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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. Members of the Krüppel-like 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. Here 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.

Keywords

KLF; endometrial pathologies; progesterone; Notch; Wnt

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

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Declaration of interest

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steroid hormones estrogen (E) and progesterone (P), working through their cognate nuclear receptors [estrogen receptor (ESR) 1 and ESR2; progesterone receptor (PGR) -A and PGR-B isoforms] are major regulators of uterine development and function (Hamilton *et al*., 2014, Kim *et al*., 2013). Their multi-faceted transcriptional pathways involve interactions with numerous nuclear co-regulators (Sangupta & O'Malley, 2014) and result in altered levels of signaling molecules that act through paracrine and autocrine circuits. 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 select uterine pathologies. The association of KLFs in ovarian carcinoma is similarly presented since the ovary is the major source of the nuclear receptor ligands E and P 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.

Kruppel-like Factors

The Specificity Protein (SP)-related Krüppel-like factors (KLFs) is a 17-member family of DNA-binding transcriptional regulators of cellular proliferation, survival, differentiation, pluripotency and epithelial-mesenchymal interactions (Suske *et al*., 2005). We refer the reader to recent excellent reviews on this family (Tetrault *et al*., 2013; Knoedler & Denver, 2014; Limame *et al*., 2014), which now also includes multiple biologically active KLF splice isoforms (Camacho-Venegas *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 and 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, Sin-3 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 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 and tissue expression (discussed below), reflecting their distinct regulation, transcriptional activator or repressor roles, and the likely diversity of their interacting proteins under tissue-specific contexts.

KLFs in Uterine and Ovarian Pathologies

Early studies suggested a potential role for KLFs in female reproductive tissues, with our laboratory's initial report on the cloning and expression of KLF9 in the pregnant pig uterus (Wang *et al*.,1997). Subsequent investigations using *Klf9* null mice showed that the global loss of KLF9 expression while non-embryo lethal, caused a subfertility phenotype characterized by reduced numbers of post-implantation embryos (Simmen *et al*., 2004) and which was associated with decreased proliferation and increased apoptosis (glandular and luminal epithelial, stromal) and partial P-resistance (stromal) of endometrial cells during the peri-implantation window (day *post-coitum* 2.5 to 3.5) when compared to wildtype counterparts (Velarde *et al*., 2005). By using WT and *Klf9*-null mice endometrial tissues 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 component *BMP2*, *PGR* (specifically the *PGR-B* isoform), and *IGFBP1* (Pabona *et al*., 2010). These collective findings provide robust support for the relevance of KLF9 and raise the likelihood for the participation of other KLFs, in uterine PGR and Wnt signaling, both of which are major regulators of cellular proliferation, survival and differentiation.

Table 1 lists uterine and ovarian pathologies that have now been linked to deregulated expression of KLF family members in women and in mouse models. It is worth noting that: 1) the attenuated expression of multiple KLFs (KLFs 2, 4, 5, 6, 9 and 11) with a few exceptions are relevant to ovarian, endometrial, cervical and/or myometrial pathologies; 2) 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) suggests roles for multiple KLFs in maintaining homeostasis in female reproductive tissues; 3) the loss of specific KLFs in various disease states occurs irrespective of their phylogenetic categories (e.g., KLF6 (Group 1), KLFs 2 and 4 (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 to underlie common pathologies; and 4) KLF13 does not appear to be associated with any of the disorders attributed to KLF9, suggesting these proteins' distinct molecular regulation and function. In this regard, KLF13 expression was not altered in endometrial tumors relative to adjacent non-tumor 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. Further, the human endometrial stromal cell line HESC, generated by overexpression of human

telomerase and shown to be P-responsive (Krikun *et al.*, 2004) is commonly used as a paradigm for human endometrial stromal cells during early pregnancy, due to their ability to decidualize *in vitro* after treatments with a cocktail of cAMP, E and P, and can be evaluated for poor decidual response upon specific *KLF* siRNA targeting (Pabona *et al*., 2010; Shen *et al.*, 2013). To mimic the labor dysfunction observed with *Klf9* null mice (Zeng *et al*., 2007), the response of a recently generated human uterine smooth muscle cell line HutSMC was tested in E+P-treated cells without or with *KLF9* siRNAs (Pabona *et al*., 2014). While such studies have resulted in the identification of common and distinct pathways for KLFs (Fig. 2), there are acknowledged limitations to the use of cell lines for extending relevance to the whole organism, providing impetus for generating new and reproductive system-targeted mouse models to further understand the dynamics of KLF actions *in vivo*.

