

Original Article

## Cadmium up-regulates transcription of the steroidogenic acute regulatory protein (StAR) gene through phosphorylated CREB rather than SF-1 in K28 cells

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**ABSTRACT** — Cadmium is a widely used heavy metal in industry and affects the male reproductive system of animals, including humans, as a result of occupational and environmental exposures. However, the molecular mechanism underlying its effect on steroidogenesis in gonads remains unclear. In this study, we demonstrated that exposure of K28 mouse testicular Leydig tumor cells to cadmium led to a significant increase in the mRNA level, promoter activity and protein level of the steroidogenic acute regulatory protein (StAR), an essential factor for steroid biosynthesis. It has been well documented that *StAR* gene transcription is regulated by multiple transcription factors, including cAMP-responsive element binding protein (CREB) family members and SF-1. Cadmium treatment caused an increase in CREB phosphorylation but did not alter the CREB protein level in the nucleus. EMSA studies revealed that cadmium-induced phosphorylated CREB formed specific complexes with the proximal region of the *StAR* gene promoter. Furthermore, co-transfection with a CREB expression plasmid significantly increased cadmium-induced StAR promoter activity. However, the nuclear level and the affinity of SF-1 protein for the StAR proximal promoter were dramatically decreased upon exposure to cadmium. Taken together, these results suggest that cadmium up-regulates *StAR* gene expression through phosphorylated CREB rather than through SF-1 in mouse testicular Leydig cells.

**Key words:** Cadmium, Steroidogenic acute regulatory protein, Leydig cells, CREB, SF-1

### INTRODUCTION

Cadmium is one of the most pervasive and persistent environmental contaminants, and it is found in soil, air and water. Humans and other mammals are exposed to cadmium in the environment through several routes, and the chance of exposure is rising accordingly with the increase in industrial usage of batteries, pigments, and electroplating equipment as well as several other applications. Because of its low excretion rate, cadmium has an extremely long biological half-life in the body, and it accumulates over time in the blood and in specific organs such as the liver and kidney as well as in the reproductive organs, including the placenta, ovaries and testes (Varga *et al.*, 1993; Massanyi *et al.*, 1995; Henson and Chedrese,

2004). The International Agency for Research on Cancer classified cadmium as a human carcinogen in 1993 (IARC, 1993), and numerous studies over several decades have provided evidence that cadmium is involved in carcinogenesis in diverse organs including the lung, liver, kidney and prostate (Waalkes, 2003; Goyer *et al.*, 2004; Thompson and Bannigan, 2008).

The effects of cadmium on reproductive endocrinology have been reported; however, the results of these studies varied depending on the experimental models and dosages used. It was shown that cadmium interfered with normal gonadal function, as a significant reduction was observed in hormonal levels *in vivo* (Piasek and Laskey, 1994; Paksy *et al.*, 1997; Piasek *et al.*, 2002; Sen Gupta *et al.*, 2004a) as well as *in vitro* (Mgbonyebi *et al.*, 1998;

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Piasek and Laskey, 1999; Piasek *et al.*, 2002). Cadmium has also been shown to stimulate the synthesis of ovarian luteal progesterone at low doses; however, at high doses, the synthesis was inhibited (Henson and Chedrese, 2004). Similarly, the opposing dual actions of cadmium have been reported in granulosa cells, in which low levels of activated P450scc mRNA expression and progesterone synthesis were noted, but the effect was abolished at high doses (Smida *et al.*, 2004). Although the mechanism by which cadmium affects steroidogenesis remains elusive, it is suggested that cadmium might be involved in the steroidogenic pathway at multiple sites.

The delivery of substrate cholesterol to the inner mitochondrial membrane, in which the P450scc enzyme is located, is considered to be the rate-limiting step in the steroidogenic cascade (Stocco and Clark, 1996). The steroidogenic acute regulatory (StAR) protein has been shown to perform a critical function at this step via the transfer of cholesterol (Lin *et al.*, 1995). In trophic hormone-stimulated steroidogenesis in the adrenal glands and gonads, the initial step is the conversion of cholesterol to pregnenolone by the P450scc enzyme. Then, the pregnenolone can be transformed into progesterone by 3 $\beta$ -HSD (Stocco and Clark, 1996). Little is known about the effects of cadmium on the regulation of the *StAR* gene. A few *in vivo* studies have been conducted in which male rats have been exposed to cadmium via a single subcutaneous injection; in these studies, considerable inhibition of StAR expression was observed at the mRNA level (Sen Gupta *et al.*, 2004b) as well as at the protein level (Gunnarsson *et al.*, 2004). However, the exact mechanism responsible for this phenomenon has yet to be clarified.

The expression of the StAR protein can be induced in the adrenals and gonads by trophic hormone or cAMP analogues (Stocco and Clark, 1996). This hormonal stimulation is followed by the formation of cyclic adenosine monophosphate (cAMP), which in turn activates PKA. PKA then phosphorylates the relevant transcription factors (Reinhart *et al.*, 1999), including cAMP response element binding (CREB) protein and cAMP response element (CRE) modulator protein (CREM), which could bind to the three CRE half sites of the *StAR* promoter, resulting in the induction of *StAR* gene transcription (Manna *et al.*, 2002). A few studies have suggested that cadmium stimulates the production of cAMP (Cook *et al.*, 1985; Kumar and Bhattacharya, 2000). The steroidogenic factor 1 (SF-1) has also been demonstrated to play important role in mediating *StAR* gene expression. SF-1 is a member of the orphan nuclear receptor superfamily and is expressed in steroidogenic tissues (Reinhart *et al.*, 1999; Manna *et al.*, 2003; Sugawara *et al.*, 2006).

Cadmium has been shown to enhance the expression of many classes of genes. It stimulates the expression of immediate early genes such as c-fos, c-jun and c-myc (Matsuoka and Call, 1995), apoptosis-inducing orphan nuclear transcription factors such as Nur77 (Shin *et al.*, 2004), the tumor suppressor gene p53 (Zheng *et al.*, 1996), genes encoding the protective molecules including metallothioneins (Imbert *et al.*, 1990), glutathione (Hatcher *et al.*, 1995) and stress-related (heat shock) proteins (Lee *et al.*, 2002). The mechanisms responsible for cadmium-induced modulation of gene activity usually involve interference with cellular signaling at the levels of cell surface receptors, cellular calcium and zinc homeostasis, modulation of protein phosphorylation, and modification of transcription factors. However, the molecular mechanism underlying the effect of cadmium on steroidogenesis remains unclear, especially in testicular Leydig cells. In our present study, we found that cadmium activated *StAR* gene transcription in mouse testicular Leydig cells through phosphorylated CREB rather than SF-1.

## MATERIALS AND METHODS

### Materials

Cadmium chloride (CdCl<sub>2</sub>) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and antibiotics were all obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). [ $\alpha$ -<sup>32</sup>P]-deoxy-CTP was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Complete protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Polyclonal anti-StAR antibody was obtained from Affinity BioReagents (Golden, CO, USA), and anti-CREB antibody was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phosphorylated-CREB and anti-SF-1 antibodies were purchased from Upstate (Lake Placid, NY, USA). All other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co.

