ERα-mediated repression of pro-inflammatory cytokine expression by glucocorticoids reveals a critical role for TNFα and IL1α in lumen formation and maintenance.

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SUMMARY

Most glandular tissues are comprised of polarized epithelial cells organized around a single central lumen. Although there is active research investigating the molecular networks involved in the regulation of lumenogenesis, little is known about the extracellular factors that influence lumen formation and maintenance. Using a three dimensional culture system of epithelial endometrial cells, we revealed a new role for pro-inflammatory cytokines such as TNFα and IL1α in formation and, more importantly, maintenance of a single central lumen. We have further studied the mechanism by which glucocorticoids repressed TNFα and IL1α expression. Interestingly, regulation of pro-inflammatory cytokine expression and subsequently lumen formation is mediated by Estrogen Receptor α but not by Glucocorticoid Receptor. Finally, we have investigated the signalling pathways involved in the regulation of lumen formation by pro-inflammatory cytokines. Our results demonstrate that activation ERK/MAPK signalling pathway, but not PI3K/Akt signalling pathway, is important for the formation and maintenance of a single central lumen. In summary, our results suggest a novel role for ERα-regulated of pro-inflammatory cytokine expression in lumen formation and maintenance.
INTRODUCTION

In glandular epithelial tissues such as the mammary gland or the endometrium, epithelial cells interact with neighboring cells and the underlying extracellular matrix to develop polarized and well-organized glands. Increasing evidences indicate that appropriate three dimensional (3D) organization is critical for tissue homeostasis (Bryant and Mostov, 2008). Maintenance of cell polarity, cell-to-cell contacts and cell-to-matrix adhesion play a pivotal role in the regulation of glandular homeostasis and epithelial cell proliferation, differentiation and survival.

Over the past years, development of 3D cultures derived from epithelial cells has provided important advances in the knowledge of the cellular and molecular mechanisms underlying epithelial cell morphogenesis and oncogenesis. Three dimensional (3D) cultures of epithelial cells were first established from different epithelial tissues or cell lines using collagen-based matrices (Elsdale and Bard, 1972; Emerman et al., 1979; Emerman and Pitelka, 1977; Hall et al., 1982). More recently, growth of Madin-Darby canine kidney (MDCK) epithelial cell line or MCF-10 mammary epithelial cell line over reconstituted basement membranes, are the most widely used in vitro 3D models to investigate cell polarity during epithelial morphogenesis as well as mechanisms involved in epithelial oncogenesis (Debnath et al., 2003a; Hebner et al., 2008; Kim, 2005; Kim et al., 2004; Mailleux et al., 2008; Martin-Belmonte and Mostov, 2008; Shaw et al., 2004; Yamada and Cukierman, 2007).

During formation of glandular epithelial tissues, epithelial cells surround a single central lumen with their apical surface facing on it. Two mechanisms of lumen formation have been reported: hollowing and cavitation (Bryant and Mostov, 2008; Mailleux et al., 2008; Martin-Belmonte and Mostov, 2008; Martin-Belmonte et al., 2008). In the first model, lumen is formed by membrane separation of glandular cells; in the second model, death of central cells by apoptosis (Debnath et al., 2002; Mailleux et al., 2007) is the main mechanism for lumen formation.

Different intracellular signalling pathways have been shown to participate in the establishment of epithelial cell polarity and lumen formation. Among them, activation of Phosphatidylinositol 3-Kinase (PI3K)/Akt has been extensively involved in the control of lumenogenesis. Overexpression of Akt in MCF10 cells (Debnath et al., 2003b) and either exogenous expression of phosphatidylinositol-3-phosphate (PIP3) (Gassama-
Diagne et al., 2006) or downregulation of PTEN in MDCK cells (Martin-Belmonte et al., 2007; Martin-Belmonte and Mostov, 2007) disrupts acinar architecture, leading to formation of multiple lumened glands. On the other hand, the extracellular regulated-kinase (ERK)/Mitogen-Activated Protein Kinases (MAPK) transduce different extracellular signals that are important for normal morphogenesis as well as for tumorogenesis of epithelial tissues such as the mammary gland (Fata et al., 2007; Whyte et al., 2009).

Pro-inflammatory cytokines such as Interleukin-1α (IL1α) and Tumour Necrosis Factor α (TNFα) play a pivotal role in inflammation and immunity. However, in addition to their function as chemical mediators of inflammation, pro-inflammatory cytokines regulate a wide range of cellular processes. This multiple functionality has been particularly studied in the case of TNFα. TNFα was initially identified as an inductor of apoptosis in tumoral cells (Carswell et al., 1975) but, over the past years, it has been shown that TNFα plays additional roles. At the cellular level, the best-characterized function of TNFα is related to its ability to induce apoptosis but, depending on cell type and cellular environment, it can also promote cellular proliferation, survival, or differentiation (Aggarwal, 2003; Gaur and Aggarwal, 2003). Furthermore, increasing evidences demonstrate that TNFα plays an important role in morphogenesis by regulating cytoskeleton organization and remodelling, which result in changes in cell shape, adhesion or migration (Mathew et al., 2009). These multiple functions of TNFα are translated to complex intracellular signalling pathways. Apart from the apoptotic arm of TNFα signalling, multiple signalling pathways such as Nuclear Factor kappa B (NF-κB), PI3K/akt,p38 MAPK, ERK/MAPKs or Jun-N terminal Kinases (JNK) (Aggarwal, 2003; Baud and Karin, 2001; Mathew et al., 2009; Wajant et al., 2003) have been shown to transduce TNFα signals.

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Formation and maintenance of polarized glands with a central lumen are key processes in morphogenesis and homeostasis of epithelial tissues. Although many works have investigated the intracellular signalling networks involved in establishment of epithelial cell polarity and lumenogenesis, little is known about the extracellular molecules such as growth factor, hormones or cytokines involved in the control of those processes. Here, we have demonstrated that the anti-inflammatory properties of glucocorticoids disrupt endometrial glandular architecture, revealing an important role for pro-inflammatory cytokines such as IL1α and TNFα in the formation of well-polarized glands and, more importantly, the maintenance of a single central lumen.
surrounded by epithelial cells displaying appropriate cell polarity. Furthermore, we
demonstrate that repression of TNFα and IL1α expression is mediated by ERα but not
by GR. Finally, we show that ERK/MAPK is required downstream of TNFα or IL1α to
transduce their effects on correct formation of a central lumen and establishment of
epithelial cell polarity.
RESULTS

Glucocorticoids induce multiple lumen formation in endometrial glandular cultures.

We have recently established a novel 3D culture of primary endometrial epithelial cells that leads to the development of polarized 3D glandular structures (Eritja et al.). In this 3D culture system, most of glands display a single central lumen and a correct apico-basal cell polarization. Surprisingly, addition of glucocorticoids, such as hydrocortisone or dexamethasone in, the culture medium caused the development of morphologically aberrant glands (Fig 1A). Double immunostaining of glands with laminin and phalloidin (to evidence apical actin cytoskeleton) revealed that glands contained multiple lumens (Fig 1B). Moreover, glands cultured in presence of glucocorticoids displayed aberrant morphological features. An immunofluorescence study using markers of apico-basal polarity revealed an aberrant positioning of Golgi apparatus (evidenced by GM130 immunostaining) as well as an aberrant location of adherens and tight junctions (evidenced by E-cadherin and ZO-1 immunostaining) (Fig 1B). Quantification of glands displaying one lumen versus glands displaying multiple lumens demonstrated that glucocorticoid treatment dramatically increased the number of glands with multiple lumens, yielding a total of 75-90% of multiple-lumened glands in the culture (Fig 1C).

