

# Ovarian steroids, stem cells and uterine leiomyoma: therapeutic implications

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Submitted on March 3, 2014; resubmitted on July 16, 2014; accepted on July 22, 2014

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**BACKGROUND:** Uterine leiomyoma is the most common benign tumor in women and is thought to arise from the clonal expansion of a single myometrial smooth muscle cell transformed by a cellular insult. Leiomyomas cause a variety of symptoms, including abnormal uterine bleeding, pelvic pain, bladder or bowel dysfunction, and recurrent pregnancy loss, and are the most common indication for hysterectomy in the USA. A slow rate of cell proliferation, combined with the production of copious amounts of extracellular matrix, accounts for tumor expansion. A common salient feature of leiomyomas is their responsiveness to steroid hormones, thus providing an opportunity for intervention.

**METHODS:** A comprehensive search of PUBMED was conducted to identify peer-reviewed literature published since 1980 pertinent to the roles of steroid hormones and somatic stem cells in leiomyoma, including literature on therapeutics that target steroid hormone action in leiomyoma. Reviewed articles were restricted to English language only. Studies in both animals and humans were reviewed for the manuscript.

**RESULTS:** Estrogen stimulates the growth of leiomyomas, which are exposed to this hormone not only through ovarian steroidogenesis, but also through local conversion of androgens by aromatase within the tumors themselves. The primary action of estrogen, together with its receptor estrogen receptor  $\alpha$  (ER $\alpha$ ), is likely mediated via induction of progesterone receptor (PR) expression, thereby allowing leiomyoma responsiveness to progesterone. Progesterone has been shown to stimulate the growth of leiomyoma through a set of key genes that regulate both apoptosis and proliferation. Given these findings, aromatase inhibitors and antiprogestins have been developed for the treatment of leiomyoma, but neither treatment results in complete regression of leiomyoma, and tumors recur after treatment is stopped. Recently, distinct cell populations were discovered in leiomyomas; a small population showed stem-progenitor cell properties, and was found to be essential for ovarian steroid-dependent growth of leiomyomas. Interestingly, these stem-progenitor cells were deficient in ER $\alpha$  and PR and instead relied on the strikingly higher levels of these receptors in surrounding differentiated cells to mediate estrogen and progesterone action via paracrine signaling.

**CONCLUSIONS:** It has been well established that estrogen and progesterone are involved in the proliferation and maintenance of uterine leiomyoma, and the majority of medical treatments currently available for leiomyoma work by inhibiting steroid hormone production or action. A pitfall of these therapeutics is that they decrease leiomyoma size, but do not completely eradicate them, and tumors tend to regrow once

treatment is stopped. The recent discovery of stem cells and their paracrine interactions with more differentiated cell populations within leiomyoma has the potential to provide the missing link between developing therapeutics that temper leiomyoma growth and those that eradicate them.

**Key words:** leiomyoma / estrogen / progesterone / aromatase / stem cells

## Introduction

Uterine leiomyomas represent the most common category of solid pelvic tumors in women, occurring in up to 80% of all women of reproductive age, with up to 30% of women experiencing severe enough symptoms to seek treatment (Cramer and Patel, 1990; Marshall et al., 1997; Myers et al., 2002; Parker, 2007). Leiomyomas can cause a range of symptoms, including abnormal uterine bleeding, pressure-related symptoms, recurrent pregnancy loss and infertility. Interestingly, leiomyoma cause many of these symptoms not only by virtue of the size and mass effect of the tumor itself, but also by modulating gene expression in the endometrium (Cakmak and Taylor, 2011; Sinclair et al., 2011). Women can have one or multiple leiomyomas, and they can achieve a wide range of sizes (Bulun, 2013). African-American women develop leiomyomas more frequently and at earlier ages than Caucasian women. Moreover, tumors in African-American women are more aggressive, as they present with larger leiomyomas and more significant symptoms than their Caucasian counterparts (Day Baird et al., 2003; Walker and Stewart, 2005). In addition to racial differences, leiomyomas show a high degree of heterogeneity in growth even within the same woman, and especially in women with multiple tumors (Peddada et al., 2008).

Despite the high prevalence of these tumors, relatively little is known about their specific etiology. X chromosome-linked clonality studies (using glucose-6-phosphate dehydrogenase) suggest that leiomyomas are monoclonal tumors derived from a single myocyte (Linder and Gartler, 1965; Townsend et al., 1970). The neoplastic transformation of a myocyte is likely due to some sort of cellular insult, although the exact etiology of this transforming event is currently unknown. Factors proposed to play a role in the conversion of a myocyte into a leiomyoma include genetic mutations, epigenetic aberrations and altered responses to hypoxia; however, the sequence of events in transformation and clonal expansion remains unclear (Bulun, 2013; Mehine et al., 2013; Tal and Segars, 2014). Regardless of the nature of the cellular insult, a common salient feature of leiomyomas is their responsiveness to steroid hormones, thus providing an opportunity for intervention. Estrogen and progesterone lead to tumor expansion by stimulating a modest rate of cellular proliferation and the production of copious amounts of extracellular matrix, which is predominantly collagen (Flake et al., 2013; Kim et al., 2013; Fig. 1). The excess collagen accumulation is also thought to contribute to the ultimate involution of leiomyomas (Flake et al., 2013).

Over 200 000 surgical procedures are performed annually in the USA to remove or destroy uterine leiomyomas, with an estimated annual cost of \$5.9–34.4 billion (Cardozo et al., 2012). Hysterectomy or myomectomy are still commonly utilized for leiomyoma treatment, however, more recently, less invasive procedures have been employed, such as uterine artery embolization and magnetic resonance guided focused ultrasound surgery (Al Hilli and Stewart, 2010; Freed and Spies, 2010).