### Cell culture, treatments and viability analysis

The K28 mouse testicular Leydig tumor cell line was a gift from Dr. C. Finanz (INSERM, CNRS, France). The K28 cells were maintained in DMEM containing 15% heat-inactivated FBS and antibiotics in humidified air containing 5% CO<sub>2</sub> at 37°C. The cells were treated with different doses of CdCl<sub>2</sub> or 0.5 mM cAMP in serum-free DMEM for different time periods as indicated in the Results section.

For the evaluation of cell viability, the cells were plat-

ed at concentration of  $5 \times 10^4$  cells/well in a 24-well plate. After culture for 48 hr, the medium was replaced with serum-free medium followed by treatment with different doses of  $\text{CdCl}_2$  for 8 hr. Then, the cells were washed with phosphate-buffered saline (PBS), and serum-free medium containing 1 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma) was added to each well. The cells were then incubated for a further 4 hr. The supernatants were carefully removed, 200  $\mu\text{L}$  of dimethyl sulfoxide was added to each well, and the plate was agitated to dissolve the purple formazan product for 20 min. After complete solubilization, the absorbance values were measured at 562 nm using an Elx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

### Real-time PCR analysis

Total RNA was extracted from  $\text{CdCl}_2$ -treated K28 cells using Trizol reagent (Invitrogen). Briefly, after collecting the cells in Trizol reagent, the lysate was mixed with chloroform (5:1, v/v), shaken strongly for 30 sec and then centrifuged at 13,000 rpm for 15 min at  $4^\circ\text{C}$ . The RNA in the upper phase was precipitated by adding isopropyl alcohol, washed once with 70% ethanol, and air-dried for 15 min. The RNA was then dissolved in water containing 0.1% diethyl pyrocarbonate (v/v). Reverse transcription was performed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). First, 2  $\mu\text{g}$  of total RNA was denatured at  $65^\circ\text{C}$  for 10 min followed by incubation at  $42^\circ\text{C}$  for 1 hr in a 20  $\mu\text{L}$  reaction mixture containing 1.0 U MMLV reverse transcriptase, 0.5  $\mu\text{g}$  oligo (dT)<sub>15</sub> primer, 0.5 U/ $\mu\text{L}$  RNase inhibitor, and 1 mM dNTPs. The reverse-transcribed cDNA (1  $\mu\text{L}$ ) was used as a template for real-time PCR. PCR reactions were carried out using a real-time PCR machine (Rotor-Gene 3000; Corbett Research, Sydney, Australia) and QuantiTect SYBR Green PCR Kits (Qiagen, Hilden, Germany) with mouse StAR primer pairs (forward: 5'-CCAGGAGCTGTCCTACATCCAG-3'; reverse: 5'-GTCCGGAACACCTTGCCCACA-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pairs (forward: 5'-AGAA CATCATCCCTGCATCC-3'; reverse: 5'-TTGTCATTGAGAGCAATGCC-3') as a quantitative control. Each 20  $\mu\text{L}$  reaction mixture containing 1  $\mu\text{L}$  of cDNA, 10 pM primers and 10  $\mu\text{L}$  of reaction buffer was incubated for 3 min  $95^\circ\text{C}$  for denaturation, followed for 50 cycles of  $95^\circ\text{C}$  for 20 sec,  $60^\circ\text{C}$  for 10 sec, and  $72^\circ\text{C}$  for 20 sec. The fluorescence signals were analyzed with the Delta-Delta C<sub>t</sub> Reactive Quantitation method using Rotor-Gene 6 software.

### Preparation of whole-cell and nuclear extracts (NEs)

Preparation of whole cell lysate was carried out as follows. Briefly, the cells from different treatment groups were washed two times with ice-cold PBS followed by scraping and collection by centrifugation. The pellets were lysed for 30 min in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.2% SDS, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$  and protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation at 13,000 rpm for 15 min, the supernatant was assayed for whole-cell proteins.

NEs were prepared from cells as previously described (Dignam *et al.*, 1983) with minor modifications. In brief, the cells were washed with PBS and collected by scraping. The cells were pelleted by centrifugation at 3,000 rpm for 5 min and submerged in ice-cold lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM  $\text{MgCl}_2$ , 0.5 mM KCl, 0.5 mM dithiothreitol, 1 mM PMSF, 5 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$  and protease inhibitor cocktail on ice for 15 min. Subsequently, 0.1% Nonidet P-40 was added, the suspensions were gently mixed, and the nuclei were immediately collected by centrifugation at 4,000 rpm 10 min. The supernatant was discarded, and the crude nuclear pellet was washed twice with lysis buffer, omitting Nonidet P-40. The pellet was resuspended in extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM  $\text{MgCl}_2$ , 0.4 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 1 mM PMSF, 5 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$  and protease inhibitor cocktail for 30 min in rotator at  $4^\circ\text{C}$ . After removing the debris by centrifugation at 13,000 rpm for 20 min, the supernatant was harvested and assayed for nuclear proteins. The protein concentrations of the cell extracts were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

### Immunoblotting

Forty micrograms of whole-cell lysates and 60  $\mu\text{g}$  of NEs were separated on 10% SDS-polyacrylamide gels after boiling for 5 min in Laemmli sample buffer. The proteins were then transferred to nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were blocked overnight at  $4^\circ\text{C}$  in Tris-buffered saline containing 0.1% Tween-20 (TBS-T, pH 7.6) and 5% non-fat dry milk; they were then incubated with specific primary antibodies that recognize StAR, SF-1, CREB and phosphorylated CREB for 3 hr at room temperature. After incubation with primary antibodies, the membranes were washed three times with TBS-T, incubated with horserad-

ish peroxidase-conjugated secondary antibody against rabbit IgG for an additional 2 hr, and washed again. Immunoreactive bands were visualized via chemiluminescence using Amersham ECL Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, England) followed by exposure to X-ray film (Carestream Health Inc., Rochester, NY, USA). The level of phosphorylated or total CREB was assessed by stripping and reprobing using the same membrane. Horseradish peroxidase-conjugated anti-beta-actin antibody (Santa Cruz) was used as a loading control for whole-cell proteins.

### Transient transfection and luciferase activity assay

The 5'-flanking regions of the rat *StAR* gene, -2200 StAR/Luc and -150 StAR/Luc (Lee *et al.*, 1999), were cloned into the pGL3-Basic vector. The CREB expression construct was kindly provided by Dr. Hueng Sik Choi (Chonnam National University, Gwangju, Korea). For transient transfection, the K28 cells were split into 24-well plates at densities of  $5 \times 10^4$  cells/well one day prior to transfection. Transfection studies were conducted with Lipofectamine Plus reagent (Invitrogen) according to the instructions provided by the manufacturer. In brief, the cells in each well were transfected with 0.4  $\mu$ g of StAR luciferase reporter constructs and 0.1  $\mu$ g of pSV- $\beta$ -galactosidase expression plasmid. The effects of CREB on cadmium signal transduction were tested in co-transfections with 0.4  $\mu$ g of -2200 StAR/Luc and 0.4  $\mu$ g of pcDNA-CREB expression constructs. The total amount of DNA used in the transfections was equalized with empty pcDNA3 vector. After incubation for 40 hr, the medium was replaced with serum-free medium followed by treatment with different doses for 8 hr. The cells were then harvested, and luciferase activity in the cell extracts was measured according to the standard method using a MicroLumat Plus LB 96 V (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was normalized to  $\beta$ -galactosidase activity to compensate for variations in transfection efficiency.

### Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described by Manna *et al.* (2002). The various complementary oligonucleotides from the mouse StAR promoter region were annealed by heating the sense and antisense primers to 65°C for 5 min in annealing buffer (10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 7.5). The oligonucleotides were then slowly cooled at room temperature and used as probes for EMSA. The sense strands of the oligonucleotide sequence previously reported by Manna *et al.*

(2002, 2003) were used. The double-stranded oligonucleotides with 5'-GG overhangs were end-labeled by filling in with [ $\alpha$ -<sup>32</sup>P]-dCTP using Klenow enzyme (Roche) at 37°C for 45 min. The probes were purified using Probe-Quant G50 micro columns (Amersham Biosciences, Buckinghamshire, UK), and excess unincorporated [ $\alpha$ -<sup>32</sup>P]-dCTP was removed. NE (10  $\mu$ g) was pre-incubated in 10 mM HEPES (pH 7.9), 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, 1  $\mu$ g poly dI·dC, and 40 ng/ $\mu$ L BSA for 15 min at room temperature. Subsequently, <sup>32</sup>P-labeled double-stranded DNA probe (greater than 6,000 cpm/ $\mu$ L) was added, and the mixtures were incubated for a further 15 min at room temperature. For the assay in which antibodies were used to block DNA-protein complex formation, NEs were incubated with a specific antibody against phosphorylated CREB, SF-1, for 1 hr on ice before the addition of the labeled probe. Non-immune rabbit IgG (Santa Cruz) was used as a negative control. When competitors were employed, a 50-fold molar excess of unlabeled oligonucleotide competitor was added to the reaction mixture containing NE and the labeled probe, and the mixture was incubated for 15 min. The DNA-protein complexes were then subjected to electrophoresis at 200 V for 90 min using a pre-cooled and pre-run native 5% polyacrylamide gel in 0.5 X TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0). The gel was vacuum-dried and exposed to X-ray film at -70°C overnight.

### Statistical analysis

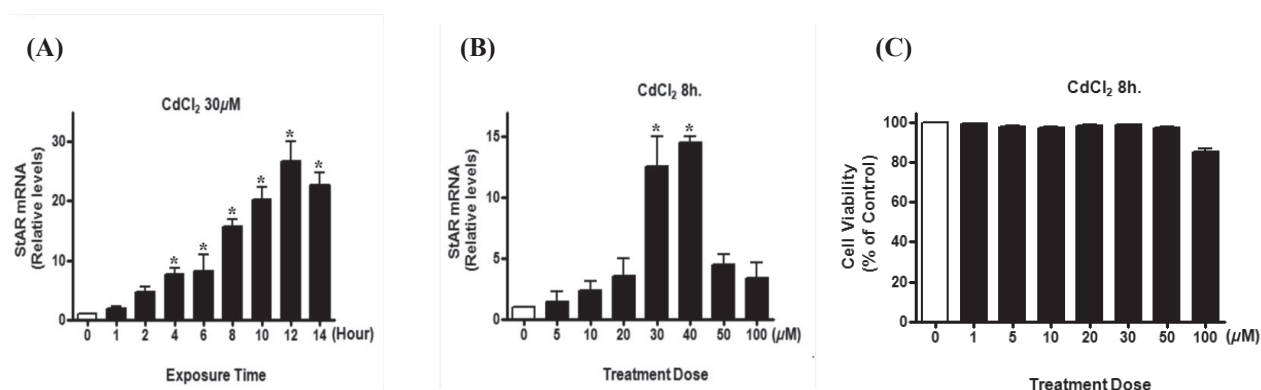
The data are expressed as the mean  $\pm$  standard error (S.E.). Experimental data were analyzed using analysis of variance (ANOVA), and significant differences among the means were determined by Duncan's multiple-range test at a 95% confidence level (SAS 9.2; SAS Institute, Cary, NC, USA).

## RESULTS

### Cadmium increases the levels of StAR mRNA and protein as well as StAR promoter activity

To evaluate the effect of cadmium on male reproductive systems, K28 mouse testicular Leydig tumor cells were incubated with various concentrations of cadmium for different time periods. After exposure to cadmium, real-time PCR analysis was performed to determine whether the StAR mRNA level was altered. As shown in Fig. 1A, cadmium significantly increased the StAR mRNA level in a time-dependent manner. The mRNA level began to increase after 1 hr and continued increasing up to 12 hr, after which it declined. To further explore

## Effects of cadmium on StAR expression



**Fig. 1.** Effects of exposure to cadmium on StAR mRNA transcription and cell viability in mouse K28 cells. Cells were treated with 30  $\mu\text{M}$   $\text{CdCl}_2$  for different time periods (0-14 hr) (A) or increasing doses of  $\text{CdCl}_2$  (0-100  $\mu\text{M}$ ) for 8 hr (B). Total RNA was isolated from the different treatment groups, and transcription of the StAR gene was estimated by real-time PCR analysis. The StAR mRNA level was normalized to the GAPDH level. At least three independent experiments were combined, and the data are expressed as the fold induction relative to the untreated control (mean  $\pm$  S.E.). (C) Cells were treated with increasing doses of  $\text{CdCl}_2$  (0-100  $\mu\text{M}$ ) for 8 hr, and cell viability was measured using the MTT assay. The absorbance value of control cells was considered as 100% viability. The results are expressed as the mean  $\pm$  S.E. from two independent experiments performed in triplicate. The exact treatment time periods and concentrations of  $\text{CdCl}_2$  in each experiment are indicated in each graph. \* indicates  $p < 0.001$ .

