genome was originally supposed to comprise only 1% coding exons and 99% junk DNA/introns with unknown functions (Venter et al., 2001). Recently, it was recognized that the transcription of such non-coding sequences results in ncRNA rather than protein (Rosalind and Ambrost, 1993; Condorelli et al., 2014). miRNAs are short (21–25 nts) ncRNAs that are typically evolutionarily conserved in different species (Bartel, 2004). To initiate their respective functions, they bind to the respective mRNAs via the seed site (6–8 nts). By the end of 2018, according to the newly published miRbase, approximately 2000 precursors and more than 2,000 mature miRNAs are known in humans (Lucas et al., 2018). Genomic sequences located between protein-coding sequences or introns of protein-coding sequences can both encode miRNAs (Bartel, 2004). Functional and mature miRNAs are synthesized after multiple processes, including transcription, nuclear maturation, export, and cytoplasmatic processing (Beermann et al., 2016).

Various miRNAs are involved in different physiological and pathological regulations of the cardiovascular system (Small and Olson, 2011). For example, miR-1 is involved in the formation of heart tubes of chick embryos and is the most enriched miRNA in adult cardiac tissues (Darnell et al., 2006). The upregulation of its expression results in the developmental arrest at E13.5 by interacting with Hand2 (Zhao et al., 2005), which was a critical transcription factor for cardiac development (Srivastava et al., 1997). miR-133 enriched in the muscles participates in cell proliferation and cell vitality, and its knockout is linked to cardiac chamber septal impairments, serious dilatation, and embryonic death (Meder et al., 2008). Lately, miR-21 has attracted attention as one of the enriched miRNAs in the cardiovascular system for its diverse effects in cardiac function (Eva Van et al., 2006; Cheng et al., 2007; Mariko et al., 2007; Sayed et al., 2007).

The hsa-miR-21 gene is located on chromosome 17q23.2, which overlays the vacuole membrane protein 1 (VMP1) gene and is conserved. The pri-miR-21 is transcribed from the introns of VMP1 by RNA polymerase II independently (Fujita et al., 2008; Selcuklu et al., 2009). Moreover, pri-miR-21 has a conserved promoter, which is located within the intron (Ha and Kim, 2014; Adams et al., 2017; Lucas et al., 2018). Data indicate that miR-21 is regulated at the transcriptional level (Cai et al., 2004; Davis et al., 2008). For example, Fujita et al. demonstrated that a promoter sequence located at 3,770–3,337 nts upstream to the miR-21 hairpin sequence exhibits many conserved enhancer elements comprising binding sites for activator protein-1 (AP-1), CCAAT/enhancer-binding protein alpha, serum response factor, tumor protein p53, and signal transducer and activator of transcription (STAT3) (Kumarswamy et al., 2011). The miR-21 expression might be regulated post-transcriptionally. The precursor miRNA-21 (pre-miR-21) (∼70 nts hairpin structure) is processed by the endonuclease Drosha from pri-miR-21 in

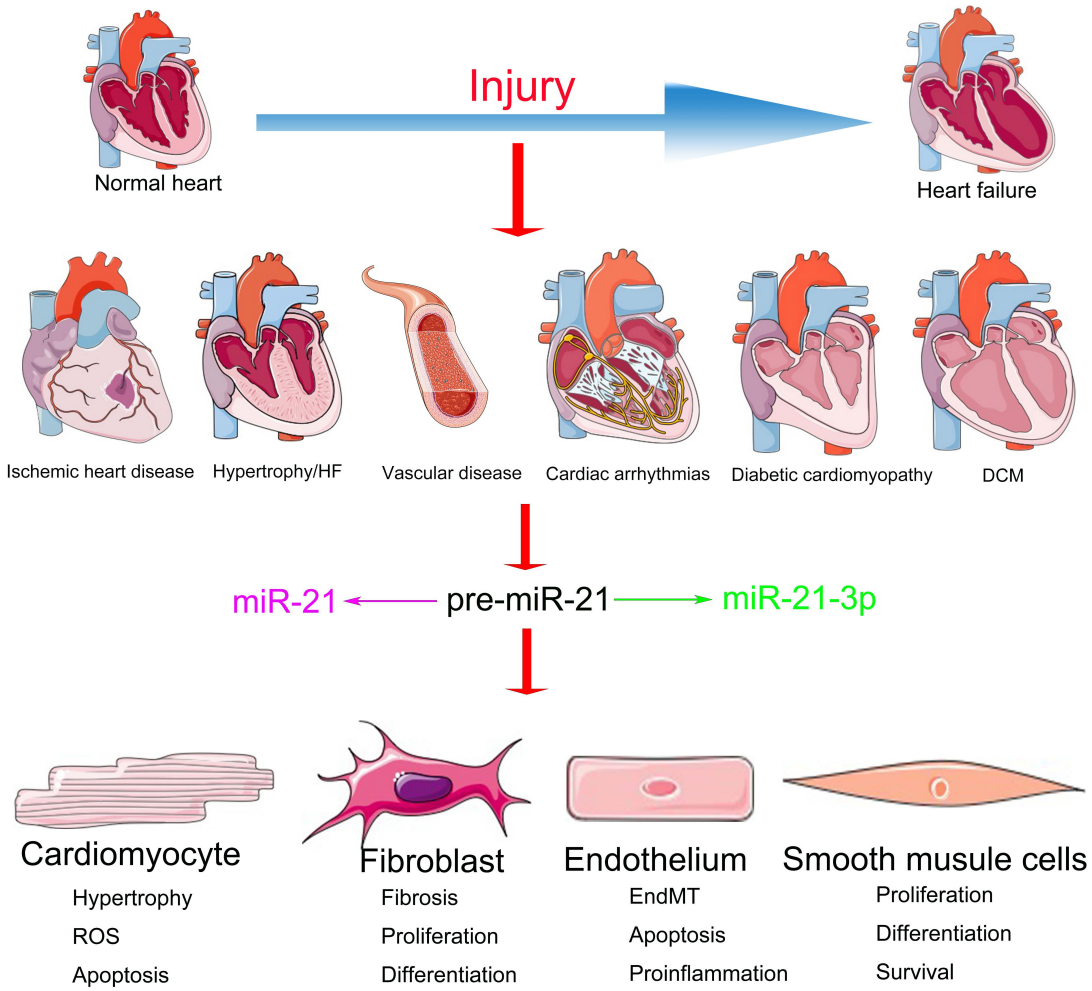

FIGURE 1 | miR-21 and miR-21-3p exert various effects on diverse cell types in multiple CVDs.

the nucleus (Lee et al., 2003), which might be upregulated upon extracellular stimulation (Jun et al., 2009). Subsequently, pre-miR-21 is exported by Exportin 5 and processed by Dicer to release mature hsa-miR-21 (also known as hsa-miR-21-5p from the 5p arm of the pre-miR-21, which is the biologically dominant arm) and hsa-miR-21-3p (formerly named hsa-miR-21* from the 3p arm of the pre-miR-21, which was previously considered less abundant than hsa-miR-21-5p) in the cytoplasm (Kumarswamy et al., 2011). Both hsa-miR-21 and hsa-miR-21-3p exhibit important functions in the cardiovascular system by targeting different mRNAs (Thum et al., 2008; Roy et al., 2009; Yan et al., 2015). In particular, the functions of miR-21 in CVDs are of vital importance (Zhang, 2008).

