

Cre transgene results in global attenuation of the cAMP/PKA pathway

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Use of the *cre* transgene in *in vivo* mouse models to delete a specific 'floxed' allele is a well-accepted method for studying the effects of spatially or temporarily regulated genes. During the course of our investigation into the effect of cyclic adenosine 3',5'-monophosphate-dependent protein kinase A (PKA) expression on cell death, we found that *cre* expression either in cultured cell lines or in transgenic mice results in global changes in PKA target phosphorylation. This consequently alters gene expression profile and changes in cytokine secretion such as IL-6. These effects are dependent on its recombinase activity and can be attributed to the upregulation of specific inhibitors of PKA (PKI). These results may explain the cytotoxicity often associated with *cre* expression in many transgenic animals and may also explain many of the phenotypes observed in the context of Cre-mediated gene deletion. Our results may therefore influence the interpretation of data generated using the conventional *cre* transgenic system.

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The ability to manipulate pluripotent embryonic stem cells through homologous recombination has revolutionized mouse genetics in the past two decades. Cre-*loxP* recombination system has been an integral part of mouse genome manipulation, initially to remove the selectable marker and thus eliminating 'the selection cassette effects'^{1,2} and subsequently to overcome certain deficiencies associated with ubiquitous gene deletions. These deficiencies include embryonic lethality and functional compensation by redundant gene products during ontogeny and can mask the effect of a particular gene deletion.³ Conditional deletion, which results in temporal and spatial ablation of the targeted genes, is achieved through the bacteriophage P1 recombinase, Cre. Cre is a member of the integrase family of site-specific recombinase that catalyzes recombination between 'lox-P' DNA elements during normal viral life cycle.⁴ In mouse gene manipulation, the targeted allele is flanked by two 34-bp lox-P sites ('floxed' allele) and the Cre recombinase is expressed by temporarily or spatially regulated promoters.³

Thus, the 'Cre-*loxP*' system has been the mainstay of mouse genetic manipulation for many years, and it has been assumed that this enzyme is inert in mammals and maintains high specificity *in vivo*. However, there have been reports of nonspecific activity on cryptic or pseudo loxP-like sites similar to the star activity observed with certain restriction enzymes.⁵ It has also been shown that Cre overexpression in the

post-mitotic spermatids results in gross chromosomal abnormalities, leading to male sterility,⁴ and chromosomal abnormality in mouse embryonic fibroblasts (MEFs), leading to growth arrest.⁶ Deletion of *prkar1a*, the ubiquitously expressed regulatory subunit of cyclic adenosine 3',5'-monophosphate (cAMP)-regulated protein kinase A (PKA), should result in increased PKA activity.⁷ During our investigation of *prkar1a*^{fl/fl} mice, we observed (contrary to expectations) a severely muted PKA response. We subsequently observed that Cre expression led to the induction of protein kinase A inhibitor (PKI) causing a global attenuation of the PKA signal transduction pathway. These observations may explain some of the phenotypes associated with Cre transgene expression, such as cellular growth retardation and cytotoxicity.

Results

Cre expression results in reduction of PKA activity. cAMP regulation of PKA is achieved via a unique three-component signaling system. The catalytic (C) and regulatory (R) subunits of PKA interact to form an inactive holoenzyme complex. Binding of cAMP induces a conformational change in the R1 α subunit (the most ubiquitously and predominantly expressed subunit) and leads to the dissociation of the holoenzyme into its constituent subunits, that is, PRKAR1A and PKAC α .⁸ The gene *prkar1a* is known to be a

