# The Krüppel-like factors in female reproductive system pathologies

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## Abstract

Female reproductive tract pathologies arise largely from dysregulation of estrogen and progesterone receptor signaling, leading to aberrant cell proliferation, survival, and differentiation. The signaling pathways orchestrated by these nuclear receptors are complex, require the participation of many nuclear proteins serving as key binding partners or targets, and involve a range of paracrine and autocrine regulatory circuits. The members of the Krüppel-like factor (KLF) family of transcription factors are ubiquitously expressed in reproductive tissues and have been increasingly implicated as critical co-regulators and integrators of steroid hormone actions. Herein, we explore the involvement of KLF family members in uterine pathology, describe their currently known molecular mechanisms, and discuss their potential as targets for therapeutic intervention.

## Introduction

The human uterus has a unique role in the successful transmission of germ line DNA to guarantee the propagation of the human species. Biologically, it is destined to provide the fertilized egg with a 'nurturing' environment for its development and maturation into a complex entity with unique capabilities to eventually function on its own. Defects in the proper development and function of the uterus present a major hurdle to reproduction. Moreover, various uterine-related pathologies, including endometrial and cervical carcinoma, endometriosis, and leiomyoma, may arise post-puberty to further contribute to infertility. The steroid hormones estrogen and progesterone, working through their cognate nuclear receptors (estrogen receptor 1 (ESR1) and ESR2; progesterone receptor A (PGRA) and PGRB isoforms) are major regulators of uterine development and function (Kim *et al.* 2013, Hamilton *et al.* 2014). Their multi-faceted

transcriptional pathways involve interactions with numerous nuclear co-regulators (Dasgupta & O'Malley 2014) and result in altered levels of signaling molecules that act through paracrine and autocrine circuits. The elucidation of the underlying mechanism(s) for the autonomous and collective behavior of the multiple cell types of the uterus to maintain function, however, continues to be a work in progress, given recent discoveries of new participants and targets.

In this review, we highlight emerging evidence documenting the participation of the multi-member Krüppel-like factor (KLF) family of transcription factors and the dynamics of their transcriptional networks and roles in cellular communication in some uterine pathologies. The association of KLFs in ovarian carcinoma is similarly presented because the ovary is the major source of the nuclear receptor ligands estrogen and progesterone

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and because ovarian-related infertility is a major problem in reproductive medicine. Disentangling the various mechanistic points of action of KLFs in these pathologies may aid in the identification of key parameters for optimal reproductive function and contribute to the development of novel treatment strategies and clinical applications to address reproductive disorders.

## Krüppel-like factors

The Specificity-Protein-related KLFs contain 17 members of a family of DNA-binding transcriptional regulators with roles in cellular proliferation, survival, differentiation, pluripotency, and epithelial-to-mesenchymal interactions (Suske *et al.* 2005). We refer the reader to recent excellent reviews on this family (Tetreault *et al.* 2013, Knoedler & Denver 2014, Limame *et al.* 2014), which now also includes multiple biologically active KLF splice isoforms (Camacho-Vanegas *et al.* 2013) and the related gene *KLF18*

that is present in the sequenced genomes of most placental mammals (Pei & Grishin 2013). KLF proteins are characterized by a conserved DNA-binding domain with three tandem $C_2H_2$-type zinc finger motifs at the carboxy-terminus, which recognizes the GT/GC box or CACCC element sites in promoter/5′ regulatory and enhancer regions (Fig. 1A). In contrast to the carboxy-termini, the amino-terminal regions of member proteins are highly variable in length and sequence and contain domains (including acidic transactivation domains, SIN3-interacting repressor domains, and CtBP2-interacting repressor domains) that interact with specific co-activators and co-repressors (Kaczynski *et al.* 2003); the diversity in this region is thought to confer unique functions to each family member. Figure 1B illustrates the sequence homologies between the two highly related family members KLF9 and KLF13, where their respective C-terminal domains display highest similarities for both mouse and human proteins. Based on their phylogenetic
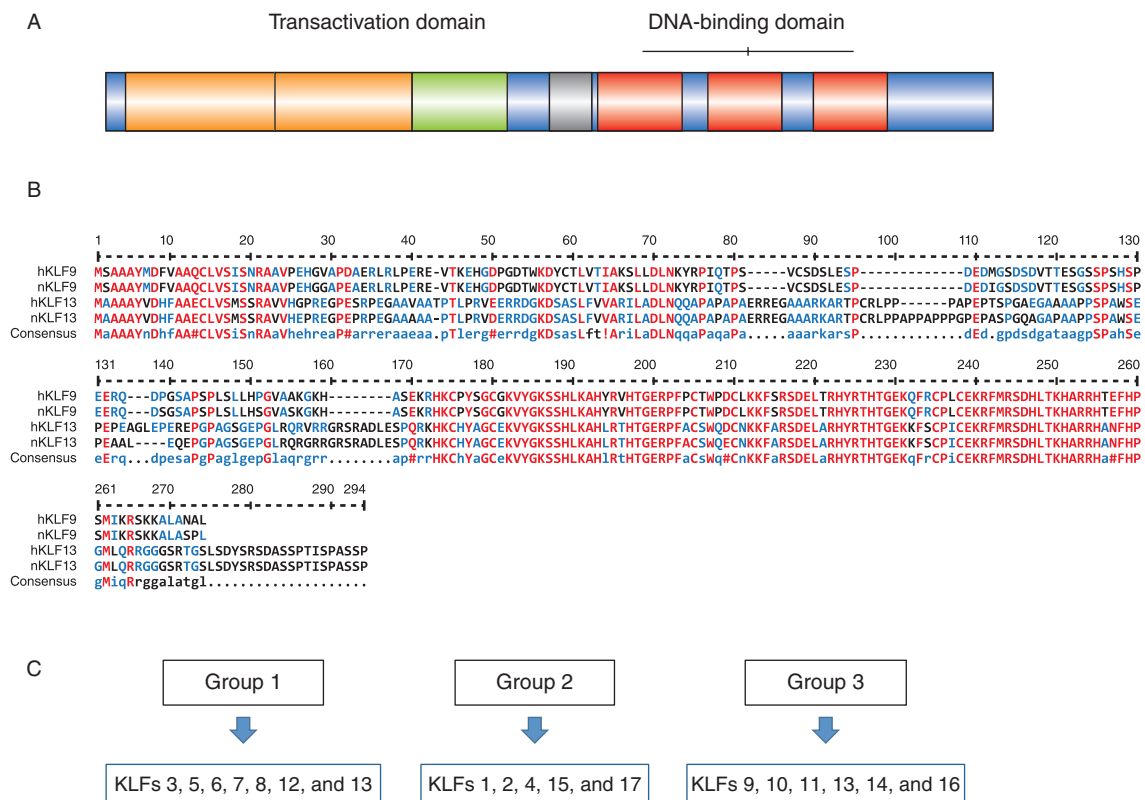


