Krüppel-like factor 4 is widely expressed in the mouse male and female reproductive tract and responds as an immediate early gene to activation of the protein kinase A in TM4 Sertoli cells

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Abstract

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor critically involved in cell proliferation, differentiation, and carcinogenesis. Recently, KLF4 has also been used for the generation of induced pluripotent stem cells. In this study, we analyzed *Klf4* expression in different mouse tissues using northern blot analysis and immunohistochemistry. Focusing on the male and female reproductive tract, we showed for the first time that KLF4 is expressed in the epithelia of the murine uterus and the vagina. In the male reproductive tract, we detected KLF4 in the epithelia of the epididymis, ductus deferens, coagulating gland, and the penis. As KLF4 is strongly inducible by FSH signaling in Sertoli cells and as this transcription factor is also involved in Sertoli cell development, we employed the mouse Sertoli cell line TM4 as a model system to investigate i) the induction kinetics of *Klf4* upon activation of the cAMP/protein kinase A pathway by forskolin and ii) the effects of *Klf4* induction on TM4 cell cycle progression. Interestingly, *Klf4* mRNA and protein were rapidly but transiently induced, reaching peak levels after 90–120 min and declining to basal levels within 4 h. Compared with the inducible cAMP early repressor, an immediate early response gene, the induction kinetics of *Klf4* is much faster. In conclusion, *Klf4* is an immediate early gene in TM4 cells and its expression in several epithelia of the male and female reproductive tract suggests an important role of *Klf4* in mouse reproductive functions.

Reproduction (2010) **139** 771–782

Introduction

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor involved in terminal differentiation and cell cycle regulation of different types of epithelial cells (Garrett-Sinha *et al.* 1996, Shields *et al.* 1996, Segre *et al.* 1999, Katz *et al.* 2002, 2005, Chen *et al.* 2003, Dang *et al.* 2003). *Klf4* has also been characterized as a tumor suppressor gene since its endodermal deletion caused precancerous changes in the epithelium of the adult stomach (Katz *et al.* 2005), and it is also involved in colorectal cancer (Dang *et al.* 2000, Zhao *et al.* 2004, Ghaleb *et al.* 2007). Moreover, *Klf4* can act as an oncogene (Rowland *et al.* 2005). Whether *Klf4* functions as a tumor suppressor or an oncogene depends on the molecular context in which KLF4 acts (Rowland & Peeper 2006). *Klf4* mRNA is highly abundant in the stomach, colon, and skin as well as in other tissues (Garrett-Sinha *et al.* 1996, Shields *et al.* 1996, Panigada

et al. 1999, Segre *et al.* 1999, Swamynathan *et al.* 2007). Lack of the mouse KLF4 protein in the skin results in loss of the skin barrier function, which leads to early postnatal death (Segre *et al.* 1999). Moreover, the absence of KLF4 in the epithelium of the developing gut leads to a dramatic reduction in the number of goblet cells in the colon (Katz *et al.* 2002).

Remarkably, KLF4 has also been shown to be crucially involved in the maintenance of pluripotency in embryonic stem cells (Li *et al.* 2005, Jiang *et al.* 2008) and in the induction of pluripotency in fibroblasts, i.e. generation of induced pluripotent stem cells (Okita *et al.* 2007, Wernig *et al.* 2007). This clearly demonstrates a not yet fully characterized ambivalent role of KLF4 in terminally differentiated cells on the one hand and in highly proliferative undifferentiated cells on the other hand.

In the male germinal epithelium, the somatic Sertoli cell represents a unique epithelial cell with highly specialized functions, since the so-called nurse cell is

responsible for germ cell survival and differentiation (Mruk & Cheng 2004). Any disturbance of Sertoli cell differentiation can impair proper Sertoli cell function and thus male germ cell development (Mason *et al.* 1986, Raymond *et al.* 2000, Myers *et al.* 2005).

Recently, we have demonstrated that *Klf4* is strongly expressed in spermatids in the mouse (Behr & Kaestner 2002, Godmann *et al.* 2005, 2009b) and human (Behr *et al.* 2007) testis. Interestingly, we found that *Klf4* is also strongly expressed in human seminoma and sometimes also in human fetal gonocytes (Godmann *et al.* 2009a). Furthermore, our results and other studies showed that *Klf4* is also expressed in testicular Sertoli cells (Hamil & Hall 1994, McLean *et al.* 2002, Sadate-Ngatchou *et al.* 2004, Godmann *et al.* 2008). Cell type-specific deletion of *Klf4* in Sertoli cells demonstrated a significant role of *Klf4* during Sertoli cell differentiation (Godmann *et al.* 2008). Interestingly, in a primary Sertoli cell culture, *Klf4* was induced several hours after FSH stimulation (McLean *et al.* 2002, Sadate-Ngatchou *et al.* 2004). FSH action is characterized by its stimulation of the adenylyl cyclase/cAMP pathway via activation of a G protein-coupled receptor (Loss *et al.* 2007). Briefly, binding of hormones to G protein-coupled receptors results in the activation of the adenylyl cyclase. This, in turn, results in an increase of intracellular levels of cAMP. cAMP binds to the regulatory subunit of the heterotetrameric protein kinase A (PKA) and causes the release of the catalytic subunit, which can migrate into the nucleus and activate target proteins by phosphorylation. Among these PKA targets are the transcription factors CREM and CREB, which are essential for many cellular processes and especially for testicular function (Don & Stelzer 2002, Monaco *et al.* 2004). The CREM isoform inducible cAMP early repressor, *ICER*, which is generated from an internal *Crem* gene promoter (Molina *et al.* 1993), has been shown to be very rapidly inducible upon activation of the cAMP/PKA signal transduction pathway in testicular Sertoli cells (Monaco *et al.* 1995). Depending on the cells investigated the peak of *ICER* mRNA levels was observed 2–4 h after activation of the cAMP/PKA pathway. Thus, the kinetics of *ICER* induction in Sertoli cells corresponds to an early response gene (Monaco *et al.* 1995).