We have previously demonstrated that, in our endometrial model, lumen formation takes place via a membrane separation event rather than by luminal apoptosis. To rule out the possibility that glucocorticoid treatment switched the mechanism of lumen formation, we performed an immunofluorescence analysis of caspase-3 activation at different time-points of gland formation. Glucocorticoid treatment did not cause any substantial increase of luminal apoptotic cells compared to control cultures (Supplementary Figure 1).

Multiple lumen formation is caused by a decrease of TNF$\alpha$ and IL1$\alpha$ expression.

To ascertain the mechanisms by which glucocorticoids induce multiple lumen formation, we first investigated whether it could be a process related to anti-inflammatory properties of glucocorticoids. For this reason, we performed a quantitative PCR array analysis of genes related to inflammation (supplementary Table 1). In this study, we found that TNF$\alpha$ and IL1$\alpha$ were the main pro-inflammatory cytokines downregulated by glucocorticoids. Results were further confirmed by single-gene quantitative real-time PCR. As we show in Figure 2A, addition of glucocorticoids for 16
hours caused a dramatic decrease in TNFα and IL1α mRNA expression. This result enabled us to check whether the decrease of pro-inflammatory cytokines was responsible for formation of multiple-lumened glands. For this purpose, we performed a complementation experiment in which we grew endometrial glands in presence of glucocorticoids alone or glucocorticoids plus increasing doses of either TNFα or IL1α. Addition of increasing doses of TNFα or IL1α caused a dose-dependent increase of glands displaying a single central lumen (Fig 2B). Finally, to demonstrate an establishment of a correct cell polarity, we performed double immunofluorescence to apical actin cytoskeleton (evidenced by phalloidin) plus either antibodies to laminin, ZO-1 or GM130. Addition of TNFα completely restored normal cell polarity in glands grown in presence of glucocorticoids (Fig 2C). The same results were obtained in case of addition of IL1α (Supplementary Figure 2).

**Expression of TNFα and IL1α is required for maintenance of a single lumen.**

Next, we investigated whether expression of TNFα or IL1α was only required for lumen formation or alternatively, cytokines were further required to upkeep one single lumen once gland was formed. To assess this point, we designed three different experimental set-ups (Fig 3A). In the first one, glandular cultures were grown in presence of hydrocortisone or dexamethasone for 6 days. At day 6, medium was replaced for medium containing glucocorticoids supplemented or not with pro-inflammatory cytokines and the cultures were grown for 6 additional days (until day 12) (see experimental set-up 1 in Fig 3A). After the first 6 days in culture with glucocorticoids most of glands displayed multiple lumens, but addition of TNFα caused a restructuration of multiple lumens into one single central lumen (Fig 3B). These results demonstrate that TNFα was able to reverse multiple-lumened glands to single-lumened glands. In the second experimental set-up, (Fig 3A) we cultured glands in BIE without glucocorticoids for 6 days, allowing the development of single lumened glands. At day 6, medium was replaced for BIE medium containing or not glucocorticoids for 6 additional days (day 12). At the end of the incubation period, glucocorticoid treatment transformed most of single-lumened glands into multiple-lumened glands (Fig 3C). In the third experimental approach (see experimental set-up 3 in Fig 1A), glandular cultures were grown in presence of hydrocortisone or dexamethasone for 6 days. At day 6, medium was replaced for medium without glucocorticoids. However, in this set-up we did not add exogenous source of pro-inflammatory cytokines. The rationale of this experimental set-up was to ascertain whether re-expression of endogenous cytokine expression was enough to change multiple lumened glands to single-lumened
ones. In this third set-up, to allow re-expression of endogenous cytokines, we extended the period of incubation without glucocorticoids to 9 days. As shown in Figure 3D, glucocorticoid withdrawal was sufficient to restore mRNA expression levels of endogenous pro-inflammatory cytokines and subsequently, reversion of multiple lumened-glands to single-lumened ones.

Finally, in order to evidence the role of pro-inflammatory cytokines in lumen dynamics, we performed a set of time-lapse experiments (Fig 3E). For this purpose, we grew 3D cultures as shown in set-up 1 and 2. The first 6 days cultures were maintained in a normal incubator and, at day 6 (just after medium switch), 3D cultures were transferred to the incubator of a time-lapse recording system. Under the first experimental set-up conditions, addition of TNFα progressively restructured multiple lumens into one single lumen (Fig 3E, supplementary movies 1 and 2). In contrast, addition of glucocorticoids to single-lumened glands resulted in the disruption of one single central lumen into multiple lumens (Fig 3E). These evidences suggest that pro-inflammatory cytokine expression is important for both formation and maintenance of luminal space.

**Glucocorticoids repress pro-inflammatory cytokine expression through ERα.**

Having ascertained that glucocorticoids regulate lumen formation and cell polarity, we decided to investigate the molecular mechanism by which glucocorticoids regulate expression of pro-inflammatory cytokines. Recent evidences demonstrate that glucocorticoids can use ERα to drive their anti-inflammatory actions (Cvoro et al.). Therefore, we analyzed the expression of both GR and ERα after glucocorticoids treatment. Addition of glucocorticoids to glandular cultures led to increased expression of both GR and ERα receptors (Fig 4A). The increase in ERα expression was further analyzed by RT-PCR. As we show in figure 4A, glucocorticoids caused a marked increase of mRNA expression of ERα, suggesting that glucocorticoids cause transcriptional activation of ERα.

To investigate the role of GR and ERα up-regulation in the control of TNFα transcription, we treated glandular cultures with the ERα antagonist ICI182170 or the GR antagonist RU486. Treatment of 3D cultures with RU486 did not restore TNFα expression (Fig 4B). In contrast, ICI182170 restored TNFα mRNA to the levels observed in control cultures (Fig 4C). To further assess the role of ERα in lumen formation, we performed experiments using two different shRNA targeting ERα.
Analysis of mRNA expression by RT-PCR revealed that ERα knock-down restored the levels of TNFα mRNA to a similar levels shown in control cultures (Fig 4D).

**Repression of pro-inflammatory expression by ERα causes multiple lumen formation and loss of cell polarity in 3D endometrial cultures.**

The above results suggest that ERα, but not GR, mediates repression of pro-inflammatory cytokine expression in response to glucocorticoid treatment. On the light of these results, we would expect that ICI182170 but not RU486 treatment should lead in the formation of single lumened glands in presence of glucocorticoids. Indeed, treatment of glandular cultures with RU485 did not block formation of multiple lumens induced by glucocorticoids (Fig 5A). In contrast, treatment of 3D cultures with dexamethasone or hydrocortisone plus ICI182170 caused a dose-dependent increase of single-lumened glands (Fig 5B). Double immunostaining with phalloidin/laminin or phalloidin/GM130 revealed that ICI182170 addition restored correct cell polarity even in presence of glucocorticoids (Fig 5C). As expected, ICI182170 caused downregulation of ERα expression (Fig 5D). To further demonstrate that inhibition of ERα expression was important to restore cell polarity, we infected 3D cultures with shRNAs targeting ERα. In agreement with the results obtained with ICI, downregulation of ERα expression by shRNA 1 and 2, prevented formation of multiple-lumened glands induced by glucocorticoids (Fig 5E).

It is important to point out that, although the effects of glucocorticoids on pro-inflammatory cytokine expression and formation of lumen were mediated by ERα, treatment of glandular cultures with estradiol did not cause reduction of pro-inflammatory cytokine expression or disruption of cell polarity (Supplementary Figure 3).