**Figure 1**

KLF members and their functional domains. (A) Schematic diagram of the highly variable amino-terminal (transactivation domain) and the highly conserved carboxy-terminal (DNA-binding domain) regions of KLF family proteins. (B) Sequence homologies among human and mouse KLF9 and KLF13 proteins. Invariant (red), conserved (blue), and variable (black) amino acid residues are indicated by single-letter codes. (C) KLFs are assigned to three sub-groups based on their phylogenetic relationships (Limame *et al.* 2014).

relationships (Limame *et al*. 2014), KLF members can be categorized into three sub-groups (Fig. 1C). Interestingly, proteins within the same categories do not typically exhibit similar functions or tissue expression (discussed below), reflecting their distinct regulation, transcriptional activator or repressor roles, and the probable diversity of their interacting proteins in tissue-specific contexts.

## KLFs in uterine and ovarian pathologies

Results from early studies indicated a potential role for KLFs in female reproductive tissues, with our laboratory's initial report on the cloning and expression of KLF9 in the uterus in pregnant pigs (Wang *et al*. 1997). Results of subsequent investigations using *Klf9*-null mice indicated that the global loss of KLF9 expression, while not lethal to embryos, caused a subfertility phenotype characterized by reduced numbers of post-implantation embryos (Simmen *et al*. 2004) and was associated with decreased proliferation and increased apoptosis (glandular and luminal epithelial, stromal) and partial progesterone-resistance (stromal) of endometrial cells during the peri-implantation window (days *post-coitum* 2.5–3.5) when compared with WT counterparts (Velarde *et al*. 2005). By using endometrial tissues of WT and *Klf9*-null mice and *KLF9*-siRNA targeting of a human endometrial stromal cell line (HESC; Krikun *et al*. 2004), the mechanistic underpinnings for the aberrant proliferative and apoptotic status with KLF9 loss-of-expression were partly attributed to disruptions in the temporal patterns of expression of the Wnt signaling

pathway components *BMP2*, *PGR* (specifically the *PGR-B* isoform), and insulin-like growth factor-binding protein 1 (*IGFBP1*) (Pabona *et al*. 2010). These collective findings provide robust support for the relevance of KLF9 and raise the likelihood of the participation of other KLFs in uterine PGR and Wnt signaling, both of which are major regulators of cellular proliferation, survival, and differentiation.

Uterine and ovarian pathologies that have now been linked to deregulated expression of KLF family members in women and in mouse models are listed in Table 1. It is worth noting that: i) the attenuated expression of multiple KLFs (KLFs 2, 4, 5, 6, 9, and 11) with a few exceptions is relevant to ovarian, endometrial, cervical, and/or myometrial pathologies; ii) the absence of several KLFs in distinct pathologies (e.g. KLF9 and KLF4 in endometrial cancer and endometriosis; KLF9 and KLF11 in endometriosis and leiomyoma) indicates roles for multiple KLFs in maintaining homeostasis in female reproductive tissues; iii) the loss of specific KLFs in various disease states occurs irrespective of their phylogenetic categories (e.g. *KLF6* (group 1), *KLF2* and *KLF4* (group 2) and *KLF9* (group 3) in ovarian cancer; *KLF4* (group 2) and *KLF9* (group 3) in endometrial cancer and endometriosis), implicating distinct KLF-interacting proteins and gene targets as underlying common pathologies; and iv) *KLF13* does not appear to be associated with any of the disorders attributed to *KLF9*, indicative of these proteins' distinct molecular regulation and function. In this regard, KLF13 expression was not altered in endometrial tumors relative to adjacent non-tumor