While strong *Klf4* expression has been reported for some adult tissues including skin, colon, and testis, numerous other cell types and epithelia have not yet been tested for KLF4 expression including the entire male (besides testis) and female reproductive tract. The first aim of the present study was to investigate KLF4 expression in distinct, not yet analyzed, adult murine epithelia of the male and female reproductive tract. Secondly, we focused on the established testicular TM4 Sertoli cell line (Mather 1980), and analyzed the kinetics of *Klf4* induction and its influence on the Sertoli cell cycle. We used the TM4 cell line to investigate i) the kinetics of *Klf4* induction on the mRNA and protein

levels; ii) whether the induction of *Klf4* indeed depends on the activation of the cAMP/PKA signal transduction pathway, as suggested by the FSH induction of *Klf4* in primary Sertoli cells; and iii) whether PKA-dependent induction of *Klf4* has an effect on cell cycle progression in TM4 cells.

Results

KLF4 is expressed in epithelia of the male reproductive tract

We have previously shown that KLF4 is expressed in round spermatids and in Leydig cells in the mouse testis (Behr & Kaestner 2002, Godmann *et al.* 2009b). In the present study, we show by immunohistochemistry that KLF4 is strongly but gradually expressed in the epididymis (Fig. 1A) reflecting the different regions of the epididymis, i.e. caput, corpus, and cauda (Johnston *et al.* 2005). Most epithelial cells of the proximal caput of the epididymis showed only very faint signals. Only few scattered cells were clearly stained. The nuclei of these cells were located rather apically (Fig. 1A and B). These cells were narrow cells, thought to be absorptive rather than secretory. The distal part of the caput epididymis showed stronger staining compared with the proximal caput. Intense cytoplasmic as well as nuclear staining was visible. Only very few cells were not stained. The corpus epididymis showed the strongest staining of all epididymal regions with very intense signals in almost all nuclei and also in the cytoplasm (Fig. 1C). Like in the distal caput, only few cells were not stained. The caudal region of the epididymis was still KLF4 positive, but the staining was less intense compared with the epididymal corpus (Fig. 1D). The cytoplasm of all cells was homogeneously stained. In contrast, there were clear differences in the nuclear signals with the minority of nuclei exhibiting only faint or even no signals (Fig. 1D). The vas deferens also showed clear staining of the epithelium (Fig. 1E). The cytoplasm of all epithelial cells was stained. In contrast, only a part of the nuclei of all epithelial cells was KLF4 positive. The stromal tissue and sperm cells in the lumen were, like in the epididymis, not stained. Figure 1F shows the corresponding negative control. Figure 1G shows the epithelium of the prostate and underlying adipose tissue. The epithelium exhibits weak staining in only a few cells, preferentially those cells lining the plicae. Interestingly, the soma and nuclei of the fat cells were strongly KLF4 positive as reported recently (Sun *et al.* 2009). In the coagulating gland, also called anterior prostate, mostly those epithelial cells lining the intraluminal folds were stained (Fig. 1H). Almost all these cells showed intensive staining of the apical cytoplasm, while the basal areas were clearly less stained. Irrespective of the cytoplasmic signals, the nuclei were either strongly stained or unstained (Fig. 1H). The seminal vesicles showed no

staining – neither in the epithelium nor in the stroma (Fig. 1I). The epithelia of the penis exhibited strong signals with restriction of the staining to the nuclei. Some of the stromal cells as well as chondrocytes were also stained (Fig. 1J–L).

KLF4 is expressed in distinct epithelia of the female reproductive tract

In the female reproductive tract, we obtained similar results with both KLF4 antibodies for the uterine epithelium (endometrium) and the vaginal epithelium (Fig. 2). Both epithelia, although histologically very different, showed clear nuclear staining in a subpopulation of cells. Interestingly, a significant population of cells in both epithelia was devoid of KLF4 antigenicity. The expression of KLF4 in the uterine epithelium may also depend on the stage of the reproductive cycle of the animals since not all specimens tested exhibited KLF4 signals (three of seven positive; Fig. 2A). However, the stage of the reproductive cycle of the mice was not determined when the tissue was fixed. Remarkably, in the vaginal epithelium, the proportion of stained cells was higher in the basal area of the epithelium than in the apical area, where differentiating cells are located (Fig. 2C). This is in contrast to other epithelia like the skin or the tongue, where the percentage of KLF4-positive cells and the intensity of the staining increased in the apical, terminally differentiating cell layers.

Using the KLF4 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), we also obtained staining in the cytoplasm of oocytes, granulosa cells, cells of the corpus luteum, in a subset of cells of the epithelium of the oviduct (cytoplasmic and nuclear staining), trophoblast cells, and some blood cells in the placenta (data not shown). However, this staining could not be reproduced with the antibody from R&D Systems (Minneapolis, MN, USA) and was therefore not considered as sufficiently validated. Furthermore, the control stainings using normal rabbit sera sometimes resulted in similar staining patterns

Figure 1 KLF4 protein expression in male reproductive tissues.

(A) Overview over the caput epididymis, where staining can be seen mainly in the epithelium of the distal caput. A clear difference in the staining intensity can be seen between different regions. (B) Magnification of the boxed detail from (A). A connective tissue string separates the area exhibiting low stain intensity from those showing high intensity. (C) Strongly stained epithelium from the epididymal corpus. Sperm cells and almost all stromal cells are not stained. (D) Cauda of the epididymis. Again, most of the epithelial cells show strong nuclear and cytoplasmic staining. Only few cells located within the epithelium are not stained. (E) Vas deferens showing staining of the epithelium. While the cytoplasm of all epithelial cells appears to be stained, only a subset of the nuclei of all epithelial cells was strongly KLF4 positive. The other half is unstained. Underlying stromal cells and sperm cells in the lumen are not stained. (F) Negative control of (E). (G) Epididymis of the prostate and underlying adipose tissue. The epididymis exhibits weak staining in a subset of cells. Interestingly, the fat cells are strongly KLF4 positive (red arrows). (H) Coagulating gland (anterior prostate). Mostly those epithelial cells lining the intraluminal folds are stained. (I) The seminal vesicle shows no staining. (J) Overview over a cross section of the penis. Boxed areas are magnified in (K) and (L) respectively. (K) Within the epithelial cells, KLF4 protein is completely restricted to the nuclei. Some of the stromal cells as well as chondrocytes (red arrow in J) in the hyaline growth cartilage next to the os penis are also stained. (L) Epithelium of the skin of the glans penis. For further details, see the Results section. Primary magnifications: 400×.

