MicroRNAs in the development and pathobiology of uterine leiomyomata: does evidence support future strategies for clinical intervention?

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BACKGROUND: Human leiomyomata (fibroids) are benign tumors of the uterus, represent the most common neoplasms of reproductive-aged women and have a prevalence of ~70% in the general population. This disorder conveys a significant degree of morbidity and remains the leading indication for hysterectomy in the USA. Prior investigations of aberrant microRNA (miRNA) expression in various malignancies have provided invaluable insight into the role of this class of small non-coding RNAs in tumor growth. Evidence of irregular miRNA expression in uterine...
fibroids has garnered recent interest for diagnostic and therapeutic applications. Since miRNA gene targets modulate several processes implicated in the genesis of uterine fibroids, more focused investigation has the potential to elucidate the functional significance of miRNA in the genesis and pathology of the disease.

**METHODS:** Comprehensive electronic searches of peer reviewed published literature in PubMed (US National Library of Medicine, National Institute of Health; http://www.ncbi.nlm.nih.gov/pubmed/) were performed for content related to the biologic functions of miRNA, the roles of miRNA in human disease and studies investigating miRNA in the context of uterine leiomyomata. Herein, this article will review the current evidence supporting the use of miRNA expression profiling as an investigative tool to assess the pathobiology of uterine fibroids and will discuss potential future applications of miRNAs as biomarkers and therapeutic targets.

**RESULTS:** Mounting evidence supports a functional role for miRNA as either indirect or direct regulators of gene expression which impacts the pathobiology of uterine fibroids. Specifically, miRNAs let-7, 200a, 200c, 93, 106b and 21 have been implicated in cellular proliferation, apoptosis, extracellular matrix turnover, angiogenesis and inflammation. Preliminary data provide evidence to suggest that respective in vitro miRNA expression in leiomyomata and myometrium is regulated by sex steroids.

**CONCLUSIONS:** Collectively, the identification of aberrantly expressed miRNAs in uterine leiomyomata and accumulating data derived from mining of gene target prediction models and recent functional studies support the concept that miRNAs might impact the genesis and progression of disease. However, the specific biologic functions of differential miRNA expression have yet to be confirmed in vivo. Further functional studies and developing miRNA technology may provide the basis for future applications of miRNAs in clinical medicine as biomarkers and therapeutic targets.

**Key words:** leiomyoma / microRNA / fibroid / biomarker / myometrium

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**Introduction**

Uterine leiomyomata (fibroids), the most common neoplasm in reproductive-aged women, has finally been recognized as a major public health issue over the past decade. By the sixth decade of life, the cumulative incidence of fibroids is nearly 70% in Caucasian (CC) women and over 80% in African-American (AA) women (Baird et al., 2003). In the USA, these sex steroid hormone responsive tumors remain the leading indication for hysterectomy (Wright et al., 2013), and result in an estimated total annual societal cost of approximately $34.4 billion (Cardozo et al., 2012). Uterine fibroids convey significant morbidity and cause a diversity of symptoms including heavy menstrual bleeding, anemia (from excessive menstrual bleeding), pelvic and abdominal pain, urinary incontinence, constipation, infertility, and recurrent pregnancy loss. As a result of the limitations of current treatment options, there is a need for a better understanding of the pathobiology of disease to develop novel approaches for the prevention and treatment of this disorder.

With the recent focus on epigenetic causes of human disease, microRNAs (miRNAs) have garnered interest for development as diagnostic and therapeutic targets. miRNAs are non-coding, stable, single-stranded RNAs ~22 base pairs long (Bartel, 2004). miRNAs regulate gene expression through gene silencing (Bartel, 2004). This post-transcriptional regulation appears to play diverse and significant roles in multiple tissues of the female reproductive system (Carletti and Christenson, 2009). In contrast to other disciplines such as cancer research, investigation of the functional role of aberrant miRNA expression in the field of fibroid biology is in its infancy. Differential miRNA expression and miRNA dysregulation in leiomyomata has been reported and implicated in the pathogenesis of this disorder by several authors (Wang et al., 2007; Luo and Chegini, 2008; Marsh et al., 2008). These findings serve as the foundation for more recent investigations of the influence of miRNAs on fibroid development, growth and recurrence and of future diagnostic and therapeutic clinical applications of miRNAs in this disorder.

This review presents current evidence supporting the concept that alterations in miRNAs can contribute directly and/or indirectly to the development and/or progression of uterine leiomyomata. This article will also discuss the potential for future clinical applications of miRNA technology.

**Methods**

A comprehensive online search of peer reviewed published literature in Entrez Pubmed (US National Library of Medicine, National Institute of Health; http://www.ncbi.nlm.nih.gov/pubmed/) was performed for content related to the biologic function of miRNA, the role of miRNA in human disease and studies investigating miRNA in the context of uterine leiomyomata. References for this review were identified with the use of the following terms: microRNA, uterus, fibroid, fibroids, leiomyoma, leiomyomata, myometrium, genetic, epigenetic, leiomyosarcoma, estradiol, progesterone, sex steroid, biomarker, therapeutic. Gene targets of miRNAs associated with uterine leiomyomata were identified with the same search methodology with Entrez Pubmed. Additional references were also identified in the bibliographies of articles identified in the primary query. The original manuscripts of all cited articles were reviewed in their entirety.

**Genetic regulation of uterine leiomyomata**

The formation and growth of these tumors is thought to be, in part, the result of several genetic and epigenetic mechanisms and the regulation by sex steroids, growth factors, cytokines, chemokines and extracellular matrix mediators (Ciarmela et al., 2011). Approximately 50% of fibroids demonstrate karyotypic or cytogenetic abnormalities (Rein et al., 1991; Meloni et al., 1992; Islam et al., 2013b). A wide array of chromosomal abnormalities (including chromosomes 2, 3, 6, 7, 8, 10, 11, 12, 13, 14, 22) and gene mutations (including MED12, HMGAI, FH, BHD, TSC2, PCOLCE, ORC3L and LHFP3) have been implicated in the early...
development of uterine fibroids (Islam et al., 2013a). Some of the more frequently reported genetic alterations of uterine fibroids include deletions of chromosome 7 and chromosome 13 and translocations of chromosomes 12 and 14 (Kiechle-Schwarz et al., 1991; Reif et al., 1991; Meloni et al., 1992; Islam et al., 2013b). A limited number of germline mutations have been associated with familial uterine fibroid syndromes (Hodge and Morton, 2007). Recently, single genetic alterations resulting in a complex array of multiple chromosomal breaks and random assembly have been proposed as the inciting event during the de novo formation of a fibroid tumor from the myometrium (Bulun, 2013; Mehine et al., 2013). However, it is still unclear if the underlying genetic mechanism(s) involved in uterine fibroid development are heritable, acquired, or a complex array of congenital germline and acquired somatic cell mutations.

Specific genes consistently implicated in the genesis of uterine fibroids include Mediator Complex Subunit 12 (MED12) and High-Mobility-Group AT-Hook 2 (HMGA2) (Bulun, 2013; Islam et al., 2013b). MED12 is one of the most common genes mutated in fibroids. MED12 is a part of the mediator complex that interacts with RNA polymerase II to regulate gene transcription (Taatjes, 2010). MED12 mutations have been found at a frequency of ∼70% in leiomyomata of Finnish women (Makinen et al., 2011b). Similar results were reported in a population of South African women (Makinen et al., 2011a). In an analysis of 1862 tumors of different varieties, investigators surmised that specific MED12 exon 2 mutations were consistently associated with leiomyomata, may play a role in their genesis, and serve as a target for the development of future therapeutic agents (Je et al., 2012).

Interestingly, inactivation of MED12 results in up-regulation of transforming growth factor β (TGF-β) (Guo and Wang, 2012; Bulun, 2013). TGF-β, a growth factor with many biologic functions including connective tissue formation, has been shown to have 3-fold greater expression in fibroids compared with matched myometrium (Leppert et al., 2006). The increased TGF-β expression in leiomyoma cells likely has an effect on the genes encoding proteins directly or indirectly involved in collagen formation and therefore plays a role in the excessive and dysregulated extracellular matrix formation (ECM), characteristic of fibroids (Leppert et al., 2006). Notably, in vitro immortalized fibroid and myometrial cells treated with TGF-β result in increased mRNA and protein production of collagen I A1, fibronectin I and connective tissue growth factor in both cell types, with expression increased in treated myometrial cells nearly to the levels found in leiomyoma cells (Joseph et al., 2010).