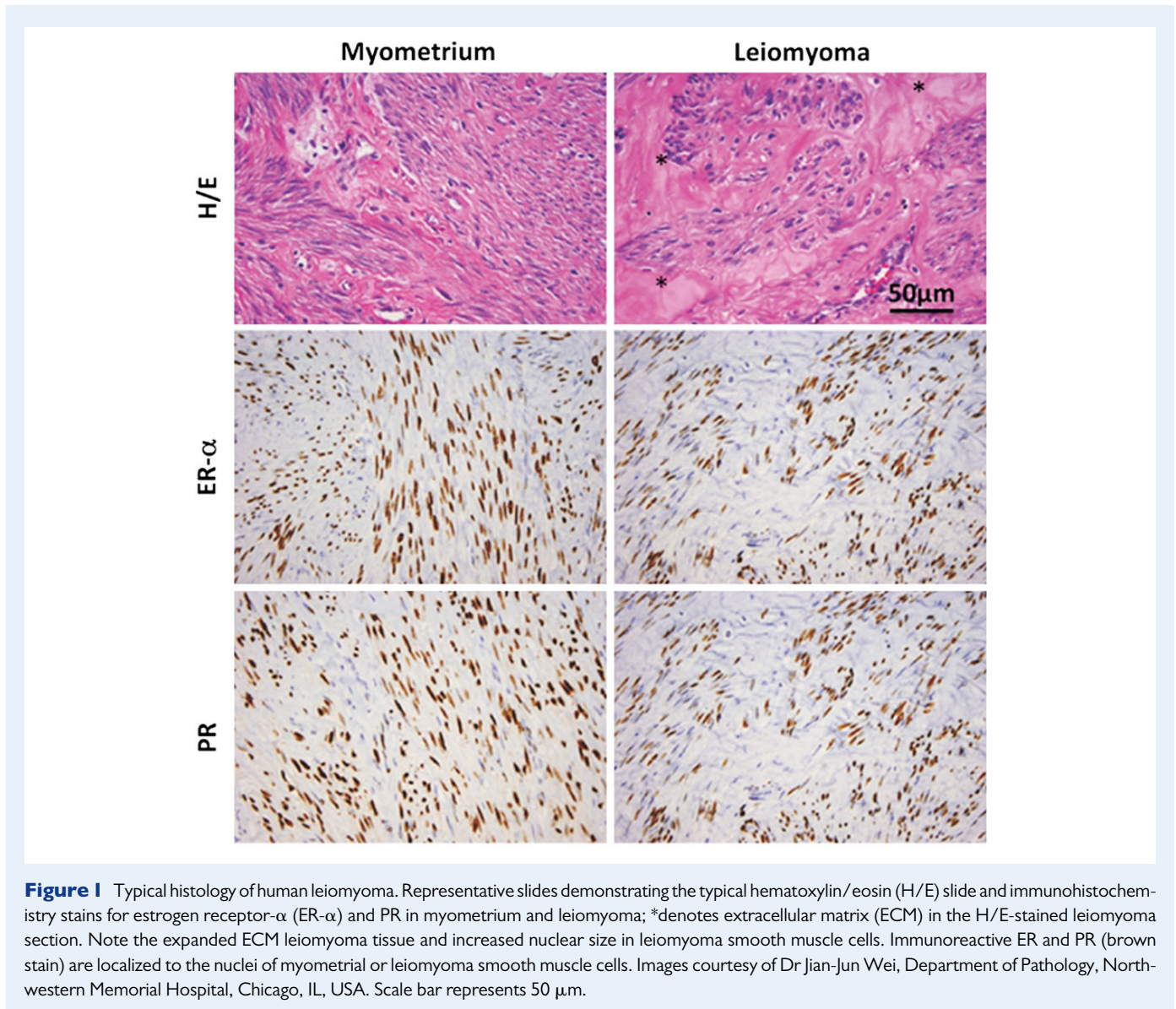
While GnRH agonists have traditionally been the mainstay of medical treatment for uterine leiomyoma, other classes of medications are being investigated with promising results, including aromatase inhibitors and selective progesterone receptor modulators (SPRMs) with primarily anti-progestogenic activity (Sabry and Al-Hendy, 2012a, b; Table I). Additionally, there is encouraging research on the role of supplements, such as vitamin D and green tea extract, as potential treatments for uterine fibroids (Halder et al., 2012, 2013; Sabry and Al-Hendy, 2012a, b; Roshdy et al., 2013). Medical treatment for uterine leiomyoma is ideally preferable to surgical management for many patients due to the inherent risks of surgery or desire to maintain future fertility. However, many of the medical options also have undesirable side effects, which often limits the beneficial duration of therapy. More importantly, leiomyoma growth also typically rebounds once treatment is stopped (Walker and Stewart, 2005). The recent discovery of a small population of stem-progenitor cells, important in leiomyoma pathophysiology, may provide the opportunity for new therapeutic targets (Mas et al., 2012; Ono et al., 2012). This review focuses on the role of aromatase, ovarian steroids and stem-progenitor cells in the pathogenesis of uterine leiomyoma, and discusses the therapeutic implications of the current state of knowledge of these pathways.

## Methods

A comprehensive search of PUBMED was conducted to identify peer-reviewed literature published since 1980 pertinent to the roles of steroid hormones and somatic stem cells in leiomyoma, including literature on therapeutics that target steroid hormone action in leiomyoma. The search included combinations of the following key words: leiomyoma, aromatase, aromatase inhibitors, anastrozole, letrozole, estrogen, estrogen receptor, progesterone, progesterone receptor, SPRMs, asoprisnil, mifepristone, ulipristal, telapristone, stem cells and progenitor cells. Reviewed articles were restricted to English language only. Studies in both animals and humans were reviewed for the manuscript. The reference lists of included articles were also reviewed to identify additional relevant studies.

## Aromatase, estrogen and estrogen receptor $\alpha$

The observation that leiomyomas occur primarily in women of reproductive age, grow during early pregnancy and regress during menopause supports the widely held view that ovarian steroids stimulate leiomyoma growth. Moreover, disruption of ovarian estrogen or progesterone production during treatment with GnRH agonist results in size reduction of leiomyomas, an effect that is reversed once treatment is stopped (West et al., 1987). Leiomyomas are exposed to estrogen not only through ovarian steroidogenesis, but also through local conversion of androgens by aromatase within the tumors themselves (Fig. 2; Bulun et al., 1994).



**Figure 1** Typical histology of human leiomyoma. Representative slides demonstrating the typical hematoxylin/eosin (H/E) slide and immunohistochemistry stains for estrogen receptor- $\alpha$  (ER- $\alpha$ ) and PR in myometrium and leiomyoma; \*denotes extracellular matrix (ECM) in the H/E-stained leiomyoma section. Note the expanded ECM leiomyoma tissue and increased nuclear size in leiomyoma smooth muscle cells. Immunoreactive ER and PR (brown stain) are localized to the nuclei of myometrial or leiomyoma smooth muscle cells. Images courtesy of Dr Jian-Jun Wei, Department of Pathology, Northwestern Memorial Hospital, Chicago, IL, USA. Scale bar represents 50  $\mu$ m.

