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Molecular mechanism of regulation of the calcium-binding protein calbindin-D_{9k}, and its physiological role(s) in mammals: a review of current research

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Abstract

Calbindin-D_{9k} (CaBP-9k) is a cytosolic calcium-binding protein that is expressed in a variety of tissues, such as uterus, placenta, intestine, kidney, pituitary gland and bone. At present, the precise role(s) of CaBP-9k remains to be clarified. CaBP-9k-null mice are normal, which indicates that other calcium-transporter genes can compensate for the lack CaBP-9k. Uterine CaBP-9k has been shown to be involved in the regulation of myometrial activity by intracellular calcium. In the uterus and placenta, *CaBP-9k* expression is regulated by the sex steroid hormones oestrogen (E2) and progesterone (P4). Intestinal CaBP-9k is involved in intestinal calcium absorption, and is regulated at the transcriptional and post-transcriptional levels by 1,25-dihydroxyvitamin D3, the hormonal form of vitamin D. Thus, evidence to date suggests that *CaBP-9k* may be regulated in a tissue-specific manner. In this review, we will summarize current data on the molecular mechanism of regulation of CaBP-9k in mammals, including recent research data generated in our laboratories.

Keywords: calbindin-D_{9k} • knockout • uterus • placenta • duodenum • kidney • pituitary

Introduction

Calcium elicits the activation of complex intracellular signalling cascades and intracellular messengers. Cytosolic-free calcium plays a crucial role in maintaining homeostasis in living organs and tissues, however, the mechanisms that couple membrane receptor activation to calcium signalling, and the pathways of calcium entry into the cytosol, are only in partly understood [1]. The 9-kilodalton cytosolic calcium-binding protein

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CaBP-9k has two calcium-binding domains, and belongs to a family of intracellular proteins with a high affinity for calcium [2]. The full-length cDNA for human *CaBP-9k* has been cloned using reverse transcription (RT)-PCR. It consists of 600 nucleotides in length, and contains the coding region for 79-amino acid, 57 nucleotides 5′- and 159 nucleotides 3′-noncoding region and a poly(A) tail [3]. We have shown that the *CaBP-9k* locus spans approximately 5.5-kb on the X-chromosome, and consists of three exons and four Alu repeats [4]. We have also shown that a sequence of 50 nucleotides downstream from the human *CaBP-9k* promoter has extensive homology to the oestrogen response element (ORE) of rat *CaBP-9k*, and that the lack of expression of *CaBP-9k* in the uterus and possibly placenta in humans may be due to a two-nucleotide change within this region [4].

CaBP-9k is expressed in a variety of mammalian tissues, that is, uterus, placenta, intestine, kidney, pituitary gland and bone [5–8]. It is involved in intestinal calcium absorption, and is regulated at the transcriptional and post-transcriptional levels by 1,25-dihydroxyvitamin D3, the hormonal form of vitamin D [9, 10]. However, CaBP-9k alone is not sufficient to mediate 1,25-dihydroxyvitamin D3-mediated increases in intestinal calcium absorption in rats [11], and in the uterus, CaBP-9k appears to be involved in the regulation of myometrial activity by intracellular calcium [7]. Thus, the physiological role(s) of CaBP-9k is still a question for further study. Recently, we demonstrated that uterine CaBP-9k is responsive to exogenous E2, and may represent a potential biomarker for exposure to environmental oestrogenic chemicals, the so-called 'endocrine disruptors' [12–16]. In this review, we will summarize current findings related to the molecular mechanism of regulation of CaBP-9k in mammals, and introduce research data from recent studies by us and others.

Phenotype of *CaBP-9k* knockout mouse

The mechanism of regulation of active calcium transport in calcium-related disorders, such as hypocalcaemia, rickets and osteomalacia, in vitamin-D-receptor (VDR)-null mice and 1α-hydoxylase-deficient mice has been examined [17–19]. To more precisely define the role of CaBP-9k in active calcium transport, we generated CaBP-9k-null mice. *CaBP-9k*-knockout (KO) mice had a normal phenotype, and did not display any of the abnormalities of *VDR*-KO mice [20], which indicated that there is compensatory gene induction in these animals [21–23]. In addition, *CaBP-9k*-KO mice were phenotypically normal at birth and survived for at least 1 year [20]. Thus, CaBP-9k appears to differ from other calcium-transporter proteins, which have been shown to be rate-limiting factors in active calcium transport. In *CaBP-9k*-KO mice, there were no detectable differences in calcium absorption or serum calcium levels compared to wild-type (WT) mice, which indicates that CaBP-9k is not required for duodenal calcium absorption, or that other factors can functionally substitute for CaBP-9k [20].