Another proposed genetic modulator of uterine fibroids, HMGA2 gene mutation, involves a (12;14) chromosomal rearrangement, and has been observed in ∼20% of clonally abnormal fibroids (Meloni et al., 1992; Peng et al., 2008). HMGA2 is a regulator of cell proliferation, differentiation and apoptosis and is thought to have some oncogenic properties (Peng et al., 2008). Similar to MED12, HMGA2 is one of the most dysregulated genes in fibroids, and is expressed commonly in embryonic tissue and rarely in adult tissues (Ligon and Morton, 2001). The t(12;14) translocation is associated with larger sized fibroids (Hennig et al., 1999) and repression of senescence in fibroid cells in vitro (Markowski et al., 2011). Notably, the let-7 miRNA family has been shown to regulate HMGA2 expression in prior studies (Peng et al., 2008). This will be discussed in more detail later in this review.

As evidenced by the highlighted studies, the genetic modulation of uterine fibroids involves the complex interface of aberrant genetic regulators and signaling pathways. As a result, our understanding of how genetic modifiers impact disease severity has not been completely elucidated. In light of the progress made in oncology with respect to delineating the impact of miRNA in tumorigenesis, it is anticipated that similar advances will improve our understanding of the pathobiology of uterine fibroids.

### Biogenesis of miRNA

The discovery of miRNAs (Lee et al., 1993; Wightman et al., 1993) ushered in a new paradigm for the understanding of gene expression and post-transcriptional regulation. miRNAs are members of a larger group of small RNA species which function through RNA interference (RNAi) (Erson-Bensan, 2014). miRNA-mediated gene expression regulation works predominately via inhibition of translation or degradation of target messenger RNA (mRNA). Studies have also suggested that specific miRNAs may activate target genes by binding directly to their promoter regions (Vasudevan et al., 2007; Place et al., 2008). Whether miRNAs inhibit or degrade their target miRNAs depends upon the degree of mismatch between the miRNA 3′ end seed sequence and the mRNA 3′ untranslated related (UTR) region it binds to (Meltzer, 2005). A high degree of complementarity favors degradation. Given this mechanism, a single miRNA species can inhibit the translation of multiple genes (Bartel, 2009; Ullah et al., 2013).

As shown in Fig. 1, miRNA biogenesis begins in the nucleus where it is transcribed into primary miRNA (pri-miRNA) by RNA polymerase II. The pri-miRNA is cleaved by a complex containing an RNase III enzyme (Drosha) and its cofactor DiGeorge Syndrome Critical Region 8 (DGCR8) into precursor miRNA (pre-miRNA). Exportin 5 transports the pre-miRNA into the cytoplasm where it is processed into a duplex by the RNase III enzyme Dicer in complex with TAR RNA binding protein (TRBP). The duplex is separated and the mature miRNA is loaded into the Argonaute protein complex called the RNA-induced silencing complex (RISC). RISC is the miRNA effector causing inhibition of mRNA translation via miRNA/mRNA sequence complementarity (Krol et al., 2010; Gurtan and Sharp, 2013; Erson-Bensan, 2014). Translation inhibition occurs either by translational repression or miRNA degradation. The activity of each miRNA on the RISC complex cannot be taken into account without considering the potentially shared biological effects of ‘clustered’ miRNA. Multiple miRNAs are often encoded in a single primary transcript. These clustered miRNA may not be related in structure but will often be related in function (Townley-Tilson et al., 2010; Gurtan and Sharp, 2013).

### Proposed miRNA function in human disease

Emerging evidence of aberrant miRNA expression has shed light on their function in normal biologic processes, and has provided invaluable insight into their potential functional significance in a wide array of common human disease states such as malignancy, cardiac disease, diabetes and insulin resistance (Zhu et al., 2011; Thum and Mayr, 2012; Wu et al., 2013). Select miRNA can influence genes whose protein products are part of key signaling pathways known to be involved with human diseases, such as the AKT and Notch signaling pathways (Xu and Mo, 2012; Galoian et al., 2013). The link between malignancy and miRNA has
been particularly compelling, as many studies have examined the function of miRNA in cancer pathogenesis, diagnosis, prognosis and treatment. The relationship between cancer and miRNA was first noted in chronic lymphocytic leukemia (CLL) patients, where miR 15 and miR 16 were found to be down-regulated or deleted compared with their respective normal controls (Calin et al., 2002). Since then, many miRNAs have been classified as oncogenic or tumor suppressive depending on how they affect cancer cell proliferation. Importantly, the same miRNA species can be classified as either oncogenic or tumor suppressive in different tissue types (Li and Yang, 2013).

In tandem with evolving investigations of the role of miRNA in the development and progression of disease, delineation of the impact of aberrant miRNA expression patterns on disease phenotype is also of clinical significance. Studies investigating various cancer types have reported the diagnostic value of altered miRNA expression profiles, or ‘signatures,’ associated with specific malignancies compared with controls (Danielson et al., 2010; Zadran et al., 2013). Other studies have examined miRNA expression and its association with disease prognosis (Santarpia et al., 2013; Xia et al., 2013; Ma et al., 2014). Another clinically relevant discovery, the confirmation of circulating miRNA in human serum, was first reported in 2008. Among patients with diffuse large B-cell lymphoma, changes in serum miRNA levels were associated with the presence of disease and were prognostic of treatment outcomes (Lawrie et al., 2008).

**Figure 1** MiRNA biogenesis. The miRNA gene is transcribed by RNA polymerase II into pri-miRNA. Pri-miRNA is cleaved by Drosha/DGCR8 into pre-miRNA and subsequently transported into the cytoplasm by Exportin 5. Pre-miRNA is processed by Dicer into a miRNA:miRNA duplex. The miRNA strands are then separated and the mature miRNA is loaded into the RISC protein complex. RISC is the effector complex which facilitates translational inhibition of target mRNA through miRNA:mRNA sequence complementarity. miRNA, microRNA; pri-miRNA, primary miRNA; pre-miRNA, precursor miRNA; DGCR8, cofactor DiGeorge Syndrome Critical Region 8; TRBP, TAR RNA binding protein; RISC, RNA-induced silencing complex.

**Differential miRNA expression in uterine leiomyomata**

**Implications of differential miRNA expression in the genesis of leiomyomata**

Several groups have utilized a variety of molecular methods to demonstrate differential expression of a significant number of genes in uterine fibroids compared with native uterine myometrium (Quade et al., 2004; Leppert et al., 2006; Hodge et al., 2012). Although novel and
informative when first introduced, the functional significance of differential miRNA expression in normal and disease states is not always as obvious as might be anticipated. The identification of differentially expressed miRNA species in fibroids, compared with the normal myometrium, provides an opportunity to more clearly define how they may be contributing to the genesis of this disease.

The field of reproductive biology has adopted its approach for inferring the biological function of miRNAs in human disease from prior work in cancer biology. The sentinel study by Lu et al. demonstrated the down-regulation of several miRNAs in a cohort of tumors compared with normal tissue. Notably, they were able to classify poorly differentiated cancers with a unique miRNA expression profiling, whereas the use of mRNA expression was inaccurate and less useful for this purpose (Lu et al., 2005). In initial steps to gain insight into the possible role of miRNA in fibroid pathobiology, Wang et al. (2007) and Marsh et al. (2008) were the some of the first investigators to confirm a differential miRNA expression pattern in uterine fibroids compared with matched myometrium.

Wang et al. performed microarray-based global miRNA analyses of patient-matched myometrium and fibroids from 41 subjects (Wang et al., 2007). Compared with matched myometrium, 45 miRNA species (24 up-regulated, 21 down-regulated) were differentially expressed. The most dysregulated miRNA species included let-7 family, miR 21, miR 23b, miR 29b and miR 197. Notably, strong associations of specific miRNA signatures were demonstrated with ethnicity and intraoperative fibroid size. Specifically, a hierarchical cluster subanalysis showed that fibroids of African-American (AA) women demonstrated a distinct miRNA expression profile compared with matched samples of Caucasian (CC) women. Furthermore, five members of the let-7 family were up-regulated in fibroids ≤3 cm compared with larger fibroids. Utilizing computer database predicted targets, the authors implied specific gene regulation by an exclusive miRNA species in fibroids. Namely, the protein product of the HMGA2 gene, a predicted target of the let-7 miRNA family, was studied in the context of fibroid size (Hennig et al., 1997; Klotzbucher et al., 1999; John et al., 2004). In larger fibroids (≥10 cm), overexpression of HMGA2 protein and lower expression of let-7 miRNA was observed compared with fibroids ≤3 cm. In primary cell cultures derived from fibroids fibroids ≥10 cm and with relative HMGA2 overexpression (compared with fibroids ≤3 cm), introduction of let 7c resulted in the reduction of HMGA2 protein expression (Wang et al., 2007).