Figure 2 KLF4 expression in the female reproductive tract. (A) Strong nuclear staining of epithelial cells of the uterine endometrium. (B) Negative control for (A). (C) The vaginal epithelium exhibits numerous KLF4-positive cells, especially in the basal area of the epithelium. Staining is restricted to the nuclei. Basal cells in contact with the basal lamina are mostly KLF4 negative (red arrows). (D) KLF4 western blot of protein isolated from mouse skin and stomach using the antibody obtained from R&D Systems. The antibody detects one specific band migrating around the expected size of 53 kDa.

as obtained with the Santa Cruz antibody, suggesting an unspecific binding of this antibody in immunohistochemistry on paraffin-embedded tissue sections. The specificity of the anti-KLF4 antibody obtained from R&D Systems was confirmed by western blot analysis on total protein extracts isolated from mouse skin and stomach. The antibody detected only one specific band in both tissue samples (Fig. 2D) validating its specificity.

Northern blot analysis of *Klf4* expression in the uterus further strengthened our results obtained by immunohistochemistry (Fig. 3). Although the mRNA levels are relatively low compared with the colon (which exhibited highest relative *Klf4* levels in this study), a clear and distinctive *Klf4* signal was obtained in the uterus. Since we detected *Klf4* mRNA also in the placenta, it remains to be elucidated whether the mRNA is translated and, if yes, where the KLF4 protein is localized (see above). As demonstrated previously and confirmed in Fig. 3, *Klf4* mRNA is abundant in the skin (Segre *et al.* 1999) as well as in several endodermal tissues including the stomach, the colon, and the lungs (Garrett-Sinha *et al.* 1996, Shields *et al.* 1996). By immunohistochemistry, we also detected KLF4 in the course of the present investigation in the mucosa of the oral cavity, the respiratory epithelium, the esophagus, the sigmoid, as well as in the epithelium of the cornea. Interestingly, also chondrocytes of the trachea were clearly stained (data not shown).

KLF4 protein is induced by forskolin as revealed by immunofluorescence

In the recent study, we have described *Klf4* expression in the murine male and female reproductive tract. Next, we were interested in how *Klf4* expression is induced. Since *Klf4* has been shown to be up-regulated in primary Sertoli cell culture (Hamil & Hall 1994, McLean *et al.* 2002) and in the testis of hypogonadal mice (Sadate-Ngatchou *et al.* 2004) several hours after FSH stimulation, we chose the TM4 Sertoli cell line as a model system. It has been shown that TM4 cells do not respond to FSH (Monaco *et al.* 1995). This is probably due to low FSH receptor expression in this cell line (Mather 1980) or even undetectable FSH receptor levels (own unpublished RT-PCR data). However, we used forskolin instead of simulating the FSH and activating the cAMP/PKA pathway and testing whether *Klf4* can also be induced in this epithelial TM4 Sertoli cell line (Mather 1980). Forskolin activates the adenylyl cyclase. This enzyme increases intracellular cAMP levels. High cAMP levels result in an activation of the PKA, which, in turn, phosphorylates its target proteins. As controls, we added only growth medium or growth medium plus DMSO (the solvent for forskolin) to cell cultures treated in the same way. We first checked by immunofluorescence (IF) whether KLF4 protein was up-regulated after the addition of forskolin. As shown in Fig. 4, 1 h after the addition of forskolin, we performed IF and could detect a clear induction of KLF4 protein (Fig. 4C, best visible in the merged picture), while untreated control cells

Figure 3 *Klf4* mRNA expression in distinct mouse organs. (A) Northern blot analysis on total RNA (10 µg/lane) isolated from different mouse tissues shows strong *Klf4* expression in tissues of the gastrointestinal tract, but also in the lung and reproductive organs like the testis, the uterus, and the placenta. No signal can be detected in the kidney, the liver, or skeletal muscle. Northern blots were hybridized with a probe against the *Klf4* open reading frame. (B) The graphic shows the ratio of *Klf4* mRNA over the 28S and 18S rRNA of the appropriate formaldehyde gel. S, skin day 3; C, colon; SI, small intestine; St, stomach; L, lung; K, kidney; U, uterus; P, placenta day 13; T, testis; Li, liver; M, skeletal muscle.

(Fig. 4A) or cells without forskolin but with its solvent DMSO (Fig. 4B) exhibited only considerably weaker signals showing non-induced endogenous KLF4 expression. Remarkably, in addition to the increased signal intensity within the nucleus, there was also a clear cytoplasmic signal visible after forskolin induction possibly representing newly synthesized KLF4 protein before its transport into the nucleus.

Very rapid induction of *Klf4* mRNA

After confirming the inducibility of KLF4 expression in TM4 cells by IF (Fig. 4), we wanted to investigate its expression kinetics in this Sertoli cell line in more detail. Since IF (Fig. 4) and northern blot analyses (data not shown; but provided as reviewer information) demonstrated that DMSO, the solvent of forskolin, had no obvious influence on *Klf4* expression in TM4 cells, all subsequent studies were performed using DMSO-supplemented growth medium as a control. In order to analyze the kinetics of *Klf4* expression in response to forskolin, total RNA samples were collected from cells that have been treated with forskolin or DMSO for 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 min and subjected to northern blot analyses (Fig. 5A, panels I and II).

Forskolin rapidly induced *Klf4* expression. A signal above *Klf4* basal levels in the corresponding DMSO control was already observed after 10 min of exposure.

Importantly, treatment caused a strong increase of *Klf4* mRNA expression after 30 min and signals significantly peaked out around 90 min. Thereafter, *Klf4* levels declined quickly. After 240 min, there was nearly no *Klf4* detectable indicating a very transient increase in the *Klf4* mRNA abundance upon activation of the PKA pathway (Fig. 5A and B).