**Table 1** Female reproductive dysfunctions and dysregulated KLF expression in humans (h) and mouse (m) models

| Pathology | KLF (species) | Over (↑)/under (↓) expression | References |
|---|---|---|---|
| Endometrial cancer | KLF4 (h) | ↓ | Simmons *et al*. (2011) |
| | KLF 9 (h) | ↓ | Simmen *et al*. (2008), Simmons *et al*. (2011) and Korani *et al*. (2013) |
| | KLF17 (h) | ↑ | Dong *et al*. (2014) |
| Ovarian cancer | KLF2 (h) | ↓ | Wang *et al*. (2005) |
| | KLF4 (h) | ↓ | Yoon & Roh (2012) |
| | KLF6 (h) | ↓ | DiFeo *et al*. (2006a,b) |
| | KLF9 (h) | ↓/↑ | Huang *et al*. (2014) and Zhang *et al*. (2014a) |
| Cervical cancer | KLF4 (h) | ↓ | Yang & Zheng (2014) |
| | KLF5 (h) | ↑ | Marrero-Rodríguez *et al*. (2014) |
| Endometriosis | KLF4 (h) | ↓ | Adammek *et al*. (2013) |
| | KLF9 (h, m) | ↓ | Lee *et al*. (2008), Pabona *et al*. (2012) and Heard *et al*. (2014) |
| | KLF11 (h, m) | ↓ | Daftary *et al*. (2013) |
| Leiomyoma | KLF9 (h) | ↓ | Rackow & Taylor (2010) |
| | KLF11 (h) | ↓ | Yin *et al*. (2010) |
| Implantation/pregnancy | KLF5 (m) | ↓ | Sun *et al*. (2012) |
| | KLF9 (m) | ↓ | Simmen *et al*. (2004) |
| Labor dysfunction | KLF9 (h, m) | ↓ | Zeng *et al*. (2008) and Pabona *et al*. (2015) |

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**Table 2** Human cell lines used to model reproductive dysfunctions associated with dysregulated KLF expression

| Pathology | Cell line | KLF | References |
|---|---|---|---|
| Endometrial cancer | EM/PR | KLF4 | Shimizu et al. (2010) |
| | Ishikawa, EEC, and HEC-1A | KLF9 | Simmen et al. (2008) and Simmons et al. (2011) |
| | EEC | KLF17 | Dong et al. (2014) |
| Ovarian cancer | OV202 and SKOV3 | KLF2 | Wang et al. (2005) |
| | SKOV3 and OVCAR3 | KLF4 | Yoon & Roh (2012) and Chen et al. (2014) |
| | SKOV3 | KLF5 | Dong et al. (2013) |
| | T80 and SKOV3 | KLF8 | Lu et al. (2012) |
| | SKOV3 and OVCAR3 | KLF9 | Zhang et al. (2014a) |
| Endometriosis | 12Z | KLF4 | Adammek et al. (2013) |
| | HESC | KLF9 | Pabona et al. (2012) |
| Implantation defects | HESC | KLF9 | Pabona et al. (2010) |
| | HESC | KLF12 | Shen et al. (2013) |
| Labor dysfunction | HutSMC | KLF9 | Pabona et al. (2015) |

tissue in women with endometrial cancer (Simmons et al. 2011). Moreover, *Klf13*-null mice did not exhibit the subfertility and prolonged labor phenotypes found for *Klf9*-null mutants (Heard et al. 2012).

Given the paucity of currently available mouse models and limited access to human tissues for studying KLF function in the uterus and ovary, human cell lines that model reproductive disease states have been used to dissect mechanisms of action of particular KLFs. These cell lines are summarized in Table 2. The human Ishikawa, endometrial endocarcinoma (EEC), and human endometrial carcinoma-1A (HEC-1A) cell lines have been investigated as models for endometrial carcinoma. The ovarian cancer cell lines OV202, SKOV3, OVCAR3, and, to a limited extent, T80 have been employed to model ovarian cancer. Furthermore, the HESC line, generated by overexpression of human telomerase and shown to be progesterone-responsive (Krikun et al. 2004), is commonly used as a paradigm for HESCs during early pregnancy, due to their ability to decidualize *in vitro* after treatments with a cocktail of cAMP, estrogen, and progesterone, and can be evaluated for poor decidual response upon targeting specific *KLF* siRNA (Pabona et al. 2010, Shen et al. 2013). To mimic the labor dysfunction observed with *Klf9*-null mice (Zeng et al. 2008), the response of a recently generated human uterine smooth muscle cell line HutSMC was tested in estrogen+progesterone-treated cells without or with *KLF9* siRNAs (Pabona et al. 2015). While such studies have resulted in the identification of common and distinct pathways for KLFs (Fig. 2), there are acknowledged limitations of the use of cell lines with regard to extending relevance to the whole organism, providing impetus for generation of new and reproductive-system-targeted mouse models to further elucidate the dynamics of KLF actions *in vivo*.