KLFs and Targeted Signaling Pathways in Uterine Pathologies

Since 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-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 KLF actions is not completely understood, albeit limited reports support 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 P-sensitivity, display reduced KLF9 (Pabona *et al*., 2012) and enhanced Notch 3 (Tamaresis *et al*., 2014) expression. Reduced P-sensitivity with loss of KLF9 is due in part to KLF9's role as a PGR-interacting protein (Zhang D *et al*., 2002; Zhang XL *et al*., 2003) and its promotion of E-dependent ESR1 down-regulation (Velarde *et al*, 2007). As regards to P/PGR and the Notch and Hh signaling pathways, studies have demonstrated their opposing and complementary associations in endometrial cells. For example, transcript levels of the Notch ligand, Delta-like 4 are reduced by medroxyprogesterone acetate in primary cultures of human endometrial glandular and stromal cells (Mazella *et al.*, 2008), Moreover, the Hh ligand Indian Hedgehog is a negatively-regulated downstream target of P/PGR (Simon *et al.*, 2009). On the other hand, Notch 1 has been shown to mediate P-dependent uterine stromal cell differentiation in primates and mice (Ashar *et al*., 2012a; Ashar *et al*., 2012b). Additionally, P increased the levels of transcriptionally active Notch 1 intracellular domain, which can form a functional complex with PGR (Afshar *et al*., 2012a). The potential complexity of the regulatory networks involving PGR and Notch/Hh signaling suggests that no single mechanism may fully account for each KLF acting through these pathways.

A core KLF circuitry comprised of KLF2, KLF4 and KLF5 has been recently implicated in regulating 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, Notch), KLFs may similarly control the regenerative capacity of endometrial and myometrial stem/progenitor cells. In this regard, endometriosis (Sassoon & 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 (Tetrault *et al*., 2013). KLF4 also mediates PGR action 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*., 2014), similar to that of KLF9 (Pabona *et al*., 2012), is reduced in 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). Recent studies have shown 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 above studies provide causative support for loss of KLF9 expression in the aberrant promotion of 'stemness', direct evidence for KLF9 and KLF4 involvement in uterine diseases remains to be fully characterized.

Several KLFs have been directly linked to 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, whose attenuated expression is linked to uterine leiomyoma, has been reported to inhibit prostaglandin E_2 synthesis by transcriptionally silencing the gene promoter for phospholipase $A_{2\alpha}$, the key enzyme for prostaglandin biosynthesis (Buttar *et al.*, 2010). Further, 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), suggesting 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 down- and up-regulation of several pro-inflammatory and anti-inflammatory genes, respectively (Pabona *et al.*, 2014). Given that a number of inflammation-associated genes are direct PGR targets (e.g., IL11, CXCL1) (Cordeaux *et al*., 2010; Kavandi *et al*., 2012), data suggest 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 (Chinenoy *et al.*, 2014) described the functional cooperation between the glucocorticoid receptor and KLFs 2 and 9 in macrophages during inflammation. Since the glucocorticoid receptor can mediate progestin effects on uterine inflammatory response (Lei *et al.*, 2012; Guo *et al.*, 2012), KLF interaction with P-dependent transcriptional circuitry is a possible node by which KLFs may exert their control of inflammatory events in the uterus.

Additional pathways that have been linked to KLFs and which may underlie a number of uterine pathologies when these KLFs are aberrantly expressed include: KLF17 promotion of epithelial-mesenchymal transitions through induction of TWIST1 in endometrial cancer (Dong *et al.*, 2014); KLF6-coactivation of NF-κB signaling via its induction of cytokines