**PTEN deficiency does not disrupt epithelial cell polarity and lumen formation**

Recent evidences have identified PTEN as critical phosphatase in the establishment of cell polarity and lumen formation in three dimensional cultures (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007; Martin-Belmonte and Mostov, 2008). Moreover, deregulation of PI3K/Akt signalling is a common event in endometrial carcinogenesis (Bussaglia et al., 2000; Llobet et al., 2009; Velasco et al., 2006). Such evidences enabled us to analyze the role of PTEN in establishment of cell polarity and lumen formation in primary 3D cultures of endometrial epithelial cells. To assess the effect of PTEN deletion on cell polarity cytoarchitecture, we isolated primary endometrial epithelial cells from PTEN +/+ or PTEN +/- mice. Isolated epithelial cells were grown in BIE to develop polarized 3D glandular structures. Surprisingly, after 10 days in culture
PTEN+/- endometrial glands displayed normal morphology, with a single lumen and no apparent changes when compared to PTEN +/- glands (Fig 6A). Moreover, we performed immunofluorescence analysis of polarity and epithelial architecture markers on PTEN+/+ and PTEN+/- glands treated with or without glucocorticoids. PTEN+/- glands cultured in BIE without glucocorticoids displayed normal apical actin cytoskeleton (Phalloidin staining) and correct E-cadherin positioning as observed in PTEN +/- glands, indicating no alterations on gland formation. Addition of either hydrocortisone or dexamethasone disrupted normal acinar morphology regardless of PTEN status (Fig 6A). Quantification of single-lumened versus multiple-lumened glands revealed no differences between PTEN+/+ and PTEN+/- glands regardless of glucocorticoids treatment (Fig 6B). As a control for correct deregulation of PI3K/Akt signalling in PTEN deficient glands we checked that that PTEN +/- displayed increased levels of phosphorylated Akt (Fig 6C). Similar results were obtained in epithelial cells treated with 10 μM of PTEN inhibitor bpV(pic) (Fig 6D) or infected with lentiviruses carrying PTEN shRNA (supplementary Figure 4). Accordingly, glucocorticoids did not change phosphorylation status of Akt albeit they completely disrupted lumen formation (Fig 6E). Finally, we wanted to investigate whether inhibition of PI3/Akt signalling would result in disruption of lumen formation. Increasing doses of the PI3K/Akt inhibitor did not disrupt the formation of normal single central lumen glands (Fig 6F). Addition of glucocorticoids plus LY294002 did not modify the effect of glucocorticoids alone on lumen formation (Fig 6G). In concordance with all these results, neither PTEN deficiency nor LY294002 treatment modified the decrease of TNFα and IL1α expression caused by glucocorticoids (Fig 6H, 6I).

**Activation of ERK/MAPK downstream of pro-inflammatory cytokines is required for correct lumen formation.**

As we have shown in Figure 6, neither activation nor inhibition of the PI3K/Akt signaling caused abnormalities in lumen formation. Consistently, glucocorticoids did not change the levels of Akt phosphorylation. In contrast, glucocorticoids caused a marked decrease of ERK phosphorylation (Fig 7A). This result enabled us to investigate the role of ERK activity in lumen formation. For this purpose, we grew 3D cultures in BIE, BIE plus TNFα supplemented with increasing doses of the MEK inhibitors UO126 or PD0325901. Both ERK inhibitors caused a dose-dependent increase of the number of glands bearing multiple lumens in either glands grown in BIE alone or glands grown in BIE supplemented with TNFα (Fig 7B). To rule out the possibility that ERK inhibition
lead to formation of multiple-lumened glands by changing ERα repression of pro-inflammatory cytokines, we analyzed the levels of ERα by western blot and the levels of TNFα and IL1α mRNA expression by Real-Time PCR. Noteworthy, although UO126 caused an efficient inhibition of ERK phosphorylation it did not change the expression of ERα (Fig 7C). Consistently, ERK inhibitors did not cause repression of pro-inflammatory cytokine mRNA levels (Fig 7D), supporting that ERK activation is required downstream of pro-inflammatory cytokine expression to promote correct lumen formation. Accordingly, addition of TNFα or inhibition of ERα expression by ICI182170 or ERα shRNA prevented the decrease of ERK phosphorylation caused by glucocorticoids (Fig 7E). Moreover, ERK inhibition prevented correct lumen formation in cultures treated with glucocorticoids plus TNFα (Fig 7F), suggesting that ERK regulates lumen formation downstream pro-inflammatory cytokines.

Finally, to further investigate the role of ERK signaling in formation of single-lumened glands, we infected 3D cultures with lentiviruses carrying a Myc-tagged constitutively active form of MEK1 (the kinase immediately upstream of ERK commonly used to activate ERKs). CA-MEK1 expression caused a marked increase of ERK phosphorylation indicating an activation of the pathway (Fig 8A). More importantly, CA-MEK1 expression reduced the formation of multiple-lumened glands in presence of glucocorticoids (Fig 8B, 8C). The CA-MEK1 expression did not cause a complete restoration of the number of single-lumened glands. This is because 3D cultures are hard to infect with lentiviruses carrying transgenes. In our hands, around 70% of glands in the culture are efficiently infected by lentiviruses carrying transgenes. These results suggest that activation of ERK is sufficient to restore cell polarity and formation of single lumen.
DISCUSSION

Establishment and maintenance of cell polarity is important for development and homeostasis of epithelial tissues. During the past years, the development of 3D cultures of epithelial cells provided an important tool to advance in the knowledge of the molecular mechanisms that participate in correct epithelial cell polarization and the formation of lumens in epithelial glandular tissues such as the mammary gland. To this regard, we have recently developed a novel serum-free 3D culture of primary endometrial epithelial cells that can be used for both morphogenesis and oncogenesis studies. Surprisingly, we have found that addition of glucocorticoids to culture medium (a common component of 3D cultures of epithelial cells) caused the formation of multiple-lumened glands displaying aberrant polarization of epithelial endometrial cells. The dramatic effects of glucocorticoids in lumenogenesis and polarization of epithelial cells enabled us to investigate the molecular mechanisms by which they would drive these effects. It is important to point out that glucocorticoids are widely used as anti-inflammatory drugs, making even more interesting the study of glucocorticoid effects on cell polarity. In a PCR-array study we have identified TNF$\alpha$ and IL1$\alpha$ as the main cytokines regulated by glucocorticoid treatment. Thus, we have focussed our investigations on these pro-inflammatory cytokines. However, as well as it happens with other functions of pro-inflammatory cytokines, it is reasonable to think that other pro-inflammatory cytokines may contribute together with TNF$\alpha$ and IL1$\alpha$ to the regulation of cell polarity. To this regard, it is worth to mention that cytokines represent a diverse group of molecules that collectively exert a wide range of actions. Many cytokines are pleiotropic and display multiple and overlapping actions. This overlap results in a functional redundancy and compensatory mechanisms of action (Kelso, 1994; Ozaki and Leonard, 2002; Paul, 1989). Glucocorticoid treatment does not exclusively decrease of TNF$\alpha$ and IL1$\alpha$, but also a decrease the expression of other pro-inflammatory cytokines (Barnes, 2006; Hayashi et al., 2004). Therefore, it is unlikely that inhibition of TNF$\alpha$ and IL1$\alpha$ results in dramatic changes in cell polarity. Nonetheless, to analyze the specific effects of TNF$\alpha$ and IL1$\alpha$ inhibition, we performed experiments using blocking antibodies to TNF$\alpha$ and IL1$\alpha$ (data not shown). Neither inhibition of TNF$\alpha$ nor inhibition of IL1$\alpha$ was sufficient to induce the formation of multiple lumened glands. This result suggests that other cytokines apart from TNF$\alpha$ and IL1$\alpha$ can participate in the establishment of cell polarity. These compensatory effects are further supported by the fact that addition of either TNF$\alpha$ or IL1$\alpha$ separately causes a complete restoration of the number glands displaying a single central lumen.
We have found that glucocorticoids cause an increase in ERα expression. One conserved function of steroid hormone receptors is that they autoregulate the expression of their own genes (Schmidt and Meyer, 1994). Estrogens up-regulate ERα gene expression in uteri of all mammalian species examined, including rodents (Bergman et al., 1992) primates (Hild-Petito et al., 1992; Koji and Brenner, 1993) and humans (Lessey et al., 1988; Snijders et al., 1992). Thus, expression of ERα is increased after its binding to estradiol, resulting in a positive feedback mechanism that controls transcription of ERα. Similarly, increased expression of ERα upon glucocorticoid addition is likely to be the result of ERα stimulation by glucocorticoids.