## KLFs and targeted signaling pathways in uterine pathologies

As KLFs are known to regulate cell proliferation, survival, and differentiation, it is quite expected that their reduced expression in many uterine diseases (Table 1) will be associated with perturbations in signaling pathways for PGR and ESR, Wnt, Notch, Hedgehog (Hh), immune activation, and epithelial-to-mesenchymal transitions, all of which are requisite for maintenance of uterine integrity and function (Fig. 2). Whether cross-talk between these pathways is mediated by actions of KLFs is not completely understood, albeit limited reports of results supporting this possibility for PGR and the Notch/Hh signaling pathways. In one study, ectopic lesions formed from *Klf9*-null endometrial tissues in a mouse model displayed activated Notch and Hh signaling and conversely reduced PGR expression (Heard et al. 2014). Moreover, eutopic endometria of women with endometriosis, a disease state characterized by loss of progesterone-sensitivity, display reduced KLF9 (Pabona et al. 2012) and enhanced Notch 3 (Tamaresis et al. 2014) expression. Reduced progesterone-sensitivity with the loss of KLF9 is in part due to KLF9's role as a PGR-interacting protein (Zhang et al. 2002, 2003) and its promotion of estrogen-dependent ESR1 down-regulation (Velarde et al. 2007). As regards progesterone/PGR–Notch–Hh signaling pathways, their opposing and complementary associations in endometrial cells have been demonstrated. For example, the transcript levels of the Notch ligand, Delta-like 4 are reduced by medroxy-progesterone acetate in primary cultures of human endometrial glandular and stromal cells (Mazella et al. 2008). Moreover, the Hh ligand Indian Hh is a negatively regulated downstream target of progesterone/PGR (Simon et al. 2009). On the other hand, Notch 1 has been shown
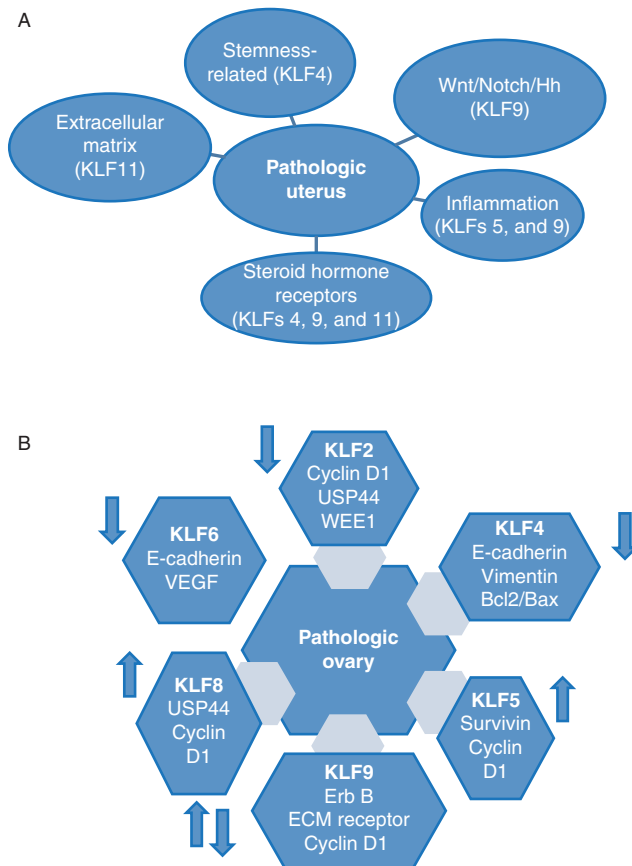
**Figure 2**
Signaling pathways and gene targets associated with dysregulated expression of KLF family members in the pathologic uterus (A) and ovary (B). Arrows in B (↑ and ↓) signify upregulated and downregulated expression of each KLF in ovarian carcinoma.

to mediate progesterone-dependent uterine stromal cell differentiation in primates and mice (Afshar *et al.* 2012*a,b*). In addition, progesterone increased the levels of transcriptionally active Notch 1 intracellular domain, which can form a functional complex with PGR (Afshar *et al.* 2012*a*). The potential complexity of the regulatory networks involving PGR and Notch/Hh signaling indicates that no single mechanism may fully account for each KLF acting through these pathways.

A core KLF circuitry comprising KLF2, KLF4, and KLF5 has been recently implicated in the regulation of the self-renewal of embryonic stem cells involving key pluripotency genes (Jiang *et al.* 2008). By regulating adult stem cell signaling pathways (e.g. Wnt and Notch), KLFs may similarly control the regenerative capacity of endometrial and myometrial stem/progenitor cells. In this regard, endometriosis (Sasson & Taylor 2008) and leiomyoma (Ono *et al.* 2014) are increasingly considered to be a

consequence of deregulated stem cell expansion. Indeed, endometrial epithelial stem/progenitor cells have been characterized from eutopic endometrium of women with endometriosis (Li *et al.* 2014), ovarian endometriotic cysts (Chan *et al.* 2011), endometrial carcinoma tissues (Hubbard *et al.* 2009), and uterine leiomyoma (Ono *et al.* 2012). KLF4 is a well-acknowledged regulator of stem cell biology and is the most highly implicated KLF in both cancer and normal stem cells (Tetreault *et al.* 2013). KLF4 also mediates the action of PGR in human endometrial epithelial cells (Shimizu *et al.* 2010), albeit unlike KLF9, KLF4 has not been shown to interact with PGR. However, KLF4 expression (Adammek *et al.* 2013), similar to that of KLF9 (Pabona *et al.* 2012), is reduced in the eutopic endometria of women with endometriosis (relative to those of women without the disease) and in endometrial tumors relative to adjacent normal tissues (Simmons *et al.* 2011). Results from recent studies have indicated that the loss of KLF9 expression promotes neurosphere formation (an *in vitro* measure of 'stemness') in neuroblastoma cells (Ying *et al.* 2011); this involved activation of Wnt signaling and KLF9 transcriptional repression of integrin-α6 expression (Ying *et al.* 2014). While the results of the studies described above provide causative support for loss of KLF9 expression in the aberrant promotion of 'stemness', direct evidence for the involvement of KLF9 and KLF4 in uterine diseases remains to be fully characterized.

Several KLFs have been directly linked to the regulation of inflammatory signaling, defects of which may contribute to uterine pathology. In particular, uterine-specific *Klf5*-null mice are infertile due to aberrant expression of the prostaglandin synthesis gene *Ptgs2*, resulting in the enhanced expression of COX2 (Sun *et al.* 2012). Similarly, KLF11, the attenuated expression of which is linked to uterine leiomyoma, has been reported to inhibit prostaglandin $E_2$ synthesis by transcriptionally silencing the promoter of the gene encoding phospholipase $A_{2\alpha}$, the key enzyme for prostaglandin biosynthesis (Buttar *et al.* 2010). Furthermore, KLF4 was shown to stimulate monocyte differentiation in the human acute myeloid leukemia cell line HL60 (Alder *et al.* 2008) and to enhance macrophage activation in the macrophage cell line J774a (Feinberg *et al.* 2005), indicating a role in immune modulation that is critical for uterine function. In women, prolonged pregnancy is associated with reduced expression of KLF9 and with aberrant downregulation and upregulation of several pro-inflammatory and anti-inflammatory genes respectively (Pabona *et al.* 2015). Given that a number of inflammation-associated genes are direct PGR targets (e.g. *IL11* and *CXCL1*;