TNFα and IL-6 (Zhang *et al*., 2014) in the pathogenesis of endometriosis; KLF5-mediated activation of the JAK-STAT signaling pathway (Tetrault *et al*., 2012), the latter a key mediator of leukemia inhibitory factor control of 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 which when dysregulated can lead to abnormal metabolism and increased risk for 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 in the uterus and ovary that 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; levels of *Klf8, Klf13* and *Klf15* transcripts only showed tendencies for differences (Fig. 3). Using a Stem Cell-Focused PCR Array, we also searched for potential differentially-regulated genes associated with the aging uterus. Transcripts for 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 this pilot 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, 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*., 2007; Heard *et al*., 2012; Daftary *et al*., 2013). This finding is not congruent with the demonstrated regulation of several key steroidogenic genes transcript levels (LDLR, StAR and CYP11A) by KLF13 in ovarian granulosa cells (Natesampillai *et al*., 2008). Interestingly, the pathologic ovary (i.e., ovarian carcinoma) is characterized by reduced (KLF2, KLF4, KLF6) and enhanced (KLF5, KLF8) expression of several KLFs; contradicting 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, survivin); epithelial-mesenchymal interactions (E-cadherin, vimentin, Extracellular matrix receptor); stem cell differentiation (USP44, ErbB), and angiogenesis (VEGF). These findings raise important questions on 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, KLF5) are similarly dysregulated, may be relevant to ovarian diseases.

KLF Networks: A Case For and Against Functional Redundancy

Since KLF expression is ubiquitous yet known reproductive system pathologies appear to involve select 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. Recent elegant studies have demonstrated this concept for KLF3 and KLF8 in a non-reproductive (i.e., erythroid) system (Eaton *et al*., 2008; Funnell *et al*., 2013). The lack of distinct uterine phenotypes in mouse knockout models for several KLF genes support this concept in the reproductive tract. A prime example involves the highly-related members KLF9 and KLF13. Albeit a definitive conclusion is limited by the lack of functional studies in mice deficient in 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 in *siKLF9*-targeted human endometrial stromal cells (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*., 2013). 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 functional hierarchy relative to KLF13. In this scenario, potential transcriptional dysregulation that may occur with KLF13 loss-of-expression is abrogated by the compensatory actions of KLF9.

The co-reduction of 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, support 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 both KLFs occurring through a linear mechanism. There is evidence for the latter possibility, at least for KLF9 and KLF4. *KLF9* siRNA knockdown in the human endometrial carcinoma Ishikawa cell line reduced *KLF4* transcript levels (Simmons *et al.*, 2011) and conversely, KLF9 over-expression in HEC-1A cells induced KLF4 gene expression (Simmen *et al*., 2008); these observations are in accord (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 both KLFs and could provide insight into 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. In regards to KLF9 and KLF11, there are limited data to support or refute redundant functions; however, they are likely to differentially mediate PGR-driven transcriptional events in uterine cells based on their distinct reproductive phenotypes upon targeted gene inactivation (*Klf11*-null mice breed normally and are fertile in contrast to *Klf9*-null mice which are subfertile) (Song *et al.*, 2010; Simmen *et al*., 2004) and the distinct mechanisms by which they mediate PGR transactivity (Zhang *et al*., 2003; Yin *et al*., 2010).

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, KLFs 4 and 15

are inversely expressed, and are found to discretely regulate initiation of DNA synthesis by virtue of their distinct responses to E- and P-treatments (Ray & Pollard, 2012). By inhibiting E-enhanced transcription of the DNA synthesis initiator protein minichromosome maintenance-2, KLF15 functions as a downstream mediator of P-inhibition of the cell cycle. What factors direct the inverse expression of KLFs 4 and 15 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

