ANALYSIS OF KAISO AS A TRANSCRIPTION FACTOR

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ABSTRACT

Recently, through reporter gene studies, the novel BTB/POZ protein, Kaiso, has been identified as a transcriptional repressor. The purpose of this study was to determine if Kaiso recruited the Histone Deacetylase Complex to mediate repression and if the previously identified Kaiso Binding Site (KBS; TCCTGCNA) is a physiological target regulated by Kaiso. The two objectives are complementary because an HDAC interaction identifies the mechanism of transcriptional regulation used by Kaiso and regulation of the KBS element identifies a novel, non-methylation dependent, physiological target under transcriptional regulation by Kaiso. Through coimmunoprecipitation and Western blot analyses, Kaiso does not interact with HDAC1, HDAC2 or mSIN3A. These results were surprising since all three of these proteins are common to a variety of repression complexes. mSIN3A is a common component of SIN3 mediated repression and HDAC1/HDAC2 are part of various repression complexes including SIN3, NuRD and CtBP. Although the remaining HDAC proteins were not assayed for an interaction, Kaiso transcriptional activity was demonstrated to be insensitive to the HDAC inhibiting drug, Trichostatin A (TSA). These results indicate either a non-HDAC mechanism of action or alternatively, transcriptional activation. Complementary to the observations of no Kaiso-HDAC interaction and TSA insensitivity was the findings that Kaiso activates transcription of the KBS *cis*-element in HCT116, HCA-7 and 293 cells, but not MDCK cells in reporter gene assays. Taken together, these results indicate that Kaiso is a dual functioning protein capable of both transcriptional activation and repression and

that the mechanism of repression is not through the direct recruitment of HDAC proteins.

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ABBREVIATIONS

ΔPOZΔAR Deleted POZ and Acid Rich Domains of Kaiso

APC **A**denomatous **P**olyposis **C**oli Protein

Arm **Arm**adillo Protein Domain

BCL-6 **B**-**c**ell **L**ymphoma **6** Protein

BTB/POZ **B**road Complex, **T**ramtrak, **B**ric a Brac/**Po**x Virus **Z**inc Finger Family

CAST **C**yclic **A**mplification and **S**election of **T**argets

CBD **C**atenin **B**inding **D**omain

CSF-1 **C**olony **S**timulating **F**actor-1

CtBP **C**-**t**erminal **B**inding **P**roteins

Del Deleted POZ domain of Kaiso

dNTP **D**eoxy**n**ucleotide **t**riphosphates

EGF **E**pidermal **G**rowth **F**actor

EMSA **E**lectrophoretic **M**obility **S**hift **A**ssay

GST **G**lutathione **S**-**T**ransferase

HDAC **H**istone **Deac**etylase

HIC-1 **H**ypermethylated **I**n **C**ancer **1** Protein

JMD **J**uxta**m**embrane **D**omain

KBS **K**aiso **B**inding **S**ite (TCCTGCNA)

MeCP2 **M**ethyl **C**pG Binding **P**rotein **2**

NuRD **Nu**cleosome **R**emodeling histone **D**eacetylase complex

PGDF **P**latelet-**D**erived **G**rowth **F**actor

PLZF **P**romyelocytic **L**eukemia **Z**inc **F**inger Protein

PCR Polymerase Chain Reaction

POZ POZ domain of Kaiso

POZ-ZF Zinc Finger subclass of BTB/POZ Family

RLU **R**elative **L**ight **U**nits

RT **R**oom **T**emperature

SDS-PAGE **S**odium **D**odecyl **S**ulfate **P**oly **A**crylamide **G**el **E**lectrophoresis

TCF/Lef1 **T** **C**ell **F**actor/**L**ymphoid **E**nhancing **F**actor **1**

TSA **T**richo**s**tatin **A**

ZF5 **Z**inc **F**inger **5** Protein

INTRODUCTION

1.1 CADHERINS-CATENINS

1.1.1 CADHERIN-CATENIN MEDIATED CELL ADHESION

Intercellular adhesion is a complex phenomenon that occurs through a variety of mechanisms. One of these mechanisms depends on a family of transmembrane glycoproteins known as cadherins. The cadherins are a large class of proteins that includes several "classical" cadherins such as E, N and P-cadherin. Adhesion occurs via intercellular Ca^{2+}-dependent homophilic cadherin-cadherin dimerization (90). These cadherins possess a cytoplasmic domain that complexes with intracellular cofactors known as catenins (90). The prototypical cadherin, E-cadherin, interacts with various catenins: β-catenin, γ-catenin and $p120^{ctn}$ through its intracellular domain (67, 68, 75, 81). Catenins can be subdivided into Armadillo catenins ($p120^{ctn}$, β- and γ-catenin) and non-Armadillo catenins (α-catenin). β- and γ-catenin form mutually exclusive complexes with the carboxyl terminal catenin-binding domain (CBD) of E-cadherin (41, 73). Interestingly, $p120^{ctn}$ can bind E-cadherin simultaneously with either β- or γ-catenin indicating that $p120^{ctn}$ binds a different site to β- or γ-catenin (81, 16). In fact, $p120^{ctn}$ binds the juxtamembrane domain (JMD) of E-cadherin and not the CBD (60, 103, 91).