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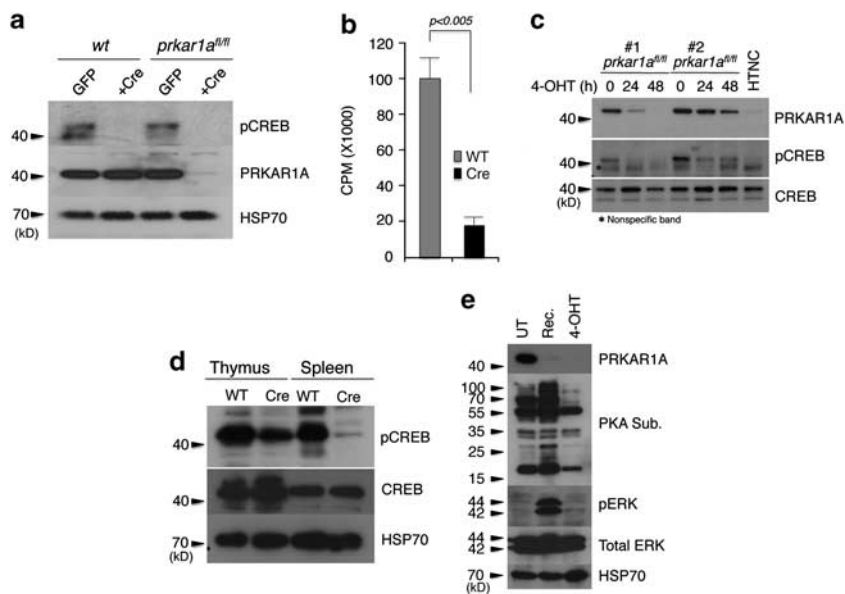


Figure 1 Cre expression downregulates PKA activity. (a) Expression of Cre in MEFs by retroviral vector downregulates PKA activity, as indicated by reduced pCREB levels even under PRKAR1A deletion. (b) PKA activity assay in crude lysates using kemptide as the substrate. (c) Deletion of PRKAR1A either by 4-OHT-regulated, lentivirally-expressed cre (clone nos 1 and 2) or by cell permeable recombinant Cre in culture results in downregulation of pCREB levels. (d) Thymocytes and splenocytes from *cre* transgenic mice show reduced levels of pCREB. (e) Titratibility of the Cre effect. MEFs (*prkar1a^{fl/fl}*) were infected with a lentivirus expressing 4-OHT-inducible Cre. Addition of 4-OHT resulted in the ablation of PRKAR1A protein in cells with concomitant reduction of PKA substrate protein in the cells (right lane). Recovery of the cells from 4-OHT results in a robust induction of PKA activity in the absence of Cre (in these cells, PRKAR1A is still ablated resulting in increased PKA activity). Error bars: \pm S.E.M., $n=3$

tumor suppressor in a variety of endocrine and non-endocrine tumors (cardiac and breast myxomas), pituitary hyperplasia, growth hormone secreting adenomas and in osteosarcomas.^{7,9,10} This is attributed to increased PKA activity. In *prkar1a^{fl/fl}* MEFs, adenoviral expression of Cre resulted in the depletion of PRKAR1A with a robust induction of both total and free PKA activity and has been attributed to the immortalization process.¹¹ However, increased PKA activity is associated with induction of apoptosis in many cell types.^{8,12,13} In an effort to understand the regulation of apoptosis by increased PKA activity, we expressed Cre in *prkar1a^{fl/fl}* MEFs by a variety of different methods. We consistently found that there was a reduction in the PKA activity following Cre expression. Expression of Cre by cells infected with a retroviral expression construct resulted in the depletion of PRKAR1A protein and reduction of PKA activity, as observed by decreased phosphorylated CREB protein and the enzyme activity (Figures 1a and b). Similarly, expression of 4-hydroxytamoxifen (4-OHT)-regulated Cre from a lentiviral vector resulted in the depletion of PRKAR1A protein and a reduction in phospho-CREB (pCREB) levels (Figure 1c). To rule out the possibility that this was an artifact of the vector DNA backbone, we transduced *prkar1a^{fl/fl}* MEFs with a recombinant, cell permeable TAT-Cre protein (with a nuclear localization signal or HTNC).¹⁴ Addition of the recombinant protein into the culture resulted in the depletion of PRKAR1A protein with a concomitant reduction in the pCREB levels (Figure 1c, lane 7). Further, T cells purified from transgenic mice expressing Cre under the T-cell-specific promoter *Ick* (*Ick-cre*) showed a reduction of pCREB in both the thymic and in the splenic T-cell populations

(Figure 1d); particularly in the splenic T cells where the reduction was much more significant.