Marsh et al. investigated respective miRNA expression patterns in premenopausal women with symptomatic uterine fibroids undergoing hysterectomy (Marsh et al., 2008). In this study, 46 miRNA species were differentially expressed in fibroids compared with myometrium. Interestingly, many of the differentially expressed miRNA species were previously implicated in other disease states with similar dependence upon either aberrant proliferation and/or regulation via sex steroid ligand and/or their receptor pathways. Notably, this group identified up-regulation of miR 225b and 34 [thyroid papillary cancer (Pallante et al., 2006)], and miR21 [glioblastoma (Chan et al., 2005; Iorio et al., 2005)], and down-regulation of miR 139 [pancreatic adenocarcinoma, (Lee et al., 2006)]. Due to the well-known regulation of uterine function by sex steroids, one of the more intriguing findings of the study was the differential expression of several sex steroid receptor associated miRNA species such as miR 21, miR 34a, miR 125b and miR 150 (Mattie et al., 2006).

In light of a reduction of HMGA2 protein expression in the setting of let 7 overexpression, and the correlation of miRNA signatures to clinical features (fibroid size and ethnicity), Wang and colleagues surmised that these genetic modifiers play a role in tumorigenesis. Although these authors implicated a potential regulatory role of the let-7 miRNA family on HMGA2 expression, it is difficult to assume that in vivo let-7 regulation of HMGA2 has a fibroid-specific functional role or is the only factor contributing to tumor genesis and/or growth. Although the specific biologic functions of the described differential miRNA species were not comprehensively investigated, the studies of Wang et al. and Marsh et al. were pivotal as the first confirmations of differential miRNA expression in fibroids and provided the necessary direction for subsequent investigations of the biologic significance of miRNA.

Putative biologic impact of differential leiomyoma miRNA expression

An initial approach in characterizing the putative function of various miRNA species in the development of fibroids was the utilization of predictive algorithm data sets (http://microrna.sanger.ac.uk/; http://www.target scan.org/; http://pictar.bio.nyu.edu/; http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk). Based upon the findings of three antecedent studies (Wang et al., 2007; Marsh et al., 2008; Pan et al., 2008) as the source of reported miRNA species in fibroids, investigators presented a data set based analysis of miRNA and their predicted gene targets (products expected to play a role in the genesis of fibroids) (Luo and Chegini, 2008). The authors relayed that the cumulative results of these three studies demonstrated a select number of miRNAs (27 miRNAs commonly expressed in at least two studies) which are irregularly expressed in leiomyomata compared with native myometrium (Luo and Chegini, 2008, Fig. 2). The putative functional implications of the majority of genes targeted by these miRNAs were associated with cell cycle regulation and apoptosis [i.e. tumor suppressors and anti-apoptotic mediators (let-7 family, miR17-92 cluster, miR 372-373, miR15-16, miR 20, miR 21, miR 26a), differentiation and hypertrophy (let 7 family, miR18b, miR 21), inflammation (miR 125, miR 155) and tissue remodeling/ECM turnover (miR 21, miR 192, miR 206, miR1, miR 133a) (Ding et al., 2004; Levens et al., 2005; Luo et al., 2006, 2007; Anderson et al., 2006; Diederichs and Haber, 2006; Meng et al., 2006; Volinia et al., 2006; Calin et al., 2007; Cho, 2007; Kato et al., 2007; McCarthy and Esser, 2007; Pan et al., 2007; Si et al., 2007; Sylvester et al., 2007; Tatsuguchi et al., 2007; Visone et al., 2007; Wang et al., 2007; Wong and Tellam, 2008)].

Although the use of predicted gene targets is a reasonable initial approach to investigating the in vivo role of miRNA in fibroids, several limitations must be realized. A single miRNA species may have several cooperating pathways, and have variable biologic functions in different tissue or cellular systems (Iorio and Croce, 2012). Moreover, it cannot be assumed that the role of a specific miRNA is similar in the same gene target in different biologic systems. This is especially true when interpreting the findings of studies which use predicted miRNA targets that have not been confirmed in the tissue/organ of interest. For example, many of the predicted targets in Luo and Chegini et al. were based upon findings of studies investigating proliferation in malignancy. At this stage, it is unclear if similar mechanisms of unrestricted growth during tumorigenesis in miRNA-linked cancer.
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models apply to benign uterine leiomyomata. However, miRNA regulation of cell proliferation, apoptosis, inflammation, angiogenesis, tissue turnover and tumor suppressor/oncogene modification, which are thought to be crucial processes in the pathobiology of human malignancy, are also thought to play significant roles in the development of uterine fibroids (Luo and Chegini, 2008). Given the incipient stage of miRNA research in fibroids, cancer miRNA models currently provide the best available guidance to investigate the biologic impact of miRNA in leiomyomata.

Impact of sex steroid hormones on miRNA expression

In light of long-standing data describing the unique endocrine, autocrine and paracrine roles of estradiol and progesterone and their respective receptors in uterine function, several investigators have capitalized on this and sought to use it as a starting point to gain further insight into fibroid-relevant miRNAs. It has been demonstrated that long-term gonadotrophin-releasing hormone (GnRH) agonist use establishes

Figure 2 The putative impact of miRNA in the pathogenesis of uterine fibroids. Proposed regulatory factors of aberrant miRNA expression include genomic instability, cellular transformation of myometrial cells, sex steroids and ethnicity. Complex regulation of a diversity of miRNAs, signaling pathways and gene targets has been implicated in the processes of ECM accumulation, cell cycle progression and hypertrophy, angiogenesis, and inflammation contributing to the development of uterine fibroids. Reproduced with permission from Luo and Chegini (2008). Thieme Publishers Inc. c-kit, stem cell factor receptor; c-myc, c-myc proto-oncogene; CDK9, cyclin-dependent kinase 9; CTGF, connective tissue growth factor; Cyr61, cysteine-rich angiogenic inducer 61; E2F1, E2F transcription factor 1; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HIF, hypoxia inducible factor; HMGA1, high mobility group AT-hook 1; HMGA2, high mobility group AT-hook 2; ICAM-1, intercellular Adhesion Molecule 1; IL-4, interleukin-4; IL-6, interleukin-6; IL-8, interleukin-8; IL-11, interleukin 11; IL-13, interleukin-13; IL-15, interleukin-15; IL-17, interleukin-17; MCP-1, monocyte chemoattractant protein-1; miRNA, microRNA; MMP, matrix metalloproteinase; MyoD, myogenic differentiation 1; Myt-1, myelin transcription factor 1; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TSP-1, thrombospondin 1; VEGF, vascular endothelial growth factor.
systemic hypoestrogenism (by down-regulation of the hypothalamic-pituitary axis and secondary reduction of ovarian production of sex steroids), reduces leiomyoma size and volume, and impedes progression of disease (Lethaby et al., 2001; Islam et al., 2013a). By virtue of binding with estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) on target cells, estradiol regulates a diversity of functions in the myometrium and leiomyomata (Andersen and Barbieri, 1995; Nilsson et al., 2001). Notably, differential (greater) expression of both ER-α and ER-β has been reported in leiomyomata compared with myometrium by several authors (Benassayag et al., 1999; Kovacs et al., 2001). In addition to its role in the activation of several signaling pathways such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and small body mothers against decapentaplegic (SMAD) for myometrial and leiomyoma cell proliferation, estradiol has also been shown to decrease expression of p53, a tumor suppressor, in leiomyoma cells in vitro (Hermon et al., 2008; Nierth-Simpson et al., 2009; Islam et al., 2013b).