One of the most important results from our investigations is the ERα-dependent regulation of pro-inflammatory cytokine expression and its link to cell polarity. Previous evidences clearly demonstrate that unligated ERα potentiates transcriptional activation of TNFα (Cvoro et al., 2006). Recently, the same group has demonstrated that ERα may drive pro-inflammatory cytokine repression caused by glucocorticoids (Cvoro et al.). Accordingly, in vivo addition of estrogen receptor antagonist ICI 182,780 inhibits the anti-inflammatory effect of glucocorticoids in mice (Cuzzocrea et al., 2007). In line with the above evidences, we have found that addition of glucocorticoids caused an expected decrease in pro-inflammatory cytokine levels through ERα. Furthermore, we found a novel function for ERα modulation of pro-inflammatory cytokine expression in the control of lumen formation, maintenance and acquisition of epithelial cell polarity. Interestingly, the effects on lumen observed after glucocorticoid treatment were not reproduced by addition of estradiol. These results can be explained by the differential gene regulation of ERα engaged by estradiol or glucocorticoids (Cvoro et al.). Since ICI182170 targets both ERα and ERβ, another reasonable explanation for the effects of ICI182170 on lumen could rely on the inhibition of ERβ rather than the inhibition of ERα. However, this possibility was ruled out by the fact that ERβ is not expressed in mouse epithelial endometrial cells (Couse et al., 1997). Furthermore, shRNA-mediated knock-down of ERα caused similar effects on lumen formation to those observed after ICI treatment, suggesting that ERα is involved in the regulation of lumen formation.

Another key finding derived from our studies is the role of the TNFα and IL1α in the maintenance of one single central lumen. Most of works have assessed the mechanisms of lumen formation but, once the lumen is formed, little is known about the extracellular factors and the molecular mechanisms that participate in maintenance of a single central lumen. To this regard, we have demonstrated that once formed, a single
central lumen is not passively maintained. Instead, maintenance of single central lumen seems to be an active process that requires active signalling.

Moreover, we have shown that endogenous pro-inflammatory cytokine expression is sufficient to maintain lumen and epithelial organization. Finally, we have assessed the signalling pathways involved in the regulation of lumen formation by pro-inflammatory cytokines. A Basic feature of cell polarity is the asymmetric organization of the plasma membrane. This organization is basically achieved through membrane trafficking along the cytoskeleton tracks under the control of signaling molecules (Bonifacino and Glick, 2004; Cai et al., 2007). Rho GTPases are molecules that control a wide range of signalling pathways decisive for the acquisition of the polarized phenotype. The function of the Rho family of small GTPases, especially cdc 42, in polarized membrane traffic, has been shown from yeast to mammalian cells (Nelson, 2009; Rodriguez-Fraticelli et al., 2005; Yu et al., 2003). Cdc 42 also controls lumen formation in MCDK cells (Martin-Belmonte et al., 2007) and more recently cdc 42 activity has been involved in regulation of the mitotic spindle orientation (Jaffe et al., 2008; Rodriguez-Fraticelli et al.). In addition to small GTPases protein, another set of membrane traffic regulators are the lipids phosphatidylinositol (PI) and its phosphorilated species. In polarized MCDK cells PI(3,4,5)P3 are localized to the basolateral membrane (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007). Studies using 3D cultures of MCDK cells, showed that PTEN localizes to the apical membrane during epithelial morphogenesis, where it excludes PI(3,4,5)P3 from this domain (Martin-Belmonte et al., 2007). It has been also demonstrated that PI(4,5)P2 recruits the adaptor protein anexin2 to the apical domain which, in turn, recruits cdc 42 which causes the organization of the subapical actin cytoskeleton and formation of the apical surface. Cdc 42 binds with Par6/aPKC complex and they promote the establishment of polarity (Martin-Belmonte et al., 2007; Martin-Belmonte and Mostov, 2007). Alterations on cdc 42, PTEN or Anexin2 expression in polarized MCDK, result in aberrant cyst, which present multiples lumen as well as an abnormal polarization (Jaffe et al., 2008; Martin-Belmonte et al., 2007). It has been also reported that E-cadherin regulates both the PTEN expression and its recruitment to cell-cell junctions to regulate lumen formation in breast cancer cells (Fournier et al., 2009).

In the present study, our results demonstrate that PTEN deficiency expression in polarized epithelial endometrial cells does not cause formation of aberrant or multi-lumenated glands. This result may initially appear to contradict the findings in MCDK
cells, but these differences can be explained in several ways. First, due to we grow our cells in a defined medium, different from that used in MDCK model, it can change microenvironment signals, and cues from the extracellular environment act directly to the positioning of apical lumen (Lipschutz et al., 2001; Martin-Belmonte et al., 2008; Myllymaki et al., ; O'Brien et al., 2001). It has been shown that depending on the microenvironment, two distinct integrin-dependent pathways can regulate epithelial cytogenesis (Myllymaki et al.). Second, PI(4,5)P2 can be distributed in equivalent densities in the apical and basolateral membranes in endometrial epithelial glands; as it happens in pancreatic acinar cells (Ozato-Sakurai et al.). Finally, these differences can be caused by intrinsic cell-specific differences in the mechanisms involved in apical lumen formation. To this regard, it is important to point out that PTEN heterozygous mice or mice with deletion of both copies of PTEN, which show dramatic susceptibility to develop endometrial carcinoma and other malignancies, do not display aberrant development of endometrial epithelium or other glandular epithelia; despite they have increased levels of phosphorylated Akt (Di Cristofano et al., 1998; Podosypanina et al., 1999). These evidences suggest that other signalling pathways can operate in the regulation of cell polarity in those epithelial tissues. Our results suggest that activation of ERK/MAPK is required for correct formation of one single lumen. The ERK/MAPK pathway has been involved in the regulation of many cellular processes including proliferation, migration, differentiation or apoptosis (Shaul and Seger, 2007). Among these functions, ERK activation has been involved in the regulation of cytoskeleton, migration and formation of cell-to-matrix and cell-to-cell adhesion (Howe et al., 2002; Pullikuth and Catling, 2007; Viala and Pouyssegur, 2004; Yee et al., 2008). Therefore, it is reasonable to hypothesize that ERK can regulate lumen formation and cell polarity by regulating cytoskeleton and cellular adhesion. We demonstrate that inhibition of ERK caused a disruption of cell polarity and let to the formation of multiple lumens without affecting TNFα or IL1α expression, suggesting that ERK/MAPK act downstream these cytokines to induce correct epithelial polarization. More importantly, the activation of ERK signalling by a constitutively active form of MEK is sufficient to prevent multiple lumen formation induced by glucocorticoids. The importance of ERK/MAPK as mediator of inflammatory signalling induced by TNFα has raised the ERK/MAPKs as putative therapeutic target for inflammation (Hommes et al., 2003; Kaminska, 2005; Karin, 2004). In summary, our investigations provide new insights in the regulation of cellular polarity and lumen formation in a 3D model of endometrial glands. We have identified a novel function for pro-inflammatory such as TNFα and IL1α in lumen formation and, more importantly, maintenance of a single central lumen
with appropriate epithelial cell polarity. We have further demonstrated that ERα plays a critical role in the regulation of cytokine expression and finally, we have shown that the ERK/MAPK signalling pathway drives the effects TNFα and IL1α on lumen formation and cell polarity.
MATERIAL AND METHODS