In order to confirm that *Klf4* expression is induced by forskolin-dependent activation of the PKA pathway, TM4 cells were treated simultaneously with forskolin and H89, a very potent PKA inhibitor. Total RNA was extracted after defined time points, and northern blot analyses demonstrated that forskolin-induced *Klf4* expression could be completely repressed by H89 (Fig. 5A, panel III). Only *Klf4* signals similar to DMSO control levels could be observed (Fig. 5A, panels II and III), indicating that *Klf4* mRNA was specifically induced by forskolin activation of the PKA pathway.

KLF4 protein is also rapidly induced by forskolin

In addition to RNA analyses, we wanted to confirm KLF4 induction at the protein level. Western blot analyses were performed on the same individual cell cultures used for northern blotting (Fig. 5). Ten minutes after treatment, only a faint KLF4 protein signal could be detected, probably presenting endogenous, non-induced levels. However, 20 min after the addition of forskolin, KLF4

Figure 4 KLF4 immunofluorescence of forskolin-treated TM4 cells. (A) A weak basal expression of KLF4 is observable in untreated TM4 cells (growth medium only) and in the DMSO control (B). Compared with the untreated (A) and the DMSO control (B), KLF4 expression is clearly increased in TM4 cells after 1 h of forskolin treatment (C). The merged picture emphasizes the increase in KLF4 signal intensity (C). (D) The negative control shows forskolin-treated TM4 cells, but no KLF4 antibody was added. KLF4 was stained with a FITC-labeled secondary antibody, shown in green. Cell nuclei were DAPI-stained (blue). The cytoskeleton was visualized by F-actin staining using TRITC-labeled phalloidin (red).

levels increased constantly and the highest protein concentrations were observed after 120 min of treatment. Thereafter, KLF4 protein levels declined rapidly and reached baseline levels already after 150 min. β -Actin was used as a loading control (Fig. 6A and B).

Klf4 is more rapidly induced than the inducible *ICER*

As a biological and kinetic control for the induction of genes by cAMP in the TM4 Sertoli cell line, we analyzed the induction of the inducible *ICER*, which is expressed in different isoforms in the TM4 cells (Monaco *et al.* 1995). *ICER* was described as an early response gene after induction by forskolin, since its mRNA was first detectable after 60 min and reached highest levels around 120 and 240 min of treatment and declined thereafter (Monaco *et al.* 1995). We probed the same northern blot shown in Fig. 5A (panels I and II), with a probe specific for *ICER* and found a very similar pattern of *ICER* induction in our experiment compared with that published previously

(Monaco *et al.* 1995): a weak induction was seen after 60 min of forskolin exposure as well as increased levels of *ICER* after 120 min (Fig. 5C, *ICER* + isoforms). Interestingly, the *Klf4* gene responds much quicker to activation of the cAMP/PKA pathway in TM4 Sertoli cells than the early response gene *ICER* (Fig. 5A, panel I). Remarkably, the quick *Klf4* response was very transient.

Activation of the cAMP/PKA pathway has no influence on the cell cycle in TM4 Sertoli cells

Since KLF4 expression is associated with cell cycle arrest (Garrett-Sinha *et al.* 1996, Shields *et al.* 1996) and since *Klf4* is quickly and strongly induced by forskolin, we investigated whether activation of the cAMP/PKA pathway in TM4 cells influenced cell cycle progression. Using fluorescence-activated cell sorting (FACS) of propidium iodide-stained cells, we analyzed synchronized TM4 cells treated with i) forskolin in 0.1% DMSO in growth medium or ii) the solvent (0.1% DMSO) in growth medium alone or iii) untreated cells (growth medium only). Cells were harvested after 0, 1, 3, 6, 12, and 24 h and analyzed for cell cycle progression by DNA content. Interestingly, we found no differences between the cultures indicating that activation of the cAMP/PKA pathway in TM4 cells and subsequent upregulation of KLF4 (and other genes/proteins) did not affect progression through the cell cycle in this experimental setup (Fig. 7). These results were confirmed by two additional independent experiments.

Figure 5 Rapid induction of *Klf4* expression in TM4 cells after forskolin exposure. (A) Northern blot analyses on TM4 cell total RNA extracts (10 µg/lane) show that *Klf4* expression is rapidly induced by forskolin (panel I). Only 10 min after treatment, there is a distinguishable and after 30 min a strong increase in *Klf4* signal intensity compared with control conditions (TM4 cells cultured in growth medium + DMSO, panel II). Highest *Klf4* mRNA levels could be detected after 90 min of forskolin exposure, whereas the DMSO control exhibits constantly weak signals. Panel III shows the repression of forskolin-induced *Klf4* expression by the PKA inhibitor H89 (forskolin + H89). The corresponding gel photos below indicate RNA loading. (B) Relative *Klf4* mRNA expression shown as the ratio of *Klf4* mRNA over 28S rRNA and 18S rRNA of the corresponding formaldehyde gel. The graphic demonstrates the quick response of *Klf4* expression to forskolin compared with DMSO (control) and to cells that have been simultaneously treated with forskolin and the PKA inhibitor H89. Results are depicted as mean $\pm$ S.E.M. N indicates the number of independent experiments. Bars labeled with different letters depict statistical significant differences ($P < 0.05$; for all, except 90 vs 180, 210, or 240 min respectively, where $P < 0.01$). (C) In order to arrange *Klf4* induction chronologically, we analyzed the expression of *ICER* (inducible cAMP early repressor). *ICER* is classified as an immediate early cAMP response gene. *ICER* mRNA levels increase 60 min after forskolin exposure and peak 240 min after treatment. Notably, compared with *ICER*, *Klf4* expression is clearly induced within the first 30 min and reaches its maximal concentration after 90 min. *Klf4* thus responds much quicker to the activation of the cAMP/PKA pathway than *ICER*. DMSO-treated control TM4 cells showed almost no *ICER* signal.