Investigations of the role of progesterone in the pathogenesis and progression of fibroids have not shown any evidence of a reduction of fibroid volume and uterine size, and have only demonstrated a reduction in heavy menstrual bleeding when progesterone is inhibited (Tristan et al., 2012; Shen et al., 2013). Higher expression of progesterone receptors A and B (PR-A and PR-B, respectively) have been reported in leiomyoma compared with myometrium. Progesterone and its receptors have been shown to influence a wide variety of cell growth and survival factors, such as epidermal growth factor (EGF), transforming growth factor beta-3 (TGF-β3), insulin-like growth factor-1 (IGF-1), B cell lymphoma protein 2 (Bcl-2), tumor necrosis factor-α (TNF-α) and the phosphatidylinositol-3-kinase/v-Akt murine thymoma oncogene (PI3/AKT) pathway (Matsuo et al., 1997; Fujimoto et al., 1998; Shimomura et al., 1998; Arici and Sozen, 2000; Kurachi et al., 2001; Yamada et al., 2004; Hoekstra et al., 2009). Progestins activate the AKT pathway and promote survival in leiomyoma cells, while inhibition of the AKT pathway promotes caspase independent apoptosis and inhibition of fibroid cell growth (Hoekstra et al., 2009; Sefton et al., 2013).

Since differential effects of ovarian sex steroids and their receptors in leiomyomata and myometrium have been implicated as a significant factor in the pathology of fibroids, the regulation of miRNA expression by estradiol and progesterone has been investigated by several groups. The majority of studies examining the effect of sex hormones on miRNA expression have been conducted with in vitro treatment of human cell lines with estradiol. The first report to imply estradiol regulation of miRNA was in 2005 with the correlation of specific aberrant miRNA signatures with estrogen and progesterone receptor status in breast cancer (Iorio et al., 2005). Although there are numerous in vitro studies analyzing the effect of ovarian sex steroids on various cytokines, growth factors, ECM-related molecules, and on cell proliferation and death in primary and immortalized leiomyoma cell lines, there is limited data on the regulation of miRNA by estradiol and progesterone in the setting of intact uterine leiomyomata.

Pan et al. evaluated the effect of estradiol and progesterone on miRNA expression profiles in matched myometrial smooth muscle cells (MSMC), leiomyoma smooth muscle cells (LMSC), transformed LMSC (T-LMSC) and a leiomyosarcoma line (SK-LMS-1) (Pan et al., 2008). In short-term studies (24 h end-point), estradiol and progesterone regulated miRNA expression patterns specific to the cell type and steroid hormone (Pan et al., 2008; Nothnick, 2012). Specifically, estradiol reduced the expression of miR 21 and miR 26a in MSMC and LMSC, while the anti-estrogen compound, ICI-182780, increased the expression of miR 20a and miR 21 in both MSMC and LMSC and miR 26a in MSMC, and inhibited expression of miR 26a in LMSC. Conversely, the progesterone analog, medroxyprogesterone acetate, increased both miR 21 and 26a in MSMC and LMSC, respectively, and inhibited miR 26a in LMSC. Although the study samples were derived from a limited number of patients (N = 7), these findings provide a preliminary insight into the potential fibroid-specific impact of sex steroids on miRNA expression and sex steroid/miRNA interactions in leiomyomata (Pan et al., 2008).

**Ethnic-specific differential miRNA expression**

When investigating the underlying etiologies of fibroids, a typical starting point includes the ethnic disparity of disease severity. It has been well established that leiomyomata disproportionately affect African-American women, with a 9-fold increased risk of developing fibroids compared with Caucasian women (Faerstein et al., 2001). A greater prevalence of fibroids in African-American women is seen even in the early reproductive years before women are symptomatic (Marsh et al., 2013) and incidence rates peak at an earlier age in this ethnic group (Marshall et al., 1997). When compared with Caucasian women, African-American women are often diagnosed with fibroids at a younger age (Kjerulf et al., 1996), have more severe disease (Kjerulf et al., 1996) and demonstrate greater rates of fibroid growth in the perimenopausal years (Peddada et al., 2008). Because African-American women have a disproportionately greater prevalence, severity of disease, and increased recurrence rates following surgical treatment compared with Caucasian women, there has been speculation of underlying ethnic-specific differences in the genetic regulation of disease (Laughlin and Stewart, 2011).

The reasons for the ethnic disparity are incompletely understood, and are likely multifactorial. Several underlying racial genetic differences have been reported and include greater aromatase activity (Isikawa et al., 2009; Bulun, 2013) and increased cytoplasmic carbonic anhydrase (CAII) gene expression in rapidly growing fibroids of African-American women (Davis et al., 2013). Collectively, these findings suggest that leiomyomata in African-American women are composed of cells which may demonstrate increased survival in acidic, hypoxic environments, and are resistant to apoptotic pathways (Davis et al., 2013). Additionally, prior reports have suggested a role of ethnicity-associated polymorphisms in the estrogen receptor alpha (ER-α) gene in African-American women (Al-Hendy and Salama, 2006a, b). Similarly, the high-activity COMT (Catechol-O-methyltransferase, an enzyme used in estrogen metabolism) Val/Val genotype is found more frequently in African-American women and is associated with a 2.5 times increased risk of developing uterine fibroids (Al-Hendy and Salama, 2006a, b).

Genetic factors associated with ethnicity have been viewed as a prime opportunity to characterize ethnic-specific modifiers of leiomyomata, and to functionally define the contribution of ethnic-specific genetic dysregulation of disease (Al-Hendy and Salama, 2006a, b; Ishikawa et al., 2009; Malik et al., 2010; Catherino et al., 2013). Data from several investigators have demonstrated ethnic-specific differential miRNA expression signatures between myometrium and leiomyomata. Pan et al. reported reduced levels of miR 181a expression in leiomyomata (compared with ethnicity-matched myometrium) in African-American...
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women, whereas miR 181a expression was increased in leiomyomata in Caucasian women compared with matched myometrium (Pan et al., 2008). miR 142-5p expression was reduced in leiomyomata compared with matched myometrium exclusively in Caucasian women (Pan et al., 2008). Wang et al. demonstrated >2-fold increased relative expression of miR 23a/b, let 7 s, miR 145, miR 197, miR 411 and miR 412 in fibroids of African-American women compared with Caucasian women. Most recently, Chuang et al. showed that miR 200c expression was reduced in both myometrium and leiomyomata in African-American women compared with Caucasian women. Most surprisingly, miR 200c expression was reduced in both myometrium and leiomyomata in African-American women compared with Caucasian women and other ethnicities to definitively confirm if an ethnic-specific miRNA expression pattern exists in fibroids. If differential expression is confirmed, it will be interesting to see if functional studies demonstrate whether ethnic-specific differential miRNA expression conveys ethnic-specific biologic functions and impacts differential disease phenotypes among races.

Since ethnicity-based differences in genetic regulation of human disease and outcomes have been suggested, many investigators have argued the physiologic significance of race/ethnicity and the validity of race as a biological classification. Following completion of the human genome project, greater genetic variations have been found within racial/ethnic groups than between them (Feldman et al., 2003). Moreover, there is no consensus for racial/ethnic-specific phenotypic characteristics, and race is subject to biases of subject perception during self-reporting (Hahn et al., 1992). Although the limitations of racial classification may pose challenges to the study of ethnic-specific miRNA expression signatures in the setting of fibroids, the identification of the most severe phenotypes of fibroid disease, which have an association with race, may still have the potential to guide future translational investigations of the continuum from miRNA gene dysregulation to ethnic-specific disparities in fibroids.

**Functional impact of miRNA in uterine leiomyomata**

With continued confirmation of differential gene and miRNA expression signatures and prediction of miRNA gene targets, our understanding of the downstream regulatory roles of miRNA is expanding. Although the impact of specific miRNA species on the progression of disease has been proposed, it is challenging to infer biological function from database predictive models of miRNA/gene interactions or differential miRNA expression profiles. Due to the complex post-transcriptional modification and biologic niche-specific regulation of miRNA, gene expression profiling and in vitro and in vivo functional analyses of miRNA regulation of specific gene targets have become essential to defining the role of fibroid-specific gene regulation on disease development and regression. Herein, an update of relevant integrated gene target profiling and miRNA functional studies is presented.