Reagents and antibodies- The recombinant basement membrane Matrigel® was purchased from BD Biosciences (San Jose, CA). Epidermal Growth Factor, hydrocortisone, dexamethasone, RU-486, ICI 182170 and LY 294002 were obtained from Sigma (St Louis, MO), Insulin-Transferrin-Sodium Selenite supplement was obtained from Invitrogen (Invitrogen, Inc., Carlsbad, CA, USA), bpV(pic), PD0325901 and UO 126 were purchased from Calbiochem (Calbiochem-Novabiochem, UK, Ltd). Antibodies to E-Cadherin and anti-GM130 were from BD biosciences; ZO-1 was from Zymed; bisBenzimide H 33342 trihydrochloride (Hoechst), Rhodamine conjugated-Phalloidin and antibodies to Laminin and Tubulin were obtained from Sigma. Alexa-Flour anti-Rabbit and anti-mouse antibodies were from Invitrogen. Anti-phospho Akt and Phosho-ERK antibodies were from Cell Signalling Technology (Beverly, MA). Anti-Glucocorticoid Receptor and anti-Estrogen Receptor α antibody were obtained from Santa Cruz biotechnology (Santa Cruz, CA.), Inc. Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Jackson ImmunoResearch Europe Ltd (Suffolk, UK). All other regents were obtained from Sigma unless specified.

Animals and Isolation of endometrial epithelial cells- Pten Knockout mice (strain nomenclature B6.129-Pten^{tmRps}) were obtained from NCI mouse repository. The C57BL6 and Pten Knock-out mice used to isolate endometrial cells were maintained in temperature and light controlled conditions and fed ab libitum. The Institutional Animal Care Committee of the IRBLleida approved all experimental procedures. The isolation of endometrial epithelial cells was processed as described previously (Eritja et al, 2010). In brief, uterine horns were dissected from 3-4 weeks old C57BL6 mice. Uterus were washed with HBSS and digested with Trypsin (Invitrogen). After trypsin digestion epithelial sheets were squeezed-out of the uterine pieces. Epithelial sheets were washed twice with PBS and resuspended in 1 ml of DMEM/F12 (Invitrogen) supplemented with 1mM HEPES (Sigma), 1% of penicillin/streptomycin (Sigma) and fungizone (Invitrogen) (basal medium). Epithelial sheets were mechanically disrupted in basal medium. Cells were diluted in basal medium containing 2% of dextran-coated charcoal-stripped serum (Hyclone) and plated in culture dishes (BD Falcon). Cells were cultured for 24 hours in a incubator at 37ºC with saturating humidity and 5% CO₂.

Three dimensional glandular cultures- Growth of endometrial epithelial cells in 3D cultures was performed as described previously (Eritja et al, 2010). 24 hours after plating in plastic, cells were washed with HBSS and incubated with trypsin/EDTA.
solution (Sigma) for 5 min at 37ºC. Trypsin was stopped by adding DMEM 10% FBS and clumps of 2-8 cells were obtained. Cells were centrifuged at 1000 rpm for 3 minutes and diluted in basal medium containing 3% of matrigel to obtain 4 x 10⁴ cell clumps/ml. For immunofluorescence, cells were seeded in a volume of 40 µl/well in 96 well plates black with micro-clear bottom (Greiner Bio-one). For western blotting, cells were placed in a volume of 200 µl in 24-well plates (BD Biosciences). In all cases, 24 hours after plating, medium was replaced by Basal medium supplemented with 5ng/ml EGF and 1/100 dilution of Insulin-Transferrin-Sodium Selenite (ITS) Supplement (Invitrogen) and 3% of fresh Matrigel (this medium is referred as BIE). Medium was replaced every 2-3 days.

**Immunofluorescence** – 3D cultures were fixed with formalin for 5 minutes at room temperature (RT), washed twice with PBS. Depending on primary antibody, cells were permeabilized with 0,2% triton X-100 in PBS for 10 minutes or permeabilized with 100% methanol for 2 minutes. Next, cultures were incubated overnight at 4ºC with the indicated dilutions of antibodies: anti-Laminin 1/500(T), Rhodamine conjugated-Phalloidin 1/500(T), E-cadherin 1/250, ZO-1 (1/250) and anti-GM130 1/100(M). After one day, cells were washed twice with PBS and incubated with PBS containing a 5 µg/ml of Hoechst dye and 1/500 dilution of Alexa Fluor secondary anti-mouse or anti-rabbit antibodies for 2 hours at RT. For double immunofluorescence staining, cells were incubated with the second round of primary and secondary antibodies. We would like to point out that in all double immunofluorescence stains, first and second primary antibodies were from different isotope. Immunofloourescence staining was visualized and analyzed using a confocal microscopy (FV1000, Olympus) using the oil-immersion X60 magnification objective. Analysis of images was made with Fluoview FV100 software.

**Confocal imaging and evaluation of lumen and cell polarity**– Endometrial epithelial glands were analyzed on a confocal microscope Fluoview FV1000. The presence of one or multiple lumens (more than two lumens) was revealed by GM130/phalloidin immunostaining. For each experiment we quantified the number of single lumen versus multiple lumens in at least 100 glands. Cell polarity of epithelial cells forming glandular structures was evidenced by double immunostaining as indicated in each figure.

**Time-lapse experiments** – Endometrial glands were grown for 6 days in BIE plus glucocorticoids. After 6 days, medium was replaced for medium containing BIE supplemented with TNFα or IL1α. At this point, cultures were incubated in a Zeiss Axiovert inverted microscope equipped with a motorized slide and a CO₂ and
temperature incubation modules. AxioVision Rel. 4.6 software was programmed to take a picture of the selected glands once a day or every 3 hours for 6 consecutive days.

**Lentiviral production and infection** - Oligonucleotides to produce plasmid based shRNA were cloned into the FSV vector using AgeI-BamHI restriction sites. shRNA target sequences were: PTEN, ATATAGGTCAAGTCTAAGTCG; ERα-1, GGTGCCCTACTACCTGGAA; ERα-2 GTGCAAGGAGACTCTCGCTATC. Lentiviral particles were produced as previously described with some modifications (Llobet et al., 2008). 293T human embryonic kidney cells were co-transfected by polyethylenimine (PEI) method with the virion packaging elements (psPAX2 and pMD2G) and the shRNA producing vector (FSV). 293T cells were allowed to produce lentiviral particles during 3-4 days in same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 minutes at 1000 rpm and filtered through a 0.45 µM filter (Millipore). The medium was concentrated by centrifugation through 100,000 MWCO Vivaspin20 columns (Sartoriusstedium Biotech). Freshly isolated or thawed mouse endometrial cells were diluted in DMEM/F12 plus 2% DCC-S as described above and plated into 24-well plastic dishes. 2-3 hours after plating 10-20, μl of concentrated lentiviral particles plus 8 μg/ml of hexadimethrine bromide (polybrene) (Sigma) were added to the cultures and incubated for 24 hours. After these infection period cells were processed to establish 3D culture as described above.