MiR-21 is typically abundant in the dominating cellular subtypes of the cardiovascular system (Zhang, 2008), including CMs (Cheng et al., 2007), ECs (Suarez et al., 2007), and VSMCs (Ji et al., 2007) and especially CFs (Roy et al., 2009). Previous studies have demonstrated the important characteristics of miR-21 in the cardiovascular system by experiments with gain- and loss-of-function mutations (Eva Van et al., 2006; Cheng et al., 2007; Mariko et al., 2007; Sayed et al., 2007; Thum et al., 2007b). However, the expression patterns and functions of miR-21 reported in various CVDs are controversial (**Supplementary Table 1**). For instance, researchers observed that during HF, the expression of miR-21 is particularly upregulated in CFs, which, in turn, activates the extracellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK) pathway by inhibiting sprouty homolog 1 (SPRY1) protein. The increased expression of miR-21 promotes cardiac remodeling by improving the viability of CF and the accumulation of hypertrophy-inducing factors. Furthermore, the inhibition of miR-21 in mice resulted in the inactivation of the ERK-MAPK pathway and the prevention of CM hypertrophy along with CF activation (Thum et al., 2008). On the contrary, transgenic mice with miR-21 overexpression demonstrated a reduction in the infarct area and CF fibrosis as well as the downregulation of phosphatase and tensin homolog (PTEN) protein and Fas ligand (FasL) in ischemic diseases (Sayed et al., 2010). These contradictions might be a result of the abundance and diverse targets of miR-21 in different cell types, which ultimately leads to miscellaneous effects on the

cardiac function during different stages and processes of CVDs (**Figure 1**).

Ischemic Heart Disease: The Role of miR-21 in CMs and CFs

According to the CVD epidemiological update in Europe in 2016 (Townsend et al., 2016), plaque damage after coronary and sequent hypoxia results in AMI, which was the major cause of morbidity and mortality. CM death (Francis Stuart et al., 2016; DeLeon-Pennell et al., 2017; Frangogiannis, 2017), the activation of EC and CF, and necrotic cell removal are involved in the progression of ischemia (Ma et al., 2014). Despite the availability of therapeutic approaches, AMI is still associated with high rates of acute death and long-term complications, such as HF. Early diagnosis and intervention are pivotal in reducing the damage caused by AMI. Currently, the most frequently used diagnostic biomarker is a highly sensitive group of cardiac proteins known as troponins (Roffi et al., 2016). However, these markers are not without limitations. Elevated troponin levels could be observed in congestive HF patients or individuals with long-term kidney diseases as well (Rubini Gimenez et al., 2014).

Recently, miRNAs were evaluated as potential markers of AMI (Fiedler and Thum, 2013). Corsten et al. (2010) investigated 32 citrate plasma samples from patients with AMI (obtained at the time of mechanical reperfusion) and 36 plasma samples from patients with atypical chest pain and positive stress testing; however, normal coronary angiograms, using RT-PCR arrays, helped in observing that the AMI patients demonstrated higher levels of circulating miR-21 as compared to those of the control group. Oerlemans et al. (2012) also used RT-PCR arrays to explore the potential diagnostic value of circulating microRNAs as novel early biomarkers in 332 suspected acute coronary syndrome (ACS) patients (Oerlemans et al., 2012). The serum samples were collected before treatment interventions. They observed that miR-21 in combination with miR-1, miR-499, and high-sensitive troponin T (hs-cTnT) performed well in diagnosing ACS (AUC = 0.90, n = 106). Elevated miR-21 could be observed even in patients with ACS who tested negative for hs-cTnT initially or showed symptom onset in $<$3 h (Oerlemans et al., 2012). However, using TaqMan PCR arrays, Liebetrau et al. (2013) demonstrated that miR-21 did not increase in patients undergoing transcoronary ablation of septal hypertrophy (TASH), which was used as a model for mimicking AMI (13 males and 8 females with the average age of 59.0 $\pm$ 13.29 years). In this study, venous blood samples were collected before and at different time points after the induction of MI for the determination of miRNAs. The variation in the conclusions derived in these studies may be due to the differences in the methods of sample collection (when and how the samples were collected), clinical and demographic parameters (age, sex, and medical history), and detection methods (RNA isolation and PCR arrays).

In addition to the possible utilization of the circulating miR-21 as a biomarker for AMI, its intracellular effects might help improve the cardiac function post-AMI. Roy et al. (2009) first reported that ischemia reperfusion markedly induced the expression of miR-21 in the infarct region of the heart. Increased miR-21 resulted in fibroblast survival and triggered fibrotic infarct remodeling by the inhibition of PTEN in CFs (Roy et al., 2009). Another study also indicated that miR-21 mediated the activation of fibroblasts caused by transforming growth factor-β (TGF-β) by targeting Jagged1 (Bronnum et al., 2013) and SMAD family member 7 (SMAD7) (Yuan et al., 2017). However, Dong et al. (2009) demonstrated the upregulation and downregulation of miR-21 expression in the infarct and border region, respectively, in the AMI rat model. Overexpression of miR-21 protected the cultured CMs against apoptosis by regulating programmed cell death 4 (PDCD4) and the AP-1 pathway (Dong et al., 2009). Sayed et al. (2010) showed that protein kinase B (AKT) upregulated the miR-21 expression. miR-21 transgenic mice exhibited a smaller infarct area and suppressed HF by downregulating the enhanced PTEN and FasL expression in the ischemic heart in CMs (Sayed et al., 2010). miR-21 increased cardiac fibrosis, which led to increased cardiac function in the CFs. It also reduced apoptosis and demonstrated a protective role in the cardiac function in CMs.

Interestingly, exosomal miR-21 participated in angiogenesis (Wang et al., 2017), cell proliferation (Xiao et al., 2016), cardiac remodeling, and metabolic regulation via the paracrine signaling networks in the target cells as well (Luther et al., 2018). For example, Chen et al. (2019) demonstrated that the knockout of exosomal miR-21 in CMs cultured with an oxygen-glucose–deprived media increased the ROS-induced apoptosis in CMs by targeting PDCD4 and decreased the activation of CFs and angiogenesis mediated by ECs, demonstrating the interaction between CMs and other cell types in the heart.

In conclusion, increased miR-21 expression caused by AMI protected the CMs from apoptosis along with enhancing the activation of CFs (**Figure 2**).

Cardiac Hypertrophy and Heart Failure: The Roles of miR-21 in CMs, CFs, and ECs

Pathological cardiac hypertrophy is primarily caused by chronic hemodynamic overload, ischemic injury, altered metabolism, or neuroendocrine activity (Barry et al., 2008). Loss of CM and excessive deposition of the extracellular matrix are major contributors to the development of cardiac hypertrophy to HF (Creemers and Pinto, 2010). Preventing cardiac hypertrophy and fibrosis is of vital importance in the prevention of HF (Frangogiannis, 2012).