**miRNA gene target prediction**

Genomic and transcriptomic integrative analyses have identified driver genes responsible for tumorigenesis in human cancer (Staaf et al., 2010; Curtis et al., 2012). Notably, Arslan et al. performed microarray gene expression studies to demonstrate altered expression of genes, whose targets were predicted to be associated with ECM production, differentiation and modulation of cell proliferation (Arslan et al., 2005; Cirilo et al., 2013). Global gene analyses have identified nonrandom cytogenetic abnormalities with associated copy number alterations (CNAs) and gene dysregulation in a large proportion of fibroids (Vanharanta et al., 2005; Hodge et al., 2009). However, fewer than anticipated genes mapping to CNA have been identified. Preliminary studies by Klemke et al. assessed the loss of let-7 binding sites resulting from truncation of the 3′ UTR of HMGA2 in uterine leiomyomata (Klemke et al., 2010). Specifically, the group assessed fibroid samples with 12q14-q15 chromosomal rearrangements with loss of miR let-7 3′ UTR binding sites on HMGA2 and HMGA2 truncated transcripts. Approximately one-third of samples demonstrated truncated HMGA2 transcripts and overexpression of the cognate protein, thus implicating reduced regulation by let-7 and amplification of HMGA2 as a potential factor in fibroid growth and regression.

To our knowledge, the most comprehensive integrated global genetic analysis to identify cell proliferation-specific gene targets in uterine fibroids has been conducted by Cirilo et al. This group performed an integrative genomic and transcriptomic analysis with comparative genomic hybridization (CGH) and integrated data analysis using the CONEXIC algorithm (Akavia et al., 2010; Cirilo et al., 2013). This large-scale expression analysis was employed in 51 fibroid samples of 34 patients to identify pathways and molecular markers associated with leiomyomata. Based upon *in silico* analyses with integrated genomic and transcriptomic profiles, fibroblast growth factor receptor 1 (FGFR1) and insulin-like growth factor binding protein 5 (IGFBP5) were noted to be aberrantly expressed in fibroids compared with myometrium, and were identified as possible therapeutic targets. Importantly, these molecules have been previously validated as targets in breast cancer and have been associated with tumor proliferation (Becker et al., 2012; Gozgit et al., 2012; Cirilo et al., 2013). With prior evidence of IGFBP5 overexpression in uterine fibroids, an estrogen-dependent association of IGFBP5 in hormone-dependent tumors, and the putative role of both FGFR1 and IGFBP5 in ECM accumulation in hepatic fibrosis models, these findings highlight the potential utility of global genomic and transcriptomic target prediction models to delineate relevant genes involved in the development of fibroids and to guide miRNA functional studies.

With the aim of investigating miRNA and mRNA interactions, Zavadi et al. performed global pattern analyses (CGH) with predicted gene target models in primary fibroid (all >10 cm from a cohort of AA women) and myometrial samples (Zavadi et al., 2010). Firstly, the group compared aberrant miRNA expression to predicted gene targets, and showed that 249 down-regulated miRNAs were the predicted targets of the five most up-regulated leiomyoma miRNAs and 97 up-regulated miRNAs were the predicted targets of the five most down-regulated fibroids miRNAs (Zavadi et al., 2010). Patterns of inverse association of miRNA expression with mRNA (miRNA predicted targets) expression in leiomyomata involved signaling pathways including MAP kinase, WNT, JAK-STAT and TGF-B, as well as dysregulation of genes involved in cell proliferation, cell adhesion, cell
matrix formation and transcriptional reprogramming (Zavadil et al., 2010).

**In vitro miRNA functional studies**

Analyses of the impact of underexpression and overexpression of specific miRNA species on their gene targets and fibroid-relevant end-points have expanded our understanding of the functional role of these molecules. Typically, miRNA overexpression (‘mimics’) involves the use of short-term transfection or viral infection of double-stranded small RNAs into primary or immortalized cell lines in order to simulate specific native miRNA species (Varga et al., 2013). Conversely, inhibition of miRNA activity may involve the use of a synthetic anti-miRNA oligonucleotide or locked nucleic acid oligonucleotides complementary to the specific miRNA target by either mispairing at the RISC degradation site or by base modification to inhibit RISC degradation. Through steric blockade following transfection, miRNA inhibitors prevent miRNA from binding to the same site, prevent the degradation of the target mRNA and facilitate underexpression of a specific miRNA species (Krutzenfeld et al., 2005). An alternative strategy to inhibiting miRNA activity is to target saturation of target mRNAs (miRNA sponges) (Iorio and Croce, 2012). Sponges express mRNA with multiple binding sites for the miRNA of interest and prevent this miRNA from binding with its gene target (Ebert et al., 2007; Iorio and Croce, 2012).

A prime example of in vitro and in vivo functional analyses of the role of aberrant miRNA expression in disease states is the hepatocellular carcinoma (HCC) model (Yau et al., 2013). This group demonstrated overexpression of miR 106b in HCC tumor tissue (compared with adjacent normal tissue), in two metastatic HCC cell lines compared with primary tumor cell lines, and an increased risk of high tumor grade associated with overexpression of this miRNA species. Cells that underwent lentiviral induction for miRNA 106b overexpression displayed significantly increased cell migration and stress fiber migration. Functional studies in an orthotopic SCID mouse model showed that tumor induction in animals with cells stably overexpressing miRNA 106b had significantly greater metastatic disease compared with controls (Yau et al., 2013). Studies such as this eloquently demonstrate the functional impact of differential miRNA expression on disease phenotype and provide support for the manipulation of miRNA expression in primary cell culture or immortalized cells lines to assess function.

As a functional application of HMG2 gene expression, profiling data of Klemke et al. and Peng et al. characterized the in vitro and in vivo repression of HMG2 by let-7 microRNAs (Peng et al., 2008; Klemke et al., 2010). They initially confirmed that noncultured leiomyomatous expressed a primary HMG2 mRNA transcript, HMG2a, and two minor isoforms, HMG2b and HMG2c. Semi-quantitative analysis of five HMG2 expressing fibroid samples, ranging in size from 1 to 10 cm, revealed a relationship between HMG2 and let 7c expression to fibroid size. Larger (5, 7 and 10 cm) fibroids contained lower relative levels of let 7c and higher HMG2 levels compared with smaller (1 and 2 cm) fibroids and vice versa. The proliferation index (Ki-67 immunohistochemical staining) was inversely proportional to let 7c expression and directly proportional to fibroid size. Primary cultures derived from these five samples were transfected with either a pGL3 construct with luciferase and a HMG2a 3’UTR or pGL3 luciferase construct with a HMG2a 3’UTR and let 7c inhibitor. Two days after transfection, the reduction in luciferase activity was greater in the cultures derived from smaller fibroids (higher relative endogenous let 7c expression) compared with cultures of larger fibroids (lower relative endogenous let 7c expression). Cotransfection of the let 7c inhibitor rescued luciferase activity up to a threshold of 80% of initial levels (Peng et al., 2008). Since HMG2 overexpression and loss of let 7 miRNA were more common in larger fibroids, let-7 miRNA expression was inversely proportional to the Ki-67 index, and endogenous let 7 repressed luciferase activity at the HMG2 3’UTR in primary leiomyoma cell cultures, the authors concluded that let 7 miRNA regulation of HMG2 may play a significant role in fibroid development and growth.

In recent notable in vitro functional studies for uterine fibroids, the impact of miR-200c on several predicted targets was assessed (Chuang et al., 2012b). With the use of primary myometrial smooth muscle cells (MSMC) and leiomyoma smooth muscle cells (LSMC) from patient-matched myometrial and leiomyoma samples from surgical specimens, overexpression and reduction of miRNA 200c was induced via transfection. Overexpression of miR 200c resulted in the alteration of cell morphology, a reduced rate of proliferation and cellular viability, and an alteration of cell morphology in MSMC and LSMC. Additionally, miR 200c overexpression resulted in concurrent reduction of vimentin and zinc finger E-box-binding homeobox 1/2 (ZEB1/ZEB2) mRNA and protein expression and an increase in E-cadherin (Chuang et al., 2012b). These authors validated tissue inhibitor of metalloproteinase 2 (TIMP2), fibrulin 5 (FBN5) and vascular endothelial growth factor A (VEGFA) as direct targets of miR200c by demonstrating reductions of miRNA and protein expression with overexpression of this miRNA species in both MSMC and LSMC. Notably, the regulatory function of miR 200c on expression of these targets was implied to occur by direct binding at the 3’ UTR as seen by luciferase reporter activity (from cotransfection with plasmid of pre-miR-200c and luciferase reporter plasmid containing 3’ UTR sequences of TIMP2, FBNL5, VEGFA) (Chuang et al., 2012b). In light of the observed impact of miR 200c on regulators of epithelial to mesenchymal transition and tumorogenesis (ZEB1/2, FBNL5), cellular transition (E-cadherin), angiogenesis (VEGFA), and matrix remodeling (TIMP2 and FBNL5), the experimental approach and findings of this study revealed the complexity of miRNA gene dysregulation and implicated miR200c as a possible regulator of several cellular processes contributing to the genesis of fibroids (Korpal et al., 2008; Bendoraitė et al., 2010; Guadall et al., 2011; Chuang et al., 2012b).