**Western Blot analysis** – Glandular endometrial 3D cultures stimulated for the indicated periods of time, were washed with HBSS and incubated with trypsin/EDTA solution for 5 min at 37°C. Incubation with trypsin was done to allow us to separate the glandular structures from Matrigel. Trypsin was stopped by adding DMEM10% FBS and the cells were lysed with lysis buffer (2% SDS, 125 mM Tris-HCL pH6.8). Relative protein concentrations were determined loading an 8% acrylamide gel, transferred to PVDF membranes and blotted with anti-tubulin antibody. Band density was determined using Quantity One software (Bio-Rad, Richmond, CA). Equal amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Non-specific binding was blocked by incubation with TBST (20 mM Tris-Hcl pH7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4°C followed by 1 hour incubation with secondary antibody 1/10000 in TBST. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

**Real-time PCR** – Total RNA was prepared using the Rneasy mini kit (Qiagen) according to the manufacturer’s protocol. Reverse transcription reaction were
performed using a total 1μg total RNA with TaqMan® Reverse Transcription Kit from Applied Biosystems. Quantitative real-time PCR detection of gene expression was performed with the ABI Prism 7000 Sequence Detection System using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The sequences of primers used for PCR were obtained commercially from Applied biosystems Assay-on-demand Gene: (TNF-alpha) Mm00443258_m1, (IL1α) Mm00439620_m1 and (Gadph) Mm99999915_g1. Relative expression was determinates from cycle threshold (Ct) values, which were normalized to Gadph as the endogenous control. Experiments were performed at least three times and statistical significance was determined by student’s test with p. value ≤0.05.

**Statistical analysis** - Experiments were performed at least three times and statistical significance was determined by student’s test with p. value ≤0.05. Unless otherwise indicated asterisks indicate p. value ≤0.05.
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REFERENCES


FIGURE LEGENDS

Figure 1. Glucocorticoids induce multiple lumen formation in endometrial glandular cultures. Endometrial epithelial cells were grown in 3D conditions for 7 days in BIE medium alone (BIE), BIE plus 500 ng/ml hydrocortisone (BIE+HC) or BIE plus 200 ng/ml dexamethasone (BIE+DEX). A) Representative images of 3D cultures showing glands with single lumen or multiple lumens. B) Double immunostaining of glands with laminin (green) and phalloidin (red), GM-130 (green) and phalloidin (red) or ZO-1 (green) and E-cadherin (red). Nuclei were counterstained with Hoechst. C) Quantification of the number of single-lumened glands versus glands displaying two or more lumens. Results are expressed as the percentage of total number of glands. Scale bars indicate 20 μm. Asterisks indicate a p-value ≤0.05.

Figure 2. Multiple lumen formation is caused by a decrease of TNFα and IL1α expression. A) Glucocorticoids reduce pro-inflammatory cytokine expression. Endometrial epithelial cells were grown in 3D conditions for 7 days in BIE, BIE plus hydrocortisone (HC) or BIE plus dexamethasone (DEX). Real-time PCR analysis of TNFα (left) and IL1α (right) expression. Results are expressed as relative mRNA levels. B) Addition of either TNFα (50ng/ml) or IL1α (50ng/ml) cause a dose-dependent blockage of multiple lumen formation induced by glucocorticoids. Quantification of single-lumened glands grown for 7 days in BIE medium, BIE plus hydrocortisone (BIE+HC) or BIE plus dexamethasone (BIE+DEX) containing increasing doses of either TNFα (left) or IL1α (right). C) Addition of TNFα restore correct glandular architecture. Glands were cultured in presence of hydrocortisone (HC), dexamethasone (DEX), hydrocortisone plus 50ng/ml TNFα (HC+TNF) or dexamethasone plus 50ng/ml TNFα (DEX+TNFα). Double immunostaining of glands with phalloidin (red)/laminin (green), phalloidin (red)/GM130 (green) or E-cadherin (red)/ZO-1 (green). Nuclei were counterstained with Hoechst. Scale bars indicate 20 μm. Asterisks indicate a p-value ≤0.05.

Figure 3. TNFα and IL1α are required for single lumen maintenance. A) Diagram showing the three different experimental set-ups to study the role of pro-inflammatory cytokines in maintenance of single central lumen. In the first set-up (top diagram), epithelial 3D cultures were grown in BIE supplemented with glucocorticoids (BIE+DEX or BIE+HC) for 6 days. After 6 days, TNFα or IL1α were added to the culture and the percentage of multiple-lumened versus single-lumened glands was scored after 6 additional days. In the second set-up (bottom diagram) epithelial 3D cultures were
grown in BIE for 6 days. After 6 initial days, medium was supplemented with glucocorticoids (HC or DEX) and glands were cultured for 6 additional days. In the third set-up, epithelial 3D cultures were grown in BIE supplemented with glucocorticoids (BIE+DEX or BIE+HC) for 6 days. At this point, medium was replaced for BIE medium with or without glucocorticoids and grown for 9 additional days B) Reversible switch from multiple lumen to one single lumen phenotypes. Representative images (left) and quantification (right) of single versus multiple-lumened glands at day 6 and at the end of the first experimental set-up (day 12). C) Representative images (top) and quantifications (bottom graphs) of single versus multiple-lumened glands at day 6 and at the end of the second experimental set-up (day 12). D) Glucocorticoid removal is sufficient to restore single-lumened glands and expression of pro-inflammatory cytokines. Left, quantification of single versus multiple-lumened glands at the end of the third experimental set up (day 15). Real-time PCR analysis of TNFα (middle) and IL1α (right) expression at the indicated days of treatment. Results are expressed as relative mRNA levels. E) Representative images of time-lapse experiments corresponding to experimental set-up 1 (top) and experimental set-up 2 (bottom). Scale bars indicate 50 μm. Asterisks indicate a p-value ≤0.05.

Figure 4. Glucocorticoids repress pro-inflammatory cytokine expression through ERα. A) Glucocorticoids increase expression of both GR and ERα. Left, western blot from cultures treated for 7 days with BIE alone or BIE supplemented with either hydrocortisone (HC) or dexamethasone (DEX) showing increased expression of both ERα and GR Membranes were reproved with tubulin to ensure equal protein loading. Quantification of band intensity in three different western blot (Bottom). Right, Real-time PCR analysis of ERα expression in cultures treated with BIE alone or BIE supplemented with hydrocortisone (HC) or dexamethasone (DEX). Results are expressed as relative mRNA levels. B) GR does not to repress TNFα expression. Left, endometrial epithelial cells were grown in 3D conditions for 7 days in BIE, BIE plus hydrocortisone (BIE+HC) or BIE plus Hydrocortisone and 0,5 μg/ml RU-486 (BIE+HC+RU). Right, endometrial epithelial cells were grown in 3D conditions for 7 days in BIE, BIE plus dexamethasone (BIE+DEX) or BIE plus dexamethasone and 0,5 μg/ml RU-486 (BIE+DEX+RU). mRNA was extracted and TNFα expression was analyzed by Real-Time PCR. Results are expressed as relative mRNA levels. C) ERα is required to repress TNFα expression. Left, endometrial epithelial cells were grown in 3D conditions for 7 days in BIE, BIE plus hydrocortisone (BIE+HC) or BIE plus hydrocortisone and 10 nM ICI182170 (BIE+HC+ICI). Right, endometrial epithelial cells
were grown in 3D conditions for 7 days in BIE, BIE plus dexamethasone (BIE+DEX) or BIE plus dexamethasone and 10 nM ICI1821700 (BIE+DEX+ICI). mRNA was extracted and TNFα expression was analyzed by Real-Time PCR. Results are expressed as relative mRNA levels. D) ERα shRNA restore pro-inflammatory cytokine expression. Endometrial epithelial cells were infected with lentiviruses carrying ERα shRNA1 or ERα shRNA2 and grown in 3D conditions in presence or absence of dexamethasone (DEX). Western blot showing downregulation of ERα expression by two different ERα shRNAs (top). mRNA was extracted and TNFα expression was analyzed by Real-Time PCR. Results are expressed as relative mRNA levels (bottom). Asterisks indicate a p-value ≤0.05.