1.1.2 CATENIN FUNCTION

The catenin cofactors regulate cadherin-mediated intercellular adhesion and are involved in various intracellular functions. One primary function is to stabilize the intercellular cadherin-mediated cell-cell contact by anchoring E-cadherin to the actin cytoskeleton. This occurs via an E-cadherin-β-catenin/γ-catenin-α-catenin complex that associates with actin through α-catenin, a protein that is homologous to the actin-binding protein vinculin (40, 56, 68, 72, 84). Armadillo repeat proteins, β-catenin and γ-catenin, bind to the catenin-binding domain (CBD) of E-cadherin through their Arm domain. The Arm repeat is a 42 amino acid motif involved in protein-protein interaction that was originally described for the *Drosophila* protein, Armadillo (77, 83). Interestingly, if the CBD of E-cadherin is deleted or truncated no cell-cell aggregation occurs (53, 67). This aberrant phenotype is attributed to a break in the interaction between E-cadherin and the catenins (75). Corroborating studies on the importance of cadherin-catenin interaction demonstrated that i)cells lacking α-catenin, but expressing normal E-cadherin, have reduced cell-cell adhesion (87) and ii)exogenous expression of α-catenin cDNA can rescue the reduced cell-cell adhesion phenotype (42, 97).

One other major role of catenins is signaling. β-catenin, for example, is also involved in cell signaling through the Wnt-1 cascade, which is important in cancer (57). Using its Arm domain, β-catenin binds to either E-cadherin, the tumor suppressor Adenomatous polyposis coli (APC) or T cell factor/Lymphoid enhancing factor 1 (TCF/Lef-1) (8, 86). Through interactions with these diverse proteins, β-catenin localizes to the plasma membrane (E-cadherin), cytosol

(APC) or nucleus (TCF/Lef-1), respectively (8, 73, 86). Through Wnt-1 signaling, β-catenin eventually accumulates in the nucleus and regulates gene expression as a ternary complex with the transcription factor Lef-1 and DNA (8). APC is a tumor suppressor protein that targets β-catenin for degradation and competes with E-cadherin for β-catenin binding (44, 86, 88). Aberrant functioning of either β-catenin or APC results in the cytosolic and nuclear accumulation of β-catenin and culminates in overexpression of target gene(s), such as *matrilysin* and *cyclin D1*, through Lef-1 activation (57, 66, 85).

The most recently classified catenin p120^{ctn} differs from the prototypical β-catenin in that it does not bind APC or α-catenin and it binds the E-cadherin JMD instead of the CBD (16, 91). The significance of ablating E-cadherin-p120^{ctn} interactions is evident from studies where the juxtamembrane domain (JMD) of E-cadherin is deleted. These studies revealed that the JMD regulates cell adhesion by preventing lateral dimerization (74), strengthening adhesion (91, 103) and it is responsible for the recruitment of cadherins to sites of intercellular contact (4, 71). While these studies do not define a specific function for p120^{ctn} in cell adhesion, they do provide insight for a potential role in cellular aggregation. Further research has demonstrated that ablated or abnormal p120^{ctn} expression in colon (32), prostate (48), bladder (89), gastric (50), breast (26) and skin (107) carcinomas. Although current research is defining a clearer role for p120^{ctn} in tumourigenesis, the exact mechanism remains unclear.

Similar to β-catenin, p120^{ctn} also localizes to the nucleus in some cells (95). This implies a role in cell signaling which is not surprising since p120^{ctn} was first

identified as a substrate for the Src tyrosine kinase (82). Ligand-induced signaling through Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF) or Colony Stimulating Factor-1 (CSF-1) receptors result in the phosphorylation of $p120^{ctn}$ (29). One of the ongoing projects to further understand the role of $p120^{ctn}$ in cell signaling was to identify $p120^{ctn}$-specific binding partners.