We also wanted to test the reversibility of this effect. First, we treated *prkar1a^{fl/fl}* MEFs with 4-OHT (5 nM) to induce Cre expression.⁸ This resulted in the deletion of *prkar1a* gene and a dramatic reduction of phospho-PKA substrate¹⁵ levels compared with untreated cells. We then released the cells, from 4-OHT treatment, resulting in a very significant induction of phosphorylated PKA substrates. These results are consistent with the notion that deletion of *prkar1a* leads to increased PKA activity in the absence of any Cre expression (Figure 1e). In addition to PKA activation, deletion of *prkar1a* also leads to increased phospho-ERK activation in the absence of PKA, indicating cross-talk between these signal transduction pathways.¹⁶

Cre expression induces PKI. To understand the mechanism behind PKA inhibition, we tested three possible scenarios. First: activation of a phosphatase by Cre protein. To address this, we measured the total phosphatase activity in cellular extracts of WT MEFs and MEFs expressing Cre protein using EnzCheck Phosphatase assay kit (Invitrogen, Carlsbad, CA, USA). This kit is capable of continuous measurement of phosphatase activity in a wide range of pH. However, we failed to detect any difference in phosphatase activity between WT and Cre-expressing MEFs (Figure 2a). Second: Cre protein acting as a surrogate substrate for PKA. KinasePhos (<http://KinasePhos.mbc.nctu.edu.tw>) analysis of the Cre protein identified Ser¹⁰⁸ as a potential PKA phosphorylation site. Indeed, *in vitro* kinase assay of bacterially expressed and purified Cre protein was readily phosphorylated by PKA

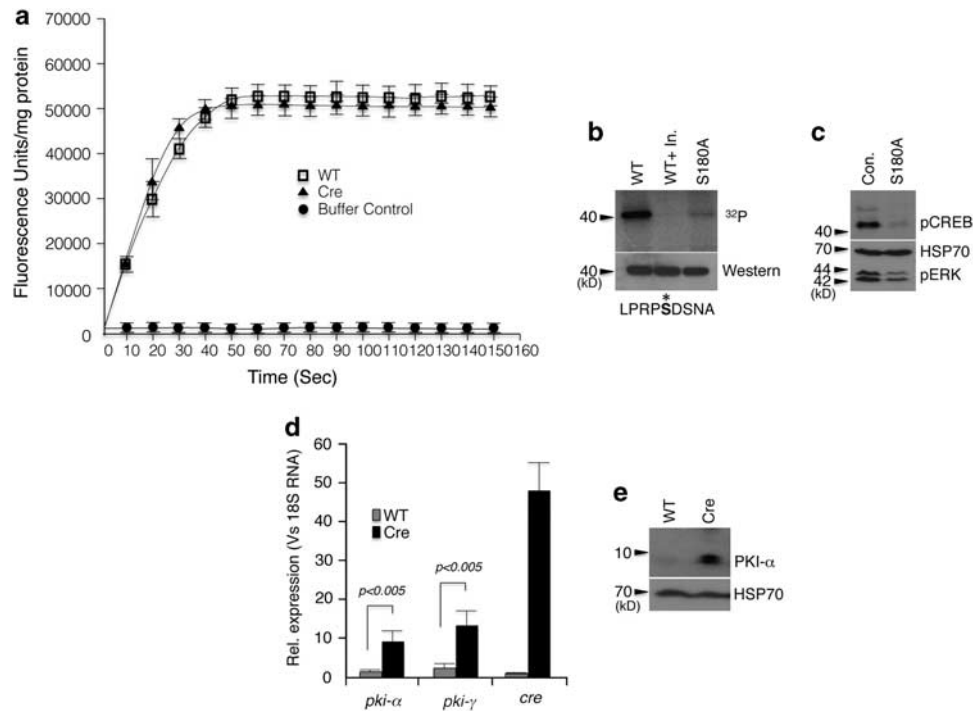


Figure 2 Possible mechanism of PKA inhibition in cre-expressing cells. (a) Phosphatase assay in cell lysate from MEFs. (b) Cre is a PKA substrate in *in vitro* kinase assay and the phosphorylation can be abolished by S180A mutation. The middle lane is the assay performed in presence of PKA-specific inhibitor (Upstate). The sequence shown below is the potential phosphorylation site. (c) Phosphorylation-deficient mutant of Cre still downregulates pCREB and pERK when expressed in MEFs. (d) Quantitative PCR analysis of PKI α and PKI γ in MEFs. (e) Western blot analysis of MEFs expressing Cre showing PKI α induction. Error bars: \pm S.E.M., $n = 3$