To identify other factors that may substitute for CaBP-9k in calcium absorption, we examined calcium-transporter gene expression in CaBP-9k-null mice during pre-weaning (mice aged 1 day to 4 weeks), when there is a dramatically higher level of intestinal absorption of calcium due to both increased food consumption and elevated fractional calcium absorption [24]. CaBP-9k-null mice expressed higher levels of duodenal *TRPV6* mRNA compared to WT mice, and *PMCA1b* mRNA levels were significantly induced in KO mice at weaning, whereas they remained constant in WT mice [20]. Others have shown that intestinal *TRPV6* mRNA levels increase 30-fold at weaning, coincident with the induction of *CaBP-9k* expression, and that duodenal *PMCA1b* is induced during pre-weaning [25, 26]. These results suggest that in the absence of CaBP-9k, the absorption of milk calcium may mediated by compensatory induction of *TRPV6* and *PMCA1b* in *CaBP-9k*-KO mice [20].

CaBP-9k-null mice maintained normal serum calcium concentrations and showed no abnormal symptoms, which suggests that a normal diet supplies sufficient calcium for *CaBP-9k*-KO mice [20]. To examine the regulation of calcium homeostasis in *CaBP-9k*-KO and WT mice, mice were fed low-, normal-, and high-calcium diets during the growth period from 3 to 10 weeks-of-age. Duodenal *TRPV6* and *CaBP-9k* mRNA levels in WT mice increased inversely with the calcium concentration of their food [27]. Furthermore, *TRPV6* mRNA levels in the low- and normal-calcium-diet groups were higher in WT mice compared to *CaBP-9k*-KO mice, which raises the possibility that CaBP-9k directly regulates *TRPV6*

gene transcription. However, additional studies are needed to determine whether CaBP-9k, or other factors are involved in the regulation of genes involved in calcium transport. The levels of duodenal *PMCA1b* and *NCX1* mRNA fluctuated with dietary calcium levels, but the differences were not significant between WT and *CaBP-9k*-KO mice. Duodenal *PMCA1b* mRNA levels in *VDR*-KO Leuven mice are reduced by a low-calcium diet [25]. We did not observe specific differences in *PMCA1b* mRNA levels in our study, which suggests that other duodenal calcium-transporter genes may participate in calcium absorption in the absence of CaBP-9k [20]. Renal TRPV6 and TRPV5 may also compensate for CaBP-9k in renal calcium re-absorption [20].

In summary, there is mounting evidence that CaBP-9k is an important factor in active calcium transport, but that its function can be compensated for by other calcium-transporter genes in CaBP-9k-null mice, including intestinal *TRPV6* and *PMCA1b* during pre-weaning, and renal *TRPV6* and *TRPV5* during growth and development [20]. CaBP-9k may also be a rate-limiting factor in duodenal and renal active calcium transport by regulating the expression of *TRPV6*, *TRPV5*, and *PMCA1b*. Because the roles of the calcium-transport proteins within any one cellular compartment are complex, it appears that organisms have evolved mechanisms of functional compensation such that inactivation of one gene does not disrupt whole-body calcium homeostasis. The generation of double- or triple-KO mice with deletions of *CaBP-9k* and other calcium-transporter genes may shed further light on the mechanism of active calcium transport.

Uterine expression and regulation of *CaBP-9k*

CaBP-9k is expressed mainly in the endometrial stroma and myometrium of the uterus in non-pregnant rats [28, 29], whereas in pregnant rats, expression is also observed in the epithelium of the uterus [30]. Bovine *CaBP-9k* is expressed only in the luminal and glandular epithelium of the endometrium, not in the myometrium or in the stromal cells of the endometrium in non-pregnant animals [31]. In contrast to the regulation of intestinal *CaBP-9k*, uterine *CaBP-9k* is not regulated by vitamin D, despite the presence of vitamin D receptors in this tissue. Rather, it is regulated by the sex steroid hormones [29, 32, 33].