1.2 KAISO

1.2.1 IDENTIFICATION AND CHARACTERISTICS OF Kaiso

To further elucidate the role of $p120^{ctn}$, a yeast two-hybrid screen was performed to identify $p120^{ctn}$ specific binding partners. This led to the discovery of a novel transcription factor Kaiso (15). This interaction occurs between the first seven Armadillo repeats of $p120^{ctn}$ and the C-terminal region of Kaiso that encompasses DNA-binding ZF domain (15). Kaiso binds specifically to $p120^{ctn}$ and none of the other proteins involved in cadherin-catenin adhesion, i.e. not β-catenin, α-catenin, E-cadherin or APC (15). Furthermore, this $p120^{ctn}$-Kaiso interaction was observed exclusively in epithelial cells, although $p120^{ctn}$ and Kaiso are expressed in a variety of cell lines and tissues including among others, heart, brain, spleen and liver (15). Kaiso was also observed to localize predominantly to the nucleus within a cell (15).

1.2.2 Kaiso AND THE BTB/POZ FAMILY

Kaiso is a member of the **B**road complex, **T**ramtrak, **B**ric a brac/**Po**x virus and **Z**inc finger (BTB/POZ) family of proteins. The BTB/POZ family is subdivided into two main classes. One class is involved in actin binding while the other class, which possess carboxyl terminal zinc fingers, is involved in DNA binding (reviewed in 2, 5, 6). The importance of BTB/POZ proteins is becoming clearer as more proteins are being discovered and characterized. Currently, these proteins are known to be involved in a broad range of functions including transcriptional regulation, chromosome rearrangement, pattern formation, metamorphosis, and development (7, 21, 28, 31, 36, 59, 65, 93, 98, 100). Kaiso is a 671 amino acid protein with an N-terminal POZ domain and three Kruppel-like C_2H_2 zinc fingers situated at the C-terminus (15). These two domains are the defining components of the BTB/POZ family (2, 6). In addition, Kaiso has two highly acidic regions, of unknown function, located between the POZ domain and the zinc finger domain (Figure 1). The C-terminal zinc fingers of the protein are involved in DNA binding while the highly conserved, hydrophobic POZ domain mediates homo- or hetero-dimerization between POZ-ZF proteins (2). This domain is believed to target POZ-ZF proteins to the nucleus (23) and serves as the transcriptional regulation domain in most family members (45). Consistent with the established role for the POZ domain, Kaiso also homodimerizes and translocates to the nucleus (6, 15).

1.2.2.1 POZ-ZF PROTEINS INVOLVED IN TUMOURIGENESIS

Interestingly, many of the mammalian POZ-ZF family are known to be involved in tumourigenesis. The three most studied mammalian POZ-ZF proteins are Promyelocytic Leukemia Zinc Finger (PLZF), B-cell Lymphoma 6 (BCL-6) and Hypermethylated in Cancer-1 (HIC-1). Aberrant functioning or misexpression of any one of these three proteins results in neoplasmic transformation (10, 33, 34, 51, 96). Through a chromosomal translocation, t(11;17), the DNA binding domain of the Retinoic Acid Receptor (RARα) is fused to the POZ domain of PLZF (12). This dominant negative chimeric transcription factor is responsible for Acute Promyelocytic Leukemia (13). Translocation, point mutations and small deletions in the major translocation cluster of *bcl-6* result in deregulation of expression culminating in lymphomagenesis (63, 104). Finally, *hic*-1 is transcriptionally silenced by hypermethylation at a CpG island. This occurs in a variety of human cancers (96). These examples give a clear indication of the importance of POZ-ZF proteins in tumourigenesis.

1.3 ELUCIDATING KAISO FUNCTION

1.3.1 POZ-ZF PROTEINS REGULATE TRANSCRIPTION

Typically, POZ-ZF proteins are transcription factors that can positively or negatively regulate gene transcription via their POZ domain. PLZF, BCL-6, and HIC-1 are all transcription factors that are known to be transcriptional repressors (19, 22, 43). Kaiso was recently added to this list of transcriptional repressors by two independent studies. One study demonstrated that mouse Kaiso is a

methylation-dependent transcriptional repressor (80) while the other study demonstrated that *Xenopus* Kaiso could also repress transcription in an artificial Gal4 assay (52). More recent data has established that mouse Kaiso can also bind sequence-specific DNA distinct from the previously identified methylated sequence (17). **This then raises the question, how does Kaiso regulate unmethylated DNA?**