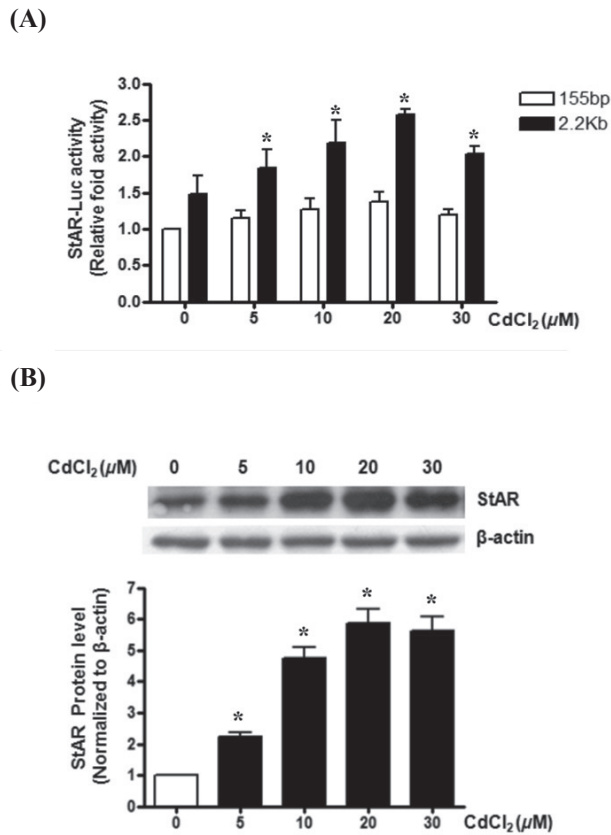
the induction pattern of StAR mRNA upon cadmium administration, cells were incubated with increasing doses of cadmium for 8 hr. Incubation with 5  $\mu\text{M}$  cadmium slightly increased the StAR mRNA level, and it was elevated maximally at cadmium concentrations between at 30 and 40  $\mu\text{M}$ ; subsequently, the StAR mRNA level declined (Fig. 1B). These results indicated that cadmium exposure induced *StAR* gene expression in both a dose- and time-dependent manner. Because treatment with 30  $\mu\text{M}$  cadmium for 8 hr caused a maximal increase in the StAR mRNA level, this treatment condition was chosen for the subsequent experiments. To analyze the effect of cadmium on cell viability, K28 cells were incubated with increasing doses of cadmium for 8 hr, and cell survival was determined using the MTT assay. As shown in Fig. 1C, exposure of cells to up to 50  $\mu\text{M}$  cadmium for 8 hr did not cause any significant change in viability. Treatment with 100  $\mu\text{M}$  cadmium caused a slight reduction in viability (85% cell survival) compared with the untreated cells, which indicated that the cadmium treatment conditions (30  $\mu\text{M}$  and 8 hr) chosen for this study have no effect on cell viability.

To confirm that the cadmium-induced up-regulation of StAR mRNA was attributable to the transcriptional activation of the gene, a luciferase activity assay using two constructs containing -2,200 bp and -150 bp regions of the *StAR* gene (Lee *et al.*, 1999) was performed. The results showed that the luciferase activity of both reporter constructs was increased by cadmium concentrations of up

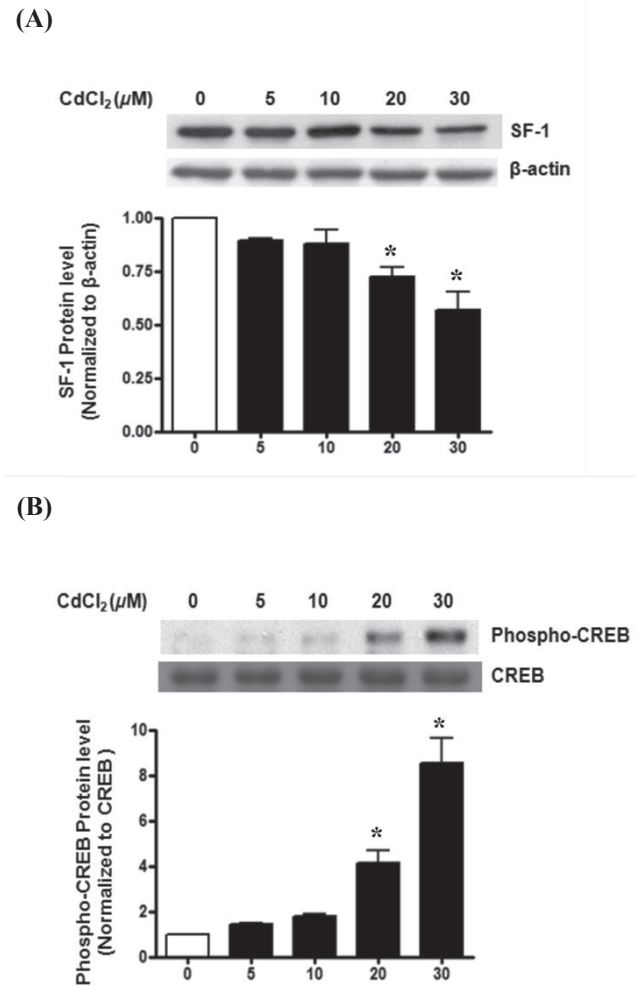
to 20  $\mu\text{M}$  compared to the basal level. A slight decrease was observed at 30  $\mu\text{M}$ ; however, the luciferase activity remained elevated over the basal level (Fig. 2A). In addition, immunoblotting was performed to determine whether cadmium also induced the expression of StAR protein. Similar to its effects on StAR mRNA expression, cadmium markedly increased StAR protein levels in a concentration-dependent manner. As shown in Fig. 2B, 30  $\mu\text{M}$  cadmium induced a 6-fold increase in StAR protein. This result, together with that of real-time PCR analysis and the luciferase activity assay, demonstrates that cadmium induces StAR protein expression by transcriptional activation of the gene in mouse testicular Leydig cells.

### Cadmium regulates the proteins involved in StAR gene transcription

To further examine the up-regulation of *StAR* gene expression by cadmium at the transcriptional level, we focused on SF-1 and CREB because they are both crucial transcriptional regulators of *StAR* gene expression in response to trophic hormones. K28 cells were incubated with increasing doses of cadmium for 8 hr, and then whole-cell lysates and NE were prepared for immunoblotting. The results shown in Fig. 3 indicated that both SF-1 and CREB proteins were maintained at a high level in K28 cells. The constitutive expression of SF-1 was considerably decreased in a dose-dependent manner by cadmium administration, and SF-1 expression was inversely correlated with the StAR level (Fig. 2B). In contrast



**Fig. 2.** Effects of exposure to cadmium on promoter activity and protein expression of StAR in K28 cells. (A) Cells were transfected with 5'-deleted StAR promoter/luciferase constructs (-2200 StAR/Luc or -150 StAR/Luc) in the presence of β-galactosidase expression plasmids. After 40 hr of transfection, the cells were incubated with increasing doses of CdCl<sub>2</sub> (5, 10, 20, 30 μM) for a further 8 hr. Luciferase activity in the cell lysates was determined, and β-galactosidase activity was used as an internal control for transfection efficiency. The results are shown as the mean ± S.E. from three independent experiments performed in quadruplicate and are expressed as fold activation over cells transfected with the -150 StAR/Luc construct without cadmium treatment. (B) Cells were treated with increasing doses of CdCl<sub>2</sub> (5, 10, 20, 30 μM) for 8 hr. StAR protein in whole-cell lysates was determined by immunoblotting. Immunoblots of the upper panel are representative of at least five independent experiments. The amount of total protein loaded in each lane was determined by reprobing with anti-β-actin antibody. For both proteins, the densities of the bands were quantified in three independent experiments. The values were normalized to the corresponding β-actin band and expressed as fold induction compared with the untreated control (mean ± S.E.). \* indicates *p* < 0.001.