and mutation of Ser¹⁰⁸ to Ala abolished most of this phosphorylation (Figure 2b). However, expression of this mutant protein in MEFs still attenuated PKA activity, ruling out the possibility that Cre protein acts as a surrogate substrate for PKA (Figure 2c). Third: Cre protein expression leads to a transcriptional induction of *pki* genes. PKI is an endogenous thermo-stable peptide that modulates cAMP-dependent PKA function. It selectively inhibits the activity of the free catalytic subunit of cAMP-dependent PKA following cAMP-mediated dissociation of the regulatory and catalytic subunits of the enzyme, and thus regulates the basal level of cAMP/PKA activity^{17,18}. Quantitative PCR analysis of cDNA from *wt* and *cre* transgenic MEFs showed a significant increase in both *pki-α* and *pki-γ* transcripts in the *cre* transgenic MEFs (Figure 2d). Western blot analysis also revealed a significant induction of PKI α protein in the *cre* transgenic MEFs, corroborating the finding that *cre* transgene expression leads to a transcriptional induction of *pki* genes (Figure 2e).

Global attenuation of signal transduction pathways in the *cre* transgenics. PKA is one of the main downstream effectors of increased cAMP levels in the cell (the other being exchange protein activated by cAMP or EPAC).¹⁹ The cAMP-dependent PKA pathway regulates a variety of cellular process including cellular proliferation, differentiation and apoptosis.²⁰ Furthermore, the PKA pathway forms part of complex network of signal transduction pathways including MAPK and mTOR.^{21,22} We wanted to test whether down-regulation of PKA activity in the Cre transgenic cells had any effect on the level of phosphorylation of other cellular proteins.

To this end, we conducted a total phosphoprotein analysis using a Kinexus antibody microarray (Kinexus Bioinformatics, Vancouver, BC, Canada). Comparison of phosphoprotein levels between WT and Cre-expressing MEFs revealed a substantial difference between these cells (Figure 3a). These results were further validated for a set of 13 proteins by western blot analysis (Figure 3b). In agreement with the western blot data, where Cre-expressing cells had reduced levels of ERK activation (Figure 1e), Kinexus analysis also showed a substantial reduction in the phosphorylation of both forms of ERK (pERK40/42).

Transcriptional profiling of Cre transgenic MEFs. In addition to the direct transcriptional effect mediated through the transcription factor CREB, PKA can exert its effect by altering calcium mobilization in the cell.²³ In eukaryotic cells, calcium ions are also involved in adaptive responses that involve global regulation of a wide variety of transcription factors.²⁴ In this work, we limited our studies to qPCR analysis of *nur77*, *areg*, *nr4a2*, *il-6*, *rgs2* and *dusp1*, which are known targets of PKA.²⁵ As evident from Figure 4a, in Cre-expressing MEFs, all these transcripts (except for *dusp1*) were substantially downregulated. Of these genes, *il-6* transcripts in particular were substantially downregulated. IL-6 is one of the preeminent cytokines involved in the inflammatory response. To validate this finding, we measured IL-6 secretion both in MEFs and in *in vitro* con A-induced T-cell blasts. Both in MEFs and in Con-A-activated T-cell blasts, Cre expression resulted in three- to fivefold reduction in secreted IL-6 (Figures 4b and c).