Figure 6 Induction of KLF4 protein expression in TM4 cells after forskolin treatment. (A) Western blot analyses of KLF4 expression on total TM4 cell protein extracts after stimulation with forskolin (30 μ g/lane). β -Actin served as a loading control and was used for normalization purpose. (B) KLF4 signals from proteins isolated 10 min after forskolin stimulation match the endogenous (non-stimulated) protein levels and served as base – the corresponding ratio of KLF4 to β -actin was defined as 1. Fold change indicates the ratio of KLF4 protein normalized to β -actin after time of forskolin treatment over the base. Four independent experiments are depicted. An increase of the KLF4 expression is observable 20 min after forskolin exposure, and KLF4 protein levels peak at 120 min. Thereafter, the KLF4 signal intensity declines quickly. The y-axis indicates the fold change of relative KLF4 protein expression over the base in arbitrary units and the x-axis shows the timeline of forskolin exposure in minutes.

Discussion

Lack of KLF4 in the epidermis, the epithelium of the stomach, and colon or the epithelium of the tongue has tremendously impairing effects on the differentiation and/or function of these tissues, as has been proven *in vivo* by *Klf4* gene inactivation studies in mice (Segre *et al.* 1999, Katz *et al.* 2002, 2005, Swamynathan *et al.* 2007). Since deletion of *Klf4* had also significant effects on leukocyte development and function (Feinberg *et al.* 2007, Klaewongkram *et al.* 2007) and on maturation and maintenance of the epithelial ocular surface (Swamynathan *et al.* 2007), it is evident that KLF4 is an important player during the differentiation of many adult cell types. We have shown in this work that KLF4 is expressed in several tissues constituting the adult male and female reproductive tract. Therefore, it is conceivable and likely that KLF4 might also be an important factor for the development and function of the reproductive tract in both sexes in mice. Future studies applying cell type-specific deletion of *Klf4* in the respective cell types will reveal the functions of KLF4 during mouse reproduction. Especially, the strong KLF4 expression in the uterine epithelium, the vagina, and in the epithelia of the male excretory ducts suggests an important function in the respective cell types. However, we have recently shown that deletion of *Klf4* in spermatids did not impair spermiogenesis and that the

male mutant mice were fertile (Godmann *et al.* 2009b). This finding may suggest that KLF activity is dispensable for spermiogenesis or alternatively that the lack of KLF4 can be compensated by other KLF family members as has already been shown in mouse embryonic stem cells (Jiang *et al.* 2008). Therefore, further studies also will have to elucidate whether other KLF family members, e.g. KLF2 and KLF5, are co-expressed together with KLF4 in those cells studied in the present work.

The CREM gene isoform *ICER* (inducible cAMP early repressor) has been described as an immediate early response gene in TM4 Sertoli (Monaco *et al.* 1995) and in WEHI7.2 thymoma cells (Mao *et al.* 1998). In both cell lines, cAMP-induced expression of *ICER* exhibited almost identical kinetics with peak levels around 3 h after induction. Interestingly, as shown in this work, *Klf4* gene expression is significantly earlier induced after PKA activation, i.e. a strong induction of *Klf4* expression could be detected after 30 min, peaking out at 90 min. Moreover, the mRNA as well as the protein levels also decreased very quickly after the forskolin stimulus. According to our knowledge, this makes *Klf4* one of the fastest responders to cAMP described so far. An intensive literature search revealed only a limited number of mRNAs and/or proteins with similar induction characteristics after PKA activation. For instance, the transcription factor *Egr1* exhibits comparable induction

Figure 7 Measurement of the TM4 cell DNA content after forskolin stimulation and subsequent *Klf4* induction. The cell cycle status of synchronized and forskolin or DMSO-treated or untreated TM4 cells was determined according to the DNA content of the cells by propidium iodide staining (PI) and fluorescence-activated cell sorting (FACS) analysis. Three-dimensional diagrams show cell cycle progression over a period of 24 h of untreated (only medium, black dotted line), forskolin-stimulated TM4 cells (black line) and cells treated with DMSO (grey line). Y-axis, relative cell number; X-axis, relative DNA content indicated by propidium iodide staining. G₀/G₁ peaks at 200 and G₂/M at 400.

kinetics (Tai *et al.* 2001). After forskolin stimulation of PC12 cells, EGR1 protein was strongly induced after 30 min, peaked after 45 min, and was back to the unstimulated level after 90 min. Such findings are in agreement with the kinetics of an immediate early gene. However, they are in part in contrast to ICER since ICER protein was much more stable than KLF4 and EGR1, and a decline of ICER protein was detectable only after 36 h (Monaco *et al.* 1995). Compared with ICER, the transient induction of KLF4 protein in TM4 cells can execute only short-term primary effects as has been postulated earlier for immediate early genes (Herschman 1991).

There were two genes described with similar induction kinetics on the mRNA level after serum stimulation of BALB/c-3T3 cells, i.e. *c-Fos* and *c-Myc* (Greenberg *et al.* 1986). *C-Fos* was strongly induced after 15 min and almost back to the unstimulated level after 60 min. *C-Myc* showed slightly increased mRNA levels after 30 min and remained high for at least 4 h. This has been confirmed by others (Lau & Nathans 1987). Moreover, this latter report described five additional immediate early mRNAs in BALB/c-3T3 cells after serum stimulation of quiescent cells. Two of these not further characterized mRNAs were also clearly induced after 10 min after stimulation. Additionally, in PC12 pheochromocytoma cells, *c-Fos* proto-oncogene was rapidly induced within 5 min after the addition of nerve growth factor (Greenberg *et al.* 1985). Thus, *Klf4* belongs to a small group of genes responding really immediately, i.e. within <15 min, to activation of signal transduction cascades.