Factors that contribute to the aberrant expression and activity of KLFs in the reproductive tract leading to 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, reinforcing these KLFs' function in stem cell renewal (Hall *et al.*, 2009). KLF4 expression was suppressed by 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 IGF-1 in human colon cancer cell lines (Bentov *et al*., 2008) and the binding of carbohydrate response element-binding protein (ChREBP), 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 for KLF9 in human endometrial stromal cells; in these cells, BMP2 inhibited KLF9 expression indirectly through KLF13 (Pabona *et al.*, 2010) while E and P had no influence on its expression (Pabona *et al*., 2012). In ovarian granulosa cells, IGF1 and LH were reported to increase KLF13 expression (Natesampillai *et al.*, 2008). The comprehensive analyses of cellular components responsible for maintaining KLF expression will be required 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 described KLF effects to health outcomes and disease states remain 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 to be gleaned when one KLF is absent. Thus, it is imperative to establish which subsets of KLFs compensate for each other, using relevant cell lines *in vitro* by siRNA targeting and by characterizing uterine (or ovarian)-targeted KLF-combination knockouts *in vivo*. Many of the mouse mutants for KLFs have modest or no reproductive phenotypes when they survive through 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; Matsumoto *et al*., 2006; Laub *et al*., 2006; Ema *et al*., 2008). For these KLFs, therefore, conditional mutations using uterine epithelial, stromal and myometrial-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 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 P/PGR signaling pathway alone, distinct KLFs are involved either as regulators or integrators of P/PGR transactivity, albeit not necessarily under the same physiological contexts (Fig. 4). To date, the proliferative, survival, and pro-/anti-inflammatory molecular signatures elicited by each KLF family member when null-mutated in specific uterine compartments have not been defined. 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? The data presented in this review documenting: 1) their association with many reproductive disorders, whose etiologies remain not well-understood; 2) their control of a plethora of signaling pathways; and 3) the considerable diversity of their target genes due to their ability to act as transcriptional activators or repressors, collectively suggest 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, estimated to occur ~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 (~1% of tumor cells) showing stem-progenitor properties was found to be essential for E+P-dependent growth of uterine leiomyomas (Ono *et al.*, 2012). Interestingly, the growth of this cell population involves ESR/PGR and Wnt signaling pathway cross-talk via E+P-induced β-catenin translocation, leading to *Axin2* promoter activation (Ono *et al.*, 2013). Since 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 treatments with these agents can result in drug resistance, with disease recurring often times after cessation of treatment. If current data indicating that KLFs integrate P/PGR and E/ESR cross-talk with Notch and 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, that promote the survival of this subpopulation and hence,

progression/recurrence of uterine pathologies. Thus, targeting Notch signaling with γ-secretase inhibitors that inhibit the intracellular localization of transcriptional mediator Notch intracellular domain may offer a viable therapeutic strategy. A proof-of-concept for the latter has been recently demonstrated 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 were identified by high-throughput 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 since uterine pathologies are mostly associated with reduced KLF expression (Table 1). Such approaches could yield novel research outcomes valuable for translation into the clinic.

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 regulating Hedgehog pathway components (Leow *et al*., 2009); and in stem cell signaling involving the Notch pathway (Oklem *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 systemwide analyses of KLF signaling. Other reproductive pathologies including preeclampsia, fallopian tube cancers, and recurrent pregnancy loss as well as male infertility may similarly benefit from an understanding of KLF biology.

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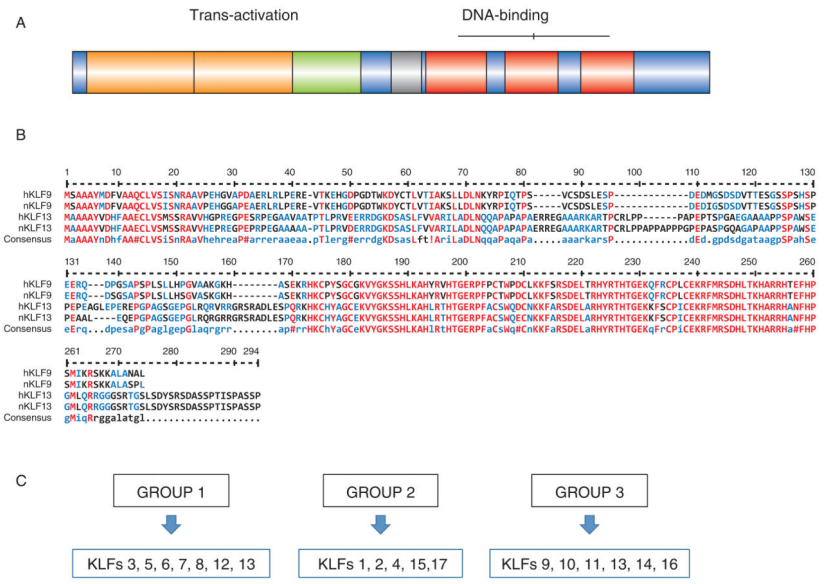

Figure 1.

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 member proteins. (B) Sequence homology among human and mouse KLF9 and KLF13 proteins. Invariant (Red), conserved (blue) and variable (black) amino acid residues are depicted as single-letter codes. (C) KLFs are assigned to three sub-groups based on their phylogenetic relationships (Limame *et al*., 2014).