Figure 5. ERα but not GR expression prevents multiple lumen formation. A) Inhibition of GR does not block formation of multiple-lumened glands. Endometrial epithelial cells were grown for 7 days in BIE, BIE plus hydrocortisone (BIE+HC), BIE plus dexamethasone (BIE+DEX) alone or with 0.5 μg/ml of RU-486. Quantification of the percentage single-lumened versus multiple-lumened glands (Left). Representative images showing phalloidin immunostaining to evidence the number of multiple-lumened. Nuclei were counterstained with Hoechst (Right). B) Dose-dependent inhibition of ERα leads to increasing number of single-lumened glands. Endometrial epithelial cells were grown for 7 days in BIE, BIE plus hydrocortisone (BIE+HC), BIE plus dexamethasone (BIE+DEX) alone or with increasing doses of ICI1821700. Quantification of the percentage single-lumened glands (Left). Representative images showing phase contrast plus phalloidin immunostaining (red) to evidence the switch from multiple-lumened to single-lumened glands (Right). Nuclei were counterstained with Hoechst (blue). C) ICI1821700 restores epithelial cell polarity and the formation of single lumen glands. Endometrial epithelial cells were grown for 7 days in BIE, BIE plus hydrocortisone (BIE+HC), BIE plus dexamethasone (BIE+DEX) alone or with ICI1821700 (BIE+ICI; BIE+HC+ICI; BIE+DEX+ICI). Double immunostaining of glands with phalloidin (red) and either laminin (green) or GM130 (green) to evidence cell polarity and lumen morphology. Nuclei were counterstained with Hoechst in all samples. D) Glucocorticoids increase ERα expression. Western blot showing increase of ERα protein levels in glands cultured for 7 days in BIE, BIE+HC or BIE+DEX. Membranes were reproved with tubulin to demonstrate equal protein loading. E) Endometrial epithelial cells were infected with lentiviruses carrying ERα shRNA1 or ERα shRNA2 and grown in 3D conditions in presence or absence of dexamethasone (DEX) or hydrocortisone (HC). Left, quantification of the percentage of single-lumened
versus multiple-lumened glands. Right, Representative images showing phalloidin immunostaining to evidence the number of multiple-lumened glands. Nuclei were counterstained with Hoechst. Scale bars indicate 50 μm. Asterisks indicate a p-value ≤0.05.

Figure 6. PTEN downregulation or PI3K inhibition did not affect lumen formation or maintenance. Endometrial epithelial cells isolated from PTEN+/+ or PTEN+/− mice were grown in 3D conditions for 10 days in defined BIE medium or in BIE plus hydrocortisone (HC), BIE plus dexamethasone (DEX). A) Double immunostaining of PTEN+/− and PTEN+/− glands with E-cadherin (green) and phalloidin (red). Nuclei were counterstained with Hoechst in all samples. B) PTEN deficiency does not modify number of lumens. Percentage of single-lumened glands in PTEN+/+ and PTEN−/− cultures. C) Western blot from PTEN+/+ and PTEN+/− glands showing decreased PTEN levels and increased Akt phosphorylation in PTEN+/− mice. D) Quantification (left) and representative images (right) of single versus multiple-lumened glands grown for 7 days with BIE or BIE plus 10 mM of PTEN inhibitor bpV(pic). in BIE. E) Glucocorticoids do not affect Akt phosphorylation. Western blot from BIE, BIE+HC and BIE+DEX treated cultures showing no differences in Akt phosphorylation after 7 days in culture. F) Inhibition of PI3K does not affect lumen formation. Quantification of single versus multiple-lumened glands grown for 7 days with the indicated doses of PI3K inhibitor LY294002 (Top). Double laminin (green)/phalloidin (red) immunostaining showing representative images of single-lumened glands (Bottom). G) Quantification of single versus multiple-lumened glands grown for 7 days with BIE or BIE plus LY294002 in BIE medium or medium containing hydrocortisone (HC) or dexamethasone (DEX). H) PTEN status does not affect the reduction of cytokine expression caused by glucocorticoids. Endometrial epithelial cells from PTEN+/+ or PTEN+/− mice were grown in 3D conditions for 7 days in defined BIE medium alone (BIE) or supplemented with hydrocortisone (HC) or dexamethasone (DEX). Real-time PCR analysis of TNFα (top graphs) and IL1α (bottom graphs) expression. Results are expressed as relative mRNA levels. I) PI3K/Akt inhibition does not affect the reduction of cytokine expression caused by glucocorticoids. Endometrial epithelial mice were grown in 3D conditions for 7 days in defined BIE medium alone (BIE) or supplemented with hydrocortisone (HC) or dexamethasone (DEX) in presence or absence of the PI3K inhibitor LY294002 (LY). Real-time PCR analysis of TNFα (top graphs) and IL1α (bottom graphs) expression. Results are expressed as relative mRNA levels. Scale bars indicate 50 μm.
Figure 7. Inhibition of ERK/MAPK signaling results in multiple lumen formation downstream of cytokine expression.  

A) Glucocorticoids reduce ERK phosphorylation. Western blot from 3D culture grown for 7 days in BIE, BIE plus dexamethasone (BIE+DEX) or BIE plus hydrocortisone (BIE+HC) showing no modification of Akt phosphorylation, increased GR expression and reduction of ERK phosphorylation. Membranes were reproved with tubulin to show equal protein loading. 

B) Inhibition of ERK/MAPK causes a dose-dependent increase of multiple-lumened glands. Endometrial epithelial glands were grown for 7 days in BIE medium alone (BIE) or BIE plus TNFα (BIE+TNFα) and increasing doses of the ERK inhibitors UO126 or PD0325901. Top, quantification of single-lumened glands in cultures treated for 7 days in BIE or BIE+TNFα with increasing doses UO126 (left) or PD0325901 (right). Bottom, representative images of phalloidin immunostaining (red) showing glands cultured for 7 days in presence of increasing doses UO126 (left) or PD0325901 (right). Nuclei were evidenced by Hoechst staining (blue). 

C) Inhibition of ERK/MAPK does not change ERα expression. Western blot from 3D cultures grown for 7 days in BIE plus increasing doses of UO126 showing an effective inhibition of ERK phosphorylation and no modification of ERα protein levels. Membranes were reproved with tubulin to show equal protein loading. 

D) Inhibition of ERK/MAPK does not repress TNFα or IL1α expression. Endometrial epithelial glands were grown for 7 days in BIE plus increasing doses of UO126. mRNA was extracted and TNFα (left) and IL1α (right) expression was analyzed by Real-Time PCR. Results are expressed as relative mRNA levels. 

E) TNFα, ICI182170 and ERα shRNA restore ERK phosphorylation in glucocorticoid treated cells. Top, western blot showing ERK phosphorylation of cultures treated with BIE or BIE+DEX and 50 ng/ml TNFα, 10 nM ICI182170 (ICI) or no additives (-) for 7 days. Bottom, western blot showing ERK phosphorylation in cultures infected with FSV vector or ERα shRNA1 treated with BIE or dexamethasone (DEX). Membranes were reproved with ERα antibodies to ensure correct inhibition of ERα. Membranes were also reproved with tubulin and total ERK to ensure equal loading. 

F) Percentage of single versus multiple-lumened glands grown for 7 days in BIE plus dexamethasone (DEX) or BIE plus TNFα with (+) or without (-) 5 μM UO126. Scale bars indicate 50 μm. Asterisks indicate a p-value ≤0.05.

Figure 8. Activation of ERK is sufficient to prevent multiple lumen formation induced by glucocorticoids. Glandular cultures were infected with vector expression of CA-MEK1 or the empty vector and grown for 7 days in BIE. 

A) Western blot showing
increased phosphorylation of ERK in cells infected with constitutively active form of MEK1 (CA-MEK1). Membranes were reproved with Myc to ensure expression of the construct and with total ERK and tubulin to ensure equal protein loading. B). Quantification of single lumened versus multiple lumened glands. C) Representative images of 3D cultures infected with empty vector or vector carrying CA-MEK1 stained with phalloidin (red). Nuclei were evidenced by Hoechst staining (blue). Scale bars indicate 50 μm.
LEGENDS TO SUPPLEMENTARY FIGURES.