Sumitani *et al.* (2000) showed that in cultured leiomyoma cells, the addition of androstenedione leads to production of estrone, which is then converted to the more potent estradiol ( $E_2$ ) by 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD). Furthermore, the addition of androstenedione led to similar rates of cellular proliferation as the addition of  $E_2$ , leading the authors to conclude that leiomyomas are capable of producing enough estrogen to sustain their own growth (Sumitani *et al.*, 2000). The addition of aromatase inhibitors to the cultured leiomyoma cells decreased proliferation, further supporting that aromatase is the key enzyme mediating *in situ* estrogen production (Sumitani *et al.*, 2000). Accordingly, leiomyomas have remarkably higher levels of aromatase and 17 $\beta$ -HSD type I compared with adjacent myometrium, which presumably leads to the higher tissue levels of estrogens observed in leiomyoma compared with the surrounding myometrium (Folkerd *et al.*, 1984; Bulun *et al.*, 1994; Sumitani *et al.*, 2000; Shozu *et al.*, 2004). Moreover, aromatase transcripts are not found in the myometrium of leiomyoma-free uteri (Bulun *et al.*, 1994). Further evidence for the

pathological role of aromatase is the quantifiable increase in aromatase expression found in the leiomyomas of African-American women, who typically have larger and greater numbers of leiomyomas, and the exhibited effectiveness of aromatase inhibitors in decreasing leiomyoma size clinically (Bulun, 2013).

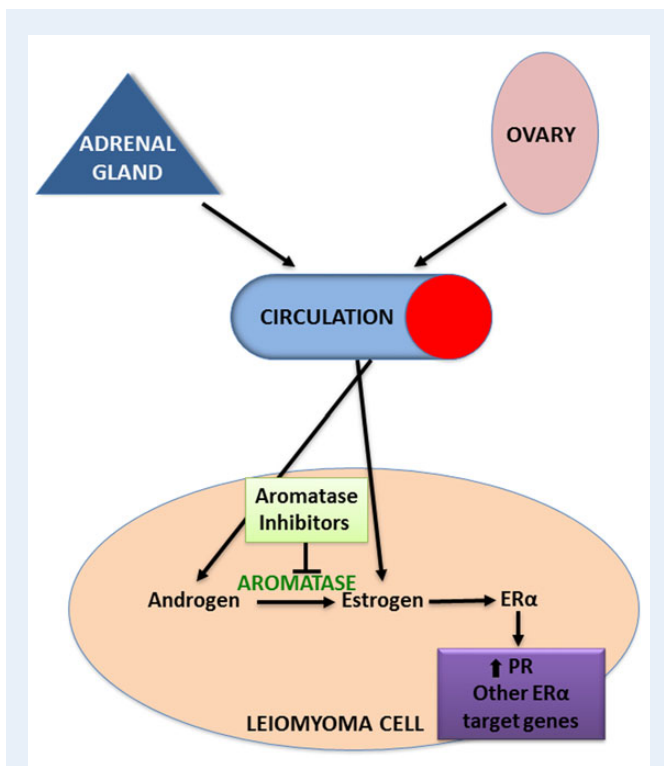
Aromatase is a member of the cytochrome P450 family and is encoded by the gene *CYP19A1*. *CYP19A1* expression is sophisticatedly regulated through multiple tissue- and cell-specific promoters and transcription factors (Bulun *et al.*, 1994; Imir *et al.*, 2007; Ishikawa *et al.*, 2008). The primary aromatase promoters utilized by leiomyoma cells are remarkably similar to breast cancer cells, being predominantly the proximal promoters I.3/II, which are activated by prostaglandin  $E_2$  or cAMP analogs (Imir *et al.*, 2007). However, in Asian women, aromatase expression is primarily regulated by the distal promoter I.4, which is activated by a glucocorticoid and a class I cytokine (Shozu *et al.*, 2002; Imir *et al.*, 2007). Although the molecular mechanisms underlying regulation of aromatase have not been completely elucidated, Ishikawa *et al.*

**Table 1** Medications that result in leiomyoma shrinkage and their effects in women.

| Class                | Examples                 | Mechanism of action                            | Side effects                       | Pregnancy category | FDA approved for leiomyoma                   | Tumors recur after cessation | Comments  |
|----------------------|--------------------------|--|------------------------------------|--------------------|--|------------------------------|---|
| GnRH agonists        | Leuprolide, Triptorelin  | Abolishes GnRH pulsatility                     | Menopausal symptoms, bone loss     | X                  | Yes, leuprolide for preoperative anemia only | Yes                          | Initial flare effect; Requires add-back therapy after 6 months                  |
| GnRH antagonists     | Ganirelix, Elagolix      | Competitive inhibition of GnRH                 | Menopausal symptoms, bone loss     | X                  | No   | Yes                          | Avoids flare effect of GnRH agonists; Elagolix is the only oral GnRH antagonist |
| SPRMs                | Ulipristal, Mifepristone | Varied progesterone antagonism                 | Endometrial thickening/hyperplasia | X                  | No   | Yes                          |   |
| Aromatase inhibitors | Letrozole, Anastrozole   | Competitive inhibition of the aromatase enzyme | Bone loss                          | D                  | No   | Yes                          | Can cause follicular stimulation  |

FDA, Food and Drug Administration.

Pregnancy categories are based on FDA classifications (A–D, X), D = established evidence of fetal risk, but potential benefits may outweigh risks in select patients; X = established evidence of fetal risk, with risks of use in pregnant women clearly outweighing the benefits.



**Figure 2** Role of estrogen in leiomyoma pathogenesis. Although leiomyomas are exposed to circulating estrogens from the ovary, it appears that substantial amounts of estrogen are also produced *in situ* via aromatization of androgens from the adrenal gland and ovary. The biologically active estrogen, estradiol, acts primarily through ER $\alpha$  to induce transcription of genes involved in proliferation and ECM formation, but its principal function is up-regulation of PR expression, thereby increasing leiomyoma responsiveness to progesterone. Aromatase inhibitors effectively block the *in situ* production of estradiol, thus decreasing leiomyoma responsiveness to both estrogen and progesterone signaling.

(2008) reported that the transcription factor CCAAT/enhancer-binding protein  $\beta$  is a key inducer of aromatase expression via regulating its proximal promoter I.3/II region. Further investigation into these molecular mechanisms may help guide the development of new therapeutics that could lead to leiomyoma-specific aromatase inhibition (Ishikawa et al., 2008) and a resultant decrease in action of locally produced estrogen.