Interestingly, *Klf4* has also been described as an immediate early gene in recent papers basically confirming the induction kinetics of *Klf4* demonstrated in the present study (Granberg *et al.* 2006, Liu *et al.* 2006, Maallem *et al.* 2008, Dijkmans *et al.* 2009). However, only the study by Dijkmans *et al.* (2009) measured the induction already after 15 min, while the other studies measured gene induction first after 45 (Maallem *et al.* 2008) or 60 (Granberg *et al.* 2006) min respectively. The inductive stimuli were nerve growth factor (Dijkmans *et al.* 2009), adenoviral infection (Granberg *et al.* 2006), and systemic hypertonicity in rats (Maallem *et al.* 2008). Since nerve growth factor has pleiotropic effects (Dijkmans *et al.* 2009 and references therein), in neither study the particular biochemical signal transduction pathways resulting in *Klf4* induction have been determined. In contrast, in the present study, we could clearly show that PKA signaling was causative for the induction of *Klf4* mRNA (see also below).

During recent years, it became evident that not all effects of increased intracellular cAMP can be attributed to PKA action (Richards 2001, Walker & Cheng 2005). For example, the kinases protein kinase B, phosphoinositide 3-kinase/PDK1, and SGK also appear to be responsive to increased cAMP levels. Previous studies on FSH-inducible genes did not reveal the identity of the intracellular signal transduction pathway, which led to

the induction of *Klf4*. In this work, we could show that indeed the cAMP/PKA pathway is responsible for *Klf4* induction. While forskolin is an activator of the adenylyl cyclase, which can activate several intracellular signaling pathways through increased cAMP levels, the protein kinase inhibitor H89, which was used in this study and completely blocked *Klf4* induction, does not inhibit any of these kinases found to be activated by increased cAMP levels except the PKA. The fact that *Klf4* induction is abolished by the protein kinase inhibitor H89 shows that indeed the cAMP/PKA pathway is responsible for *Klf4* gene induction.

There are earlier studies that demonstrate the inducibility of *Klf4* mRNA in primary Sertoli cells upon an FSH stimulus (Hamil & Hall 1994, McLean *et al.* 2002, Sadate-Ngatchou *et al.* 2004). Nevertheless, the earliest time points investigated in these studies on the Sertoli cell were 2 and 2.5 h after stimulation respectively, thus not covering the time range typical of an immediate early response gene (Herschman 1991). Moreover, these studies did not investigate KLF4 protein induction. It is still unclear how this very rapid gene induction is achieved. However, we propose two models. The first model involves the transcription factor CREB, which can be phosphorylated by the PKA (Sassone-Corsi 1998). Activated CREB could bind to the *Klf4* gene promoter and activate it immediately after activation of the cAMP/PKA signal transduction pathway. Indeed, a conserved CRE, the binding site of the transcription factors CREB and CRE modulator in cAMP-responsive genes, has been found in the *Klf4* gene promoter (Zhang *et al.* 2005). However, this CRE is much further upstream (~3 kb) from the transcription start site than most CREs found to be functional. Therefore, it remains to be proven whether this CRE is important for the rapid induction of *Klf4*. Another model for the rapid *Klf4* gene induction is an auto-regulatory mechanism. The KLF4 protein has four probable PKA phosphorylation sites (Blom *et al.* 1999). Since it has been shown that KLF4 can activate its own promoter (Mahatan *et al.* 1999) and since even in the uninduced state there are basal levels of KLF4 protein present (see Fig. 4), it is conceivable that KLF4 itself is the primary target of the PKA and binds upon activation by the PKA to its own promoter. A third possibility is that the *Klf4* gene promoter can primarily be activated by an as yet unknown PKA-activated transcription factor.

Interestingly, KLF4 protein levels decline very quickly after peaking at 120 min. Although *Klf4* mRNA signals are still moderate after 150 min of forskolin exposure, the protein concentrations reach a level that is similar to what is observed initially after 10 min of treatment and which probably represents endogenous KLF4 basal expression. Owing to the fact that this behavior was consistently reproduced, it is highly likely that the KLF4 protein is less stable and rapidly degraded in TM4 cells. Recent findings in the literature support this

explanation: i) [Chen et al. \(2005\)](#) demonstrated that KLF4 exhibited a half-life of ~ 120 min and that it was rapidly degraded by the ubiquitin–proteasome complex. Furthermore, ii) KLF4 contains a PEST domain ([Shields et al. 1996](#)), which is characteristic of proteins with very short half-lives of < 2 h ([Rogers et al. 1986](#), [Babon et al. 2006](#)).

Moreover, we have previously shown that *Klf4* mRNA contains some sequence motifs in its 3'-UTR, which strongly resemble or perfectly match binding sites for proteins repressing translation ([Godmann et al. 2005](#)). Thus, it is conceivable that translation of the *Klf4* mRNA is also strictly controlled in TM4 cells so that KLF4 protein levels can decline even before the corresponding mRNA has been degraded. However, it is beyond the scope of the present study to unravel the molecular mechanisms responsible for the KLF4 protein degradation.

KLF4 has been implicated in cell cycle regulation by controlling the G₁/S and the G₂/M cell cycle checkpoints ([Shields et al. 1996](#), [Yoon & Yang 2004](#)). Upregulation of KLF4 caused an arrest of cell cycle progression. Therefore, we were interested in whether forskolin-induced upregulation of KLF4 in the TM4 cell line would have an effect on cell cycle progression of these cells. Interestingly, we found no such effect, neither a total block nor a delay. This was somewhat surprising since other systems including *in vitro* cell culture systems showed such a KLF4-mediated cell cycle block, for instance by down-regulating the M-phase cyclin B ([Yoon & Yang 2004](#)). An explanation for the finding in the present study could be that the induction of *Klf4* expression in the TM4 cells was too transient to mediate long-term effects on the cell cycle. Another possibility is that the immortal TM4 cell line overrides the normal cellular response to KLF4 induction by its aberrant cellular program that enables the cells to grow indefinitely *in vitro*.

Furthermore, it is also possible that KLF4 is not involved in cell cycle regulation in Sertoli cells, since our *in vivo* studies demonstrated that the lack of KLF4 in Sertoli cells caused a functional delay in Sertoli cell differentiation but did not affect their proliferative behavior ([Godmann et al. 2008](#)).