Supplementary Figure 1

**Glucocorticoid treatment does not induce luminal apoptosis during gland formation.**

Parallel endometrial glands were grown for the indicated number of days in BIE plus either hydrocortisone (HC) or dexamethasone (DEX). At every time point, cultures were fixed and immunofluorescence to phalloidin (red) and anti-active caspase-3 (green) were performed. Nuclei were counterstained with Hoechst in all samples. Scale bars indicate 20 μm.

Supplementary Figure 2

**Addition of IL1α restores correct glandular architecture.** Glands were grown in presence of BIE, BIE plus IL1α (50ng/ml), hydrocortisone (HC), hydrocortisone plus IL1α (HC+ IL1α), dexamethasone (DEX) or dexamethasone plus IL1α (DEX + IL1α). Double immunostaining of glands with Phalloidin (red) and Laminin (green) or Phalloidin (red) and GM 130 (green). Nuclei were counterstained with Hoechst in all samples.

Supplementary Figure 3

**Treatment of glandular culture with estradiol did not cause lumen disruption or reduction of pro-inflammatory cytokine expression.**

A) Addition of 1nM, 10nM, 100nM of estradiol did not cause disruption of glandular polarity. Representative images showing phase contrast and double immunostaining with E-cadherin (green) and Phalloidin (red) are shown. Nuclei were counterstained with Hoechst in all samples. B) Quantification of the percentage single-lumened glands and multiple-lumened glands treated with different doses of estradiol. C) Endometrial epithelial cells were grown in 3D conditions for 7 days in BIE, and then were treated for 16 hours with BIE or estradiol 100nM. mRNA was extracted and TNFα expression was analyzed by Real-Time PCR. Results are expressed as relative mRNA levels.

Supplementary Figure 4

**PTEN downregulation using lentiviruses carrying PTEN shRNA did not affect lumen formation.**
Endometrial epithelial cells were obtained and infected with lentiviruses carrying shRNA PTEN or shRNA FSV (empty vector) and were grown in 3D conditions for 10 days. **A)** Knock-down of PTEN did not cause defects in cell polarity. Double immunostaining of FSV or PTEN knock-down glands with Laminin (green) and Phalloidin (red) or ZO-1 (green) and E-cadherin (red). **B)** PTEN deficiency did not modify number of lumens. Percentage of single-lumened glands in shRNA FSV and PTEN shRNA cultured glands. **C)** Western blot from infected glands carrying FSV and PTEN shRNA showing decreased PTEN levels and increased Akt phosphorylation after 10 days in culture. Membranes were reproved with tubulin to ensure equal protein loading.

**Supplementary movies**

Endometrial glands were grown for 6 days in BIE plus glucocorticoids. After 6 days medium was replaced for medium containing BIE supplemented with TNFα ([Supplementary movie 1](#)) or IL1α ([Supplementary movie 2](#)). At this point were incubated in a Zeiss Axiovert inverted microscope equipped with a motorized slide and a CO₂ and temperature incubation modules. AxioVision Rel. 4.6 software was programmed to take a picture of the selected glands every 3 hours for 6 consecutive days.
FIGURE 1

A

BIE
BIE+DEX
BIE+HC

B

Phalloidin/Laminin
Phalloidin/GM130
E-cadherin /ZO -1

BIE
BIE+DEX
BIE+HC

C

% of Glands

SINGLE LUMEN
MULTIPLE LUMEN

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FIGURE 2

A

Relative IL1α mRNA expression

B

% Single-lumened glands

C

Phalloidin/Laminin
Phalloidin/GM130
E-cadherin/ZO-1

BIE HC DEX

Relative TNF-α mRNA expression

BIE HC DEX

BIE HC DEX

BIE HC DEX

BIE HC DEX

BIE HC DEX

BIE HC DEX
FIGURE 3

A

SET-UP 1

6 days BIE+HC or BIE+ DEX

Day 6

Multiple lumened glands

Add TNF α or IL1α

Day 12

Score single - lumened vs Multiple-lumened glands

SET-UP 2

6 days BIE

Day 6

Single lumened glands

Add HC or DEX

Day 12

Score single - lumened vs Multiple-lumened glands

SET-UP 3

Glucocorticoid withdrawal

9 days BIE alone

Day 6

Multiple lumened glands

6 days BIE+HC or BIE+ DEX

Day 12

Score single - lumened vs Multiple-lumened glands

Day 8

Day 6

Day 7

Day 10

Day 9

Day 11

Day 12

B

Day 6

DEX

Day 12

+/- TNF α

+/- DEX

Day 6

BIE

Day 12

BIE

% of Glands

MULTIPLE LUMEN

SINGLE LUMEN

% of Glands

MULTIPLE LUMEN

SINGLE LUMEN

BIE  6 days DEX  12 days

+ BIE  15 days DEX  15 days

+ BIE  6 days DEX  12 days

BIE + DEX 6 days

BIE  12 days

+ BIE + DEX + TNF α 12 days

BIE + HC 12 days

BIE + HC + TNF α 12 days

BIE  15 days

HC  15 days

+ BIE  9 days

HC  15 days

+ BIE  9 days

Relative TNF α mRNA expression

Relative IL α mRNA expression

% of Glands

BIE  15 days

DEX  15 days

+ BIE  15 days

DEX  12 days

+ BIE  9 days

HCBIE

Day 6

Day 12

BIE+DEX Add TNF α

Day 6

Add DEX

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FIGURE 6

A. Immunofluorescence images showing expression of PTEN, p-Akt, and total Akt in PTEN+/+ and PTEN+/- glands treated with BIE, HC, and DEX.

B. Bar graph showing the percentage of single-lumen glands in PTEN+/+ and PTEN+/- mice treated with BIE, HC, and DEX.

C. Western blots showing expression of PTEN, p-Akt, and total Akt in PTEN+/+ and PTEN+/- glands treated with BIE, HC, and DEX.

D. Graph showing the percentage of glands with single vs. multiple lumens in PTEN+/+ and PTEN+/- mice treated with BIE and BIE + 10 μM bpV (pic).

E. Western blots showing expression of GR, p-Akt, total Akt, and tubulin in PTEN+/+ and PTEN+/- glands treated with BIE, HC, and DEX.

F. Bar graph showing the percentage of single-lumen glands in PTEN+/+ and PTEN+/- mice treated with BIE and LY294002 at 1, 5, and 10 μM.

G. Bar graph showing the percentage of single-lumen glands in PTEN+/+ and PTEN+/- mice treated with BIE, HC, and DEX.

H. Graph showing relative TNF-α mRNA expression in PTEN+/+ and PTEN+/- mice treated with BIE, HC, and DEX.

I. Graph showing relative IL-6 mRNA expression in PTEN+/+ and PTEN+/- mice treated with BIE, HC, and DEX.
FIGURE 7

A

BIE

- HC DEX

GR

p-AKT

Total AKT

p-ERK

Total ERK

Tubulin

B

% Single lumen glands

BIE - TNF

BIE + TNF

UO126 (μM)

0 1 2.5 5

0 100

% Single lumen glands

BIE - TNF

BIE + TNF

PD0325901 (nM)

0 1 10 100

D

Relative TNFα mRNA expression

UO126 (μM)

0 1 2.5 5

0.2 0.4 0.6 0.8 1 1.2 1.4

Relative IL-1α mRNA expression

UO126 (μM)

0 1 2.5 5

0.2 0.4 0.6 0.8 1 1.2 1.4
FIGURE 8

A

Empty Vector CA-MEK1

Myc

p-ERK

Total ERK

Tubulin

B

% of Glands

Empty vector CA-MEK1

MULTIPLE LUMEN SINGLE LUMEN

C

Empty vector CA-MEK1

BIE HC DEX BIE HC DEX

* *