**Fig. 3.** Effect of exposure to cadmium on the expression of proteins involved in steroidogenesis in K28 cells. Cells were treated with increasing doses of CdCl<sub>2</sub> (5, 10, 20, 30 μM) for 8 hr. (A) Whole-cell lysates were prepared, and the level of SF-1 was determined by immunoblotting. Equal loading was confirmed using β-actin. The experiment was repeated four times, and typical results are shown (mean ± S.E.). (B) Nuclear extracts were isolated as described in "Materials and methods", and phosphorylated CREB was detected by immunoblotting. The membrane was stripped and reprobbed with an anti-CREB antibody. The density of each band was quantified, and the ratios of phosphorylated CREB to CREB were calculated from three independent experiments (lower panel, mean ± S.E.). The results are expressed as fold induction compared with the untreated control. \* indicates *p* < 0.001.

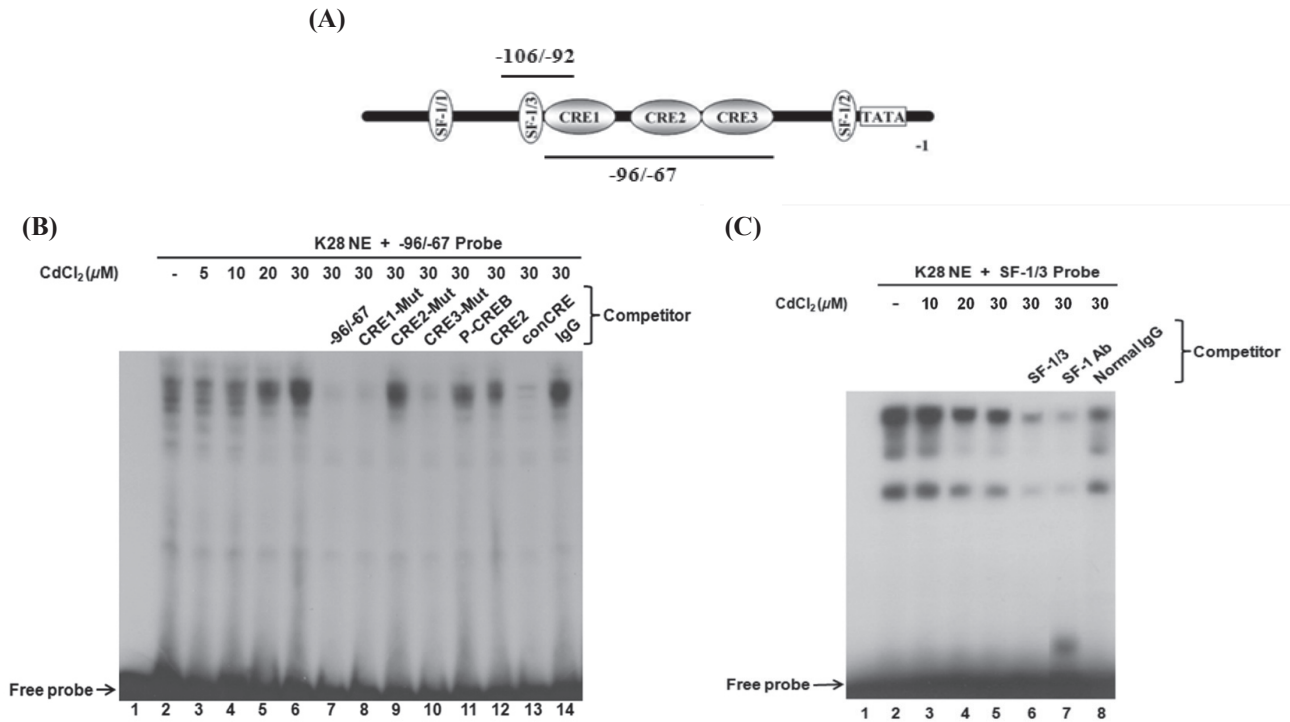
## Effects of cadmium on StAR expression

to the change in the SF-1 level, cadmium treatment did not significantly alter the nuclear CREB level. Interestingly however, cadmium induced the phosphorylation of CREB, as shown in Fig. 3B. The phosphorylated form of the protein was not detected in untreated cells, whereas a slight increase in phosphorylation occurred when the cells were treated with cadmium between 10  $\mu\text{M}$  and 20  $\mu\text{M}$ . This induction was remarkably increased upon treatment with 30  $\mu\text{M}$  cadmium. Analysis of the phosphorylated CREB to CREB ratio revealed significant accumulation of phosphorylated CREB in cells treated with 30  $\mu\text{M}$  cadmium, and these data demonstrate that activation of CREB by cadmium involved protein phosphorylation rather than protein synthesis.

### Cadmium increases binding of phosphorylated CREB, but not SF-1, to the StAR promoter

The direct involvement of the transcription factors described above in cadmium-induced up-regulation of StAR was explored next. To determine whether phosphor-

ylated CREB and SF-1 proteins bind to the StAR promoter and to investigate the contribution of cadmium to the binding of these proteins to the StAR promoter, EMSA was performed using nuclear proteins extracted from cadmium-treated K28 cells. Earlier studies have shown that the -150/-20 bp region was considerably conserved among the StAR promoters of different species including mice, rats, and humans, and this region had three 5'-canonical CRE half-site sequences and three putative SF-1 recognition motifs (Sugawara *et al.*, 1997; Wooton-Kee and Clark, 2000; Manna *et al.*, 2002, 2003). As shown in Fig. 4B, a  $^{32}\text{P}$ -labeled -96/-67 probe containing the three CRE half-sites (as depicted in Fig. 4A) formed specific binding complexes with NEs from K28 cells (lane 2), and the levels of these complexes were increased in NEs from cells treated with cadmium at 20 and 30  $\mu\text{M}$  (compare lane 2 with lanes 5 and 6). The DNA-protein complexes were diminished following addition of a phosphorylated CREB antibody, as shown in the immunoblotting experiment (lane 11), compared to the addition of nonimmune



**Fig. 4.** Binding of proteins present in cadmium-treated K28 NEs to the CRE and SF-1 regions. (A) Schematic diagram revealing the presence of three putative CRE half-sites and SF-1 binding sites in the mouse StAR promoter (-151 to -1 bp). The oligonucleotide probes used for EMSA are illustrated. K28 cells were treated for 8 hr with increasing doses of  $\text{CdCl}_2$  (5, 10, 20, 30  $\mu\text{M}$ ) and NEs were assessed in EMSA to analyze protein-DNA binding using a  $^{32}\text{P}$ -labeled CRE element (-96/-67 bp) (B) or SF-1 (-106/-92 bp) (C) as described in "Materials and methods". Cold competitors were used at 50-fold molar excess. An antibody competition assay was carried out with specific antibodies against phosphorylated CREB and SF-1. Nonimmune IgG antibody was used as a negative control. The experiments were repeated three times, and representative results of one experiment are shown.