In summary, we show for the first time expression of KLF4 in distinct cell types of the male and female murine reproductive tract including the epididymis, the vas deferens, the penis, the uterus, and the vagina. Keeping the relevance of KLF4 in many different mouse cell types in mind, the strong expression of KLF4 in several cell types of the reproductive tract may suggest an important role of KLF4 also during reproductive functions in mice. In TM4 Sertoli cells, *Klf4* mRNA and protein are very rapidly and specifically induced by the cAMP/PKA signal transduction pathway. Moreover, the induction was very transient, classifying *Klf4* as an immediate early gene in response to PKA activation in TM4 Sertoli cells.

Materials and Methods

Immunohistochemistry

Bouin's-fixed and paraffin-embedded mouse tissue specimens were sectioned at 6 μ m. Sections were rehydrated and an antigen retrieval step was performed by microwaving the sections in 0.01 M sodium citrate buffer (pH 6.0) for 10 min. Unspecific binding of the first antibody was blocked by a 60-min incubation step in 5% (w/v) BSA in 0.05 mol/l Tris–HCl, 0.15 mol/l NaCl, pH 7.6 (TBS). A rabbit polyclonal antibody raised against the 180 N-terminal amino acids of KLF4 of human origin (SC-20691; Santa Cruz Biotechnology) was used at a 1:600 dilution in 5% BSA in TBS. Additionally, a KLF4 affinity-purified polyclonal antibody directed against recombinant full-length mouse KLF4 (AF3158; R&D Systems) was used in a 1:50 or 1:100 dilution. All incubation steps were done in a humid chamber and incubations with the primary antibodies were performed overnight at 4 °C. DakoCytomation Universal LSAB 2 System-AP (K0674) including biotinylated secondary antibody polymer and alkaline phosphatase (AP)-conjugated streptavidin or DakoCytomation Universal LSAB 2 System-HRP (K0672) including biotinylated secondary antibody polymer and HRP was employed for detection of bound primary antibody. Fuchsin (K0624) chromogen or 3-3' diaminobenzidine was used as a substrate for the AP or HRP respectively. Mayer's hematoxylin served as counterstain. Control stains were carried out using normal rabbit or goat sera respectively, instead of the primary antibody. Tissue samples from two to seven different animals were analyzed.

Cell culture

TM4 cell culture

TM4 cells (kindly provided by Dr Thomas Walther, Berlin) were cultured in growth medium consisting of 46% DMEM (with 4500 mg/l glucose, with pyridoxine HCl, without sodium pyruvate), 46% Ham's F12 (with 10 mg/l phenol red, with 1.176 g/l NaHCO₃, without L-glutamine), 6.5% FCS, 15 mM HEPES, 25 000 Units penicillin per 500 ml, and 25 mg streptomycin per 500 ml.

Stimulation and inhibition of the adenylyl cyclase pathway in TM4 cells

TM4 cells (5×10^5) were grown on cell culture dishes (diameter: 10 cm) for 24 h. Afterwards, cells were treated with 10 μ M forskolin (Sigma–Aldrich) in growth medium or 10 μ M forskolin and 75 μ M H89 (*N*-[2-*p*-bromocinnamylamino]ethyl]-5-isoquinoline-sulfonamide-2HCl; Biosource, Camarillo, CA, USA) in growth medium or 0.26% DMSO in growth medium or growth medium alone. RNA was isolated after 10, 20, 30, 60, 90, 120, 180, and 240 min incubation time.

Synchronization of TM4 cells

TM4 cells (5×10^4 cells/well) were grown on six-well plates for 24 h, washed twice with Moscona's buffer containing 13.6 mM NaCl, 4 mM KCl, 12 mM NaHCO₃, 10 mM D-glucose,

0.36 mM NaH_2PO_4 , and 0.18 mM KH_2PO_4 (pH 7.4), and cultured for 24 h in growth medium without fetal bovine serum. Then, cells were treated with 10 μM forskolin (Sigma–Aldrich), which were dissolved in DMSO (final concentration in the medium 0.1%), in growth medium, 0.1% DMSO in growth medium, or growth medium alone. The DNA content of the stimulated TM4 cells was analyzed as described below.

Immunofluorescence

TM4 cells (2×10^4) were grown on chamber slides ($4.2 \text{ cm}^2/\text{well}$) overnight and then stimulated with 10 μM forskolin dissolved in DMSO for 1 h under cell culture conditions as described above. Incubation of TM4 cells with 0.1% DMSO in growth medium or growth medium alone served as controls. After stimulation, cells were rinsed twice with Moscona's buffer, fixed with 4% paraformaldehyde/Moscona's buffer for 30 min, and permeabilized with 0.05% Triton in ddH_2O for 5 min, both at room temperature. Cells were rinsed twice with Moscona's buffer. Non-specific binding sites were blocked with 0.5% BSA in Moscona's buffer for 1 h at 4 °C. Then, cells were incubated with an anti-KLF4 antibody (H180, sc-20691, Santa Cruz Biotechnology, 1:500 in 0.5% BSA/Moscona's buffer) overnight at 4 °C. Next day, TM4 cells were washed thrice in 0.5% BSA/Moscona's buffer and incubated with a mixture consisting of secondary antibody (Alexa Flour 488, A11008, Molecular Probes/MoBiTec, Göttingen, Germany; 1:200 in 0.5% BSA/Moscona's buffer) and 5 $\mu\text{g}/500 \mu\text{l}$ (7.66 μM) tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma) for F-actin staining, for 1 h at room temperature in the dark. After washing, cell nuclei were DAPI stained (1:200 in 0.5% BSA/Moscona's buffer; 15 min at room temperature in the dark), cells were rinsed once with 0.5% BSA/Moscona's buffer, twice with ddH_2O , and mounted with DAKO Faramount aqueous medium (DAKO Cytomation, Hamburg, Germany).

Confocal microscopy was performed using a Zeiss Axiovert 100 M microscope attached to a confocal laser scanning microscopy system (model LSM 510; objective: Plan-Apochromat 20×0.75 ; Carl Zeiss, Jena, Germany) as described previously (Heneweer *et al.* 2002). As excitation sources, an argon laser with output at 488 nm, a helium–neon laser with output at 543 nm, and an Enterprise laser with output at 364 nm were available. To improve the signal-to-noise ratio, each slice was scanned eight times followed by averaging.