1.3.1.1 MECHANISM OF TRANSCRIPTIONAL REPRESSION

PLZF and BCL-6 are transcriptional repressors that are known to recruit the histone deacetylase (HDAC) repression complex through their POZ domain (18, 24, 33, 43, 62). Both proteins directly interact with Class I and Class II Histone Deacetylases (HDAC1, 4, 5 and 7), mSIN3A and NCoR/SMRT (24, 33, 38, 43, 45, 62, 99). Class I HDACs (HDACs1-3, 8) are grouped together based on homology with yeast HDAC Rpd3 while Class II HDACs (HDACs 4-7, 9, 10) have homology with yeast Hda1 (35). The recruitment of the HDAC complex by PLZF and BCL-6 occur through multiple physical interactions between the HDAC members (HDAC1, 4, 5 and 7 and mSIN3A) and various regions on the two POZ-ZF proteins (18, 24, 33, 43, 45, 62, 99). Recently it has been demonstrated that BCL-6 also recruits histone deacetylases through a conserved 17 amino acid region located in the central portion of the protein independent of the POZ domain (106). Due to this interaction with HDAC complex members, PLZF and BCL-6 exhibit sensitivity to the HDAC inhibiting drug, Trichostatin A (TSA) and both fail to repress transcription in its presence (25, 62). A variety of transcription factor classes including nuclear hormone receptors (39, 69), Mad and Mxi (3, 39)

(37, 58), YY1 (101), Rb (9) and methyl CpG binding protein 2 (MeCP2) (47, 70) utilize the Histone Deacetylase (HDAC) complex to repress transcription. **The recruitment of HDAC complex by POZ-ZF proteins and many other transcriptional repressors suggest that Kaiso may also recruit the HDAC macromolecular complex to mediate transcriptional repression.**

However, the studies of Kaiso repressing transcription do not eliminate the possibility that Kaiso may also function as a transcriptional activator. Moreover, some POZ-ZF proteins display both repression and activation activities. This is exemplified by ZF-5. This POZ-ZF protein most similar to Kaiso, functions as both an activator and repressor on target gene promoters. This dual function is attributed to an acidic domain upstream of the amino terminal zinc fingers (49, 78). Since Kaiso also possesses an acidic region upstream of its three zinc fingers (15), it may also activate target genes.

1.3.1.2 METHODOLOGY TO ASSAY TRANSCRIPTIONAL REGULATION

Reporter gene assays have been used extensively to determine the transcriptional activity and mechanism of function for a variety of POZ-ZF proteins. These assays have characterized PLZF, BCL-6 and HIC1 and identified them as transcriptional repressors (19, 24, 62). Reporter gene studies have also mapped activation/repression domains of various proteins (23, 61, 62, 106).

Coimmunoprecipitation/Western blot analysis is an ideal technique to detect protein-protein interactions *in vivo*. Through this technique, interactions between PLZF and BCL-6 with HDAC complex members have been detected (62, 99).

The simplicity and vast amount of information provided by reporter gene assays and coimmunoprecipitation/Western blot analyses make these techniques ideal for characterizing a novel transcription factor such as Kaiso.

1.3.2 FUNDAMENTALS FOR FURTHER KAISO RESEARCH

While studying methylation–dependent gene silencing, Prokhortchouk *et al.* discovered a potential DNA-recognition site for Kaiso. In reporter gene assays, Kaiso was observed to preferentially repress the methylated sequence 'MGMGCCAAMG' (where M represents a methylated cytosine) over an unmethylated form of this sequence. Electromobility Shift Assays (EMSA) confirmed the specificity for this sequence (80).

Independent studies in our lab have revealed that Kaiso binds DNA via its zinc finger region, but also have identified another Kaiso binding site (KBS), TCCTGCNA. This Kaiso specific sequence was first identified through cyclic amplification and selection of targets (CAST) analysis. The specificity for this sequence was later confirmed by EMSA studies using GST-Kaiso-ZF fusion proteins and oligonucleotides derived from the *matrilysin* promoter (17). The KBS sequence has been identified within the promoter of both human and murine *matrilysin* genes implying *matrilysin* as a potential target gene under the regulation of Kaiso. The binding specificity of Kaiso to both human and murine

matrilysin was confirmed by EMSA analysis (17). **These studies have identified a nonmethylation dependent sequence (KBS) that may be regulated by Kaiso in reporter gene assays.**

The following experimental outline will be followed in the course of this project to address each of the objectives. First, coimmunoprecipitation and Western blot analysis will be used to detect an interaction between Kaiso and HDAC complex members. Next, reporter gene assays will be conducted in the presence of Trichostatin A, a known inhibitor of HDAC-mediated repression, to either confirm previously established interactions or identify an interaction with outstanding HDAC proteins. Finally, reporter gene assays will be conducted to determine if the 4xKBS sequence is a *bone fide* physiological target and assess the function of Kaiso on this nonmethylated sequence. A proposed model for Kaiso function is depicted in Appendix 30. It is hypothesized that Kaiso interacts with members of the HDAC complex and represses transcription of the 4xKBS *cis*-element.