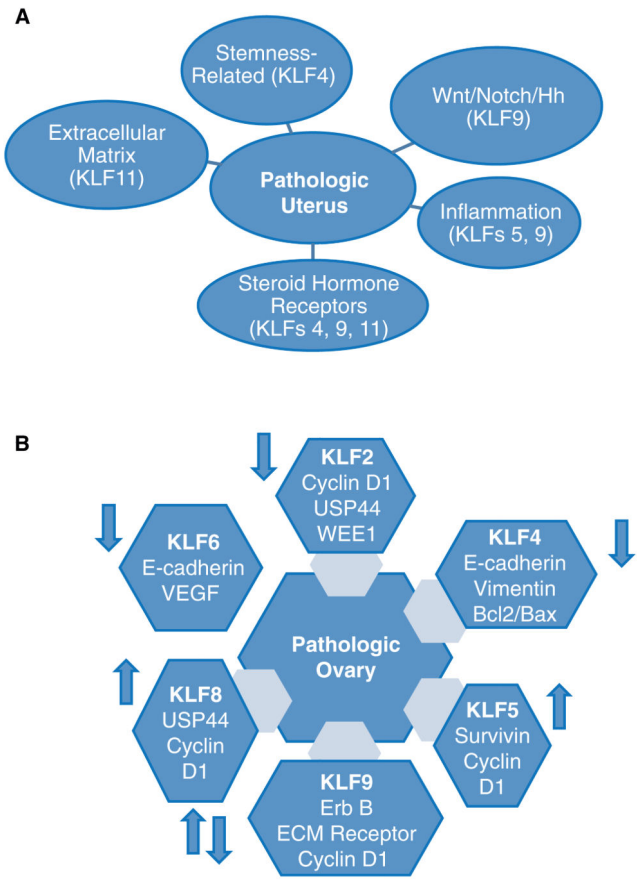

Figure 2.

Signaling pathways and gene targets associated with dysregulated expression of KLF family members in the pathologic uterus (A) and ovary (B). Arrows in panel B (↑ and ↓) signify up- and down-regulated expression of each KLF with ovarian carcinoma.