RNA isolation and northern blotting

Total RNA was extracted from different mouse tissues and the TM4 Sertoli cell line using peqGOLD-TriFast (peqLab Biotechnology GmbH, Erlangen, Germany) following the manufacturer's instructions. Performing northern blot analysis, total RNA was additionally purified using the RNeasy Mini Kit (Qiagen). Ten (Fig. 3) or five (Fig. 5) micrograms respectively of purified total RNA per sample were electrophoresed in 1% agarose/3-(*N*-morpholino)propanesulfonic acid/formaldehyde gels, blotted onto nylon membranes (Amersham Pharmacia), and cross-linked by u.v. irradiation. Filters were prehybridized

at 68 °C for 60 min in ExpressHyb buffer (BD Biosciences Clontech). α -[^{32}P]-dCTP was incorporated into the probe using the High Prime solution from Roche Molecular Biochemicals. Hybridizations were performed at 68 °C for 16 h. Membranes were washed thrice for 20 min each time in $2 \times \text{SSC}/0.1\%$ SDS at room temperature and twice for 30 min each time in $0.1 \times \text{SSC}/0.1\%$ SDS at 50 °C. The membranes were exposed to an X-ray film (Kodak) for 16–72 h at -80 °C in an exposure cassette with intensifier screens. To generate the *Klf4* open reading frame probe, the entire coding region was amplified from mouse testis cDNA by PCR using the following primer combinations: Ex1-fw 5'-cct tct ggg ccc cca cat ta-3'; Ex5-re 5'-gtc aca tcc act acg tgg gat-3'. The ICER probe was amplified using: mmlCER-fw1 gac tgt ggt acg gcc aat aag; mmDBD2-ICER-re1 gtt ctg aac ttc cag cac tgc. PCR products were subcloned into the pCRII vector (Invitrogen) and sequenced. Ten micrograms of each plasmid DNA were digested with 20 Units of EcoRI, separated by electrophoresis and gel purified (MinElute, Qiagen). Approximately, 100 ng of each probe were radiolabeled with α -[^{32}P]-dCTP (Hartmann Analytic, Braunschweig, Germany) using High Prime Solution (Roche Molecular Biochemicals). Prior to rehybridization with a different probe, northern blots were dehybridized by boiling the membranes in $0.1 \times \text{SSC}/1\%$ SDS for 15 min.

Immunoblotting

After isolation of total RNA from TM4 cells, proteins of the same individual cultures were also extracted using the peqGOLD-TriFast system (peqLab Biotechnology) following the manufacturer's instructions. Total protein was quantified using Micro BCA Protein Assay Reagent Kit from Pierce Biotechnology/Perbio Science (Bonn, Germany). Protein extracts (30 $\mu\text{g}/\text{lane}$) from TM4 cells were separated by SDS-PAGE (10% SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk powder/PBS/0.1% Tween 20 for 1 h at room temperature. Then, the anti-KLF4 primary antibody (Rowland *et al.* 2005, H180, sc-20691, Santa Cruz Biotechnology, Heidelberg, Germany) was diluted 1:4000 in 5% milk powder/PBS/0.1% Tween 20, and applied to the membranes, which were incubated overnight at 4 °C. After washing, the protein–antibody complexes were detected with a HRP-conjugated secondary antibody (Goat anti-Rabbit IgG-HRP, Santa Cruz Biotechnology), diluted 1:20 000 in 5% milk powder/PBS/0.1% Tween 20 for 1 h at room temperature using the ECL plus detection system (GE Healthcare, Uppsala, Sweden). Total protein extracts from mouse skin and stomach were used as a positive control and subjected to western blot analyses in order to evaluate the functionality of the KLF4 antibody from R&D Systems (AF3158). The primary antibody was used in a 1:1000 dilution, and the secondary antibody (Santa Cruz SC-2922) was used in a 1:5000 dilution.

DNA content analysis

To explore a potential role of KLF4 in cell cycle progression of TM4 cells, 5×10^4 cells/well were grown on cell culture dishes

(diameter: 3.5 cm) and treated with 10 μ M forskolin in 0.1% DMSO in growth medium, 0.1% DMSO in growth medium, or growth medium alone after synchronization as described above. Cells were harvested after 0, 1, 3, 6, 12, and 24 h of stimulation, washed once in PBS, and fixed and permeabilized in 100 μ l Cytofix/Cytoperm solution (Becton Dickinson, San Jose, CA, USA) overnight at 4 °C. The fixation/permeabilization solution was removed by the addition of 1 ml Perm/Wash buffer and centrifuged at 173 *g* in a Heraeus Megafuge for 5 min at room temperature. After the addition of 0.25 mg/ml RNase A and 20 μ g/ml propidium iodide in PBS, the cell suspension was incubated in the dark for 30 min at 4 °C. Cell cycle analysis was done by using FACS Calibur flow cytometer (Becton Dickinson). The data were collected with Cell Quest and analyzed using FlowJo Software (Becton Dickinson).

Analyses of relative mRNA expression and protein levels

The analyses of relative mRNA expression (northern blotting) and relative protein levels (immunoblotting) were done using the AlphaDigiDoc analysis system (Alpha Innotech Corporation, San Leandro, CA, USA). The *Klf4* mRNA signals were normalized to the corresponding 28S rRNA and 18S rRNA (formaldehyde gel), and the KLF4 protein was normalized to β -actin (SC-1616, Santa Cruz). If not otherwise indicated, each experiment was repeated a minimum of three times. All values are expressed as means $\pm$ S.E.M. To detect significant effects of forskolin treatment, a one-way ANOVA was performed and to identify differences between means, a Student–Newman–Keuls test means comparison was applied.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) to R Behr (Be2296/4).

Acknowledgements

We appreciate the support of Dr Renate Renkawitz-Pohl, Dr Hans-Werner Denker, and Dr Trevor Cooper. The technical assistance of Isabell Kromberg, Marion Niebeling, Nicole Umland, and Vera Liebau is gratefully acknowledged.

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Received 27 November 2009

First decision 5 January 2010

Accepted 5 January 2010