HYPOTHESIS

The findings in the current literature led to the hypothesis to be tested in this thesis.

Kaiso is a transcriptional repressor and functions like other POZ-ZF proteins by recruiting the HDAC complex and interact with the various components *in vivo.*

Specific Objectives: 1. Determine if Kaiso interacts with the HDAC complex
2. Determine if Kaiso mediates repression through KBS.

MATERIALS

DNA molecular marker, BenchMARK prestained protein size marker, *Taq* DNA polymerase I, 10x PCR buffer, 50 mM $MgCl_2$, dNTP and Kodak film were all purchased from InVitrogen Life Technologies in Carlsbad, CA, USA. Restriction enzymes were purchased from New England Biolabs Ltd. Beverly, MA, USA while Calf Intestine Phosphatase and T4 DNA ligase were purchased from MBI Fermentas Hanover, MD, USA. DNA purification kits (mini, midi, and maxi) and Superfect transfection reagent were purchased from Qiagen in Valencia, CA, USA. DNA purification kits (mini and midi) and the Dual Luciferase Assay System were purchased from Promega Corporation, Madison, Wisconsin, USA. Bradford Reagent was purchased from Bio-Rad in Mississauga, Ontario while the HDAC1(H51), HDAC2(H54), mSIN3A(K20) antibodies were all purchased from Santa Cruz Biotechnologies Inc in Santa Cruz, California, USA. Rabbit anti-Mouse IgG was purchased from Jackson Laboratories Inc. in West Grove, Pennsylvania, USA and Protein A Sepharose beads were purchased from Pharmacia Biotechnologies in Basking Ridge, New Jersey, USA.

2.1 DNA VECTORS

Mouse Kaiso cDNA was cloned into pcDNA3 (InVitrogen) in the following variations: full length, P*oz*, *ΔPoz* (Del) and ΔPozΔAR (deleted POZ and acidic region). A putative Kaiso responsive reporter vector, 4xKBS-pGL3 control, was created by cloning four Kaiso binding sites (KBS) upstream of the SV40 promoter in the multiple cloning site of a pGL3 control vector (Figure 2).

2.2 CLONING 4xKBS-pGL3

Using a previously synthesized 4xKBS-pGL2 vector (created and provided by Dr. Howard Crawford), the 4xKBS sequence was amplified by PCR. The exact sequence of the 4X element is 5'-CTCAGATCTGCACTAATCCTGCTAACCGCTCAGATCT-3' with the core binding sequence underlined. The 5' primer, AB26015 (5'-ATTC**GGTACC**CGAGCTCTTACGCGTGCTA-3') has a KpnI restriction site (bold) while the 3' primer, AB26016 (5'-ATTC**CCCGGG**GGTTGCTGACTAATTGAGATGC-3') terminated with a SmaI restriction site (bold). The PCR reaction was performed using 20 ng 4xKBS-pGL2 DNA, 0.5 μM of each primer, 0.2 mM dNTP, 1 mM $MgCl_2$, 1xPCR buffer, 29 μl dH_2O and 1 μl Taq DNA polymerase I. The PCR mixture minus enzyme, was heated to 95^{o}C for 5 min then the Taq enzyme was added. A total of 30 cycles were conducted using an Eppendorf Master Cycler Gradient thermocycler. For each cycle, the denaturing temperature was 95 ^{o}C for 1 min, annealing temperature of 55 ^{o}C for 30 sec and extension temperature of 75^{o}C for 45 sec. After the 30 cycles were complete the reaction was held at 4^{o}C overnight. The PCR product was stored at -20^{o}C. After the PCR reaction, the product was first purified using a commercial kit (Qiagen) and electrophoresed on an agarose gel to confirm the 180 bp size. The product was then digested with KpnI (NEB) and SmaI (NEB), sequentially. A total of 15 μl of the cleaned PCR reaction was digested. Using a 8:1 ratio of insert to vector, the 4X KBS element was ligated

with pGL3 vector using T4 ligase (MBI Fermentas) and 1x ligation buffer (MBI Fermentas). The reaction was placed at 16^{O}C overnight in a water bath. The product was then verified via automated sequencing by the MOBIX Central Facility (Hamilton, ONT.) using the primer AB27010 (5'-GCAGGTGCCAGAACATTTCT-3')

(Figure 2).