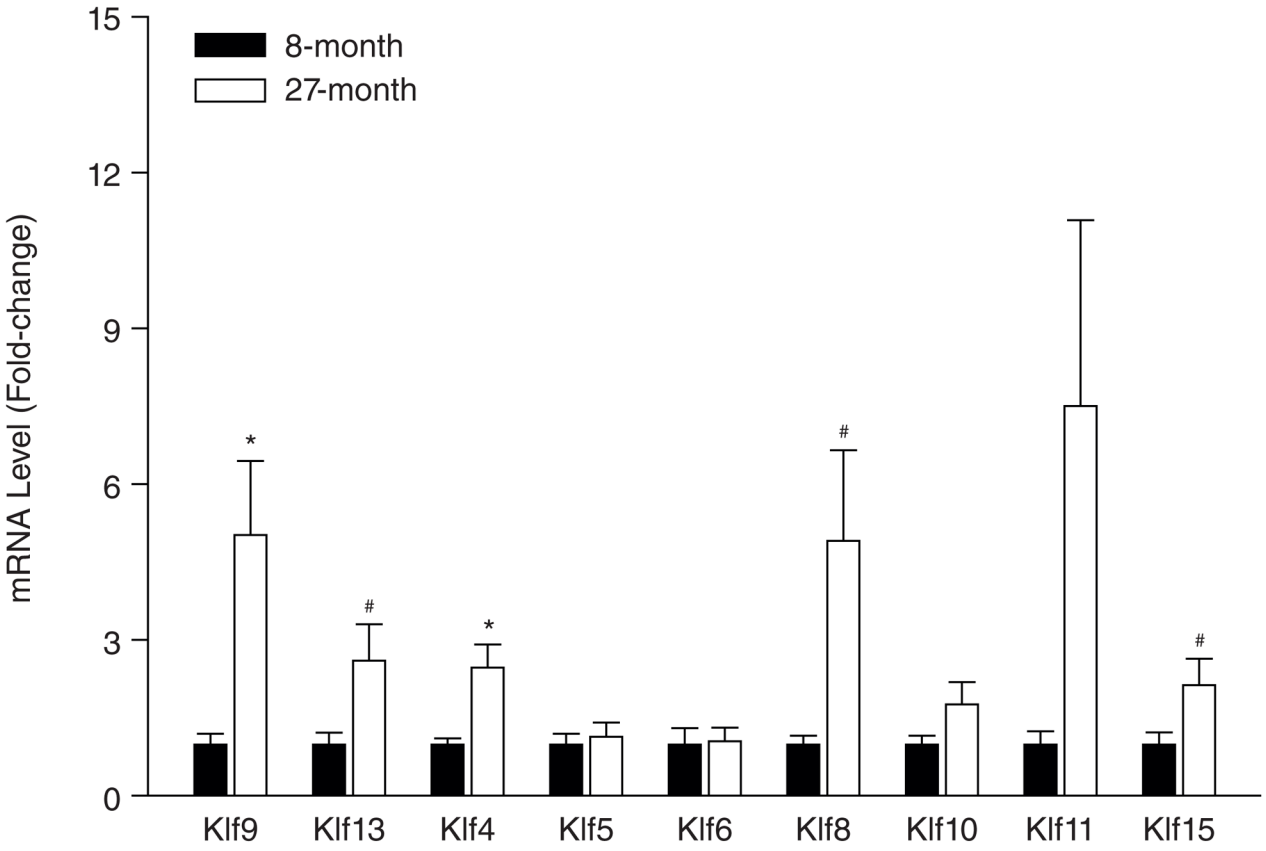

Figure 3.

Expression of select KLFs in young and aging mouse uteri. Transcript levels of various KLFs were quantified in 8- and 27-month old C57BL/6 mouse uteri by quantitative RT-PCR. Data (mean ± SEM) are expressed as fold-change and were obtained from n=7 individual mice per age group. Transcript levels were normalized to corresponding levels of 18S, and then to control (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 $0.05<P<0.10$