In line with the findings of miR 200c by Chuang et al., Zavadil et al. investigated the functional role of miR 200a. Since loss of miR 200 family members is associated with the epithelial/mesenchymal transition in invasive ovarian cancer (Cochrane et al., 2010), and miR 200a is down-regulated in primary fibroid samples, this group performed a functional validation of this miRNA and its predicted gene targets. Overexpression of miR 200a in immortalized leiomyoma cells resulted in reduced expression of tubulin beta (TUBB), cytochrome p450 1B1 (CYP1B1) and c-terminal binding protein 2 (CTBP2), in vitro growth inhibition, and reversal from a fibroblastoid phenotype to an epithelial phenotype (Zavadil et al., 2010). Taken together with the findings of Chuang et al., these findings suggest that miR 200 family members impact on fibroid growth and may contribute to the mesenchymal character of fibroids (Zavadil et al., 2010).

With the aim to define the functional roles of a single miRNA species cluster and its host gene in cell cycle progression, inflammation, angiogenesis and tissue turnover, Chuang et al. conducted an in vitro analysis of the
miR93/106b cluster and their host gene, MCM7 (minichromosome maintenance complex component 7), in paired myometrial and fibroid primary cell cultures from African-American and Caucasian patients (N = 63) exposed to hormonal therapies (Chuang et al., 2012a). MCM7, a DNA helicase, is located on chromosome 7q22, co-transcribes miR 106b ~25 cluster, is overexpressed in several malignancies, and is thought to play a significant role in DNA replication, progression of the cell cycle, and cellular proliferation and transformation (Li et al., 2005; Forsburg, 2008; Lau et al., 2010; Ota et al., 2011; Chuang et al., 2012a). Aberrant expression of miR 106b ~25 is evident in several malignancies, has been predicted to target genes involved in proliferation, apoptosis and tissue remodeling, and is thought to act as a proto-oncogene through association with MCM7 or Myc (Kan et al., 2009; Poliseno et al., 2010; Chuang et al., 2012a). In primary tissue, miR 93 expression was reduced while MCM7 expression was elevated in leiomyomata compared with myometrium in both groups of women. A similar expression pattern was observed in women with abnormal uterine bleeding and in samples collected during the luteal phase. Following transduction of TF324 cells (spontaneously transformed uterine leiomyoma smooth muscle cells) with doxycycline inducible lentiviral vector containing miR 106b ~25 cluster and induction with doxycycline, the predicted gene targets, IL-8 and F3 were differentially expressed compared with untreated cells. Compared with matched myometrium, leiomyomata demonstrated reduced F3 expression and increased IL-8. F3 was increased in the luteal phase, decreased in the follicular phase and displayed an inverse relationship to miR 93 during the menstrual cycle (Chuang et al., 2012a). Immunoblot analysis confirmed reduced expression of F3, and elevated IL-8 and MCM7. Following transfection of MSCC and LSMC (with pre-miR 93 and pre-miR 106b), a dose-dependent regulatory function of miR 93 and miR 106b on F3 and IL8 through direct interactions with their respective 3’UTR was demonstrated via luciferase reporter assay gain of function (miR 93 and miR 106b) and a reduction in connective tissue growth factor (CTGF), plasminogen activator inhibitor (PAI-1), and pentraxin-related protein (PTX3) (all known downstream products of F3) expression in LSMC. Because F3 regulates IL8 in other cell types, the results revealed that F3 and IL8 are direct targets of miR93/106b, and implicated regulation of IL8, CTGF, PAI-1 and PTX3 through F3. Additionally, overexpression of miR 93 and miR 106b in LSMC resulted in reduced cellular viability, proliferation, and increased caspase activity and apoptosis (Chuang et al., 2012a). Collectively, these findings demonstrated specific miRNA regulatory function on an associated gene and several downstream gene targets and their protein products. Most importantly, this study provided invaluable insight into the potential mechanisms of miRNA regulation of transcription and translation events involved in proliferation, apoptosis and ECM turnover during the de novo development of leiomyomata in myometrium.

Table I presents a summary of commonly identified miRNA in leiomyomata with results of functional studies, proposed biologic function, and predicted and validated miRNA gene targets.

**miRNA applications in translational medicine**

With the transition from the laboratory bench to the clinical bedside, the paradigm of improved diagnosis and treatment of human disease via investigation has been realized in translational medicine. Moreover, the development of biomarkers, which can detect the presence of disease (diagnostic), indicate the progression of disease (prognostic) and predict the response of disease states to a specific therapeutic regimen (predictive), has been highly sought after. In disease states such as uterine fibroids, which are diagnosed following tumor development and have a significant recurrence rate, the development of miRNA as diagnostic, prognostic and predictive biomarkers has the potential to change the standard of care. Herein, the potential application of miRNA as biomarkers and targets for therapeutic intervention in uterine fibroids will be discussed.

**Diagnostic biomarker approaches**

An ideal biomarker would provide an accurate noninvasive method to detect fibroids in their earliest stage prior to the typical detection period with clinical examination and/or imaging. Unfortunately, an accurate and noninvasive diagnostic miRNA biomarker for uterine fibroids has not come to fruition and is not at a stage of imminent development or clinical application (Levy et al., 2013). In contrast to the infancy of diagnostic miRNA biomarker development in the field of uterine biology, the investigation and development of miRNA biomarkers for the detection of very early stage disease has been realized in the field of cancer research. Since miRNA expression alterations are the rule and not the exception in human cancers, several investigators have utilized aberrant miRNA profiles to detect disease, to categorize site of origin (malignancy of unknown primary site), to predict metastatic potential and to assess the risk of recurrence for several malignancies (Volinia et al., 2006; Rosenfeld et al., 2008; Iorio and Croce, 2012). For example, the up-regulation of a single miRNA species, miR 21, in the extracellular vesicles of cerebrospinal fluid has been shown to accurately distinguish patients with glioblastoma from those with benign brain tumors (Akers et al., 2013). In the realm of biomarkers for early diagnosis, du Rieu et al. demonstrated overexpression of miR 205 and miR 21 in early pancreatic ductal adenocarcinoma precursor lesions compared with later stage cancer (du Rieu et al., 2010).

Since uterine fibroids are a benign disease process, and treated primarily via surgical or procedural means, many have questioned whether the development of diagnostic biomarkers is necessary in the clinical management of this disease (Levy et al., 2013). Physical examination and transvaginal ultrasound are inexpensive means for initial diagnosis. Furthermore, delayed diagnosis does not carry a risk of mortality as seen in the delay of diagnosis of malignancy. One notable circumstance in which a diagnostic biomarker, such as a characteristic miRNA expression signature would be useful, is in differentiating uterine leiomyomata from leiomyosarcomas. Since both tumors have similar radiographic appearances, leiomyosarcomas are not typically diagnosed until the time of surgery. Due to the risk of accidental dissemination of the malignant leiomyosarcoma during removal or morcellation of a presumed benign uterine fibroid, the development of a preoperative diagnostic biomarker would facilitate referral to a gynecologic oncologist for the proper initial surgical procedure (Levy et al., 2013). As in the case of du Rieu et al. in which a miRNA biomarker detected precursor pancreatic adenocarcinoma lesions, a similar diagnostic miRNA biomarker may also be useful to signal the initial de novo transition from myometrium to fibroid. Ultimately, an early detection biomarker may have the potential...
to guide the development of an effective long-term therapeutic agent to prevent progression of disease.

Although the use of biomarkers in bodily fluids (saliva, sera, plasma, urine, feces) may offer the possibility of noninvasive early detection, there is no direct evidence that many solid tumor diseases, such as uterine leiomyomata, secrete miRNA locally or systemically (Khan et al., 2013). As an initial approach to justify research for the investigation of noninvasive diagnostic biomarkers for uterine fibroids, future research efforts may consider a multicenter comparison of respective miRNA expression patterns in patient-matched serum, myometrium and leiomyoma to provide supporting evidence for the existence of circulating diagnostic miRNA biomarker(s) of disease.