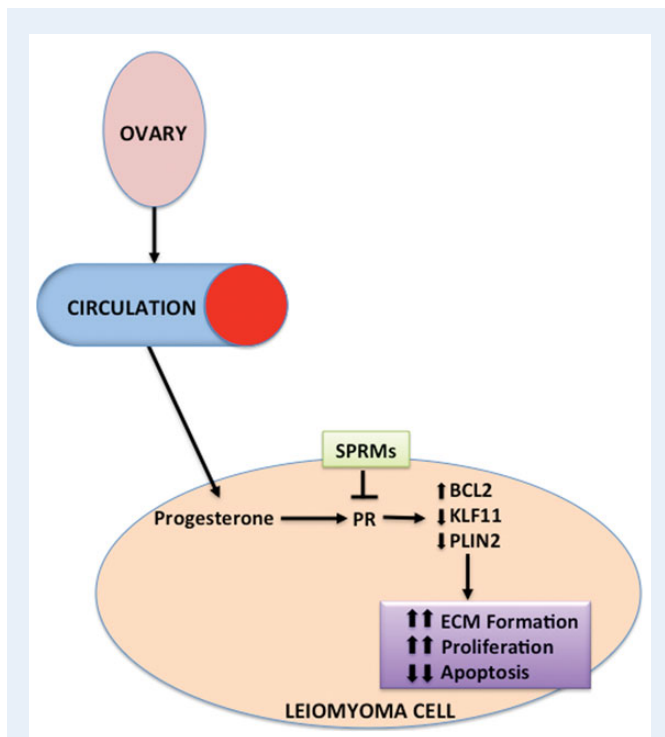
The product of aromatase, estrogen, up-regulates the expression of several genes thought to play a role in leiomyoma pathogenesis, including multiple growth factors, collagens, and the estrogen and progesterone receptors (ER, PR; Fig. 2; Andersen et al., 1995; Li and McLachlan, 2001; Maruo et al., 2004). Estrogen acts primarily via two nuclear receptors, ER $\alpha$  and ER $\beta$ , which are expressed in both the myometrium and in leiomyoma where they uniquely coordinate gene transcription (Andersen and Barbieri, 1995; Pedeutour et al., 1998; Benassayag et al., 1999). Some studies have reported increased ER expression in leiomyoma compared with the surrounding myometrium, whereas other studies report no such difference (Wilson et al., 1980; Tamaya et al., 1985; Jakimiuk et al., 2004). Although the exact roles and interplay of ER $\alpha$  and ER $\beta$  have not been fully elucidated in the pathogenesis of leiomyoma, it is thought that ER $\beta$  may regulate the transcriptional activity of ER $\alpha$ , which is the stronger activator of transcription of the two (Jakimiuk et al., 2004). Additionally, several studies have suggested that polymorphisms of the ER $\alpha$  gene may increase susceptibility to leiomyoma (Al-Hendy and Salama, 2006; Feng et al., 2013). In addition to activation from estrogen binding, ER $\alpha$  is also activated through its phosphorylation by the mitogen-activated protein kinase (MAPK) pathway and possibly via other kinases (Hermon et al., 2008). Based on these findings, Hermon et al. (2008) hypothesized that estrogen-bound ER $\alpha$  induces growth factor expression, which can then stimulate the MAPK pathway and further activate ER $\alpha$  via phosphorylation in an autocrine fashion.

Although estrogen was traditionally thought of as the primary stimulus of leiomyoma growth, clinical studies, as well as a xenograft mouse model, have demonstrated that progesterone is necessary for estrogen-

related leiomyoma growth, suggesting that estrogen alone is necessary, but not sufficient for proliferation (Lamminen *et al.*, 1992; Ishikawa *et al.*, 2008). Ishikawa *et al.* (2010) showed that estrogen/ER $\alpha$  regulates expression of PR and that estrogen alone is not a mitogen *in vivo*. Moreover, Hassan *et al.* (2007) reported that disruption of the estrogen signaling pathway by transfecting leiomyoma cells with an ER mutant that suppresses the activity of wild-type ER diminishes both ER- and PR-gene expression. These findings suggest a more permissive role for estrogen, acting via induction of PR expression, and thereby allowing leiomyoma responsiveness to progesterone (Ishikawa *et al.*, 2010; Bulun, 2013).

## Progesterone and progesterone receptor

Progesterone is a steroid hormone essential for coordinating normal mammalian female reproductive physiology (Lydon *et al.*, 1995; Graham and Clarke, 1997; Lee *et al.*, 2006). The physiologic actions of progesterone are mediated by interaction with PR, a member of the nuclear hormone superfamily of ligand-activated transcription factors (Mangelsdorf *et al.*, 1995; Robinson-Rechavi *et al.*, 2003). There are two predominant PR isoforms, designated PR-A and PR-B, which are transcribed from the same gene by two distinct promoters, with the only difference being that human PR-B is larger by an additional 164 amino acids at the amino terminus (Lessey *et al.*, 1983; Kastner *et al.*, 1990; Gronemeyer *et al.*, 1991). As a result, PR-A and PR-B may have distinct transcriptional activities (Tung *et al.*, 1993, 2006; Vegeto *et al.*, 1993; McDonnell *et al.*, 1994; Tetel *et al.*, 1999; Edwards, 2000).



**Figure 3** Role of progesterone in leiomyoma pathogenesis. Ovarian progesterone acts via PR to regulate transcription of key genes for apoptosis, proliferation and ECM formation. Effective SPRMs act as progesterone antagonists at PR and block transcription of these genes.

Evidence from clinical and experimental studies indicates that progesterone and PR play key roles in uterine leiomyoma growth and development (Cermik *et al.*, 2002; Fig. 3). Several studies have reported increased expression of both PR-A and PR-B in leiomyoma tissue compared with adjacent normal myometrial tissue (Fig. 1; Brandon *et al.*, 1993; Englund *et al.*, 1998; Nisolle *et al.*, 1999). Interestingly, Ishikawa *et al.* demonstrated that PR mRNA levels were significantly higher in leiomyomas in Japanese women compared with African-American or Caucasian women (Ishikawa *et al.*, 2009). Studies in mice with selective ablation of PR isoforms revealed that PR-A is necessary for ovulation and modulates the anti-proliferative effects of progesterone in the uterus, and PR-B is required for normal mammary gland development and function (Mulac-Jericevic *et al.*, 2000, 2003). Little is known about the specific roles of each PR isoform in uterine leiomyoma.

*In vivo*, the proliferation marker proliferating cell nuclear antigen and mitotic counts are highest in leiomyoma tissue during the luteal/secretory phase (Kawaguchi *et al.*, 1989; Lamminen *et al.*, 1992). Clinical studies indicate that leiomyoma proliferative activity in postmenopausal women increases significantly with combined estrogen plus progestin replacement but not with estrogen replacement alone (Lamminen *et al.*, 1992). Most importantly, in an *in vivo* human leiomyoma xenograft model where human leiomyoma cells dissociated from fibroid tissues were grafted underneath the renal capsules of immunodeficient mice, progesterone and its receptor directly stimulated tumor growth, whereas the key action of estrogen and its receptor was to maintain PR expression in leiomyoma tissue (Ishikawa *et al.*, 2010). These data suggested that progesterone might be the primary hormone driving the growth of uterine leiomyoma. In this model, estrogen plus progesterone not only stimulated leiomyoma cell proliferation, but also extracellular matrix formation, which was abolished by co-treatment with the progesterone antagonist mifepristone (RU-486; Ishikawa *et al.*, 2010).