There is a strong body of evidence that *CaBP-9k* is regulated by sex steroid hormones in the rat uterus. In particular, oestrogen-dependent regulation of *CaBP-9k* has been demonstrated. Injection of oestrogen into 21-day-old rats increased the expression of *CaBP-9k* mRNA up to 300-fold compared to non-treated rats. Furthermore, *CaBP-9k* mRNA has been shown to fluctuate during the estrous cycle in the rat uterus, when serum oestrogen levels also fluctuate [34]. The expression of *CaBP-9k* mRNA in the rat uterus is not detectable at dioestrus, increases at prooestrus and reaches its highest level at estrous, after which expression decreases as animals enter metoestrus. Using a CaBP-radioimmunoassay, it was demonstrated that CaBP-9k synthesis decreases drastically in the uterus of ovariectomized (OVX) rats, whereas it is greatly enhanced by low physiological doses of E2 in a dose-dependent manner [29]. Slot blot and Northern blot analysis have been used to demonstrate that oestrogen stimulates the transcription of *CaBP-9k* in the uterus of mature (OVX and immature rats [35]. The expression of *CaBP-9k* is completely repressed during dioestrus in the rat uterus, when the concentration of 17β-estradiol (E2) is at its lowest, and increases at prooestrus in response to the rise in plasma E2 [32, 34]. It has also been shown that E2 causes the rapid accumulation of *CaBP-9k* mRNA in the uterus of OVX rats [36]. In oestrogen-primed OVX rats, P4-inhibited oestrogen-induced *CaBP-9k* expression, and this effect was completely abrogated by co-administration of the progesterone antagonist RU486 [32]. In pregnant rats, it has been shown that P4 down-regulates *CaBP-9k* expression in the uterus during early pregnancy [37].

In OVX gilts, E2 treatment induced an increase in *CaBP-9k* mRNA levels, whereas administration of P4 to OVX pigs decreased *CaBP-9k* mRNA levels [38]. In contrast, P4 has been shown to have a positive role in the regulation of *CaBP-9k* expression in the bovine uterus [31]. In the luteal phase of the oestrus cycle, the concentration of uterine CaBP-9k protein and *CaBP-9k* mRNA was threefold higher compared to that in follicular phase of cows. Recently, we demonstrated that *CaBP-9k* mRNA and protein are prominently expressed during the luteal phase in the pig uterus [39]. This result was in contrast to the rat uterus, and indicated that P4 may play an important role in the up-regulation of *CaBP-9k* during the luteal

phase in the porcine uterus. In addition, *CaBP-9k* appears to be predominantly expressed in the epithelium and glandular structures of the pig uterus during the luteal phase, which suggests that *CaBP-9k* may be differentially regulated during this cycle, presumably by steroid hormones, and in particular, by high P4 levels in this tissue [39].

In contrast to rats, the expression of uterine *CaBP-9k* is not under strict oestrogen regulation in mice. *CaBP-9k* is expressed in the mouse uterus, and the level of expression increases during early pregnancy and implantation [40, 41]. *CaBP-9k* mRNA is expressed in the luminal and glandular endometrial epithelia in the uterus at the time of implantation, and in the luminal, but not in the glandular, epithelia early in pregnancy (day 5 of pregnancy). In oophorectomized adult mice, P4-enhanced *CaBP-9k* mRNA expression in the uterus, whereas E2 had no effect [40]. *CaBP-9k* mRNA is expressed at high levels in the uterus of mice at dioestrus and metoestrus, and at basal levels at prooestrus and oestrus [41]. This particular study also showed that E2 alone fails to induce uterine *CaBP-9k* mRNA expression. Taken together, these results suggest that *CaBP-9k* expression in the uterus is under complex hormonal regulation in different species. To date, there has been no evidence that *CaBP-9k* is regulated by oestrogen in tissues or cells other than those of the female reproductive tract in mice. Recently, we demonstrated that RU486, a P4 antagonist, induces a significant decrease in *CaBP-9k* mRNA expression, whereas tamoxifen and ICI182780, which are E2 antagonists, have no effect on the expression of *CaBP-9k* mRNA [42]. These results indicate that P4, and not E2, is a key regulator of *CaBP-9k* expression during late pregnancy and lactation in the mouse uterus.