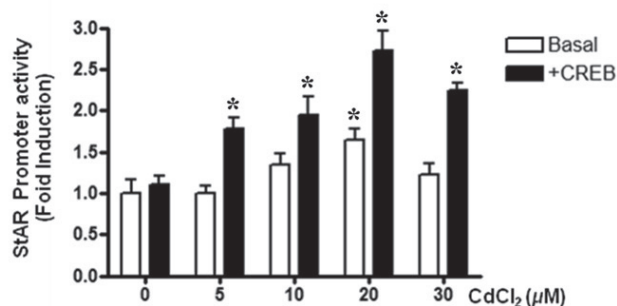
IgG (lane 14). The specific complexes nearly disappeared after addition of a 50-fold molar excess of unlabeled oligonucleotides (lane 7). To identify the cadmium-responsive CRE half-site, a competition study was performed with cadmium-treated K28 NEs using oligonucleotides with mutations in each CRE half-site. The DNA-protein complexes competed with oligonucleotides with mutations in the CRE1 site (lane 8) and the CRE3 site (lane 10), while oligonucleotides with mutations in the CRE2 site were not able to compete with these complexes (lanes 9). The DNA-protein binding specificity was also assessed by competition with CRE2 and consensus CRE sequences (Manna *et al.*, 2002). After the cold CRE2 was added, the amount of complex formed (lane 12) seemed to be decreased by about 50% compared to lane 6 and when the consensus CRE sequences added, the formation of the complex was greatly decreased (lane 13). These results suggest the critical involvement of the CRE2 site in DNA-protein complex formation, which is in agreement with previous findings demonstrating that, among the three CRE half-sites, CRE2 is the major site involved in StAR promoter activation (Manna *et al.*, 2002, 2003).

The -150/-20 bp region of the *StAR* gene was shown to contain three putative SF-1 recognition motifs

(Manna *et al.*, 2003 and as illustrated Fig. 4A). Notably, the SF-1/3 region located between -106 and -92 bp upstream of the transcriptional start site was previously demonstrated to play a more important role in StAR promoter activation compared with the SF-1/1 and SF-1/2 sites (Manna *et al.*, 2003). As indicated in Fig. 4C, the  $^{32}\text{P}$ -labeled SF-1/3 probe formed specific binding complexes with NEs of K28 cells (lane 2); however, the levels of these complexes were significantly decreased by cadmium in a dose-dependent manner (compare lanes 2 and 5). The binding was greatly diminished by addition of a 50-fold molar excess of unlabeled oligonucleotides (lane 6), and the specific antibody against SF-1 also markedly decreased DNA-protein complex formation (lane 7). The specificity of the complexes was further verified using a nonimmune IgG antibody that did not affect SF-1/3 site-NE binding (lane 8).

#### Over-expression of CREB protein enhances cadmium-induced StAR promoter activity

Additionally, we attempted to determine whether an increased level of phosphorylated-CREB can elevate StAR promoter activity. K28 cells were transfected with the -2200 bp StAR reporter construct in the absence or presence of CREB expression plasmid; they were then treated with or without increasing doses of cadmium for 8 hr (Fig. 5). Over-expression of CREB did not have a significant effect on StAR promoter activity in untreated cells when compared with mock-transfected cells (pcDNA3). However, as expected, cadmium treatment further augmented the CREB-mediated induction of StAR promoter activity in a dose-dependent manner, suggesting that the CREB is phosphorylated.



**Fig. 5.** Effects of exposure to cadmium on StAR promoter activity in K28 cells over-expressing CREB. Cells were transfected with a -2200 StAR promoter/luciferase construct in the presence of  $\beta$ -galactosidase expression plasmids with or without CREB expression plasmids. The total amount of transfected DNA was kept constant using empty vector. After 40 hr of transfection, the cells were incubated with increasing doses of CdCl<sub>2</sub> (5, 10, 20, 30  $\mu\text{M}$ ) for an additional 8 hr. Luciferase activity in the cell lysates of each group was determined, and  $\beta$ -galactosidase activity was used as an internal control for transfection efficiency. Luciferase activity values in untreated cells were set to 1.0, and the relative luciferase activity in CdCl<sub>2</sub> treated cells was calculated (mean  $\pm$  S.E.). The experiments were performed in duplicate and repeated three times. \*indicates  $p < 0.001$ .

#### DISCUSSION

The purpose of this study was to determine the effect of the heavy metal cadmium on *StAR* gene expression in steroidogenic cells. These results indicated that cadmium induced the transcription of the *StAR* gene in mouse testicular Leydig cells in both a time- and dose-dependent manner (Fig. 1). Cadmium was also shown to stimulate the activity of the StAR promoter (Fig. 2A), thus suggesting that this heavy metal induces the transcription of the *StAR* gene. Cadmium was previously reported to increase the intracellular cAMP level (Cook *et al.*, 1985; Kumar and Bhattacharya, 2000) and was also demonstrated to bind to the CRE and promote transcriptional activity of stress genes in HepG2 cells (Tchounwou *et al.*, 2001). Additionally, earlier reports suggested that cadmium can directly activate PKC (Long, 1997), and recent studies have dem-



onstrated the possible role of PKC in StAR expression in mouse testicular Leydig cells (Jo *et al.*, 2005; Manna *et al.*, 2009). These results suggested that the cAMP/PKA and PKC pathways might be involved in the cadmium-induced transcriptional activation of the *StAR* gene in testicular Leydig cells. However, Gunnarsson *et al.* (2004) and Sen Gupta *et al.* (2004b) have reported that cadmium caused the inhibition of testosterone production via lowered expression of StAR in rat testicular Leydig cells *in vivo*. It might be possible that the effects of cadmium on male reproductive system would vary depending on the experimental systems. Thus we are currently conducting experiment on the levels of hormones as well as the proteins involved in hormone biosynthesis in cadmium-treated mouse Leydig cells *in vivo* in order to test whether or not cell line study is consistent with *in vivo* study.

The response to cAMP at the molecular level is typically mediated by a palindromic conserved sequence (5'-TGACGTCA-3') commonly referred to as the cAMP response element (CRE) (Lalli and Sassone-Corsi, 1994). However, similar to cAMP-regulated steroid hydroxylase genes, the *StAR* gene lacks a consensus CRE (Waterman, 1994). Members of a large family of basic leucine zipper (bZIP) CRE binding factors, including the CRE binding protein (CREB), the CRE modulator protein (CREM), and the activating transcription factor (ATF-1), have all been shown to interact with this sequence. Members of the CRE binding factor family can serve as activators or repressors of transcription, and these proteins can homodimerize and heterodimerize via a specific interaction domain (Manna *et al.*, 2002). Three genes, *CREB*, *CREM* and *ATF-1*, share a profound homology and constitute the CREB/CREM subfamily (CREB/CREM). These genes encode proteins that mediate transcriptional activation of the *StAR* gene (Manna *et al.*, 2002). Previous reports have shown that 150 nucleotides from the transcriptional start site were critical for cAMP and trophic hormone-induced *StAR* gene activation (Manna *et al.*, 2003) and the three CRE half sites (-96/-67 bp) have been characterized within the cAMP-responsive region (-151/-1 bp) of the mouse *StAR* gene (Manna *et al.*, 2002). In this study, treatment of 10 mM cadmium induced 2-fold increase in the phosphorylated CREB protein, while the StAR protein level was increased 5-fold. The result could suggest that cadmium-induced StAR expression may be caused by more transcription factors including CREM and ATF-1, as well as CREB protein. Further study is required to identify all transcriptional factors participating in cadmium-induced StAR expression.