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Cordeaux *et al.* 2010, Kavandi *et al.* 2012), results indicate that the deregulated expression of numerous inflammatory mediators may be a direct outcome of aberrant PGR signaling involving KLFs. In a recent study, Rogatsky and colleagues (Chinenov *et al.* 2014) have described the functional cooperation between the glucocorticoid receptor and KLF2 and KLF9 in macrophages during inflammation. As the glucocorticoid receptor can mediate the effects of progestin on uterine inflammatory response (Guo *et al.* 2012, Lei *et al.* 2012), The interaction of KLF with progesterone-dependent transcriptional circuitry is a possible node by which KLFs may exert their control over inflammatory events in the uterus.

Additional pathways that have been linked to KLFs and that may underlie a number of uterine pathologies when these KLFs are aberrantly expressed include: the promotion by KLF17 of epithelial-to-mesenchymal transitions through induction of TWIST1 in endometrial cancer (Dong *et al.* 2014); the coactivation by KLF6 of NFκB signaling via its induction of the cytokines tumor necrosis factor α and interleukin 6 (Zhang *et al.* 2014*b*) in the pathogenesis of endometriosis; KLF5-mediated activation of the JAK–STAT signaling pathway (Tetreault *et al.* 2012), the latter being a key mediator of leukemia inhibitory factor that controls embryo implantation and hence successful pregnancy (Rosario *et al.* 2014); and KLF14- (de Assuncao *et al.* 2014) and KLF11- (Zheng *et al.* 2014) mediated activation of lipid and metabolic signaling, respectively, processes that when dysregulated can lead to abnormal metabolism and increased risk of endometrial cancer.

Reproductive aging is a natural biological process and does not fall into the category of a uterine pathology (Nelson *et al.* 2013); however, societal demands based on a woman's choice to time her pregnancy have raised the need to further understand age-related co-morbidities of the uterus and ovary, which can be modified for successful pregnancy outcome. To begin to evaluate a potential role for KLFs in this process, we measured the transcript levels of several KLF family members in uteri of young (8 months; $n=7$) and old (27 months; $n=7$) C57BL/6 mice by quantitative RT-PCR. Of the nine KLFs analyzed, only the mRNAs for *Klf9* and *Klf4* displayed significant differences as a function of age; the levels of *Klf8*, *Klf13*, and *Klf15* transcripts only showed tendencies towards differences (Fig. 3). Using a stem-cell-focused PCR array, we also searched for potential differentially regulated genes associated with the aging uterus. The transcripts of a number of cell-cycle regulators, cytokines, and self-renewal markers were distinctly regulated during aging (Table 3). While it is premature to establish a correlation between aging and the KLFs based on the results of this pilot
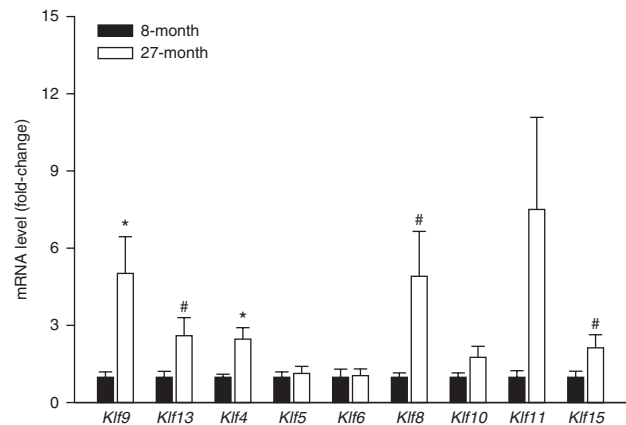


**Figure 3**
Expression of selected KLFs in uteri of young and aging mice. Transcript levels of various KLFs were quantified in uteri of 8- and 27-month-old C57BL/6 mice by quantitative RT-PCR. Data (mean ± s.e.m.) are expressed as fold-change and were obtained from $n=7$ individual mice/age group. Transcript levels were normalized to corresponding levels of 18S RNA, and then to controls (8-month-old uteri) and were calibrated to a standard curve using pooled cDNA stocks. *Significant at $P<0.05$, by *t*-test; #, tending to significance with $0.05<P<0.10$.

study, the well-recognized reductions in the reservoir of uterine stem cells with aging together with the suggested roles of KLFs in stem cell self-renewal provide a reasonable basis for utilizing the aging uterus as a unique model to further evaluate a potential link between stem cell biology and KLFs.