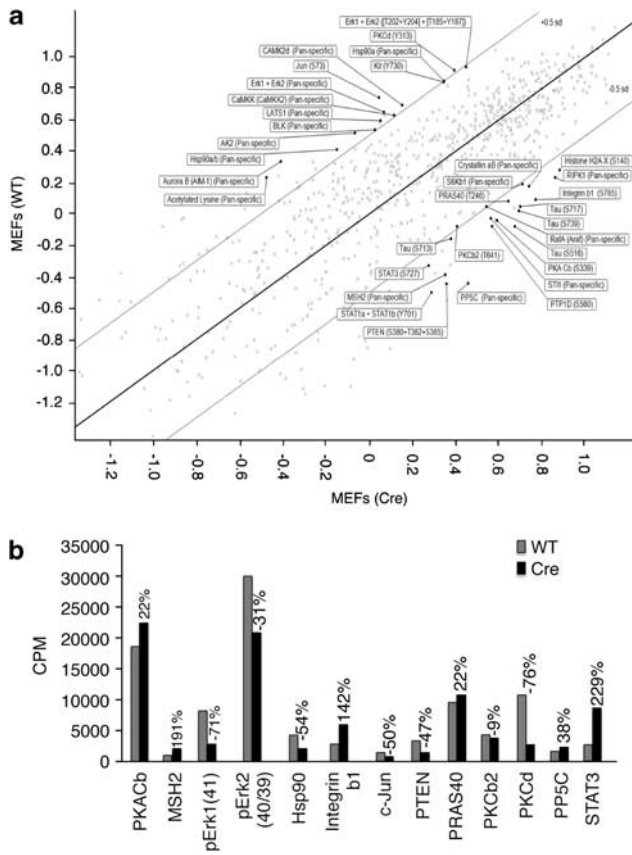


Figure 3 Cre transgene alters global phosphorylation pattern in MEFs. (a) Phosphoprotein profiling by Kinex antibody microarray (806 pan-specific and phospho-specific antibodies) of WT and *cre* transgenic MEFs. Antibodies with very low signals (Z score less than -0.5) were removed from further consideration. (b) Antibodies whose signals differed substantially (where the difference in Z scores was greater than 0.5) were selected for secondary validation through immunoblotting

Although Cre recombinase binds to its cognate DNA sequence with very high affinity, it can also bind to non-target DNA with 20-fold lower affinity.²⁶ Therefore, we were interested to know whether the nuclease activity associated with its promiscuous DNA binding could explain Cre's pleiotropic effects. To this end, we created an active site mutant of Cre Y325A.²⁷ Retroviral infection of MEFs (*c-myc*^{fl/fl}) with the wild-type cre resulted in site-specific recombination and the removal of the target allele (Figure 5a, lower panel) whereas the mutant Y324A failed to do so. However, the wild-type Cre recombinase expression with the nuclease activity resulted in the downregulation of pCREB whereas the mutant had no effect, suggesting that the pleiotropic effect of Cre is associated with binding to its cognate DNA sequence (Figure 5a, lower panel).

Next, we tested the new-generation Cre recombinase fused to a mutant estrogen hormone-binding domain (ER^T).²⁸ In this system, ER^T-Cre expression is regulated by the ROSA26 (*R26*) locus and is therefore ubiquitous. ER^T-Cre protein remains inactive in the cytoplasm until addition of the non-steroidal estrogen analog 4-OHT. To test whether the ER^T-Cre cre could lessen its effect on PKA activity, we used