Thum et al. (2007b) showed that the altered expression of miRNAs and mRNAs in the human fetal heart was closely associated with HF and the overexpression of several fetal miRNAs in CMs, including miR-21, which led to cellular hypertrophy and alternated gene expression, giving rise to the conditions mimicking HF. Eva Van et al. (2006) discovered that miR-21 expression increased in the hypertrophic heart but did not change in patients with HF. Further, by using animal models, Cheng et al. (2007) and Mariko et al. (2007) reported the upregulation of miR-21 expression with the progression of hypertrophy in the heart and had a negative effect with respect

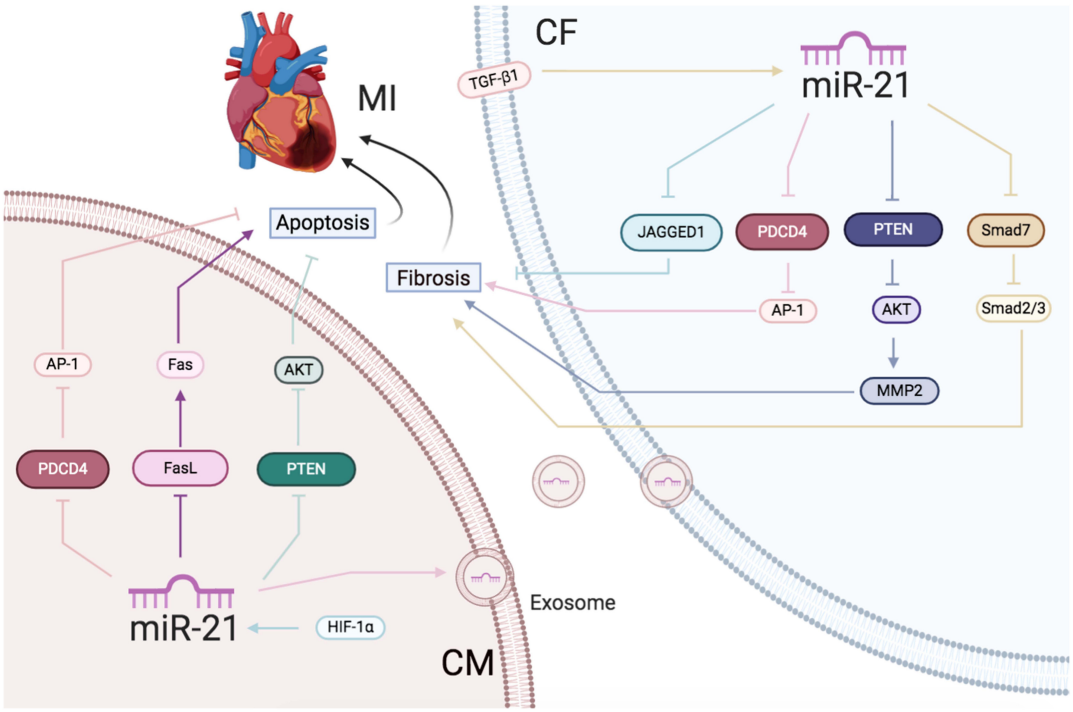

FIGURE 2 | Ischemic heart disease: the roles of miR-21 in CMs and CFs.Reported major signals in CMs. Pink lines: miR-21 decreases PDCD4/AP-1 to inhibit apoptosis. Blue lines: HIF-1α-induced miR-21 downregulates PTEN/AKT to reduce apoptosis. Purple lines: miR-21 decreases FasL/Fas to reduce apoptosis. Reported major signals in CFs. Yellow lines: TGF-β-induced miR-21 downregulates Smad7 and activates Smad2/3 to increase fibrosis. Blue lines: miR-21 inhibits JAGGED1 to increase fibrosis. Gray lines: miR-21 inhibits PTEN/AKT/MMP2 to increase fibrosis. Pink lines: exsomal miR-21 in CMs inhibits PDCD4/AP1 pathway to promote CFs fibrosis.

to the size of CM. The varied patterns of miR-21 expression in these studies might be due to the variation in the sampling intervals (the stage of hypertrophy and heart failure) and the medical history of patients (the cause of heart failure should be taken into account). Moreover, the sources influencing miR-21 expression are complex. The expression of miR-21 might be varied in multiple cell types in the heart during different stages of disease progression. Indeed, several studies have emphasized different influences of miR-21 on CMs and non-CMs, especially CFs. Thum et al. (2008) showed that the expression of miR-21 in CMs was low in the base state. It increased selectively in CFs rather than CMs during heart failure. miR-21 activates the ERK-MAPK pathway via the inhibition of SPRY1 and mediates the structural and functional deterioration of cardiac function (Thum et al., 2008). In addition, the activation of the transcription factor AP-1 and subsequent miR-21 expression mediated Ang II-induced cardiac fibrosis. miR-21 led to the inhibition of the antiapoptosis and antifibrosis targets PTEN and SMAD7, ultimately leading to the proliferation of CFs (Lorenzen et al., 2015). The administration of antagomir that acts against miR-21 in mice with left ventricular pressure overload attenuated the endothelial-to-mesenchymal transition in ECs *in vivo* (Kumarswamy et al., 2012).

Exosomes replicated the redeeming function of the host cells in target cells, partly by paracrine action. The composition and biological activities of exosomes primarily depend on the cells secreting the exosomes. Qiao et al. (2019) observed that the exosomes in patients with HF derived by explant heart tissue demonstrated decreased CM proliferation and angiogenesis mediated by ECs leading to cardiac dysfunction. miR-21 overexpression in the exosomes suppressed PTEN/AKT signaling to improve CM vitality and angiogenesis (Qiao et al., 2019). The fate of miR-21-3p has also garnered interest due to its influence on the progression of cardiac hypertrophy to HF (Duygu and Da Costa Martins, 2015). Deep RNA sequencing demonstrated increased expression of miR-21-3p during HF in humans (Yang et al., 2014). Exosomal miR-21-3p in CFs could induce CM hypertrophy by communications between the two cell types with paracrine signaling (Bang et al., 2014). Meanwhile, our group identified that miR-21-3p exerted an antihypertrophic effect on CMs by targeting HDAC8 and observed reduced expression of miR-21-3p in hearts after 2 weeks, which was increased after 4 weeks following transverse aortic constriction (TAC) (Yan et al., 2015). The contrasting roles of miR-21-3p might be attributed to different cell types. Patients often showed abnormalities in cardiac structure and function with high blood pressure, such as left ventricular hypertrophy and HF (Lip et al., 2000). In our previous studies, we demonstrated that the systemic delivery of miR-21 in animals decreased the blood pressure and improved cardiac hypertrophy in the spontaneously hypertensive rat (SHR) by upregulating mitochondrial cytochrome B (CYTB). The miR-21 expression in hypertensive patients was increased with the rise in blood pressure (Li et al., 2016).