## KLFs and targeted signaling pathways in ovarian pathologies

It is notable that for those mice with global null-mutations of specific KLFs (e.g. KLF9, KLF11, and KLF13) and surviving through adulthood, an ovarian phenotype characterized by dysfunctions in steroid hormone synthesis is not manifested throughout the reproductive years (Simmen *et al.* 2004, Zeng *et al.* 2008, Heard *et al.* 2012, Daftary *et al.* 2013). This finding is not congruent with the demonstrated regulation of transcript levels of several key steroidogenic genes (*LDLR*, *STAR*, and *CYP11A*) by KLF13 in ovarian granulosa cells (Natesampillai *et al.* 2008). Interestingly, the pathological ovary (i.e. ovarian carcinoma) is characterized by reduced (*KLF2*, *KLF4*, and *KLF6*) and enhanced (*KLF5* and *KLF8*) expression of several KLFs; contradictory results have been reported for *KLF9* (Fig. 2B). Analyses of currently identified target genes associated with dysregulation of distinct KLF expression in ovarian cancer cells revealed perturbations in those related to proliferation and differentiation (cyclin D1); apoptosis (*Bcl2*, *Bax*, and survivin); epithelial-to-mesenchymal

**Table 3** Differentially expressed genes in the aging uterus

| Genes[a] | Fold-change[b] |
|---|---|
| **Cell cycle regulators/Wnt signaling** | |
| *Apc* | −2.10 |
| *Axin1* | −2.75 |
| *Ccna2* | −8.40 |
| *Ccnd1* | −7.36 |
| *Ccne1* | −4.99 |
| *Myc* | −2.48 |
| **Cytokines/growth factors** | |
| *Bmp1* | −6.06 |
| *Cxcl12* | −4.56 |
| *Fgf1* | 2.17 |
| *Fgf2* | −2.64 |
| *Igf1* | −3.81 |
| **Self-renewal markers** | |
| *Cd44* | −9.19 |
| *Msx1* | −7.26 |
| *Jag1* | −1.64 |
| *Hspa9* | −2.58 |
| *Myst1* | −3.48 |

[a]Identified using stem cell signaling qPCR array.
[b]Aging versus young uterus: (−) down-regulation.

interactions (E-cadherin, vimentin, and extracellular matrix receptor); stem cell differentiation (USP44, ErbB); and angiogenesis (VEGF). These findings raise important questions regarding how KLFs alone or together may integrate the physiological processes in the ovary and whether pathways defined for uterine pathologies in which multiple KLFs (e.g. KLF4 and KLF5) are similarly dysregulated may be relevant to ovarian diseases.

## KLF networks: a case for and against functional redundancy

As KLF expression is ubiquitous, yet known reproductive system pathologies appear to involve selected subsets of KLFs (Table 1), functional redundancies and compensatory regulation among KLFs must exist to ensure robust physiological responses to cellular perturbations for maintaining homeostasis. Results of recent elegant studies have been used to demonstrate this concept for KLF3 and KLF8 in a nonreproductive (i.e. erythroid) system (Eaton *et al.* 2008, Funnell *et al.* 2013). The lack of distinct uterine phenotypes in mouse knockout models for several genes encoding KLFs supports this concept for the reproductive tract. A prime example involves the highly related members KLF9 and KLF13. Although the ability to draw a definitive conclusion is limited by the lack of functional studies of mice deficient for both KLFs, support for a KLF9–KLF13 genetic interaction comes from findings that *Klf9*-null mouse uteri at peri-implantation displayed increased *Klf13* expression, which

was confirmed using *siKLF9*-targeted HESCs (Pabona *et al.* 2010). Moreover, *Klf13*-null mice are reproductively normal, perhaps due to the accompanying increase in nuclear KLF9 protein levels shown for *Klf13*-null endometrial cells (Heard *et al.* 2012). Thus, the absence of an association between KLF13 and any reproductive dysfunctions reported to date (Table 1) may be a consequence of the placement of KLF9 at a higher level of the functional hierarchy relative to KLF13. In this scenario, potential transcriptional dysregulation that may occur with loss of KLF13 expression is abrogated by the compensatory actions of KLF9.

The co-reduction in KLF9 and KLF4 expression noted in endometrial cancer and in endometriosis and those of KLF9 and KLF11 in endometriosis and in leiomyoma (Table 1) on the other hand supports the concept of distinct programs of gene expression being controlled by these KLFs. Alternatively, this may indicate that there is an obligatory pathway that is mediated by two KLFs occurring through a linear mechanism. There is evidence for the latter possibility, at least for KLF9 and KLF4. Knockdown of *KLF9* with siRNA in the HEC Ishikawa cell line reduced the levels of *KLF4* transcripts (Simmons *et al.* 2011) and conversely, overexpression of *KLF9* in HEC-1A cells induced the expression of *KLF4* (Simmen *et al.* 2008); these observations are in accordance with the hypothesis (albeit yet to be proven) that KLF4 serves as a downstream target of KLF9 either directly or indirectly. Parallel transcriptome and ChiP-Seq analyses of uterine cells subjected to *siKLF9* and *siKLF4* targeting, alone and in combination, will be required to identify unique and shared networks regulated by the two KLFs and could provide an insight regarding whether KLF4 is an early target of KLF9. Importantly, such studies may allow the identification of an obligate response (gene target, signaling pathway) mediated by both KLFs. With regards to KLF9 and KLF11, there are limited data to support or refute redundant functions; however, based on their distinct reproductive phenotypes upon targeted gene inactivation (*Klf11*-null mice breed normally and are fertile in contrast to *Klf9*-null mice that are subfertile; Simmen *et al.* 2004, Song *et al.* 2005) and the distinct mechanisms by which they mediate PGR transactivity (Zhang *et al.* 2003, Yin *et al.* 2010), they are likely to differentially mediate PGR-driven transcriptional events in uterine cells.

The opposing actions of KLF4 and KLF15 in uterine epithelial cells constitute additional support for non-redundant functions of KLF family members. In these cells, KLF4 and KLF15 are inversely expressed, and are found to discretely regulate initiation of DNA synthesis by virtue of their distinct responses to estrogen- and

progesterone-treatments (Ray & Pollard 2012). By inhibiting estrogen-enhanced transcription of the DNA synthesis initiator protein minichromosome maintenance 2, KLF15 functions as a downstream mediator of progesterone-inhibition of the cell cycle. The factors that direct the inverse expression of KLF4 and KLF15 and their opposing responses to steroid hormones in the uterine epithelium have yet to be determined. Clearly, the biology underlying optimal uterine function involving KLF regulatory networks is wide-open for further investigations.