The mechanism of regulation of uterine *CaBP-9k* by steroids is relatively well understood in rats. In the rat uterus, the expression of *CaBP-9k* is up-regulated by oestrogen, and down-regulated by progesterone during the estrous cycle and early pregnancy [32, 34, 36, 37]. The level of expression of *CaBP-9k* mRNA fluctuates during the estrous cycle, but exhibits a very different pattern in the uterus of the rat and mouse [41]. In the mouse uterus, *CaBP-9k* is expressed at high levels during P4-dominant dioestrus and metoestrus, and at basal levels during E2-dominant prooestrus and oestrus. Furthermore, treatment with E2 or P4 alone results in no induction, or a moderate up-regulation of expression of *CaBP-9k* mRNA, respectively, whereas combined treatment with both steroids induces a high level of expression of *CaBP-9k* [41]. The mechanisms that underlie these distinct patterns of regulation of *CaBP-9k* in the rat and mouse are not clear. The effect of E2 on the regulation of *CaBP-9k* appears to be mediated by an imperfect ERE in intron A of the mouse *CaBP-9k* gene [9, 36]. In rats, regulation of *CaBP-9k* is mediated by an ERE located in the first intron [43]. Intron A of mouse *CaBP-9k* was recently cloned, and it contains a single base change in the ERE compared to rat *CaBP-9k* [44]. This may partially explain the observed differences in the hormonal regulation of *CaBP-9k*, the rat and mouse uterus. Porcine *CaBP-9k* in the uterus does not contain a functional ERE in intron A, which may reflect yet another mechanism of regulation of *CaBP-9k* in pigs [38]. However, we cannot rule out the possible involvement of other as-yet unknown cell-, tissue-, and species-specific factors in the regulation of *CaBP-9k* expression. For example, the regulation of *CaBP-9k* expression by oestrogen in the rat was uterine-specific, and a similar pattern was not observed the intestine [29]. Recently, we demonstrated that the putative ERE of *CaBP-9k* fails to bind to the ER in the mouse uterus [45]. When we isolated mouse genomic clones of *CaBP-9k* and analysed their expression in the mouse uterus, we found that the promoter region of *CaBP-9k* contains a putative progesterone response element (PRE), and that promoter activity is stimulated by P4. These results suggest that mouse uterine *CaBP-9k* expression is regulated by a PRE [45].

Placental expression and regulation of *CaBP-9k*

Calcium is transported to the developing foetus *via* the placenta, and CaBP-9k appears to play an important role in the regulation of Ca^{2+} transport from the mother to the foetus during pregnancy. However, the role of CaBP-9k in placental function during pregnancy is not known. Recently, it was shown that *CaBP-9k* transcripts are present in cytotrophoblast cells and syncytiotrophoblasts of the human term placenta, with a lower level of expression in cytotrophoblast cells compared to syncytiotrophoblasts [46]. CaBP-9k protein is detectable in the cytotrophoblast and syncytiotrophoblasts of placental tissue sections, as

well as in cultured cells, which indicates that *CaBP9k* is expressed in trophoblast cells in the human term placenta [46]. The expression of *CaBP-9k* in the placentas of species other than human has also been examined [38, 47–49]. High levels of CaBP-9k are present in the epithelial cells of the yolk sac and endodermal cells of the placenta [28]. The expression of *CaBP-9k* mRNA was not detectable by Northern blot analysis in porcine myometrium and placenta, but it was detectable by RT-PCR [38]. *CaBP-9k* mRNA has been detected in the trophoblasts of various species, in addition to human. It is currently hypothesized that CaBP-9k plays a role in calcium transfer *via* parallel gestational changes in placental CaBP-9k and foetal growth, and that this in turn regulates the foetal accumulation of calcium. In cattle, the level of *CaBP-9k* in the caruncular epithelium increases during the last trimester, most likely in response to the increased need for calcium during the mineralization of the foetal skeleton [49]. In the placenta of mice, there is a distinct pattern of expression of *CaBP-9k* compared to other tissues, such as intestine and kidney, which indicates that the expression of *CaBP-9k* in the mouse placenta is not dependent on the VDR [50]. Recently, we demonstrated for the first time that *CaBP-9k* is expressed in the mouse placenta and extra-embryonic membrane, and that *CaBP-9k* mRNA may be regulated by E2 and P4 and their cognate receptors through complex signalling pathways in these tissues [51].

Duodenal and renal expression and regulation of *CaBP-9k*

In mammals, a number of tissues express *CaBP-9k*, including intestine, uterus, placenta, kidney and bone [5–8, 31, 48, 49, 52]. Intestinal CaBP-9k is involved in intestinal calcium absorption, and in rodents, it is regulated at the transcriptional and post-transcriptional levels by 1,25-dihydroxyvitamin D3, the hormonal form of vitamin D [9, 10, 53]. It has also been demonstrated that this physiologically active form of vitamin D may be involved in the regulation of expression of duodenal *CaBP-9k*, but not involved in PMCA1 expression in human [54]. Furthermore, evidence suggests that vitamin D-mediated regulation of *CaBP-9k* may also be important in reproductive tissues [55, 56]. However, while intestinal *CaBP-9k* is clearly regulated by vitamin D, placental and uterine levels do not respond to vitamin D-depletion. Therefore, in the uterus, *CaBP-9k* does not appear to be regulated by vitamin D, despite the presence of VDRs in this tissue.