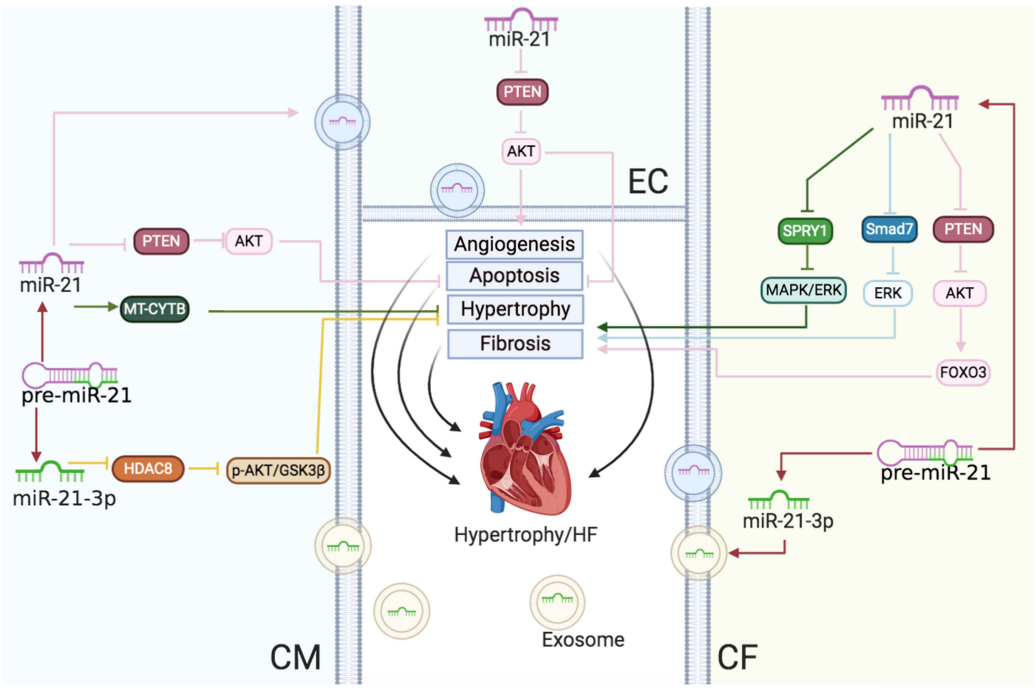

FIGURE 3 | Cardiac hypertrophy and HF: the roles of miR-21/miR-21-3p in CMs, CFs, and ECs. Reported major signals in CMs. Green lines: in hypertension, miR-21 enhances MT-CYTB mRNA translation in the mitochondria to inhibit hypertrophy. Pink lines: in HF, exosomal miR-21 suppresses the PTEN/AKT signaling to inhibit apoptosis. Yellow lines: miR-21-3p downregulates the HDAC8/p-AKT/GSK3β pathway to decrease hypertrophy. Reported major signals in CFs. Pink lines: miR-21 downregulates PTEN and enhances the AKT/FOXO3 pathway to induce fibrosis. Blue lines: miR-21 inhibits the SMAD7/ERK pathway to induce fibrosis. Green lines: miR-21 downregulates SPRY1/ERK/MAPK to increase fibrosis. Reported major signals in ECs. Pink lines: exosomal miR-21 from CMs downregulates PTEN to decrease EC apoptosis and induce angiogenesis.

In conclusion, during the progress of cardiac hypertrophy to HF, upregulated miR-21 reduced CM sizes but also mediated cardiac fibrosis (**Figure 3**).

Vascular Disease: The Role of miR-21 in ECs and VSMCs

Injuries or alterations would promote EC fragility or migration and VSMC dedifferentiation, migration, and proliferation in vessels (Leite-Moreira et al., 2013). Cellular phenotypic transformation accounted for the progress of proliferative diseases, including atherosclerosis, in-stent restenosis, and hypertension (Cahill and Redmond, 2016). This further led to a sudden heart attack and vessel disease (Small et al., 2010). Recently, miRNAs, including miR-21, were proposed to modulate their expression in response to the effector molecules in endothelial and VSMCs (Cheng et al., 2009a; Cordes et al., 2009; Davis et al., 2009; Xin et al., 2009).

The abdominal aortic aneurysm (AAA) leads to high morbidity and mortality rates worldwide (Creager et al., 2004). Maegdefessel et al. (2012) observed that miR-21 was upregulated and its target PTEN was downregulated in human aortic samples, which were obtained from patients with AAA who underwent surgical repair of the augmentative abdominal aorta (57–68 mm). miR-21 was also found to be upregulated primarily in human arteriosclerosis obliterans (ASO) samples. HIF-1α induced miR-21 targeted tropomyosin 1 (TPM1) to promote VSMC proliferation and migration during ASO (Wang et al., 2011). miR-21 was shown to be present in abundance in the failed human transplant similar to that in the animal transplant models (McDonald et al., 2013). miR-21 knockdown derepressed PTEN to reduce the neointima formation and phenotypic transition in VSMCs and CFs in a rat balloon surgery (Ji et al., 2007). According to Davis et al. (2008), miR-21 affected TGF-β and bone morphogenetic protein (BMP) expression and slowed down human VSMCs, leading to a contractile phenotype by downregulating PDCD4. Furthermore, TGF-β and the BMP pathway accelerated the cleavage of pri-miR-21 into pre-miR-21 by Drosha complex, thus elevating the mature miR-21 levels post transcription (Davis et al., 2008).

Interestingly, for the first time, our studies observed that amlodipine-activated AKT2 not only increased SP1 translocation into the nucleus, but also the cooperative binding to the miR-21 promoter, which finally targeted PDCD4 to change the phenotype of VSMCs (Fang et al., 2019). Meanwhile, we also demonstrated that miR-21-3p levels were significantly declined in both hypertensive patients and SHR plasma. miR-21-3p upregulation caused a sustained attenuation of hypertension with a significant reduction in the destruction of the target organs, including arterial and kidney fibrosis as well as cardiac hypertrophy and fibrosis in SHRs by suppressing adrenal α2β-adrenergic receptor (ADRA2B) in the arteries (Wang F. et al., 2018).

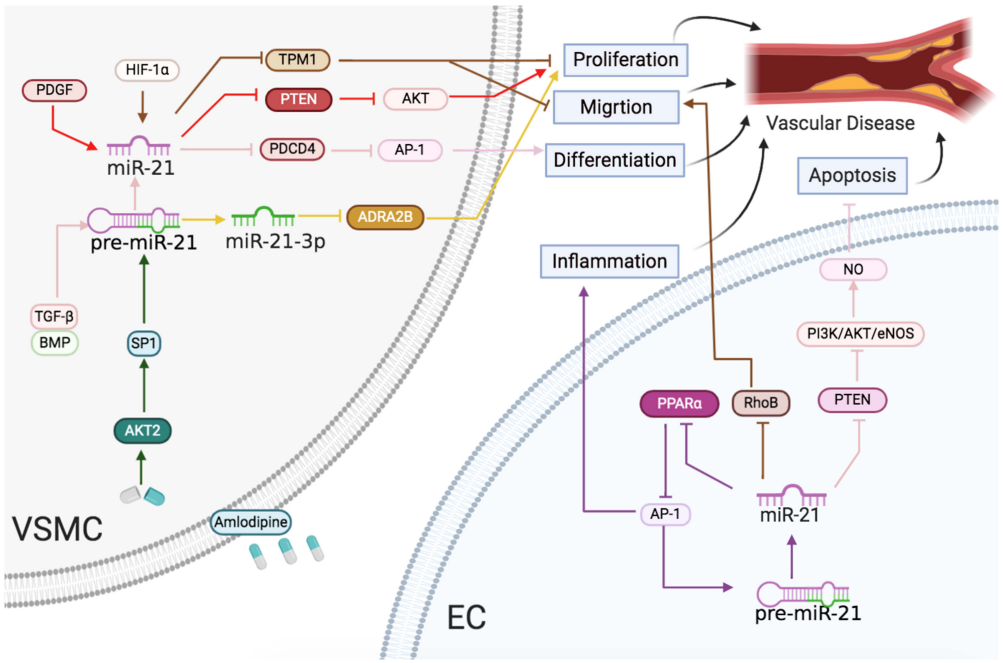

FIGURE 4 | Vascular diseases: the roles of miR-21/miR-21-3p in ECs and VSMCs. Reported major signals in ECs. Brown lines: miR-21 represses RhoB to reduce migration. Purple lines: miR-21 downregulates PPARα and activates AP-1 to accelerate inflammatory response. Pink lines: miR-21 blocks PTEN and activates the PI3K/AKT/eNOS pathway to enhance NO level and decrease apoptosis. Reported major signals in VSMCs. Red lines: in neointima formation, PDGF-induced miR-21 inhibits PTEN to induce proliferation. Brown line: in arteriosclerosis obliterans, HIF-1α-induced miR-21 downregulates TPM1 to induce proliferation and migration. Pink line: TGF-β/BMP-induced miR-21 downregulates the PDCD4/AP1 pathway to promote differentiation. Green line: in hypertension, the amlodipine-activated AKT2/SP1 pathway to induce miR-21 and upregulated miR-21 downregulates the PDCD4/AP1 pathway to promote differentiation. Yellow line: in hypertension, miR-21-3p suppresses ADRA2B to reduce proliferation.

miR-21 targeted RhoB reduced the EC proliferation and migration and decreased the ability to form tubules, thereby leading to negative regulation of angiogenesis (Capogrossi et al., 2011). However, miR-21 promoted endothelial dysfunction and atherosclerotic lesion development under shear stress (SS) (Davies, 2009). Zhou et al. (2011) verified that oscillatory SS-induced miR-21 repressed PPARα, which, in turn, reduced the inhibition of the AP-1 pathway by PPARα. Positive feedback was observed increasing the transcription of miR-21 and inflammation in ECs, suggesting that the inhibition of miR-21 might be a key treatment approach for regulating EC dysfunction (Zhou et al., 2011). In previous studies, miR-21 increased fivefold in human umbilical vein endothelial cells (HUVECs) subjected to unidirectional SS for 24 h and increased miR-21 inhibited PTEN to enhance nitric oxide (NO) production and EC viability (Weber et al., 2010).