Using the same *in vivo* human leiomyoma xenograft model, Qiang *et al.* (2014) recently demonstrated that estrogen plus progesterone induces extracellular matrix production via down-regulation of miR-29b. Using microarray-based global micro RNA expression analysis, we and others have discovered that miR-29b expression was reduced in leiomyoma tissues compared with adjacent normal myometrium tissues (Wang *et al.*, 2007; Marsh *et al.*, 2008). Although *in vitro* culture significantly alters gene expression profiles in leiomyoma smooth muscle cells (Zaitseva *et al.*, 2006), miR-29b levels are consistently lower in cultured leiomyoma cells than in myometrial cells, similar to *in vivo* tissue (Qiang *et al.*, 2014). Consistent with previous reports that miR-29b binds to the 3' untranslated region of mRNAs of multiple collagen genes and represses their expression, increasing miR-29b levels in leiomyoma cells using miR-29b lentivirus proportionally reduces the collagen I $\alpha$ 1 level (Qiang *et al.*, 2014). This observation was further confirmed *in vivo* using a xenograft model (Qiang *et al.*, 2014). Leiomyoma cells transduced with miR-29b lentiviral vector or control vector were embedded in collagen and grafted under the kidney capsule in immunodeficient mice, which were ovariectomized and supplemented with estrogen plus progesterone (Qiang *et al.*, 2014). Although grafts containing control vector-transduced cells gave rise to typical solid leiomyoma tumors underneath the kidney capsule, leiomyoma grafts with ectopic miR-29b expression failed to form solid tumors in the presence of estrogen plus progesterone (Qiang *et al.*, 2014), supporting the hypothesis that dysregulation of miR-29b plays an important role in tissue fibrosis

and tumor formation (van Rooij et al., 2008; Cushing et al., 2011; Qin et al., 2011; Roderburg et al., 2011; Zhou et al., 2011).

Simultaneously, immunofluorescence analysis demonstrated that protein levels of collagen 1a1 and 3a1 were significantly reduced in the mir-29b restoration group. Finally, estrogen plus progesterone, but not estrogen alone, suppressed mir-29b expression, indicating that down-regulation of mir-29b by estrogen plus progesterone is essential for uterine leiomyoma growth (Qiang et al., 2014). Given that SMAD3 can mediate transforming growth factor (TGF)- $\beta$ -induced down-regulation of mir-29 via binding to the mir-29 promoter, and the TGF- $\beta$  pathway plays an important role in leiomyoma growth (Lee and Nowak, 2001; Qin et al., 2011; Yin et al., 2011), it would be interesting to investigate whether progesterone/PR and TGF- $\beta$ /SMAD signaling pathways interact to regulate mir-29 expression and its effect on leiomyoma growth.

## Aromatase inhibitors

Based on the role of estrogen and aromatase in leiomyoma pathogenesis described above, aromatase is a logical target for treatment (Fig. 2). Non-steroidal aromatase inhibitors reversibly and competitively bind the aromatase enzyme and block its access to its natural substrates such as androstenedione or testosterone (Michaud and Buzdar, 1999; Chumsri et al., 2011). Over time, more specific aromatase inhibitors, with superior bioavailability and side-effect profiles, have been developed and the current 'third-generation' aromatase inhibitors (e.g. anastrozole and letrozole) have been shown to result in >98% inhibition (Chumsri et al., 2011; Lonning and Eikesdal, 2013). The role of aromatase inhibitors in the treatment of leiomyoma was first reported by Shozu et al. (2003), when they described a significant decrease in leiomyoma size and symptomatology in a perimenopausal woman treated with fadrozole. Since then, several clinical trials have demonstrated the efficacy of letrozole and anastrozole in reduction of leiomyoma volume of up to 52.5% and in providing symptomatic control (Varelas et al., 2007; Hilario et al., 2009; Parsanezhad et al., 2010; Duhan et al., 2013). Moreover, letrozole has been shown to be equivalent to GnRH agonist in reducing leiomyoma size while avoiding the side effects of the profound hypoestrogenism caused by GnRH agonists, specifically severe hot flashes (Duhan et al., 2013).

Aromatase inhibitor treatment and the subsequent decrease in circulating estrogens are not without side effects. If used in the follicular phase, aromatase inhibitors can lead to follicular stimulation and pregnancy. Therefore, women prescribed aromatase inhibitors need contraception. Additionally, there have been conflicting results regarding the level of hypoestrogenism induced by aromatase inhibitors, with some studies suggesting a more systemic effect and others a more localized effect (Duhan et al., 2013; Shozu et al., 2003). The most concerning potential consequences of long-term treatment include the possibility for increased bone loss and increased cardiovascular risk, especially in younger patients (Lonning and Eikesdal, 2013). Minor reported side effects include hot flashes and musculoskeletal pain. There is also some question as to the effectiveness of aromatase inhibitors, particularly anastrozole, in overweight and obese individuals, based on data from breast cancer trials (Lonning and Eikesdal, 2013). Moreover, developing resistance to the action of aromatase inhibitors has been reported in breast cancer (Chumsri et al., 2011; Lonning and Eikesdal, 2013). These concerns suggest that treatment with aromatase inhibitors may

only be a temporary or bridging therapy for leiomyoma, as opposed to a long-term solution. Unfortunately, once aromatase inhibitors are discontinued, leiomyomas regrow, albeit to smaller volumes than the pre-treatment size (Duhan et al., 2013).

## Selective progesterone receptor modulators

Four SPRMs have been used in clinical trials—mifepristone (RU486), asoprisnil (J867), ulipristal acetate (CDB2914) and telapristone acetate (CDB4124)—and all of these treatments were shown to reduce leiomyoma size and improve quality of life (Chabbert-Buffet et al., 2005; Chwalisz et al., 2005; Spitz, 2009; Bouchard et al., 2011; Talaulikar and Manyonda, 2012; Islam et al., 2013; Fig. 3). In 1993, it was first suggested that mifepristone might be a novel management strategy for uterine leiomyoma (Murphy et al., 1993). Since then, a large number of clinical trials looking at various doses of mifepristone have been performed to test its effect on tumor regression and symptomatic improvement. Compared with higher doses, lower doses of mifepristone appear to have the same effectiveness for symptom control and can reduce tumor size by up to 50%, and also have better safety profiles (Eisinger et al., 2009; Islam et al., 2013).