### Table 1 Summary of miRNA species commonly identified in leiomyomata.

<table>
<thead>
<tr>
<th>Differential miRNA expression (fibroid compared with myometrium)</th>
<th>Predicted target genes</th>
<th>Target genes validated in fibroids</th>
<th>Proposed function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>†miRNA 21</td>
<td>BCL2, PTEN, TPM1</td>
<td>–</td>
<td>Tumorigenesis cell survival oncomir</td>
<td>Si et al. (2007), Marsh et al. (2008) and Wang et al. (2007)</td>
</tr>
<tr>
<td>†miRNA 34a</td>
<td>GAS1, CDK4, CDK6, E2F3</td>
<td>–</td>
<td>Cellular proliferation, cell survival</td>
<td>Marsh et al. (2008), Ma et al. (2013) and Wang et al. (2007)</td>
</tr>
<tr>
<td>†Let-7 family</td>
<td>HMGA2, TSC1, IMP-1, IMP-3, IRS2</td>
<td>HMGA2</td>
<td>Cellular proliferation, tumorigenesis</td>
<td>Peng et al. (2008), Klemke et al. (2010) and Wang et al. (2007)</td>
</tr>
<tr>
<td>†miR200 family</td>
<td>ZEB1/ZEB2, TIMP2, FBLN5, VEGFA, TUBB, CYP1B1, CTBP2, MAIF, BCL2, CITED2, LASS6, PHF1A, TSC22D1, ATXN1, JUN, NFIB</td>
<td>ZEB1/ZEB2, TIMP2, FBLN5, VEGFA, TUBB, CYP1B1, CTBP2</td>
<td>Cellular transition, angiogenesis, matrix remodeling</td>
<td>Chuang et al. (2012a, b) and Zavadil et al. (2010)</td>
</tr>
<tr>
<td>†miRNA 93/106B</td>
<td>F3, IIB, CTGF, PAI-1</td>
<td>F3, IIB, CTGF, PAI-1</td>
<td>Inflammation, tissue turnover</td>
<td>Chuang et al. (2012a, b)</td>
</tr>
<tr>
<td>†miRNA 30a</td>
<td>PLLAG2, RAR, SMARCD2, SLC29A3, HLF, MAP2K5, TNXA</td>
<td>–</td>
<td>Oncomir</td>
<td>Wang et al. (2007) and Zavadil et al. (2010)</td>
</tr>
<tr>
<td>†miRNA 23b</td>
<td>PPARG, HIVEP2, GATA2, FOSB</td>
<td>–</td>
<td>Oncomir</td>
<td>Wang et al. (2007) and Zavadil et al. (2010)</td>
</tr>
<tr>
<td>†miRNA 27a</td>
<td>TSC1, RXR, RAR, IGF1</td>
<td>–</td>
<td>Tumor suppressor</td>
<td>Zavadil et al. (2010)</td>
</tr>
<tr>
<td>†miRNA 29B</td>
<td>TRAF4, COLIA2</td>
<td>–</td>
<td>Inflammation, extracellular matrix</td>
<td>Wang et al. (2007) and Zavadil et al. (2010)</td>
</tr>
<tr>
<td>†miRNA 197</td>
<td>RNPC1, TNRC5, ESRI, GLUR2, P3 K, IGFBP3, GRIP1</td>
<td>–</td>
<td>Tumor suppressor</td>
<td>Wang et al. (2007) and Zavadil et al. (2010)</td>
</tr>
<tr>
<td>†miRNA 212</td>
<td>COL1A1, E2F5, HB-EGF, CDKN1A</td>
<td>–</td>
<td>Extracellular matrix</td>
<td>Wang et al. (2007)</td>
</tr>
</tbody>
</table>

†, up regulated in fibroid versus myometrium; †↓, Down regulated in fibroid versus myometrium; BCL2, B-cell CLL/lymphoma 2; PTEN, phosphatase and tensin homolog; TPM1, tropomyosin 1 (alpha); GAS1, growth arrest-speciﬁc 1; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; E2F3, E2F transcription factor 3; HMGA2, high mobility group AT-hook 2; TSC1, tuberous sclerosis 1; IMP-1, insulin-like growth factor 2 mRNA binding protein 1; IMP-3, insulin-like growth factor 2 mRNA binding protein 3; IRS2, insulin receptor substrate 2; ZEB1, zinc ﬁnger E-box binding homeobox 1; ZEB2, zinc ﬁnger E-box binding homeobox 2; TIMP2, TIMP metallopeptidase inhibitor 2; FBLN5, ﬁbulin 5; VEGFA, vascular endothelial growth factor A; TUBB, tubulin, beta class 1; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; CTBP2, C-terminal binding protein 2; MAIF, v-maf avian musculoaponeurotic ﬁbrosarcoma oncogene homolog; CITED2, Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain; 2; LASS6, ceramide synthase 6; PHF1A, PHD ﬁnger protein 21A; TSC22D1, TSC22 domain family, member 1; ATXN1, ataxin 1; JUN, jun proto-oncogene; NF1B, nuclear factor I/B; F3, coagulation factor III; IL8, interleukin 8; CTGF, connective tissue growth factor; PAI-1, serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; PLLAG2, pleiomorphic adenoma gene-like 2; RAR, retinoic acid receptor, alpha; SMARCD2, SWI/SNF-related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2; SLC29A3, solute carrier family 29 (equilibrative nucleoside transporter), member 3; HLF, hepatic leukemia factor; MAP2K5, mitogen-activated protein kinase kinase 5; TNXA, tenascin A; PPARG, peroxisome proliferator-activated receptor gamma; HIPVE2, human immunodeficiency virus type 1 enhancer binding protein 2; GATA2, GATA binding protein 2; FOSB, FBJ osteosarcoma oncogene B; RXR, retinoid X receptor; IGF1, insulin-like growth factor 1 (somatomedin C); TRAF4, TNF receptor-associated factor 4; COL1A2, collagen, type I, alpha 2; RNPC1, RNA binding motif protein 38; TNRC5, canary FGF signaling regulator 3; ESRI, estrogen receptor 1; GLUR2, glutamate receptor, ionotrophic, AMPA 2; PPIK, phosphatidylinositol 4,5-bisphosphate 3-kinase, catalytic subunit alpha; IGFBP3, insulin-like growth factor binding protein 3; GRIP1, glutamate receptor interacting protein 1; COL1A1, collagen, type I, alpha 1; E2F5, E2F transcription factor 3, p130-binding; HB-EGF, heparin-binding EGF-like growth factor; CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Cip1).

### Prognostic and predictive biomarker applications

Underlying continued investigation of the role of aberrant miRNA expression in the pathobiology of uterine fibroids is the potential application of miRNA signatures to predict response to therapy (regression and recurrence). A prime example of the utility of miRNA to predict response to treatment (predictive biomarkers) includes miR 26 HCC and miR 21 in pancreatic cancer. Specifically, patients with hepatocellular carcinoma with reduced hepatic miR 26 demonstrated an improved response to interferon and extended survival compared with those with reference range miR 26. Similarly, patients with up-regulation of miR
21 in pancreatic adenocarcinoma were noted to have a poor response to adjuvant chemotherapy (Schetter et al., 2008).

As mentioned previously, the medical treatment of uterine fibroids involves induction of a hypoestrogenic state, disruption of progesterone receptor signaling or establishment of a progestin dominant state. Interestingly, in the previously discussed study by Pan et al., miR 21 and 26a were differentially regulated by estradiol, an estradiol antagonist, and a progestin in a tissue-specific manner in MSMC and LSMC. In light of these findings, future studies may focus on the utility of leiomyoma miRNA signatures to predict response to medical treatment. Assessment of the predictive value of fibroid and myometrial miRNA expression signatures on clinical response end-points such as duration to maximum fibroid volume reduction following GnRH agonist therapy, time for return to original fibroid volume following GnRH agonist cessation, and response of dysfunctional uterine bleeding to oral contraceptive pills, systemic progestins, or progesterone receptor modulators may be a potential area of future investigation.

Many women who undergo a hysterectomy and/or surgical intervention will have a recurrence of disease. The potential application of miRNA expression signatures to predict regression or recurrence of disease following uterine artery embolization, focused ultrasound ablation, or myomectomy may have the most clinical value. A predictive/prognostic miRNA signature may have the most benefit if it allows clinicians to provide pre-procedural risk assessment of recurrence and facilitates selection of the most effective first-line treatment option. Given the significant recurrence rate of uterine fibroids, a comparison of miRNA expression of fibroids/myometrium from the primary surgery to fibroids/myometrium removed in a subsequent surgery for recurrence may be an opportunity to expand our understanding of the natural history of disease development and mechanisms for disease progression, and potentially to identify which subset of patients may be most successful with a specific treatment option.