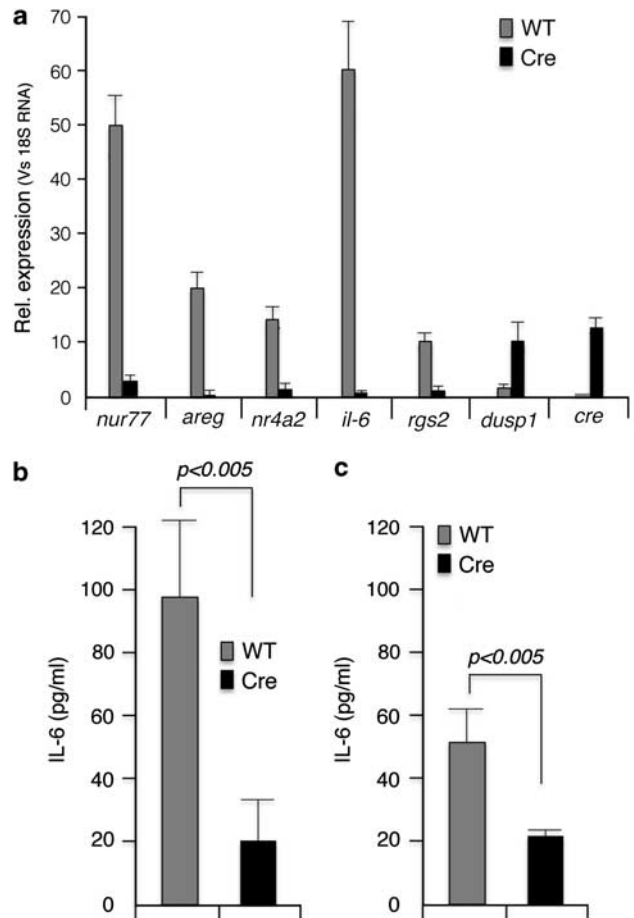


Figure 4 Cre expression results in downregulation of PKA transcriptional targets. (a) qPCR analysis of transcripts from *wt* and *cre* transgenic MEFs. (b) IL-6 measurement in the culture supernatant of MEFs. (c) IL-6 measurement in the culture supernatant of concanavalin A-activated splenic T cells. Error bars: \pm S.E.M., $n = 3$

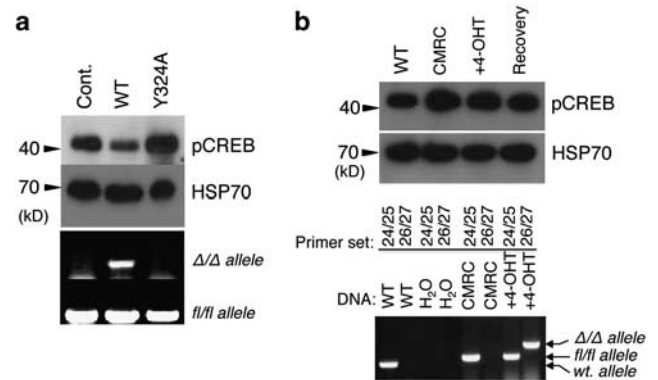


Figure 5 PKA inhibitory activity of Cre is dependant on its nuclease activity. (a) MEFs (*prkar1a*^{fl/fl}) were infected with retroviruses expressing either the wild-type- or recombinase-deficient mutant cre (Y324A). Recombinase activity was measured by the deletion of the floxed allele by PCR using specific primers (bottom two panels) and pCREB level was measured by western blot analysis (top panels). (b) ROSA-Cre expression does not result in PKA downregulation. CMRC cells (*c-myc*^{fl/fl}; *rosa26cre*) were treated with $1.0 \mu\text{M}$ 4-OHT, and cells were analyzed after 48 h and after another 48 h in the absence 4-OHT (recovery) for pCREB levels (upper panels) and for allele-specific deletion (bottom panels). Primer pair 24/25: *wt* allele (400 bp), floxed allele (500 bp); 26/27: *c-myc*-deleted allele or Δ/Δ allele (600 bp)

CMRC MEFs (*c-myc*^{fl/ml}; *r26-cre*) that were treated with 4-OHT. This led to the deletion of the *c-myc* allele without any reduction of pCREB levels in these MEFs (Figure 5b). These results demonstrate that although the nuclease activity is closely associated with its PKA inhibitory property, this problem could be mitigated by using the new-generation ER^T-Cre, which is fused to the estrogen receptor domain, and appear to have no PKA inhibitory activity.