The regulation of *CaBP-9k* in gastrointestinal tissues is not well understood. *CaBP-9k* contains a vitamin D-responsive DNA element (VDRE), which has been shown to be involved in the regulation of expression of *CaBP-9k* in the intestine [29]. *CaBP-9k* is actively expressed in enterocytes, which are the dominant epithelial cell in the duodenal mucosa [54]. Its expression level decreases further along the gastrointestinal tract, and levels are barely detectable in the distal ileum and large intestine. CaBP-9k is believed to be an important factor in calcium absorption and metabolism in the intestine. It is of interest that *CaBP-9k* is active only in the enterocytes, which are the major epithelial cells of the duodenal mucosa [25], and that its expression decreases to almost undetectable levels in the digital ileum and large intestine. These aspects of intestinal CaBP-9k warrant further study.

To better understand the function of CaBP-9k in gastrointestinal tissues in humans, we examined the relationship between the levels of *CaBP-9k* and *VDR* mRNA in the intestine, and aging and gender to determine whether *CaBP-9k* expression was related to mineral homeostasis or the development of age-related calcium disorders, such as osteoporosis [57]. *CaBP-9k* mRNA expression in the bulb and 2nd portion of the duodenum increased with age, whereas calcium levels in the blood decreased. We detected no significant age-associated changes in *VDR* mRNA expression in the duodenum. These results indicated that the function of intestinal CaBP-9k may not be related to calcium absorption in these tissues, however, the precise role of intestinal CaBP-9k remains to be elucidated [57]. Certainly, the age-related increase in intestinal *CaBP-9k* expression provides intriguing insight into the role of CaBP-9k in the intestine.

It has been widely accepted that glucocorticoids (GCs) can reduce intestinal calcium absorption by decreasing active transport and normal brush-border vesicle uptake of calcium [58, 59]. GCs have been shown to reduce the synthesis of calcium-binding proteins in chicken [60]. In mammalian duodenum and kidney, calcium ions actively enter from the

lumen *via* epithelial calcium transporter 1 or 2, and bind to the calcium-binding proteins CaBP-9k or CaBP-28k. Ions are discharged into the blood by plasma membrane-localized calcium ATPase 1, or the Na/Ca exchanger [61]. However, the target of GCs in the malfunction of renal and duodenal calcium absorption and re-absorption is not known. To determine whether endogenous factors from the adrenal glands are involved in the regulation *CaBP-9k* expression, duodenal and renal expression of *CaBP-9k* was analysed in adrenalectomized (ADX) mice [62]. In the duodenum, there was no difference in *CaBP-9k* mRNA or protein levels in ADX mice compared to sham-operated (control) mice, whereas renal CaBP-9k levels were significantly decreased in ADX mice compared to the controls. This data implies that certain factors from the adrenal gland take part in the regulation of renal *CaBP-9k*. Further studies are needed to identify and characterize these endogenous factors that are potentially involved in the modulation of renal CaBP-9k protein.

The currently accepted hypothesis is that renal *CaBP-9k* is expressed at the site of calcium re-absorption. Renal *CaBP-9k* appears to be expressed in distal convoluted tubules, where it is thought to facilitate calcium re-absorption [63]. However, mice treated with a pharmacological dose of dexamethasone (DEX) do not display altered renal expression of *CaBP-9k* mRNA or protein. Interestingly, ADX-induced suppression of renal CaBP-9k was reversed by long-term supplementation with DEX, which supports the hypothesis that endogenous GCs from the adrenal gland regulate renal CaBP-9k homeostasis. Renal *CaBP-9k* is induced at the transcriptional and translational levels by DEX administration, and then returns to control levels overtime, which suggests that exogenous GCs might also affect renal CaBP-9k homeostasis, similar to endogenous GCs [62]. The increased expression of renal *CaBP-9k* in response to DEX was completely reversed by the glucocorticoid receptor (GR) antagonist RU486, which indicates that the regulation of renal expression of *CaBP-9k* may be dependent on the GR [62].