It had been reported that cadmium treatment induced the mitogenic signaling in the 1LN prostate cell line

through the increase of transcription factors NF $\kappa$ B and CREB. (Misra *et al.*, 2003). Also Kondo *et al.* (2012) demonstrated that treatment of cadmium activate extracellular signal-regulated kinase 5 (ERK5) which is a member of the mitogen-activated protein kinase (MAPK) family. They also showed that the activation of ERK5 pathway in turn induced the phosphorylation of cell survival-transcription factors including CREB, ATF-1, and c-FOS in HK-2 human renal proximal tubular cells. Further study is necessary to see that the ERK5 pathway was also involved in the phosphorylation of CREB in our study by administering BIX02189, MAPK/ERK kinase inhibitor. The -150/-20 bp region of the *StAR* gene has been shown to harbor three SF-1-binding *cis* elements, namely SF-1/1, SF-1/2, and SF-1/3. Notably, the SF-1/3 region located between -106 and -92 bp upstream of the transcriptional start site has previously been shown to be more closely involved with both basal and cAMP-induced *StAR* gene expression (Montminy *et al.*, 1986) and StAR promoter activity in comparison with the SF-1/1 and SF-1/2 sites (Manna *et al.*, 2003).

To confirm whether CRE and SF-1/3 elements are important for the regulation of StAR expression by cadmium, EMSA was performed. The results of EMSA showed that DNA-protein complex formation with the CRE2 site of the StAR promoter (-96/-67) was increased upon cadmium exposure (Fig. 4B). However, cadmium decreased the binding of nuclear proteins to the SF-1/3 site (Fig. 4C). These results corresponded exactly to those obtained with immunoblotting. Treatment of K28 cells with cadmium resulted in an increase in the level of phosphorylated CREB and a decrease in the level of SF-1 (Fig. 3). Finally, the result shown in Fig. 5 provides strong evidence that cadmium-induced transcriptional activation of the *StAR* gene is mediated by phosphorylated CREB.

Cadmium is environmental contaminant, and mammals (including humans) can be easily exposed to cadmium in their surroundings. Accordingly, many researchers have studied cadmium, and a large body of data is available. Although the effect of cadmium on reproductive endocrinology has been reported, the molecular mechanism by which it affects steroidogenesis has remained unclear, especially in testicular Leydig cells. In this study, we have demonstrated that exposure of mouse testicular Leydig cells to cadmium results in an increase in *StAR* gene transcription. Moreover, we have presented evidence that cadmium up-regulates *StAR* gene transcription through phosphorylated CREB rather than SF-1. This study is the first report describing a molecular mechanism for cadmium-induced *StAR* gene regulation in testicular Leydig cells.

**Conflict of interest**--- The authors declare that there is no conflict of interest.

## REFERENCES

- Clem, B.F., Hudson, E.A. and Clark, B.J. (2005): Cyclic adenosine 3',5'-monophosphate (cAMP) enhances cAMP-responsive element binding (CREB) protein phosphorylation and phospho-CREB interaction with the mouse steroidogenic acute regulatory protein gene promoter. *Endocrinology*, **146**, 1348-1356.
- Cook, D.B., Dewar, J.H. and Ershadi, S.S. (1985): Stimulation of cyclic AMP production in chick renal tissue by cadmium and manganese. *J. Inorg. Biochem.*, **24**, 223-232.
- Dignam, J.D., Martin, P.L., Shastri, B.S. and Roeder, R.G. (1983): Eukaryotic gene transcription with purified components. *Methods. Enzymol.*, **101**, 582-598.
- Goyer, R.A., Liu, J. and Waalkes, M.P. (2004): Cadmium and cancer of prostate and testis. *Biometals*, **17**, 555-558.
- Gunnarsson, D., Svensson, M., Selstam, G. and Nordberg, G. (2004): Pronounced induction of testicular PGF(2 alpha) and suppression of testosterone by cadmium-prevention by zinc. *Toxicology*, **200**, 49-58.
- Hatcher, E.L., Chen, Y. and Kang, Y.J. (1995): Cadmium resistance in A549 cells correlates with elevated glutathione content but not antioxidant enzymatic activities. *Free Radic. Biol. Med.*, **19**, 805-812.
- Henson, M.C. and Chedrese, P.J. (2004): Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. *Exp. Biol. Med. (Maywood)*, **229**, 383-392.
- IARC (1993): Beryllium, cadmium, mercury, and exposures in the glass manufacturing industry. Working Group views and expert opinions, Lyon, 9-16 February 1993. *IARC Monogr. Eval. Carcinog. Risks Hum.*, **58**, 1-415.
- Imbert, J., Culotta, V., Furst, P., Gedamu, L. and Hamer, D. (1990): Regulation of metallothionein gene transcription by metals. *Adv. Inorg. Biochem.*, **8**, 139-164.
- Jo, Y., King, S.R., Khan, S.A. and Stocco, D.M. (2005): Involvement of protein kinase C and cyclic adenosine 3',5'-monophosphate-dependent kinase in steroidogenic acute regulatory protein expression and steroid biosynthesis in Leydig cells. *Biol. Reprod.*, **73**, 244-255.
- Kobayashi, M., Masaki, T., Hori, K., Masuo, Y., Miyamoto, M., Tsubokawa, H., Noguchi, H. and Takamatsu, K. (2005): Hippocampal-deficient mice display a defect in cAMP response element-binding protein activation associated with impaired spatial and associative memory. *Neuroscience*, **133**, 471-484.
- Kondo, M., Inamura, H., Matsumura, K. and Matsuoka, M. (2012): Cadmium activates extracellular signal-regulated kinase 5 in HK-2 human renal proximal tubular cells. *Biochem. Biophys. Res. Commun.*, **421**, 490-493.
- Kumar, S.V. and Bhattacharya, S. (2000): In vitro toxicity of mercury, cadmium, and arsenic to platelet aggregation: influence of adenylate cyclase and phosphodiesterase activity. *In Vitro. Mol. Toxicol.*, **13**, 137-144.
- Lalli, E. and Sassone-Corsi, P. (1994): Signal transduction and gene regulation: the nuclear response to cAMP. *J. Biol. Chem.*, **269**, 17359-17362.
- Lee, M.J., Nishio, H., Ayaki, H., Yamamoto, M. and Sumino, K. (2002): Upregulation of stress response mRNAs in COS-7 cells exposed to cadmium. *Toxicology*, **174**, 109-117.
- Lee, H.K., Yoo, M.S., Choi, H.S., Kwon, H.B. and Soh, J. (1999): Retinoic acids up-regulate steroidogenic acute regulatory protein gene. *Mol. Cell. Endocrinol.*, **148**, 1-10.
- Lin, D., Sugawara, T., Strauss, J.F.3rd, Clark, B.J., Stocco, D.M., Saenger, P., Rogol, A. and Miller, W.L. (1995): Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science*, **267**, 1828-1831.
- Long, G.J. (1997): The effect of cadmium on cytosolic free calcium, protein kinase C, and collagen synthesis in rat osteosarcoma (ROS 17/2.8) cells. *Toxicol. Appl. Pharmacol.*, **143**, 189-195.
- Manna, P.R., Dyson, M.T., Eubank, D.W., Clark, B.J., Lalli, E., Sassone-Corsi, P., Zeleznik, A.J. and Stocco, D.M. (2002): Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Mol. Endocrinol.*, **16**, 184-199.
- Manna, P.R., Eubank, D.W., Lalli, E., Sassone-Corsi, P. and Stocco, D.M. (2003): Transcriptional regulation of the mouse steroidogenic acute regulatory protein gene by the cAMP response-element binding protein and steroidogenic factor 1. *J. Mol. Endocrinol.*, **30**, 381-397.
- Manna, P.R., Huhtaniemi, L.T. and Stocco, D.M. (2009): Mechanisms of protein kinase C signaling in the modulation of 3',5'-cyclic adenosine monophosphate-mediated steroidogenesis in mouse gonadal cells. *Endocrinology*, **150**, 3308-3317.
- Massanyi, P., Toman, R., Valent, M. and Cupka, P. (1995): Evaluation of selected parameters of a metabolic profile and levels of cadmium in reproductive organs of rabbits after an experimental administration. *Acta. Physiol. Hung.*, **83**, 267-273.
- Matsuoka, M. and Call, K.M. (1995): Cadmium-induced expression of immediate early genes in LLC-PK1 cells. *Kidney Int.*, **48**, 383-389.
- Mgbonyebi, O.P., Smothers, C.T. and Mrotek, J.J. (1998): Modulation of adrenal cell functions by cadmium salts. 5. Cadmium acetate and sulfate effects on basal and ACTH-stimulated steroidogenesis. *Cell. Biol. Toxicol.*, **14**, 301-311.
- Misra, U.K., Gawdi, G. and Pizzo, S.V. (2003): Induction of mitogenic signalling in the ILN prostate cell line on exposure to submicromolar concentrations of cadmium<sup>2+</sup>. *Cell. Signal.*, **15**, 1059-1070.
- Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986): Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Nat. Acad. Sci.*, **83**, 6682-6686.
- Paksy, K., Rajczy, K., Forgacs, Z., Lazar, P., Bernard, A., Gati, I. and Kaali, G.S. (1997): Effect of cadmium on morphology and steroidogenesis of cultured human ovarian granulosa cells. *J. Appl. Toxicol.*, **17**, 321-327.
- Piasek, M. and Laskey, J.W. (1994): Acute cadmium exposure and ovarian steroidogenesis in cycling and pregnant rats. *Reprod. Toxicol.*, **8**, 495-507.
- Piasek, M. and Laskey, J.W. (1999): Effects of in vitro cadmium exposure on ovarian steroidogenesis in rats. *J. Appl. Toxicol.*, **19**, 211-217.
- Piasek, M., Laskey, J.W., Kostial, K. and Blanus, M. (2002): Assessment of steroid disruption using cultures of whole ovary and/or placenta in rat and in human placental tissue. *Int. Arch. Occup. Environ. Health*, **75**, 36-44.
- Reinhart, A.J., Williams, S.C. and Stocco, D.M. (1999): Transcriptional regulation of the StAR gene. *Mol. Cell. Endocrinol.*, **151**, 161-169.
- Sen Gupta, R., Kim, J., Gomes, C., Oh, S., Park, J., Im, W.B., Seong, J.Y., Ahn, R.S., Kwon, H.B. and Soh, J. (2004a): Effect