METHODS

3.1 CELL CULTURE

HCT116, MCF-7, HCA-7, DLD-1, HT29, SW480, Rat-1, NIH3T3, MDCK and 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) supplemented with 10% Fetal Bovine Serum (Gibco BRL), 1% penicillin/streptomycin (Gibco BRL) and 0.4% fungizone (Gibco BRL) and grown at 37^{O}C with 5% CO_2.

3.2 TRANSFORMATIONS AND DNA PURIFICATION

All transformations were conducted using chemically competent DH5α *Escherichia coli* cells and a protocol that is a derivation of the protocol outlined in Current Protocols. First, 100 ng of DNA was added to freshly thawed cells. The cells were then incubated on ice for 30 min, heat-shocked at 37^{O}C for 20 seconds and returned on ice for 2 min. Afterwards, 1 ml of Luria broth was added to the cells which were then incubated at 37^{O}C while shaking for 1 hour. The cells were then centrifuged for 10-20 sec at 14,4000g/RT and resuspended in 100 µl of Luria broth media. Using both 80 µl and 20 µl the resuspended cells were then plated onto Luria broth agar plates containing 0.1mg/ml Ampicillin. The plates were incubated for 12-15 hours at 37^{O}C. After this time, a single colony was selected and inoculated in 5 ml of Luria broth/Ampicillin media for 15 hours at 37^{O}C. The DNA was then purified using a DNA purification kit (Qiagen or Promega) as per manufacturer's protocol. The purified DNA was then

characterized via restriction enzyme digests and agarose gel electrophoresis to confirm expected band sizes. Once the constructs were confirmed as correct, 2 ml of the original 5 ml culture was inoculated in 50 ml Luria Borth/Ampicillin media for 15 hours at 37^{O}C. The DNA was then purified using a commercial kit (Promega or Qiagen) following the manufacturer's protocol.

3.3 TRANSIENT TRANSFECTIONS

Transfections were conducted on either 6 well or 24 well tissue culture plates (Falcon) using 9 μg or 3 µg, respectively, of Superfect Reagent (Qiagen). A total of 1 μg of DNA per well was used for both 6-well and 24 well plates. Cells were seeded at 2.0-8.0 x10^{4} cells per well one day prior to transfection. The DNA and Superfect were incubated in 100 μL of serum free media for 10 min. The cells were washed twice with 2 ml of cold Phosphate Buffer Saline (PBS). An additional 1.9 ml of serum free media was then added to the DNA-Superfect mixture. A 2 ml final volume of this mixture was then incubated on the cells for 2 hours at 37^{O}C and 5% CO_2.

The following amounts of DNA were transfected for the 4xKBS-pGL3 reporter assays:

Reporter [pGL3 control with an SV40 promoter and enhancer (Promega), pGL3 basic lacking a promoter (Promega) or 4xKBS-pGL3]: 800 ng.

Effector [pcDNA3, pcDNA3-*kaiso*, pcDNA3-*poz*, or pcDNA3-*del*]: 200 ng.

3.4 LUCIFERASE ASSAY

Reporter gene assays were conducted based on the principals outlined by Alam *et al.* (1). Luciferase expression was assayed 24 hours post transfection using a Dual Luciferase Assay System (Promega), as follows unless otherwise stated. The cells were washed with 2 ml cold PBS and lysed with 350 μL of Passive Lysis Buffer (Promega) for 15 min while shaking. A 20 μL volume was then assayed for Luciferase expression (relative light units, RLU) with either a LUMAT LB9501, Berthold or Luminoskan, Ascent luminometer.

3.5 PROTEIN QUANTIFICATION

Protein quantification was determined via Bradford Analysis. Using a 0-10 mg/ml range of Bovine Serum Albumin protein as a standard, 20 μL of whole cell lysate was diluted in 1 ml of 1x Bradford Reagent (Bio-Rad Braford Reagent) and the absorbance at 595 nm measured on a spectrophotometer (Eppendorf Biophotometer).

3.6 LUCIFERASE ACTIVITY COMPUTATION

The RLU obtained for the luminometer was then divided by the total protein concentration from a 20 μL aliquot. A mean and standard deviation value was calculated for the triplicate data. The basal level was set to 1 and fold activation/repression was assessed for each experiment. The positive and negative controls were not represented in the displayed data. The Luciferase

expression experiments were conducted simultaneously in 2 or 3 independent wells. The data presented represents a minimum of 3-10 independent trials.