CaBP-9k has been shown to be involved in protecting cells from the potentially cytotoxic effects of free calcium ions by mediating the intracellular transport of calcium ions to the plasma membrane [64, 65]. In gastrointestinal tissue, the GR is highly expressed in the epithelial cells of the duodenum and jejunum, and GCs appear to be essential for cellular differentiation and maintenance of mineral absorption [66–68]. Immunohistochemical analysis revealed that CaBP-9k is present in the cytoplasm of the duodenocyte, and GRs are present in the nuclei of the same cells [62]. The GR is also present in the nuclei of cells in the surrounding adipose tissue of the intestinal tract. The localization of CaBP-9k and GRs to the same cells supports the hypothesis that activation of the GR by DEX reduces the level of CaBP-9k in duodenocytes, and provides an explanation of why GR antagonists completely block DEX-induced decreases in CaBP-9k levels [62]. In the kidney, CaBP-9k localized mainly to the distal tubule, as expected [62]. The GR is mainly expressed in the glomerulus, but it is also present in the distal segments [69, 70]. Thus, although GRs do not appear to localize to the distal tubule, evidence suggests that the distal tubule is the site of renal CaBP-9k homeostasis, and the site of action of DEX in ADX mice [62].

In summary, we have examined the regulation of *CaBP-9k* by GCs in the calcium absorbing and re-absorbing organs, that is, duodenum and kidney, and have presented evidence of the potential role of CaBP-9k in these organs [62]. Sustained DEX administration suppresses duodenal *CaBP-9k* expression, but has no effect on renal *CaBP-9k*. DEX was shown to regulate mouse *CaBP-9k* expression in a tissue-specific manner. DEX inhibited *CaBP-9k* expression in the duodenum, temporally induced expression in the kidney, and had no effect on expression levels in the uterus. GR-mediated reduction of duodenal *CaBP-9k* may be involved in negative calcium absorption in GC-induced osteoporosis (GIO), whereas renal *CaBP-9k* does not appear to be involved [62].

Pituitary expression and regulation of *CaBP-9k*

Recently, it was shown that *CaBP-9k* is highly up-regulated by E2 in the somatolactotrope cell line GH3, a pituitary gland tumour cell line [71]. Recently, the expression and regulation of *CaBP-9k* mRNA and protein in the rat pituitary gland during the estrous cycle was examined [72]. At the same time, immature rats were treated with a physiological dose of E2 in the absence or presence of P4, and *CaBP-9k* expression was analysed to verify that *CaBP-9k* expression

in the pituitary gland was under hormonal regulation. The level of *CaBP-9k* mRNA was highest at prooestrus, and began to decrease at oestrus. At dioestrus and metoestrus, the expression level of *CaBP-9k* mRNA was very weak or undetectable. These results agreed with previous results showing that there were high levels of uterine *CaBP-9k* mRNA at prooestrus and oestrus, and basal levels at dioestrus and metoestrus [34]. Furthermore, uterine *CaBP-9k* expression was also dependent on E2 levels [29, 33] and ER [37, 44]. It is of interest that pituitary levels of *CaBP-9k* mRNA and protein parallel uterine levels, as it suggests that uterine and pituitary *CaBP-9k* are expressed and regulated in a similar manner during the estrous cycle in rats [72]. Taken together, these results suggest that pituitary levels of *CaBP-9k* mRNA and protein are tightly regulated during estrous cycle, and that the steroid hormones, E2 and P4, play a role in the regulation of *CaBP-9k* in the pituitary gland throughout the cycle of the rat [72].

Rat *CaBP-9k* contains an imperfect ERE between exon I and intron A, which binds to the ER [36, 43]. We and others have shown that uterine *CaBP-9k* mRNA and protein are induced by E2 treatment [12, 35, 73], and that in immature rats, this increase is mediated *via* the ER-α pathway [74]. ERα is highly expressed in the anterior pituitary, while the expression level of ERβ is very low in this tissue [75]. In addition, ERs do not appear to be expressed in the intermediate and posterior lobes of the pituitary gland [76], which suggests that an ER/ERE-dependent pathway is a key mechanism of E2-dependent regulation of uterine *CaBP-9k*. In the pituitary gland, sex steroids are known to regulate anterior pituitary functions through both direct and indirect pathways [77, 78]. After treatment with E2 alone, there was a significant increase in pituitary CaBP-9k after 48 hrs, which was sustained for 72 hrs after the final administration of E2 [72]. *CaBP-9k* expression has been shown to increase as early as 3 hrs following E2-induction, and peak approximately 48 hrs after treatment [71]. To clarify the role of P4 in the induction of *CaBP-9k* expression by E2 in rats, we treated immature rats with E2 in the absence or presence of P4. We found that P4 treatment reversed the E2-mediated induction of pituitary *CaBP-9k* expression [35]. These results were in agreement with the effect of E2 and P4 on uterine *CaBP-9k* expression. E2-induced increases in *CaBP-9k* expression were completely blocked by ICI 182,780, an ER antagonist [12, 74, 79]. Interestingly, the ERα pathway represents a major mechanism of induction of uterine *CaBP-9k* expression [74]. Uterine *CaBP-9k* expression during early pregnancy was reduced by P4. The E2-induced expression of *CaBP-9k* was also reversed by P4 treatment, which suggests that P4 is an antagonist of E2-mediated up-regulation of *CaBP-9k* [32].