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- of ascorbic acid supplementation on testicular steroidogenesis and germ cell death in cadmium-treated male rats. *Mol. Cell. Endocrinol.*, **221**, 57-66.
- Sen Gupta, R., Sen Gupta, E., Dhakal, B.K., Thakur, A.R. and Ahn, J. (2004b): Vitamin C and vitamin E protect the rat testes from cadmium-induced reactive oxygen species. *Mol. Cells*, **17**, 132-139.
- Shin, H.J., Lee, B.H., Yeo, M.G., Oh, S.H., Park, J.D., Park, K.K., Chung, J.H., Moon, C.K. and Lee, M.O. (2004): Induction of orphan nuclear receptor Nur77 gene expression and its role in cadmium-induced apoptosis in lung. *Carcinogenesis*, **25**, 1467-1475.
- Smida, A.D., Valderrama, X.P., Agostini, M.C., Furlan, M.A. and Chedrese, J. (2004): Cadmium stimulates transcription of the cytochrome p450 side chain cleavage gene in genetically modified stable porcine granulosa cells. *Biol. Reprod.*, **70**, 25-31.
- Stocco, D.M. and Clark, B.J. (1996): Regulation of the acute production of steroids in steroidogenic cells. *Endoc. Rev.*, **17**, 221-244.
- Sugawara, T., Kiriakidou, M., McAllister, J.M., Kallen, C.B. and Strauss, J.F.III. (1997): Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry*, **36**, 7249-7255.
- Sugawara, T., Sakuragi, N. and Minakami, H. (2006): CREM confers cAMP responsiveness in human steroidogenic acute regulatory protein expression in NCI-H295R cells rather than SF-1/Ad4BP. *J. Endocrinol.*, **191**, 327-337.
- Tchounwou, P.B., Ishaque, A.B. and Schneider, J. (2001): Cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells (HepG2) exposed to cadmium chloride. *Mol. Cell. Biochem.*, **222**, 21-28.
- Thompson, J. and Bannigan, J. (2008): Cadmium: toxic effects on the reproductive system and the embryo. *Reprod. Toxicol.*, **25**, 304-315.
- Varga, B., Zsolnai, B., Paksy, K., Naray, M. and Ungvary, G. (1993): Age dependent accumulation of cadmium in the human ovary. *Reprod. Toxicol.*, **7**, 225-228.
- Waalkes, M.P. (2003): Cadmium carcinogenesis. *Mutat. Res.*, **533**, 107-120.
- Waterman, M.R. (1994): Biochemical diversity of cAMP-dependent transcription of steroid hydroxylase genes in the adrenal cortex. *J. Biol. Chem.*, **269**, 27783-27786.
- Wooton-Kee, C.R. and Clark, B.J. (2000): Steroidogenic factor-1 influences protein-deoxyribonucleic acid interactions within the cyclic adenosine 3,5-monophosphate-responsive regions of the murine steroidogenic acute regulatory protein gene. *Endocrinology*, **141**, 1345-1355.
- Zheng, H., Liu, J., Choo, K.H., Michalska, A.E. and Klaassen, C.D. (1996): Metallothionein-I and -II knock-out mice are sensitive to cadmium-induced liver mRNA expression of c-jun and p53. *Toxicol. Appl. Pharmacol.*, **136**, 229-235.