Conversely, asoprisnil suppresses uterine bleeding in a dose-dependent manner, with decreased bleeding reported in 28, 64 and 83% of subjects at 5, 10 and 25 mg/day, respectively, and reduced fibroid volume by up to 36% at 25 mg/day (Chwalisz et al., 2007), possibly by decreasing uterine artery blood flow (Wilkens et al., 2008). Recently, follow-up studies have raised concerns about asoprisnil, in that it primarily functions as a progesterone antagonist and does not oppose estrogenic activity in endometrium (Madauss et al., 2007). Similarly, clinical trials of telapristone acetate were suspended due to concerns over liver toxicity; however, trials have recently resumed employing lower doses (Bouchard et al., 2011).

The newest antiprogesterin to be studied, ulipristal acetate, has shown promising results. In a clinical trial comparing the efficacy and side-effect profile of ulipristal acetate to the GnRH agonist leuprolide acetate, ulipristal acetate provided more prolonged tumor volume reduction after termination of treatment, although leuprolide acetate caused greater reduction in fibroid volume overall (Donnez et al., 2012a, b; Talaulikar and Manyonda, 2012; Islam et al., 2013; Kim et al., 2013). Importantly, patients seem to tolerate ulipristal acetate better than leuprolide acetate with a significantly lower incidence of hot flashes, little effect on bone density and less profound suppression of E<sub>2</sub> levels (Donnez et al., 2012a, b). Currently, ulipristal acetate 5 mg/day is approved in Europe for preoperative treatment of <3 months, but has not been approved in the USA by the Food and Drug Administration ([www.fda.gov](http://www.fda.gov)) for the treatment of leiomyoma.

Mechanisms underlying the therapeutic roles of these four antiprogesterins are still being investigated. Gene expression of Kruppel-like transcription factor 11 (KLF11), a tumor suppressor, is significantly lower in leiomyoma tissues compared with adjacent myometrial tissues (Yin et al., 2010). Bisulfite sequencing revealed that the CpG island in the KLF11 gene promoter is hypermethylated in leiomyoma tissue. Therefore, dysregulation of KLF11 may be a key factor involved in uterine fibroids. Using a genome-wide approach, we found a PR-binding site located 20.5 kb upstream of the KLF11 gene promoter. Importantly,

KLF11 knockdown markedly increased leiomyoma cell proliferation. Intriguingly, several investigators reported the novel role of KLF11 in cardiac and liver fibrosis through mediating extracellular matrix gene expression (Mathison *et al.*, 2013; Zheng *et al.*, 2014). In primary cultures of uterine leiomyoma cells, mifepristone treatment robustly regulates the protein and mRNA levels of KLF11 via enhancing the recruitment of Sp1, RNA polymerase II, PR and its coactivator SRC-2 to both the distal enhancer and basal promoter region of the KLF11 gene. It is unclear whether these activities can alter KLF11 methylation status. Furthermore, taking advantage of a robust and unbiased ChIP-sequencing assay, we recently discovered another novel PR target gene, perilipin 2 (PLIN2), in leiomyoma cells. PLIN2, an adipose differentiation-related protein, is ubiquitous in non-adipose lipid droplet-containing cells and plays important roles in lipid droplet formation and stabilization, but its loss is linked to the expression of fibrogenic genes in hepatic fibrosis. Moreover, in clear cell renal carcinoma, higher PLIN2 expression is associated with better cancer-specific survival and cancer-free survival. These findings strongly suggest that KLF11 and PLIN2 may regulate both cell proliferation and extracellular matrix formation in leiomyoma and provide novel gene therapeutic targets.

In cultured leiomyoma cells, treatment with ulipristal acetate, telapristone acetate and asoprisnil decreases cell proliferation by down-regulating a number of growth factors and their receptors, including insulin-like growth factor-I, epidermal growth factor, TGF $\beta$ 3, vascular endothelial growth factor (VEGF)-A and VEGF-B (Xu *et al.*, 2005, 2006; Chen *et al.*, 2006; Wang *et al.*, 2006; Maruo *et al.*, 2007; Luo *et al.*, 2010; Yoshida *et al.*, 2010). These treatments also induce apoptosis in cultured leiomyoma cells through activation of multiple differential apoptotic pathways: asoprisnil activates the tumor necrosis factor-related apoptosis-inducing ligand-mediated signaling pathway, as well as the endoplasmic reticulum stress-induced pathway (Sasaki *et al.*, 2007; Xu *et al.*, 2007; Yoshida *et al.*, 2010), while ulipristal acetate induces apoptosis by reducing the anti-apoptotic protein BCL2 and stimulating expression of cleaved caspase-3 and cleaved poly ADP ribose polymerase (Xu *et al.*, 2005; Chen *et al.*, 2006; Yoshida *et al.*, 2010). Furthermore, asoprisnil and ulipristal mediate extracellular matrix formation by increasing extracellular matrix metalloproteinase (MMP) inducer, MMP-1, MMP-8 and membrane type I-MMP protein contents, and decreasing tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, type I and type III collagen protein levels (Morikawa *et al.*, 2008; Xu *et al.*, 2008; Yoshida *et al.*, 2010). Importantly, all these observations occurred in cultured leiomyoma cells, but not in cells derived from adjacent normal myometrium, suggesting the tissue-specific roles of these compounds. Both asoprisnil and ulipristal acetate demonstrated high affinity for PR (Blithe *et al.*, 2003; DeManno *et al.*, 2003). Further studies are needed to investigate the genome-wide binding status of PR liganded with ulipristal or asoprisnil, similar to the aforementioned studies on mifepristone.

Overall, the most commonly expressed concern during treatment of uterine leiomyoma with SPRMs is endometrial thickening from blocked progesterone action in the endometrium. A study sponsored by the US National Institutes of Health specifically evaluated the endometrial histological changes following treatment with mifepristone, asoprisnil or ulipristal acetate and reported little evidence of mitosis and atypical hyperplasia; however, there was asymmetry of stromal and epithelial growth and prominent cystic, dilated glands. These novel endometrial changes represent a new morphological category designated as

progesterone receptor modulator-associated endometrial change (Horne and Blithe, 2007; Mutter *et al.*, 2008; Spitz, 2009; Williams *et al.*, 2012). Of note, most of the existing studies have described endometrial changes over a short period (months) of follow-up, with one group reporting return to normal endometrial histology 6 months after cessation of ulipristal acetate (Williams *et al.*, 2012). Although it has not yet been tested in humans, a promising new SPRM, CP8947, was shown to inhibit leiomyoma cell proliferation and extracellular matrix gene expression *in vitro* without blocking progesterone action in the endometrium in an animal model (Catherino *et al.*, 2010). More long-term studies are necessary to understand the full side-effect profile of SPRMs.