**Therapeutic applications**

The continuum of miRNA therapeutic target development encompasses the initial identification of aberrant miRNA expression in a disease state to confirmation of miRNA gene targets to assessment of the impact of miRNA loss or gain of function during *in vitro* studies with primary or immortalized cells lines to assessment of *in vitro* and *in vivo* targeted therapy (Fig. 3). miRNA-based therapy involves either a direct approach in which oligonucleotides or viral-based constructs are used to inhibit the expression of an oncogenic miRNA or reintroduce a tumor suppressor or an indirect approach to regulate miRNA expression by modulating its processing (Iorio and Croce, 2012). Due to the complexity of miRNA/gene interactions involving multiple gene targets and several signaling pathways, efficient therapeutic targeting of a specific disease, such as fibroids, may be difficult. Concerns for nonspecific ‘off target’ miRNA action and a switch to a more aggressive disease phenotype have been raised (Teague et al., 2010). Moreover, challenges to the translation of miRNA research to the clinical setting include lack of nonhuman primate animal models for fibroids and inconsistent efficacy of miRNA-based in vivo delivery systems. Notably, promising results of the first ever human trial of targeted miRNA therapy were only recently published in early 2013. In this international phase 2a, placebo controlled, double-blind, randomized clinical trial of patients with chronic hepatitis C (HCV) genotype 1 infection, investigators demonstrated safety and efficacy of a systemic anti-miR 122 oligonucleotide to significantly reduce the HCV viral load (Janssen et al., 2013).

As observed in animal and human trials with directed anti-miRNA treatment, miRNA gene targets which impact intrinsic mechanisms of disease development and progression represent potential targets for therapeutic effect. Because of the early stage of investigation into the biologic role of miRNA in uterine fibroids, the tangible development and utilization of miRNA-targeted therapy for this reproductive disorder is premature and has not been realized to date. To this end, there are several promising and common aberrantly expressed miRNAs targets which require further study prior to consideration as candidates for interventional targeted therapy in clinical medicine.

**Future investigative approaches**

**Candidate miRNA targets**

In light of the association of let-7 regulation and HMGA2 and fibroid size, and evidence to suggest that let 7 can directly repress the oncogenes RAS and MYC, let 7 may represent one of the more promising targets for the future investigation of the underlying mechanisms of fibroid progression (Hui et al., 2005; Sampson et al., 2007; Peng et al., 2008; Zhu et al., 2011). Due to its known impact on oncogenes and cell cycle regulation, this candidate miRNA family may elucidate the role of miRNA in gene targets and signaling pathways which regulate the balance between cell proliferation, survival and apoptosis. The potential impact of miRNAs let-7, 200a, 200c, 93, 106b and 21 in tumorigenesis, ECM turnover, angiogenesis, inflammation and cell cycle may provide a prime opportunity to delineate the functional role of commonly observed miRNAs in fibroids. As proof of principle, future studies may assess if gain or loss of function of these species can significantly impact the process of cellular transformation from myometrium to fibroid (Fig. 4).

Recently, miR 21 has been investigated by several investigators as a potential biomarker and therapeutic target. It is thought to be regulated by estradiol and has been cited as one of the most common differentially expressed miRNA species in leiomyomata. Since many of its targets are tumor suppressors, and its aberrant expression has been reported by several groups in a diversity of malignancies, it has been termed an oncomiR, and has emerged as a miRNA target of interest for future investigation in many neoplasms (Esquela-Kerscher and Slack, 2006; Wickramasinghe et al., 2009). Unfortunately, little is known about its role in the pathobiology of fibroids. In 2012, Fitzgerald et al. investigated the expression patterns of miR 21 and one of its well-known targets, programmed cell death 4 (PDCD-4) (Fitzgerald et al., 2012). PDCD-4 is involved in cell cycle regulation, apoptosis and cellular transformation. miR 21 was not found to be an *in vitro* regulator of PDCD-4 expression in leiomyomata and myometrial primary cell cultures, but was found to impact cellular apoptosis through unknown targets. In light of these findings as well as the continued study of miR 21 as a serum and tissue diagnostic and predictive biomarker in cancer biology, this miRNA species may be of interest in future studies to investigate the role of miRNAs in the genesis of fibroids.

**In vitro and in vivo models**

Essential to continued investigations of miRNAs as potential biomarkers and therapeutic targets for leiomyomata is the use of relevant *in vitro* and *in vivo* models which simulate human disease. Prior *in vitro* studies have...
demonstrated that mechanical stress may be a significant contributor to the excessive ECM deposition phenotype of these benign tumors (Rogers et al., 2008; Norian et al., 2012). Specifically, Norian et al. demonstrated a potential role for RhoA signaling on the viscoelastic nature of the ECM (Norian et al., 2012). Furthermore, this group provided novel in vitro evidence suggesting that the intrinsic stiffness of fibroids and the critical events of tumorigenesis may be mediated in part by aberrant mechano-transduction and RhoA expression (Norian et al., 2012). In a three dimensional in vitro model, immortalized matched myometrial and leiomyoma cell lines demonstrated the morphologic and molecular characteristics of their derivative tissues and maintain the ability to produce ECM and respond to TGFβ3 (Malik and Catherino, 2012). In line with 3D culture systems, a future area of development may also involve the use of 3D organotypic cultures and rotating wall vessel bioreactor technology. With the ability to recapitulate in vivo structure and function of a particular organ, these culture systems have the potential to provide biologically relevant models for future preclinical studies (Hjelm et al., 2010; Skardal et al., 2010; Barzegari and Saei, 2012). In light of the significance of ECM dysregulation in fibroid development, these in vitro models may provide novel approaches to expand our understanding of the role of miRNA in the genesis of this reproductive disorder.

With the advent of transgenic murine fibroid models, in vivo investigation of miRNA function may now be considered. In the transgenic murine model generated by the Teixiera group, constitutive β-catenin activation in the uterine mesenchyme of mice produces mesenchymal tumors which express TGFβ and mammalian target of rapamycin (mTOR) (Tanwar et al., 2009). Additionally, the TSC-2 deletion mouse and Eker rat models demonstrate myometrial hyperplasia and fibroid production. Interestingly these features are abrogated with inhibition of mTOR signaling or the elimination of sex steroid production (Walker et al., 2003; Prizant et al., 2013). Recently, Varghese et al. demonstrated loss of the tumor suppressor, REST (repressor element 1 silencing transcription factor) and up-regulation of G protein coupled receptor 10 (GPR10) in uterine fibroids. This confirmed findings from previous mRNA analyses of Walker et al. (Crabtree et al., 2009). This overexpression of GPR10 in mice resulted in myometrial hyperplasia and excessive ECM deposition (Varghese et al., 2013). With previously confirmed targeting of miR 21 (REST) and miR 99a/100 (mTOR), these transgenic

Figure 3 Strategies for the confirmation of miRNA function and the development of future diagnostic and therapeutic applications for uterine leiomyomata. Following confirmation of differentially expressed miRNA species and their predicted gene targets, in vivo and in vitro studies will prove necessary to define biologic function. Findings of overexpression or underexpression studies of specific miRNAs will determine if miRNA technologies may provide further insight into the pathobiology of disease or if applications as biomarkers or therapeutic targets are feasible.
models may provide invaluable insight into the in vivo role of microRNAs (Sun et al., 2013; Varghese et al., 2013).

**Conclusions**

Over the past decade, demonstration of differential leiomyoma miRNA expression has ushered in a novel perspective of the epigenetic regulation of this reproductive disorder. Since in vitro studies have shown miRNA regulation of gene targets which impact cellular processes crucial to fibroid development, comprehensive functional studies may delineate factors responsible for the genesis and recurrence of disease. In contrast to the prolific investigation of miRNA in cancer biology, confirmation of the in vivo functional role of miRNA dysregulation and miRNA gene targets in the phenotype of fibroids is still in a preliminary stage. To this end, translational applications of miRNA technologies in the clinical care of uterine fibroids are premature at the current time and require further investigation. In light of current limitations in early diagnosis, prevention and medical treatment, an improved understanding of the complex networks of miRNA gene regulation may provide promise for the future development of biomarkers of disease and targeted therapeutics.

**Authors’ roles**


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