## Regulation of KLF expression

The factors that contribute to the aberrant expression and activity of KLFs in reproductive tract pathology have not been well-characterized, in contrast to other systems. In embryonic stem cells, induction of KLF2 by *OCT4* and of KLF4 by *LIF* has been demonstrated, providing further evidence supporting these KLFs' function in stem cell renewal (Hall *et al.* 2009). KLF4 expression was suppressed by the transcription factor FOXO in B-lymphocytes (Yusuf *et al.* 2008) and by an inhibitor of Notch signaling in the mouse gastrointestinal tract (Zheng *et al.* 2009) and, conversely, was induced by Notch 1 intracellular domain in ocular surface epithelia (Zhang *et al.* 2013). KLF6 expression was stimulated by IGF1 in human colon cancer cell lines (Bentov *et al.* 2008), and the binding of carbohydrate-response-element-binding protein, a glucose-activated transcription factor, induced KLF10 promoter activity and expression in rat hepatocytes (Iizuka *et al.* 2011). The identity of factors that regulate KLF expression in uterine cells is currently limited to that of KLF9 in HESCs; in these cells, *BMP2* inhibited KLF9 expression indirectly through KLF13 (Pabona *et al.* 2010) while estrogen and progesterone had no influence on its expression (Pabona *et al.* 2012). In ovarian granulosa cells, *IGF1* and luteinizing hormone (*LH*) were reported to increase the expression of KLF13 (Natesampillai *et al.* 2008). Comprehensive analyses of cellular components responsible for maintaining KLF expression will be required in order to understand and ultimately manipulate KLF regulatory circuits for optimal reproductive function.

## The next steps: a perspective

In the last decade, multiple molecular pathways mediated by KLFs have been elucidated in uterine and ovarian cells and tissues. Nevertheless, direct evidence linking KLF effects to health outcomes and disease states remains elusive. How may we address this gap in knowledge? In most cases, the difficulty lies in the absence of mouse models that recapitulate the human disease and in the possible biological redundancies among subsets of KLFs that may prevent abnormal responses being observed when one KLF is absent. Thus, using relevant cell lines *in vitro* by siRNA targeting and by characterizing uterine (or ovarian)-targeted KLF-combination knockouts *in vivo*, it is imperative to establish which subsets of KLFs compensate for each other. Many of the mouse mutants for KLFs have modest or no reproductive phenotypes when they survive to adulthood (e.g. *Klf9-*, *Klf11-*, and *Klf13*-null mice). For other KLFs, homozygous disruptions result in early embryo (for *Klf4*, *Klf5*, and *Klf6*), *in utero* (for *Klf2*), and neonatal (for *Klf7*) lethality (Wani *et al.* 1999, Laub *et al.* 2006, Matsumoto *et al.* 2006, Ema *et al.* 2008). Therefore, for these KLFs, conditional mutations using uterine epithelial-, stromal-, and myometrial-tissue-specific promoters driving the Cre-recombinase may serve as a powerful strategy for studying gene function in each cell type. Such studies are anticipated to be labor-intensive and complex, given that the uterus has multiple cellular compartments and that several KLFs exhibit preferential cellular expression (e.g. KLF9 in endometrial stroma and myometrium; Simmen *et al.* 2004). Indeed, the complexity of 'teasing out' the details of KLF signaling in each compartment is best illustrated when one considers that for the progesterone/PGR signaling pathway alone, distinct KLFs are involved either as regulators or integrators of progesterone/PGR transactivity, albeit not necessarily in the same physiological contexts (Fig. 4). To date, proliferative, survival, and pro-/anti-inflammatory molecular signatures elicited by each KLF family member have not been defined when null-mutated in specific uterine compartments. The power of increasingly sophisticated approaches such as ChiP-Seq, various 'omics' technologies, and precise genome editing methodologies using engineered nucleases offered by the clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) proteins should be harnessed to address this question.

So why study KLFs in the face of their seeming complexity? In this review, the present data indicating i) their association with many reproductive disorders, whose etiologies are not well-understood; ii) their control of a plethora of signaling pathways; and iii) the considerable diversity of their target genes due to their ability to act as transcriptional activators or repressors, collectively indicate their prominent roles as integrators of uterine (and ovarian) biology. Perhaps an exciting direction for KLF research is one that focuses on their transcriptional roles in uterine and ovarian stem cell biology. It is well-known that the endometrium displays dramatic regenerative properties,
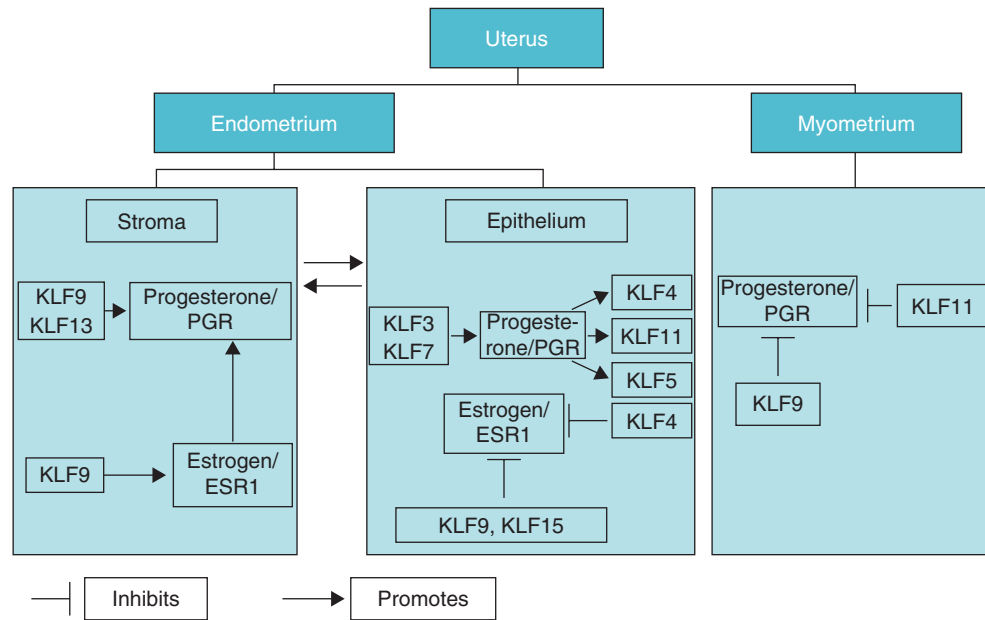
**Figure 4**