As described above, we have shown that the regulation of *CaBP-9k* by steroid hormones is similar in the pituitary and uterus [72]. In contrast to the mechanism of hormonal regulation of uterine *CaBP-9k* in rats, hormonal regulation of *CaBP-9k* in the reproductive tissues of mice is less well known [80, 81]. We examined whether *CaBP-9k* expression in the pituitary gland of mice fluctuates during the estrous cycle or in response to hormones, and found that neither *CaBP-9k* mRNA nor protein was detectable in the pituitary gland of mice [72]. Thus, while mice and rats share a high level of similarity in the genomic organization of *CaBP-9k*, the spatial expression pattern and regulation of *CaBP-9k* appear to be distinct in each animal. *CaBP-9k* is abundantly expressed in mouse kidney, but is undetectable in rat kidney [82]. In addition, we were unable to detect *CaBP-9k* in the pituitary glands of male rats or immature rats. Taken together, the data suggest that pituitary *CaBP-9k* is expressed in mature female rats, is up-regulated by E2, and antagonized by P4 in the anterior lobe of pituitary gland [72].

Transcriptional regulation of *CaBP-9k*

While it is well-established that *CaBP-9k* in the rat uterus is regulated by oestrogen, the putative ERE of *CaBP-9k* failed to bind to the ER in the mouse uterus [83]. Previously, we isolated several mouse genomic clones of *CaBP-9k* and used them to analyse CaBP-9k expression in the mouse uterus [45]. The promoter region of *CaBP-9k* contains several putative steroid hormone receptor-binding sites. We constructed several luciferase reporter plasmids that contained the *CaBP-9k* promoter, and used them to transfect T47D breast cancer cells that expressed both the ER and PR [84]. We demonstrated that *CaBP-9k* promoter activity maps to a region that contains a putative PRE, and that expression is stimulated by progesterone. In the uterus of oophorectomized mice, *CaBP-9k* was up-regulated by progesterone, but not by E2 [45]. Thus, the data suggest that mouse uterine *CaBP-9k* is regulated by a PRE.

Although there has been progress in our understanding of the transcriptional regulation of *CaBP-9k*, the mechanism of regulation of *CaBP-9k* expression is not fully elucidated, mainly due to the lack of an appropriate *CaBP-9k*-expressing cell line. GH3 cells are a rat pituitary somatolactotrophic cell line that was derived from the MtT/W5 pituitary tumour. Growth and prolactin synthesis in GH3 cells are both stimulated by oestrogen [71]. Many oestrogen-responsive reproductive tissues express ERβ and ERβ mRNA, including the pituitary gland [85]. In GH3 cells, *CaBP-9k* expression is highly responsive to E2 treatment, and the regulation of *CaBP-9k* expression in the pituitary gland in rats is highly sensitive to exogenous and endogenous E2 [71, 72]. We characterized *CaBP-9k* promoter response elements using *CaBP-9k*-expressing GH3 cells. GH3 cells were transiently transfected with various promoter constructs, and activity was assayed by luciferase reporter gene assay, electrophoretic mobility shift assay (EMSA), and mutagenesis. We identified several E2-dependent promoter elements, including an ERE and other *cis*-acting elements in the rat *CaBP-9k* promoter [84].