3.7 IMMUNOPRECIPITATION

Coimmunoprecipitation assays (54) were conducted on indicated cell lines that were cultured to a high confluency (75%-100%) on 100 mm dishes (Falcon) following the general principals outlined by Klenova *et al.* (54). Cells were lysed using 1 ml of 1%, Nonidet P40 10 mM pH 7.4 , buffer containing 1 mM Sodium Orthovanadate (Sigma), 1 mM Phenylmethylsulfonylfluoride (Sigma), 10 μg/ml Leupeptin, 2 mM EDTA (pH 8) and 10 μg/ml Aprotinin (Sigma) for 5 min as outlined by Daniel *et al.* (15). Following this time, cells were actively lysed by scraping and passage through a 26-gauge needle. The cell lysates were then vortexed at maximum speed for 2 min. The debris was then precipitated in a microcentrifuge at 14,000 g for 5 min at 4^{O}C. The supernatant was pooled, vortexed and transfered to a clean microfuge tube. As outlined by Daniel *et al.* (15) a total of 4 μg of specified antibody was added per tube of lysate and incubated end-over-end for 1 hour at 4^{O}C. For monoclonal antibodies (6F anti-Kaiso, 12CA5 anti-Hemaglutinin or 15D2 anti- p120^{ctn}), a total of 30 μl bridge (20 μl Protein A Sepharose beads (Amersham-Pharmacia) and 10 μl rabbit anti-mouse IgG (Jackson Laboratories)) was added per 1 ml whole cell lysates and rotated end-over-end at 4^{O}C for an additional hour. Whereas 30 μl of Protein A Sepharose was added to polyclonal (H11 anti-HDAC1, H54 anti-HDAC2 and K51 anti-mSIN3A) antibody precipitates. Whole cell lysates were then centrifuged for

30 seconds at 14,000g at 4 ^{O}C to pellet the beads and bound protein, then washed four times using cell lysis buffer and finally resuspended in 50 μl of 2x Laemmli Sample Buffer.

3.8 WESTERN ANALYSIS

The immunoprecipitated samples were then boiled for 3 min followed by centrifugation at 14,000 g for 2 min at RT. The samples were then subjected to SDS-PAGE as discussed by Brunette (11) for 3 hour and 45 min at a constant 35 mA using either a 7% or 10% polyacrylamide gel (11, 92). As outlined by Towbin *et al.* (92) the proteins were then transferred to a nitrocellulose membrane soaked in a 25 mM Tris, 192 mM Glycine, 2.5 mM SDS and 0.2 v/v methanol transfer buffer using a semi-dry apparatus for 2 hours. The membranes were then blotted overnight at 4^{O}C with appropriate dilution of 1^{O} antibody in 10 ml of a 3% TBS-skim milk solution. The membranes were washed 5 times in TBS for 7 min and blotted with an appropriate dilution of 2^{O} antibody in a 3% TBS-skim milk solution for 2 hours at room temperature. The membranes were then washed 5x 5 min with TBS and developed by enhanced chemilluminescence (Amersham) on film (X-Omat Blue XB-1, Kodak) as explained by Towbin *et al.* (92).

RESULTS

4.1 KAISO DOES NOT INTERACT WITH THE HDAC COMPLEX IN TRANSFORMED EPITHELIAL CELL LINES

To elucidate the mechanism of action of Kaiso as a putative transcription factor, coimmunoprecipitation/Western blot analyses were used to determine if Kaiso, like PLZF and BCL-6 POZ-ZF proteins, interacted with certain components of the HDAC complex. To address this objective, the following cell lines were chosen and assayed by coimmunoprecipitation and Western blot analysis: MCF-7 (Human breast adenocarcinoma), HCT116 (Human colorectal carcinoma), DLD-1 (Human colorectal adenocarcinoma), HT29 (Human colorectal adenocarcinoma), HCA7 (Human colon carcinoma), SW480 (Human colorectal carcinoma), Rat-1 (Rat fibroblast) and NIH3T3 (Mouse fibroblast).

In these coimmunoprecipitation and Western blot experiments, mSIN3A served as a positive control for both anti-HDAC1 and HDAC2 Western blots. This control also allowed for an assessment of a Kaiso-mSIN3A interaction with HDAC1 or HDAC2 serving as the positive control for an anti-mSIN3A blot. $p120^{ctn}$, the binding partner of Kaiso, served as a positive control in the anti-Kaiso Western blots. In all these assays, the anti-Hemaglutinnin antibody, 12CA5, served as a negative control. These studies were repeated in Rat and Mouse fibroblast cells, Rat-1 and NIH3T3, even though the Kaiso-$p120^{ctn}$ interaction has only been observed in epithelial cells. Unfortunately, a positive control for Kaiso could not be utilized in these two cell lines.