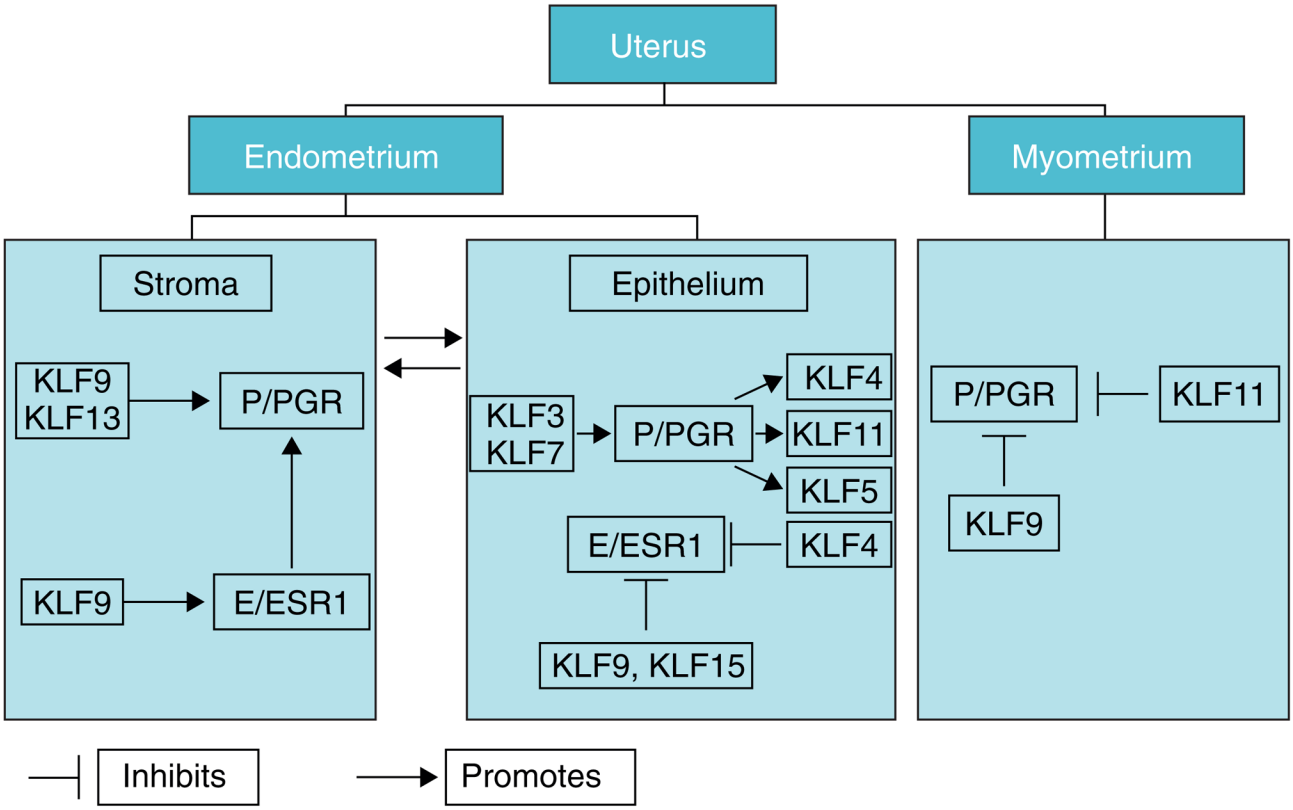

Figure 4.

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

Table 1

Female Reproductive Dysfunctions and KLF Dysregulated Expression in Humans (h) and Mouse (m) Models

PATHOLOGY	KLF (Species)	Over (↑)/Under (↓) Expression	References
Endometrial Cancer	KLF4 (h) KLF 9 (h) KLF17 (h)	↓ ↓ ↑	Simmons *et al*. (2011) Simmen *et al*. (2008) Simmons *et al*. (2011) Korani *et al*. 2013 Dong *et al*. (2014)
Ovarian Cancer	KLF2 (h) KLF4 (h) KLF6 (h) KLF9 (h)	↓ ↓ ↓ ↓/↑	Wang *et al*. (2005) Yoon & Roh (2012) DiFeo *et al*. 2006 Huang *et al*. (2014) Zhang *et al*. (2014)
Cervical Cancer	KLF4 (h) KLF5 (h)	↓ ↑	Yang & Zhang (2014) Marrero-Rodrigues *et al*. (2014)
Endometriosis	KLF4 (h) KLF9 (h, m) KLF11 (h, m)	↓ ↓ ↓	Adammek *et al*. (2013) Lee *et al*. (2008) Pabona *et al*. (2012) Heard *et al*. (2014) Daftary *et al*. (2013)
Leiomyoma	KLF9 (h) KLF11(h)	↓ ↓	Rackow & Taylor (2010) Yin *et al*. (2010)
Implantation/Pregnancy	KLF5 (m) KLF9 (m)	↓ ↓	Sun *et al*. (2012) Simmen *et al*. (2004)
Labor Dysfunction	KLF9 (h, m)	↓	Zeng *et al*. (2007) Pabona *et al*. (2014)

Table 2

Human Cell Lines Used to Model Reproductive Dysfunctions Associated with KLF Dysregulated Expression

PATHOLOGY	Cell Line	KLF	References
Endometrial Cancer	EM/PR Ishikawa, EEC, Hec-1-A EEC	KLF4 KLF9 KLF17	Shimizu et al. (2010) Simmen et al. (2008) Simmons et al.(2011) Dong et al. (2014)
Ovarian Cancer	OV202, SKOV3 SKOV3, OVCAR3 SKOV3 T80, SKOV3 SKOV3, OVCAR3	KLF2 KLF4 KLF5 KLF8 KLF9	Wang et al. (2005) Yoon & Roh (2012); Chen et al. (2014) Dong et al. (2013) Lu et al. 2014) Zhang et al. (2014)
Endometriosis	12Z HESC	KLF4 KLF9	Adammek et al. (2013) Pabona et al. (2012)
Implantation Defects	HESC HESC	KLF9 KLF12	Pabona et al. (2010) Shen et al. (2013)
Labor Dysfunction	HutSMC	KLF9	Pabona et al. (2014)

Table 3

Differentially-Expressed Genes in the Aging Uterus

Genes[a]	Fold-change[b]
Cell Cycle	
Regulators/Wnt signaling	
APC	−2.10
Axin 1	−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 vs. Young Uterus: (-) down-regulation