In summary, vessel injuries inducing miR-21 expression resulted in VSMC and EC proliferation, migration, and differentiation, leading to atherosclerosis, hypertension, and restenosis (**Figure 4**).

Cardiac Arrhythmias: The Role of miR-21 in CMs and CFs

Cardiac arrhythmias are the abnormalities or perturbations in the normal activation or the rhythms of the heart myocardium. Various factors contribute to cardiac arrhythmias, including ischemia, electrolyte disturbance, scarring, aging, and certain medications (Fu, 2015). These factors result in CM hypertrophy, abnormal electrical activity, and cell death, which might induce fibrosis. Indeed, fibrosis is a decisive factor of myocardial heterogeneity and a major marker for HF, which augmented the trend for reentry arrhythmias and diastolic stiffness (Swynghedauw, 1999). miR-21 is also essential in the regulation of CM and CF phenotype in cardiac arrhythmias.

Upregulated miR-21 in the left atria leads to the downregulation of its downstream molecule SPRY1 in atrial fibrillation (AF) patients. A previous study demonstrated that increased miR-21 expression activated Rac1-GTPase, LOX, and CTGF, which subsequently augmented ECM deposition and activated Drosha and Dicer (Adam et al., 2012). According to Barana et al. (2014), increased miR-21 induced by chronic AF downregulated L-type calcium current flow, which acts as a signal of electrical disorders in detached human atrial CMs. Further, Huang et al. (2016) revealed that heart surgery initiated a neoteric reciprocal flow of STAT3 and miR-21. Inhibition of miR-21 repressed p-STAT3 in CFs, which relieved AF and reduced atrial conduction disorders and predisposition in AF. In contrast, AF patients exhibited decreased miR-21 expression in plasma, and AF status representing the severity of AF (paroxysmal vs. persistent) was proportional to miR-21 expression. Interestingly,

miR-21 expression increased 1 month after AF disappearance (McManus et al., 2015).

Collectively, the aforementioned studies demonstrated that increased miR-21 expression during the cardiac arrhythmias not only induced CM electrical remodeling but also enhanced CF activation, leading to severe fibrosis (**Figure 5**).

Cardiomyopathy: The Role of miR-21 in CMs and CFs

Diabetic cardiomyopathy is characterized by early diastolic dysfunction, final systolic dysfunction, cardiac hypertrophy, and ventricular dilation in the heart and leads to HF (Tabak et al., 2012; Guo and Nair, 2017). High glucose levels and fatty acid utilization disorders lead to apoptosis of CMs (Tziomalos et al., 2010), fibroblast activation, and EC dysfunction (Bauersachs and Thum, 2007; Thum et al., 2007a). In recent studies, miR-21 was demonstrated to act as a potential regulator for diabetic cardiomyopathy. Most studies show a decline in miR-21 levels in the plasma of diabetic patients (Zampetaki et al., 2010; Jansen et al., 2016; Giannella et al., 2017). Moreover, bariatric surgery or exercises might restore the miR-21 level in the circulation of diabetic/prediabetic patients (Villard and Marchand, 2015; Lew et al., 2017). Furthermore, miR-21 was overexpressed in diabetes, diabetes with coronary artery disorders, and diabetes with acute HF, particularly in diabetes with acute HF according to the recent statistics. Measurement of miR-21 levels might be beneficial in predicting the occurrence of acute HF in symptomless diabetics (Al-Hayali et al., 2019).

The miR-21 expression is particularly elevated in high-glucose-cultured CFs, which results in increased collagen cross-link and cardiac fibrosis via Jun amino-terminal kinases/stress-activated protein kinases (JNK/SAPK) and p38 by regulating dual specific phosphatase 8 (DUSP8) (Liu et al., 2014). On the contrary, Zhou et al. (2018) reported that miR-21 was downregulated in palmitate-treated CMs. miR-21 protected CMs from apoptosis through inhibiting p65 and p-p38 expression. Our group also observed downregulated miR-21 in palmitate-treated CMs and db/db mice, which resulted in diabetes-induced diastolic dysfunction by increased gelsolin (GSN) levels and reduced NO production via Akt-eNOS-NO signaling (Dai et al., 2018). The reason for the varied observations might be attributed to the fact that, although high glucose and high fat contribute to the etiology of diabetic cardiomyopathy, different sources have varied effects on miR-21 expression, which could exert diverse functions in multiple cell types. Liu et al. (2014) observed that high glucose increased the levels of miR-21, whereas Dai et al. (2018) and Zhou et al. (2018) observed that miR-21 expression decreased in response to palmitate. In addition, Liu et al. (2014) observed that miR-21 increased collagen cross-link in CFs and cardiac fibrosis, whereas Dai et al. (2018) and Zhou et al. (2018) found that miR-21 exerted a protective effect in CMs.

Chronic inflammation of the heart tissue caused by the viral infection (principally by the coxsackievirus, HIV, and adenovirus hepatitis virus) is termed viral myocarditis (VMC) (Pollack et al., 2015; Fung et al., 2016; Wang Y. et al., 2018). Only 60% of pediatric patients with acute myocarditis survived for 10 years (Towbin et al., 2006), and 9% of patients suffered from dilated cardiomyopathy (DCM), and 12% of young adults died in a short interval following the onset of VMC (Fabre and Sheppard, 2006). DCM characterized by ventricular dilatation and systolic dysfunction would lead to arrhythmia and HF (Maisch et al., 1996; Arthur and Feldman, 2000). Multiple mechanisms have been implicated in the progression from VMC to DCM (Cooper et al., 2010), including direct viral injuries on CMs, cardiac fibrosis, and inflammatory responses (Xu et al., 2014). miR-21 expression increased in the heart of acute VMC patients as well as in that of coxsackie B3 (CVB3)-infected VMC mice (Corsten et al., 2012; Fung et al., 2016). More importantly, upregulated miR-21 promoted cardiac fibrosis during the progression of VCM to DCM by inhibiting SPRY1 and enhancing the MAPK signaling pathway (Xu et al., 2014). *In vivo* silencing of miR-21 in mice with VMC might reduce inflammatory lesions, suppress T helper 17 cell differentiation, and rescue heart function (Liu et al., 2013).

In conclusion, high glucose level and viral injuries induced miR-21-enhanced cardiac fibrosis, whereas reduced miR-21 expression caused by palmitate led to CM apoptosis and cardiac hypertrophy (**Figure 5**).