Complex and redundant control of PGR and ESR1 signaling by KLFs in distinct uterine compartments. Promotion or inhibition of PGR and ESR1 activity may occur by direct or indirect mechanisms. Arrows originating from progesterone/PGR (in epithelium) indicate KLFs acting as integrators of progesterone/PGR signaling. Bi-directional arrows between stroma and epithelium signify the dynamic communication between the two endometrial compartments.

estimated to occur approximately 400-times during a woman's reproductive years; these have been linked to the presence of adult stem cells displaying key properties of mesenchymal stem cells (Figueira *et al.* 2011, Spitzer *et al.* 2012). In a recent study, Taylor and colleagues (Sakr *et al.* 2014) demonstrated that mesenchymal stem cells are recruited to endometriosis lesions and that reduction of this recruitment can diminish lesion incidence. Similarly, a small population of cells (approximately 1% of tumor cells) showing stem-progenitor properties was found to be essential for estrogen+progesterone-dependent growth of uterine leiomyomas (Ono *et al.* 2012). Interestingly, the growth of this cell population involves ESR/PGR–Wnt signaling pathway crosstalk via estrogen+progesterone-induced β-catenin translocation, leading to *Axin2* promoter activation (Ono *et al.* 2013). As loss of *KLF11* expression is associated with increased PGR signaling and proliferation of leiomyoma cells (Yin *et al.* 2010), it is tempting to consider that inhibition of the aberrant expansion of myometrial smooth muscle stem cells by KLF11 may avert tumor initiation and leiomyoma.

How will understanding the biology of KLFs lead to novel and more effective therapies for female reproductive disorders? To date, treatment options for most uterine disorders involve aromatase inhibitors and progestins; however, prolonged treatment with these agents can result in drug resistance, with disease recurring often after cessation of treatment. If current results indicating that KLFs integrate progesterone/PGR and estrogen/ESR crosstalk with Notch–Wnt pathways to control aberrant stem/progenitor cell proliferation are verified, it may be possible to develop non-steroidal treatments that target specific 'stemness' factors such as the Notch ligand *JAGGED1*, which promote the survival of this subpopulation and therefore progression/recurrence of uterine pathologies. Thus, targeting Notch signaling using γ-secretase inhibitors that inhibit the intracellular localization of the transcriptional mediator Notch intracellular domain may offer a viable therapeutic strategy. A proof-of-concept for the latter has been recently performed for uterine serous carcinoma in a human xenograft model in mice (Groeneweg *et al.* 2014). In a recent report, small-molecule inhibitors of the expression of the colorectal cancer oncogene *KLF5* has been identified by high-through-put screening of compound libraries (Bialkowska *et al.* 2011). The isolated compounds, screened using a rat intestinal cell line stably expressing a luciferase reporter driven by the human *KLF5* promoter, reduced endogenous KLF5 protein levels and decreased the viability of a number of colorectal cancer cell lines. A similar strategy may also be employed to elude reproductive pathologies, although compounds promoting, rather than inhibiting, KLF expression will need to be identified because uterine pathologies are mostly

associated with reduced KLF expression (Table 1). Such approaches could yield novel research outcomes valuable for translation into clinical applications.

Finally, it is worth noting that the major male reproductive disease, namely prostate cancer, is also highly associated with dysfunctions in numerous KLFs including KLF4 (Wang *et al.* 2010), KLF5 (Frigo *et al.* 2009), KLF6 (Narla *et al.* 2001), KLF8 (He *et al.* 2013), and KLF9 (Shen *et al.* 2014). Importantly, a number of signaling pathways reported for KLF (dys)regulation of prostate epithelial cell proliferation, differentiation, and survival overlap with those elucidated for KLF-mediated uterine function. In particular, KLFs have been reported to participate in androgen-receptor-dependent signaling (Liu *et al.* 2012, He *et al.* 2013), the male counterpart of PGR/ESR signaling in females, in regulation of Hh pathway components (Leow *et al.* 2009), and in stem cell signaling involving the Notch pathway (Oktem *et al.* 2014). However, no KLFs have been demonstrated so far to be indispensable for spermatogenesis.

## Conclusion

The growing evidence for the functional and correlative association of KLFs in various female (and male) reproductive pathologies underscores the importance of extending and expanding current knowledge of this multi-faceted transcription factor family in reproductive health. New possibilities for targeting KLFs may soon be available from reproductive-system-wide analysis of KLF signaling. Treatment of other reproductive pathologies, including preeclampsia, fallopian tube cancers, and recurrent pregnancy loss, as well as male infertility, may similarly be benefited by an understanding of KLF biology.

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