## Stem-progenitor cells

Somatic stem cells (also called adult stem cells or tissue-specific stem cells) are a small group of cells present throughout the body that undergo asymmetric division, allowing self-renewal and the production of daughter cells that can go on and differentiate into tissue-specific cell types. These cells are necessary for tissue regeneration and repair, which is critical for maintaining organ function (Weissman, 2000; Li and Clevers, 2010). Similarly, tumor-initiating cells (also called cancer stem cells or tumor progenitor cells) are a small group of cells within a tumor also capable of asymmetric division, and thereby have the ability for self-renewal and tumor maintenance and growth (Schofield, 1978; Jordan *et al.*, 2006). Recently, human and mouse myometrial tissues have been found to contain a subset of cells characteristic of somatic stem cells, which self-renew and produce daughter cells in a hormone-dependent manner (Arango *et al.*, 2005; Ono *et al.*, 2007; Szotek *et al.*, 2007). Subsequently, we and others have been able to isolate a small population of leiomyoma cells consistent with undifferentiated somatic stem cells or tumor progenitor cells (Mas *et al.*, 2012; Ono *et al.*, 2012).

Stem cells derived from leiomyoma tissue, but not myometrium, carry mediator complex subunit 12 (MED12) mutations, leading some to hypothesize that at least one genetic hit may transform a myometrial stem cell into a leiomyoma tumor progenitor cell, which then interacts with the surrounding myometrial tissue to give rise to a leiomyoma tumor (Ono *et al.*, 2012). Interestingly, mutations in the MED12 gene have been reported in ~70% of uterine leiomyomas (Makinen *et al.*, 2011; McGuire *et al.*, 2012). Mutations affecting the expression of the high mobility AT-hook 2 (HMGA2) gene have also been reported, and appear to be mutually exclusive with MED12 mutations, suggesting the possibility of different pathophysiologies behind leiomyomas harboring different mutations (Bertsch *et al.*, 2014). Alterations in HMGA2 and MED12 expression and function have also been hypothesized to support leiomyoma stem-progenitor cell self-renewal and cell proliferation (Bulun, 2013), making it unclear whether these genetic alterations cause the transformation of a myometrial stem cell or simply support already existing leiomyoma stem-progenitor cells. Importantly, it has alternatively been hypothesized that uterine hypoxia, aberrant methylation or abnormal estrogen signaling could play a critical role in the transformation of a myometrial stem cell into a leiomyoma (Zhou *et al.*, 2011; Maruyama *et al.*, 2013). Further research into the cellular insult leading to this transformation event in myometrial stem cells could reveal important therapeutic targets.

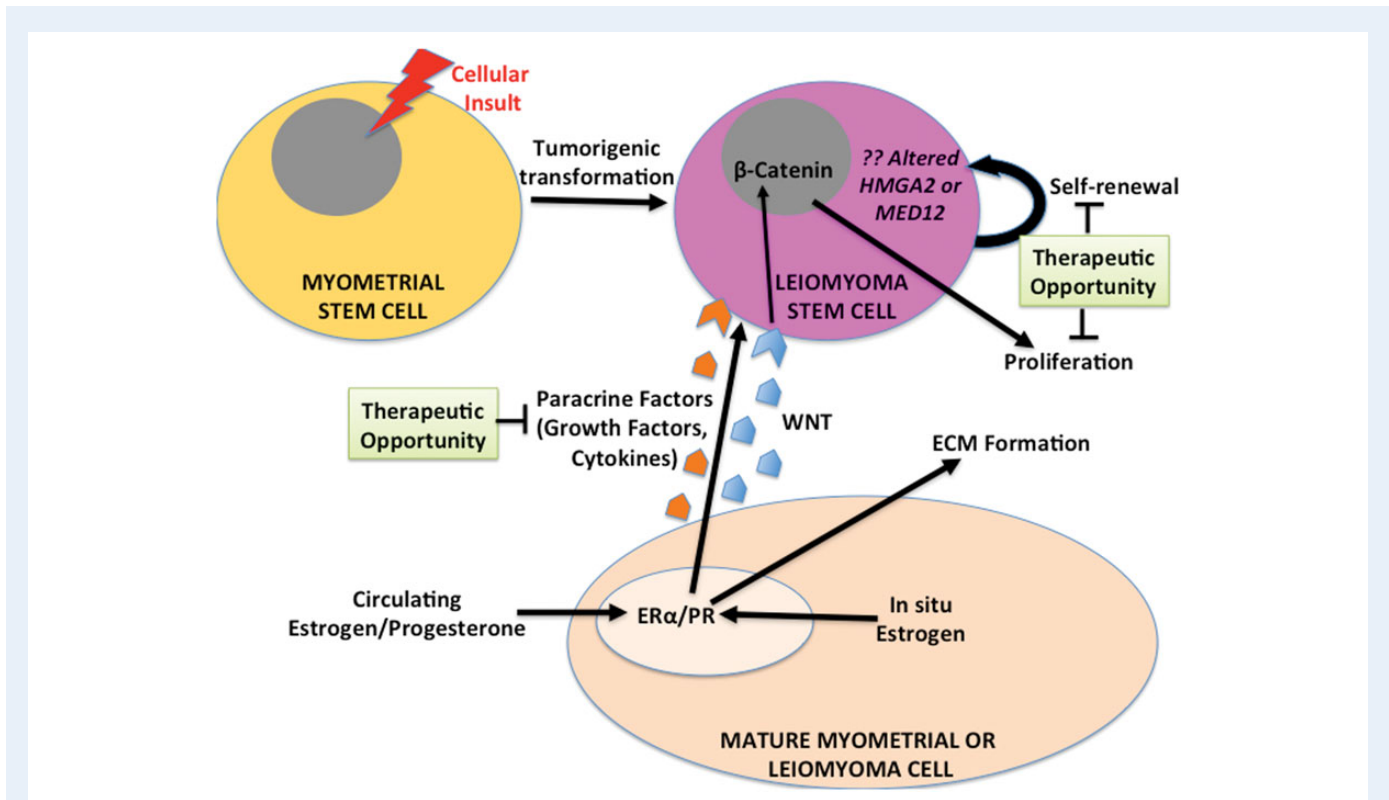
Evidence suggests that leiomyomas possess much smaller populations of stem cells compared with the myometrium (Chang *et al.*, 2010);

however, the leiomyoma stem cell population is likely essential for steroid-dependent leiomyoma growth (Mas et al., 2012; Ono et al., 2012). When cell suspensions containing leiomyoma stem-progenitor cells and mixed myometrial cells are injected under the kidney capsules of immunodeficient mice treated with estrogen and progesterone, they grow into significantly larger tumors than those containing differentiated leiomyoma cells and mixed myometrial cells. The tumors derived from leiomyoma stem-progenitor cells also have a much higher proliferation index than the tumors that do not contain these cells (Ono et al., 2012). Interestingly, leiomyoma stem-progenitor cells appear to be deficient in ERs and PRs, but have tumorigenic capabilities when stimulated by estrogen and progesterone. Moreover, leiomyoma stem-progenitor cells seem to require the presence of either mature leiomyoma or myometrial cells for proliferation and growth. We hypothesize that these cells rely on strikingly higher levels of steroid hormone receptors in surrounding differentiated myometrial and leiomyoma cells to mediate estrogen and progesterone action via paracrine signaling (Ono et al., 2012; Fig. 4).