REGULATION OF miRNA-21 IN CVDs

As highlighted above, miR-21 participates in infarction injuries, cardiac remodeling, atherosclerosis, arrhythmias, and cardiomyopathy, which are caused by infection or metabolic disorders (Thum et al., 2008; Thum, 2012, 2014; van Rooij and Olson, 2012; Fiedler and Thum, 2013; Li et al., 2016; Dai et al., 2018; Zhou et al., 2018). This indicates its potential as a clinical therapeutic target. However, miR-based treatments have challenges associated with it. At present, two major procedures are designed to alter miRNA expression. To decrease the expression of a specific miRNA, specific antisense oligonucleotides (ASOs), small interfering RNA (siRNA), miRNA sponges could be typically utilized. Genetic knockout and synthetic miRNA mimics or pre-miRNA in viral vehicles are used to enhance a specific miRNA level (**Figure 6**).

Antagomirs and LNA anti-miRs are the most prominent examples of ASOs, which perfectly match to the target miRNA in a complementary fashion to block the inhibitory function of miRNAs (Obad et al., 2011). Thum et al. (2008) reported that cholesterol-modified antagomiR-21 protected against cardiac hypertrophy and fibrosis by reacting to TAC-induced pressure overload. However, Patrick et al. showed that LNA-antimiR-21 and miR-21 knockout decreased the levels of miR-21 but did not alter the pathological process associated with pressure overload or other stimulations (Eva Van et al., 2006). Subsequently, Thum et al. (2011) compared the efficiency of the three different anti-miRs and discovered that treatment with two long antagomirs reduced cardiac fibrosis and hypertrophy, whereas tiny LNAs did not exert any beneficial effect. Recently, despite the improvements in anti-miRs [selenomethylene LNAs (Nahar et al., 2016), small RNA zippers (Meng et al., 2017), and peptide nucleic acids (Paulasova and Pellestor, 2004)], no more approaches are published exploring the potential application for CVD treatment.

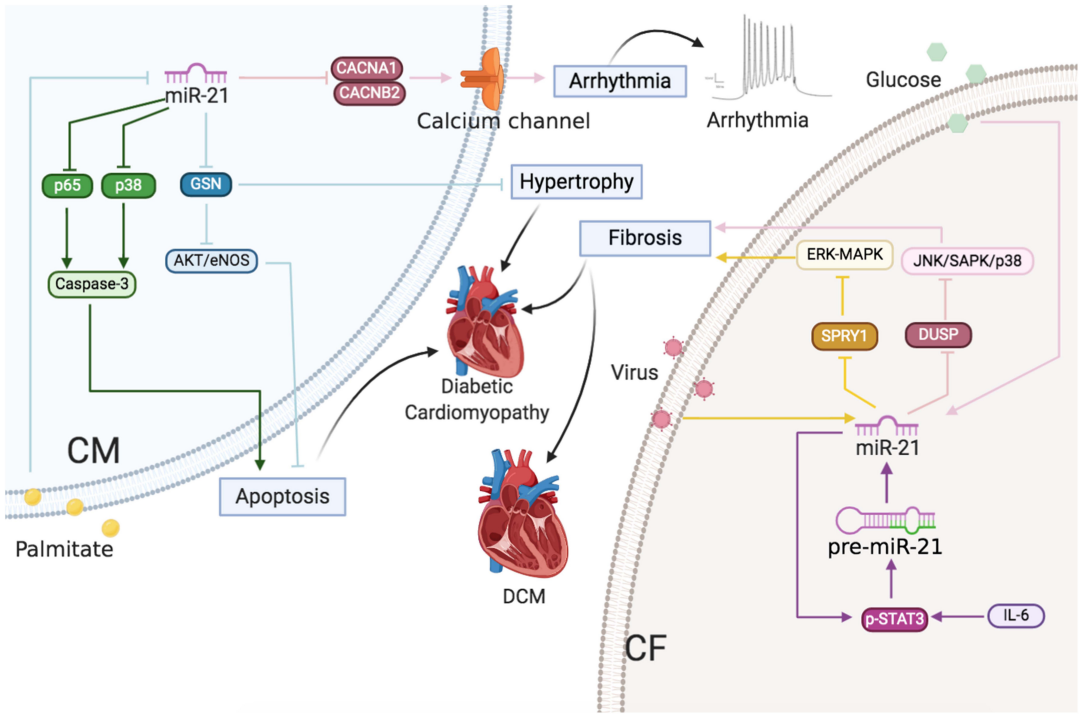

FIGURE 5 | Cardiac arrhythmias and cardiomyopathy: The roles of miR-21 in CMs and CFs. Reported major signals in CMs. Pink line: in cardiac arrhythmias, miR-21 downregulates Ca^{2+} channel subunits CACNA1C and CACNB2 to decrease $I_{Ca,L}$ density. Green line: in diabetic cardiomyopathy, palmitate decreases miR-21 and then miR-21 inhibits p65 and p38 to decrease apoptosis. Blue line: in diabetic cardiomyopathy, palmitate decreases miR-21 and then miR-21 inhibits GSN to decrease hypertrophy and apoptosis. Reported major signals in CFs. Yellow line: in DCM, virus-induced miR-21 inhibits SPRY1 to induce fibrosis. Purple line: in AF, IL-6-enhanced miR-21 via p-STAT3 positive feedback, and miR-21 inhibits SPRY1/ERK/MAPK to induce fibrosis. Pink line: in diabetic cardiomyopathy, glucose-induced miR-21 decreases DUSP8 to increase fibrosis.

SiRNAs, other inhibitors in RNA duplexes, are combined with the loop sequence of the miRNA or a particular sequence of mRNA to protect the respective miRNA targets (Behlke, 2008). The third miRNA inhibitors, known as miRNA sponges, prevented the action of a specific miRNA by sponging to miRNA seed regions or the mRNA sequence of the respective miRNA target (Messina et al., 2016). However, the proper dosage of siRNA and miRNA sponges to counter certain endogenous miRNA concentrations is difficult to define. Furthermore, Cheng et al. (2009b) have designed and added tough decoys (TuD)-miR-21 into lentiviral vehicles for long-term suppression of miR-21.

The induction of miRNA levels is even more ambitious compared to miRNA inhibition. Double-stranded RNA fragments acting as miRNA mimicked the endogenous miRNAs and replaced or enhanced miRNA density in tissues (Olson, 2014). Mature miR-21 mimics are widely used to overexpress miR-21 *in vitro* and can be easily purchased from the industry (Liang et al., 2012; Bang et al., 2014). Although high concentrations of miRNA mimics could achieve a significant increase in target miRNAs (Bader et al., 2010), miRNA mimics might also result in unanticipated side effects. Pre-miR-21 in lentivirus vector was proven to efficiently overexpress miR-21 (Wang J. et al., 2018), and could specifically be overexpressed in CM by combining with the mouse myosin-6 promoter (Thum et al., 2008). However, these viral vectors overexpressing the precursor sequence might finally result in a complex effect by synthesizing both miR-21 and miR-21-3p (Schober et al., 2014).