Discussion

The problems associated with *cre* transgene expression are well documented. These include proliferation defects, cytotoxicity and chromosomal abnormalities²⁹ and epigenetic effects.³⁰ The present study highlights yet another problem associated with the use of the *cre* transgene expression to achieve conditional deletion of desired genes, that is, attenuation of the PKA signaling pathway with downregulation of its transcriptional targets and the global changes in the phosphoprotein levels in the cell. In the present study, we expressed Cre using a variety of systems including 4-OHT regulatable lentiviral expression system, retroviral expression system, tissue-specific transgene expression (*lck-cre*) and even bacterially expressed recombinant Cre (i.e., HTNC) with consistent results (except for LysM-Cre transgenics, data not shown). These findings obviate the possibility that this effect was mediated by a specific vector backbone or due to the random integration event. Further, we also showed that the effect on the PKA pathway could be due to the upregulation of the PKA inhibitor, PKI, although the molecular basis of this effect is yet to be elucidated. However, it is clear that this effect is dependent on its nuclease activity. This is consistent with the finding that cytotoxicity observed with *cre* expression is intrinsic to its nuclease activity.²⁹ As the PKA pathway has an important role in the phosphorylation of a variety of substrates including transcription factors involved in cell activation and proliferation,³¹ it is tempting to speculate that Cre-mediated cytotoxicity may be a result of shutting down the PKA pathway.

Given the importance of the PKA signaling pathway in the regulation of transcription factors involved in cell cycle and proliferation, it is likely that its downregulation will have significant implications for cell survival and proliferation. The effect on IL-6 secretion is notable; IL-6 is an interleukin with both a pro-inflammatory and anti-inflammatory effects, and is involved in the acute phase of the innate immune response.³² Its anti-inflammatory effects are mediated (at least in part) by downregulating expression of TNF- α and MIP-2 α .³³ The deregulation of IL-6 impacts numerous disease states, including many types of cancer.³⁴

Given the present observation described here, it is worth noting that PubMed has more than 6000 entries on *cre* transgenic mice dating back to the past 25 years. The generally accepted controls for *cre* transgenic mice are non-*floxed* or non-*cre* transgenic *floxed* mice, so the observed phenotype of a particular *cre* transgenic animal might easily be a combination of gene deletion and Cre-mediated effects on PKA signaling (such as dampening of IL-6 secretion). Therefore, it may be worthwhile revisiting some of these experiments (especially those involve cAMP pathway or

cytokine signaling) using the new-generation *cre* transgenics such as the 'self-excising *cre*'²⁹ or using the ROSA-Cre system where CRE-ERT2 is integrated at the *rosa26* locus, which eliminates random integration effects and the effect on the PKA pathway by regulated administration of 4-OHT.³⁵ Even better alternative would be what Matthaei³⁰ suggested, that is, LacO-LacI^R-based repression of desired gene and regulating the gene activity by IPTG in the drinking water.

Materials and Methods

Cell culture and lentiviral infection. MEFs were cultured in DMEM supplemented with 10% fetal calf serum (Invitrogen) at 37 °C in a humidified 10% CO₂ incubator. Immortalized MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40 large T antigen expressing lentiviruses. To generate lentiviral particles, 293T cells were transfected with packaging constructs pCMV δ R8.2 and VSVg and the relevant lentiviral plasmid at a ratio of 1:0.4:0.6 using Fugene 6.0 transfection reagent (Roche Diagnostics, Castle Hill, NSW, Australia) following the manufacturer's instructions. The virus containing supernatants were harvested, filtered (0.8 μ M) and supplemented with polybrene (4 μ g/ml). Target cells were infected with virus supernatant as described.⁸

The generation of *prkar1a*^{fl/ml} mouse is already described¹⁴ and was a kind gift from Dr. Hua Gu (Columbia University, NY, USA). *c-myc*^{fl/ml} MEFs were a kind gift from Dr. Ignacio de Alboran (Madrid).³⁶

Antibodies. Phospho-(Ser/Thr) PKA substrate (#9621), anti-ERK (#4695) and phospho-ERK (#9102) antibodies were purchased from Cell Signaling (Boston, MA, USA). pCREB antibodies were from Abcam (ab5803, Cambridge, MA, USA), Prkar1a antibodies were from BD Biosciences (610609, North Ryde, NSW, Australia) and PKI α antibodies were from Santa Cruz Biotechnology (sc-50349, Santa Cruz, CA, USA). Anti mouse, anti rabbit and anti rat HRP-conjugated secondary antibodies were purchased from GE Healthcare (Rydalmere, NSW, Australia).