Uterine CaBP-9k may be involved in smooth muscle constriction at term and/or during implantation in the endometrium [72, 79, 86]. We recently reported that *CaBP-9k* expression is induced by endogenous and exogenous E2 in the pituitary glands of rats [72]. Although the expression and regulation of *CaBP-9k* in these tissues have been well described, the regulation of the *CaBP-9k* promoter remains unclear. To begin to understand the mechanism of E2-mediated *CaBP-9k* expression in the pituitary gland, we performed a series of transient transfection experiments in GH3 cells using different *CaBP-9k* promoter constructs, and used relative luciferase activity (RLA) to assess the transcriptional activity of each [84]. We identified a putative E2-responsive element in the *CaBP-9k* promoter, from nucleotides −915 to –663 (relative to the transcriptional start site at +1), and confirmed that this region contained one or more transcription factor-binding sites by EMSA. Sequence analysis identified a putative NF-κB-binding site in this region, which, when mutated, significantly decreased RLA in GH3 cells. These results confirmed that this site is an important *cis*-acting element in the E2-mediated induction of *CaBP-9k* expression. Finally, binding of the p65 subunit of NF-κB to its putative-binding site in the rat *CaBP-9k* promoter (nucleotides −848 and −834) was confirmed by EMSA. In addition, E2-mediated induction of *CaBP-9k* expression was partly inhibited by N-tosyllys chlormethyl ketone (TLCK), which blocks the dissociation of NF-κB and IκB, and inhibits translocation of NF-κB into the nucleus. These results indicated that *CaBP-9k* expression is regulated *via* a non-classical pathway involving NF-κB [84]. The ER antagonist ICI significantly inhibited E2-mediated *CaBP-9k* expression, which confirmed that the activation of *CaBP-9k* transcription in GH3 cells occurs *via* an ERα-dependent pathway.

ERα-mediated regulation of gene transcription occurs through two distinct pathways: the classical and non-classical pathway. In the classical pathway, ERα interacts with an ERE in the promoter region of its target gene, and recruits cofactors to induce or repress transcription. In the non-classical pathway, ERα regulates the transcription of genes without binding directly to an ERE. Rather, ERα alters the activities of other transcription factors, such as AP-1, Sp1 or NF-κB [87, 88]. We demonstrated that E2-mediated *CaBP-9k* activation occurs *via* a non-classical pathway that involves NF-κB binding to its cognate site in the *CaBP-9k* promoter, as well as a classical ERα-ERE pathway of transcriptional regulation [84]. However, while both classical and non-classical pathways may be involved in E2-mediated *CaBP-9k* gene transcription in GH3 cells, the ERα-ERE pathway appears to be the main signalling pathway for regulating *CaBP-9k* transcription [84].

Conclusions

Although CaBP-9k is expressed in a variety of tissues, that is, uterus, placenta, intestine, kidney and pituitary gland, in several species, the physiological role of CaBP-9k in these tissues remains a topic of intense study. The information of cellular distribution of CaBP-9k is very limited, and it appears that CaBP-9k may not be expressed in the central nerve system (CNS), except for hypothalamus. In the CNS, an alternative calcium-binding protein, calbindin-D28k (CaBP-28k), may replace CaBP-9k in the control of calcium ions [89]. It has been hypothesized that CaBP-9k plays an important role in re-sorption in the kidney and absorption in the intestine. In addition, uterine CaBP-9k is involved in the regulation myometrial activity by intracellular calcium, and that placental CaBP-9k plays a role in calcium transfer from the mother to the foetus during development. Although intestinal *CaBP-9k* is clearly regulated by vitamin D, it appears that *CaBP-9k*

is not regulated by vitamin D in the uterus, despite the presence of VDRs in this tissue. Rather, uterine *CaBP-9k* is regulated by the sex steroid hormones E2 and P4 and their receptors through complex signalling pathways. Thus, it is likely that *CaBP-9k* is transcriptionally regulated in a tissue-specific manner.

It is believed that CaBP-9k is participating in both calcium transport and buffering calcium ions in our body. Although *CaBP-9k* should be considered an important factor in active calcium transport or buffering calcium ions, other calcium-transporter genes can compensate for its function to maintain calcium ions in blood, that is, intestinal *TRPV6* and *PMCA1b* during pre-weaning and renal *TRPV6* and *TRPV5* during growth and development in our CaBP-9k-KO mouse model. This KO mouse model has provided the information that functional calcium absorption or re-sorption by these calcium related genes seems to be processed by para-cellular mechanism. In addition to this para-cellular mechanism by the calcium related genes, other active mechanism(s) for controlling and transporting calcium ions may be present in various tissues, which express these calcium-involving genes *in vivo*. The studies from our lab indicate that both the classical pathway, and a non-classical pathway that involves NF-κB, are involved in the regulation of E2-mediated *CaBP-9k* expression in GH3 cells. However, while our previous studies of the regulation of *CaBP-9k* expression and function contribute to a better understanding of the physiological role(s) of *CaBP-9k* in various tissues, additional studies are warranted, and further insight into the regulation of *CaBP-9k* in different tissues and its physiological role(s) in reproduction and development are needed.

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