In principle, there are two prime goals, namely reduction of the dose and the risk of toxicity and sequence-specific side effects. To improve cellular absorption and decrease dose-dependent toxicities, delivery strategies consisting of altering the structure to obtain resistance against RNases, adding cholesterol, and decreasing the immunoreaction and off-target effects should be concerned (Lucas et al., 2018). Obtaining tissue or cell-type specificity is another obstacle to overcome. Compared with miR-21 inhibition, anti-21-coated stents confined miR-21 in the local circulatory system and exerted fewer side effects according to Wang et al. (2015). AAV serotype 9 viruses demonstrated a preference for the heart with a specific dose, which could be applied to delivery miRNAs, shRNAs, and mRNAs in animals (Care et al., 2007; Li et al., 2009; Eulalio et al., 2012; Karakikes et al., 2013). Ramanujam et al. (2016) generated AAV9 and Moloney murine leukemia virus vectors for the deletion of miR-21 in the CM- and non-myocyte-specific manner in chronic left ventricular pressure overload models. Our group tried AAV9 and CM-specific promoter troponin T to specifically express miR-21 in CMs and proved that miR-21 demonstrated a protective role in CMs during diabetes (Dai et al., 2018). Furthermore, adding specific promoters, such as transcription factor 21 and fibroblast specific protein 1 (FSP1), to ECs and CFs, respectively, would

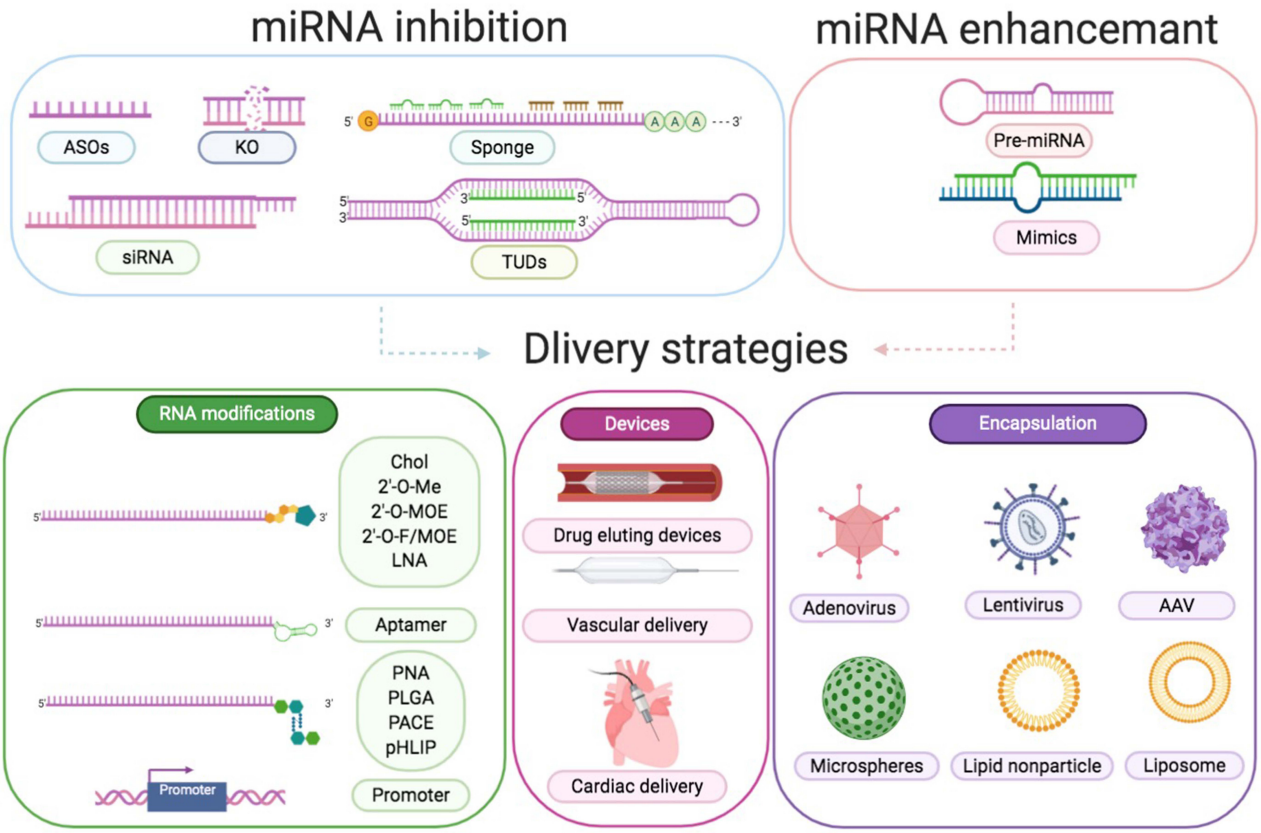

FIGURE 6 | Approaches to modulate miRNA function. Downregulation of a certain miRNA preferentially could be achieved by utilization of specific ASOs, siRNA, miRNA sponges, TUDs, or by genetic knockout. Overexpression of a specific miRNA could be achieved by using miRNA mimics or pre-miRNA expression systems. Delivery strategies are RNA modifications, devices, and encapsulation. RNA modifications include conjugation to cholesterol (Chol), 2′-*O*-methyl (MOE), LNA, aptamer, peptide nucleic acid (PNA), poly lactic-co-glycolic acid (PLGA), poly amine-co-ester terpolymer (PACE), pH low insertion peptide (pHLIP), and cell-specific promoter. Devices include drug eluting devices, vascular delivery, and cardiac delivery. Encapsulation includes adenovirus, lentivirus, AAV, microspheres, lipid nanoparticles, and liposomes.

allow specific effector molecules to be expressed (Song et al., 2012). Only miR-21 knockout mice have been preferred (Patrick et al., 2010; Lu et al., 2011) owing to the limitations of promoter strength (Lindsley et al., 2007; Qian et al., 2012) and affinity with cells and organs (Acharya et al., 2011; Kong et al., 2013). For example, β-galactosidase activity under the control of FSP1 was observed in many but not all CFs and certain endocardial and ECs as previously reported. Several novel approaches demonstrating miRNA-specific delivery have been published so far. In addition to assembled AAV particles with large polypeptides coupled to the surface and aptamer-linked miRNAs (Muik et al., 2017), new technologies, including nanoparticles and advanced biomedical materials, have been developed for the transport of particular molecules to the desired location. miR-21 mimics could increase perfusion and cell viability while reducing cardiac remodeling post-MI in the border area by delivering nanoparticles to macrophages, demonstrating a new treatment strategy (Bejerano et al., 2018). Furthermore, Alexy et al. (2014) reported that miR-21 was reduced in microparticles (MPs) released by ECs in response to tumor necrosis factor α (TNF-α). They also observed that Rho-associated coiled-coils containing protein kinase-dependent, miRNA-rich MPs could transfer their contents effectively and were antiapoptotic, whereas caspase-dependent, miRNA-poor MPs were proapoptotic. The aforementioned results demonstrated an underlying application of cell-to-cell interaction by packaging miRNAs in MPs (Alexy et al., 2014). Although the novel therapeutics described above have not been applied for clinical use, they demonstrate a great promise.

DISCUSSION

The influence of miR-21 on the cardiovascular system is not negligible. However, several controversies have been associated with it. Circulating miR-21 are potential biomarkers for CVDs, but their levels have been inconsistent in the published reports. First, the blood sample used for detection might not be subjected to standardized preparation methods, and the plasma sample might differ from the serum sample. The miR-21 levels are not constant during the course of a given disease. For example, miR-21 levels in the acute period of AMI (upregulated) would be different from those obtained after successful PCI therapy

(downregulated). Second, the number of participants as well as their past medical history, age, gender, and race may also result in differential expression levels of circulating miR-21. A multicenter independent study with a large cohort will contribute to the stability and reliability of the discovery data (Small and Olson, 2011). In addition, the source cell type of miR-21 in the circulating system is unknown, and miR-21 might be secreted from diverse cell types under different stimuli. miR-21 expression in the tissues involves the same contentions. For example, the expression of miR-21 varies with the stages of AMI (Gu et al., 2015) and localization of the cell type within the heart (Roy et al., 2009). Therefore, the expression pattern of miR-21 in the heart may change. Recently, many researchers have concentrated on the cellular-specific expression of miR-21.