Kinase assay. *In vitro* PKA assays were carried out using the PKA assay kit (Upstate, Lake Placid, NY, USA). Each assay contained 5–10 ng of the recombinant hexa-his-tagged *cre* protein in a total volume of 60 μ l reaction buffer that contained the following: 10 μ l of 1 \times assay dilution buffer, 5 μ l of 20 μ M cAMP, 10 μ l of nonspecific kinase inhibitor cocktail, 1 μ l of PKA catalytic subunit (2.5–10 U) and 10 μ l of Mg-ATP cocktail. Ten microliter of PKA inhibitor was added to the control reactions. The reactions were incubated at 30 °C for 30 min before adding 2 \times Laemmli loading buffer and separating on SDS-polyacrylamide gel electrophoresis gel (Novex, Invitrogen, Carlsbad, CA, USA), followed by transfer onto PVDF membrane. The radioactive proteins were detected in a Typhoon 9410 phosphorimager (GE Healthcare). For measuring PKA activity in crude lysates, 5 μ l of 1 mM Kemptide was used as the substrate in a reaction similar to the one used for the recombinant Cre protein. At the end of the reaction, 25 μ l of the reaction was spotted on P81 paper squares, air dried and washed three times in 0.75% phosphoric acid and once in acetone before reading in a scintillation counter.

Phosphatase assay. Total phosphatase in the cell lysate was measured using EnzCheck Phosphatase Assay Kit (E12020, Molecular Probes, Eugene, OR, USA) with 10 μ g of cell lysate following the manufacturer's instructions.

Cell permeable *cre* purification. HTNC (His-tagged, TAT-fusion Cre with nuclear localization signal) was originally gifted by K. Rajewsky obtained from Addgene (Cambridge, MA, USA). Recombinant protein from *E. coli* was purified by nickel affinity chromatography. Endotoxin was removed using Endotoxin removal beads (Miltentyl Biotech, North Ryde, NSW, Australia) and filtered through 0.8- μ filters before using in cell culture at 100 μ g/ml.

qPCR analysis. Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using Superscript III RT system (Invitrogen). Quantitative PCR was carried out using SYBRGreen reagent (Invitrogen) following the manufacturer's instructions. The gene-specific primers were as follows: *areg* (s):

5'-AGAACCTGGAGGTGGTGCATG-3', *areg* (as) 5'-TCGTTCCAAAGGTGCAC TGTG-3'; *duosp1* (s): 5'-GTACCCCTCTCTACGATCAGGGG-3', *duosp1* (as): 5'-CC AGGAGCTGATGTCTGCCTT-3'; *il6* (s): 5'-ACGGCCTTCCCTACTTCACA-3', *il6* (as): 5'-ATGGTACTCCAGAAGACCAGAGGA-3'; *nr4a2* (s): 5'-GATCCGGGCT CCCTTCACAA-3', *nr4a2* (as): 5'-CGAAGGTCTGCCATCCACT-3'; *nur77* (s): 5'-CCTGTTGCTAGAGTCTGCCCTT-3', *nur77* (as): 5'-CCTGTTGCTAGAGTCTG CCTTC-3'; *rgs2* (s): 5'-GGCCAGTAAATATGGGCTGG-3'; *rgs2* (as): 5'-GTG GTGAAGCAGCCACTTGTA-3'; *cre* (s): 5'-GGGATTGCTTATAACACCCTGTTA CG-3', *cre* (as): 5'-TATTCGGATCATCAGTACACAGAG-3'; *pki- α* (s): 5'-TG CAGATTTTCATTGCTTCAGGA-3', *pki- α* (as): 5'-CAGACTTGGCTGCTTCTCC-3'; *pki- γ* (s): 5'-GACTTCATCTCCTGCGACCG-3', *pki- γ* (as): 5'-CAGGATGAGGTG TTCGCATCA-3'.

Conflict of Interest

The authors declare no conflict of interest.

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