The mechanism of steroid hormone paracrine action on leiomyoma stem-progenitor cells has not been fully elucidated. Recently, Ono et al. (2013) reported a critical role for the wingless-type (WNT)/ $\beta$ -catenin pathway in the communication between leiomyoma stem-progenitor cells and the surrounding differentiated cells. Treatment of

mature myometrial cells with estrogen and progesterone resulted in secretion of WNT ligands, which induced nuclear translocation of  $\beta$ -catenin in neighboring leiomyoma stem-progenitor cells and ultimately activated the expression of genes critical for growth and proliferation. Moreover, selective inhibition of WNT binding or  $\beta$ -catenin in leiomyoma stem-progenitor cells, but not in fully differentiated leiomyoma cells, significantly decreased tumor growth (Ono et al., 2013). The importance of  $\beta$ -catenin in myometrial stem cell function has previously been shown in an animal model, where myometrial stem cells differentiate into adipocytes in  $\beta$ -catenin-deficient uteri (Arango et al., 2005; Szotek et al., 2007). These findings are consistent with previous reports implicating the WNT/ $\beta$ -catenin pathway in leiomyoma formation and fibrogenesis (Tanwar et al., 2009; Lam and Gottardi, 2011; Gottardi and Konigshoff, 2013). Additionally, MED12 has previously been shown to regulate  $\beta$ -catenin/WNT signaling, further supporting its role in leiomyoma pathogenesis (Kim et al., 2006). On the other hand, treatment with progesterone has previously been shown to decrease WNT expression in the ovine uterus (Satterfield et al., 2008), so the role of steroid hormones in the WNT signaling pathway needs to be further clarified.

Much remains to be explored in leiomyoma stem-progenitor cells. Thus far, the presence of leiomyoma stem-progenitor cells has been



**Figure 4** Proposed role of stem-progenitor cells in leiomyoma pathogenesis. It has been proposed that a single myometrial stem-progenitor cell goes through tumorigenic transformation following a cellular insult and gives rise to daughter leiomyoma stem-progenitor cells, which proliferate, undergo self-renewal and clonally expand in response to steroid hormones via paracrine signaling from surrounding differentiated myometrial and leiomyoma cells. Pathways involved in the paracrine signaling have not been fully elucidated, but there is recent evidence for a critical role of the WNT/ $\beta$ -catenin pathway. Treatments targeting these paracrine interactions, or the stem-progenitor cells themselves, could not only treat existing leiomyoma, but also possibly prevent the development and growth of new tumors.



identified as the leiomyoma-derived side population (SP) cells, based on the universal ability of somatic stem cells to extrude Hoechst dye (Challen and Little, 2006; Jordan *et al.*, 2006). This technique has been successfully employed in multiple tissues throughout the body (Goodell *et al.*, 1996; Gargett and Masuda, 2010), however, the pitfalls of the SP technique for stem cell isolation are its expense and high sensitivity to slight variations in staining conditions, as well as the profound toxic effects of Hoechst staining on cell survival (Golebiewska *et al.*, 2011). To avoid these consequences and to further study the function and regulation of leiomyoma stem-progenitor cells, cell surface markers should be identified and used for isolation. Additionally, the molecular characteristics of leiomyoma stem-progenitor cells remain unknown. To further examine the function and regulation of this population of cells, an unbiased genome-wide investigation of their gene expression should be undertaken and will likely lead to new therapeutic targets (Fig. 4). Finally, the pathways that mediate paracrine signaling between leiomyoma cells and surrounding differentiated cells need to be further explored. Scientific direction may be taken from the breast cancer literature, which is a better-studied model of stem-progenitor cells that are deficient in ERs and PRs, but are hormone responsive via paracrine interaction with surrounding differentiated cells (Asselin-Labat *et al.*, 2010; Fillmore *et al.*, 2010; Roarty and Rosen, 2010; Montales *et al.*, 2012; Cittelly *et al.*, 2013; Vares *et al.*, 2013). For example, Cittelly *et al.* (2013) discovered that progesterone leads to expansion of stem-progenitor cells in breast cancer via down-regulation of miR-29 and subsequent up-regulation of KLF4. These results are particularly interesting for leiomyoma researchers in light of the aforementioned findings from Qiang *et al.* (2014) that steroid hormone suppression of miR-29 is necessary for leiomyoma growth, and may indicate that a similar mechanism is at play in leiomyoma stem-progenitor cells. Moreover, research performed on breast cancer stem-progenitor cells may shed light on potential therapeutics targeting leiomyoma progenitor-stem cells. For example, Montales *et al.* (2012) showed that dietary factors targeting the phosphatidylinositol 3-kinase/Akt signaling pathway can decrease expansion of stem-progenitor cells, and therefore decrease progression and recurrence in breast cancer.

## Conclusions

Until very recently, the knowledge that leiomyoma cells contained ERs and PRs led to assumptions about their pathophysiology involving primarily sex steroid stimulation. Over the past several years, the concept of the importance of stem-progenitor cells in the tumorigenesis of many cancers has been evolving, and this has recently been applied to leiomyomas, indicating that the story behind their growth is much more complex than previously thought. Much research has been dedicated to understanding the role of ovarian steroids in the pathogenesis of leiomyoma, and has led to the development of medical treatment options, such as aromatase inhibitors and antiprogestins. Unfortunately, none of these treatments result in complete regression of leiomyoma, and tumors recur after treatment is stopped. Finding an effective, long-term treatment for leiomyoma could have great public health implications, given their high prevalence and associated medical costs. The recent discovery of stem cells and their paracrine interactions with more differentiated cell populations within leiomyoma may provide the missing link between developing therapeutics that temper leiomyoma growth and those that eradicate them.

## Authors' roles

M.B.M., P.Y. and S.E.B. drafted the original manuscript. M.B.M., P.Y., M.O., J.S.C., M.T.D., A.N., E.E.M., D.C., J.J.K., J.J.W. and S.E.B. contributed to the research and interpretations of data discussed in the manuscript, revised the manuscript and approved the final version.

## Funding

National Institutes of Health Grant NIH/NICHD P01-HD057877.

## Conflict of interest

None declared.

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