Moreover, studies have observed that miR-21 expression is important in other diseases, such as cancer (Bonci, 2010), kidney fibrosis (Chau et al., 2012), metabolic syndrome (Calo et al., 2016), and asthma (Kim et al., 2017). The targets of miR-21 in CVDs might also participate in the etiology of such diseases. For example, PTEN, which is considered as a cancer suppressor, also plays a role in CMs, CFs, and ECs by influencing cell apoptosis and proliferation. miR-21-dependent PPARα downregulation could affect fatty acid oxidation and trigger steatosis in hepatocytes (Loyer et al., 2016) as well as mediate kidney injury and fibrosis in epithelial cells (Chau et al., 2012). Meanwhile, miR-21 targeted PPARα resulted in increased inflammation owing to the ECs in the heart. Thus, when we discuss the function of miR-21, the cell types and specific pathological status must be carefully considered. Current research has also demonstrated that miR-21 might serve as a potential biomarker for the diagnosis, prognosis, and prediction of multiple diseases (Ramachandran et al., 2013; Bautista-Sanchez et al., 2020). According to Qu et al. (2017), serum miR-21 was especially upregulated in patients with pancreatic cancer with a sensitivity of 0.77 and specificity of 0.8104 in 56 pancreatic cancer patients from six medical centers in China. Ramachandran et al. (2013) have indicated that miR-21 might be a sensitive (and non-invasive) indicator of kidney damage in pooled urine samples from 6 patients with acute kidney injury (AKI) and 6 healthy controls as demonstrated by miRNA PCR array (miRBase version 18, containing 1,809 miRNAs; Qiagen). This increases the complexity in the assessment of the roles of miR-21 in CVDs. We should carefully include proper negative controls while investigating the role of miR-21 in CVD patients. Further, miR-21 expression should be combined with the results of the evaluation of clinical symptoms and lab examination of the patients for a comprehensive diagnosis of the disease.

Because the function of miR-21 is mediated by its target genes, it is important to identify its direct targets. Studies have reported various targets of miR-21 not only in different cell types but also in the same cells. We speculated that one of the reasons might be the fact that various bioinformatic tools using different algorithms were employed in different studies to predict the targets of hsa-miR-21 (**Table 1**). For example, we searched the updated miRTarBase and observed that both miR-21-5p and miR-21-3p exhibited binding sites in the 3′ UTR region of PTEN. Meanwhile, PTEN was predicted as the target of miR-21-5p by using a modified bioinformatics approach proposed by Doench and Sharp (2004), Kiriakidou (2004), Meng et al. (2006). However, PTEN was not predicted as the biological target of hsa-miR-21 or miR-21-3p by the latest Targetscan (Ver 7.2) approach. The algorithm of a certain bioinformatics website might be constantly updated (Costa et al., 2020). For example, PTEN was not predicted as the target of miR-21-3p by the latest Targetscan (Ver 7.2), but it was also predicted as the target of hsa-miR-21-3p by using TargetScan (Ver 3.1), miRWalk and miRbase (Zhu et al., 2019). Furthermore, conventionally, miRNAs always bind to the 3′ UTR of their target mRNAs. However, in recent years, it has been reported that miRNAs could also bind to the CDS, promoter, and 5′ UTR regions of the target genes (Iwakawa and Tomari, 2013; Li et al., 2019). Several binding sites of hsa-miR-21 in the CDS, promoter, and 5′ UTR regions of PTEN were also predicted by the BiBiServ using an RNA–RNA hybrid. Most algorithms use seed regions for Watson–Crick matching of the target, but the secondary structure of the mRNA may also contribute to the binding (Didiano and Hobert, 2006). Therefore, considering the complexity in the prediction of direct targets of miRNAs, biological experiments are essential to validate the bioinformatic predictions. Studies have verified PTEN as the direct target of hsa-miR-21-5p and hsa-miR-21-3p using biological experimental methods, such as luciferase reporter assay, western blot, and qRT-PCR (Sayed et al., 2010; Qiao et al., 2019; Zhu et al., 2019).

TABLE 1 | Useful web links for miRNA target prediction.

miRNA targets within 3′ UTR	
PicTar	https://pictar.mdc-berlin.de
TargetScan	http://www.targetscan.org/vert_72
miRNA targets within ORFs	
TargetScanS	https://tools4mirs.org/software/mirna_databases/targetscans
miRNAs targets for genomic	
miRanda	http://cbio.mskcc.org/microrna_data/miRanda-aug2010.tar.gz
miSTAR	http://www.mi-star.org
RNA22	https://cm.jefferson.edu/data-t
miRcode	http://www.mircode.org/index.php
miRDB	http://www.mirdb.org
Bibiserv	https://bibiserv.cebitec.uni-bielefeld.de
miRNA targets with the secondary structure of the target mRNA	
PITA	https://genie.weizmann.ac.il/pubs/mir07/mir07_data.html
miRNA targets based on experimental data	
starBase	http://starbase.sysu.edu.cn
miRTarBase	http://mirtarbase.mbc.nctu.edu.tw/php/index.php
TarBase	https://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex
miRanda	http://cbio.mskcc.org/microrna_data/miRanda-aug2010.tar.gz
Integrated miRNA predicition of mRNA targets	
Diana	http://diana.imis.athena-innovation.gr/DianaTools/index.php
miRecords	https://tools4mirs.org/software/mirna_databases/mirecords
miRWalk2	http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/
miRSystem	https://tools4mirs.org/software/target_functional_analysis/mirsystem
miRdip	http://ophid.utoronto.ca/mirDIP

Considering the comprehensive signaling network, it is possible that PTEN is not only a direct target of miR-21, but is also regulated at a "second" level by a product of a target mRNA, which makes the identification process more complex.

Several limitations need to be eliminated before the clinical application of miRNA. The phenotypes and molecule responses in mice administered with antagomirs or miRNA mimics were different from or even contrary to those detected from the knockout mice according to the aforementioned description (Lucas et al., 2018). Several possibilities might explain these findings. First, general antagomirs and miRNA mimics in all tissues may not only influence the target of interest in the given cell type but also other potential targets in different cells and tissues. For example, PTEN regulated PI3Ks and AKT to function as an antiapoptotic regulator in diverse cellular types (Oudit, 2004). The downregulation of PTEN mediated by miR-21 in the heart inhibited CM and EC apoptosis, which played a protective role in heart function while promoting VSMC and CF proliferation, resulting in the deterioration of cardiac function. Furthermore, we should also consider the fact that the target of interest may also be regulated by multiple miRNAs before the systemic intervention. PDCD4, one of the targets of miR-21, was demonstrated to be a tumor-suppressor gene and the target of procancer miRNAs (Lankat-Buttgereit and Göke, 2009). Second, anti-miRs may exert non-negligible off-target effects. The enhancement of miRNA levels might affect irrelevant targets. Finally, a short period and partial suppression of miRNA is certainly different from miRNA gene knockout. Gene knockout would cause embryonic phenotypes, which are unlike adult disease etiologies. Moreover, complete gene knockout would cause the organism to initiate a compensation response. To verify the specific cellular effect of miR-21 on cardiac function in CVDs, we must overcome the barriers to effectively and accurately express molecules in a given cell in the cardiovascular system.

AUTHOR CONTRIBUTIONS

BD and FW conceived of and designed the review and contributed to the writing of the manuscript. XN, HD, YZ, ZY, HL, JF, and ZW helped with designing the review. DW and CC conceived of and designed the experiments, supervised and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.563166/full#supplementary-material

Supplementary Table 1 | The reported expression patterns and functions of miR-21 in CVDs.

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