4.1.1 NO KAISO-HDAC1 INTERACTION IN TRANSFORMED EPITHELIAL CELLS

The first interaction tested was between Kaiso and HDAC1 in all six transformed epithelial cell lines. Figure 3 depicts an anti-HDAC1 Western blot using six different epithelial cell lines, including: HCA-7, SW480, DLD-1, HT29, HCT116 and MCF-7. It is evident that HDAC1 was immunoprecipitated only by anti-HDAC1 antibody (lane 3) and the positive control anti-mSIN3A (lane 4). All other antibodies, anti-Kaiso (lane 1), anti- $p120^{ctn}$ (lane 2) and anti-12CA5 (lane 5), failed to coimmunoprecipitate a 60 kDa protein corresponding to HDAC1. To confirm this finding the reciprocal experiment was performed and samples Western blotted for Kaiso. As seen in Figure 4, Kaiso was only coimmunoprecipitated by $p120^{ctn}$ but not HDAC1 or mSIN3A. These two studies strongly suggest that there is no interaction between Kaiso and HDAC1.

4.1.2 NO KAISO-HDAC2 INTERACTION IN TRANSFORMED EPITHELIAL CELLS

The next HDAC component assayed for an interaction with Kaiso was HDAC2. To test whether Kaiso interacted with HDAC2, I repeated the coimmunoprecipitation and Western blot experiments using HDAC2 specific antibodies. Figure 5 demonstrates that only mSIN3A (lane 4) immunoprecipitates HDAC2. HDAC2 was not coimmunoprecipitated by Kaiso (lane 1), $p120^{ctn}$ (lane 2) or the control antibody (lane 5). In the reciprocal experiment to test for Kaiso coprecipitating HDAC2, Kaiso was only precipitated by $p120^{ctn}$. HDAC2, mSIN3A and 12CA5 all failed to coimmunoprecipitate Kaiso.

Therefore, based on the results these two studies, Kaiso and HDAC2 do not coimmunoprecipitate from any of the six cell lines analyzed.

4.1.3 NO KAISO-mSIN3A INTERACTION IN TRANSFORMED EPITHELIAL CELLS

Although a direct interaction between Kaiso and HDAC1 or HDAC2 was not observed, recruitment of the HDAC complex could be mediated via an interaction with the corepressor mSIN3A. However, as seen in Figure 4 lane 4 and Figure 7 lane 1, Kaiso and mSIN3A do not coprecipitate each other. Therefore, in these six cell lines, Kaiso and mSIN3A fail to coimmunoprecipitate.

4.2 KAISO DOES NOT INTERACT WITH THE HDAC COMPLEX IN NONTRANSFORMED FIBROBLAST CELL LINES

4.2.1 NO KAISO-HDAC1 INTERACTION IN NONTRANSFORMED FIBROBLAST CELLS

To eliminate the possibility of a tumorigenic phenotype as a factor on a Kaiso-HDAC1 interaction, nontransformed, mouse and rat fibroblast cells were also assayed. The results of these experiments are presented in Figure 8A and 8C. It is clear that the Kaiso immunoprecipitation in lane 1 (8A) failed to coprecipitate HDAC1 in either cell line tested. The reciprocal blot of this is presented in 8C. This figure supports the previous finding in that the HDAC1 immunoprecipitate (lane 3) failed to coimmunoprecipitate Kaiso. These two studies are consistent with those in the transformed cell lines in that both Kaiso and HDAC1 unsuccessfully coimmunoprecipitate.

4.2.2 NO KAISO-HDAC2 INTERACTION IN NONTRANSFORMED FIBROBLAST CELLS

A Kaiso-HDAC2 interaction was also assessed in both NIH3T3 and Rat-1 cell lines to eliminate any effects caused by transformation. The results from these immunoprecipitation and Western blot analyses are presented in Figure 8B which was an anti-HDAC2 Western blot and Figure 8C which was a Western blot probed for Kaiso. It is evident from these two figures that only mSIN3A could coprecipitate HDAC2 and only $p120^{ctn}$ could coprecipitate Kaiso. These studies demonstrate that in Rat-1 and NIH3T3 cells, like the transformed epithelial cell lines, Kaiso and HDAC2 do not coimmunoprecipitate.

4.2.3 NO KAISO-mSIN3A INTERACTION IN NONTRANSFORMED FIBROBLAST CELLS

An interaction between Kaiso and mSIN3A was also assayed in the same nontransformed fibroblast Rat-1 and NIH3T3 cell lines. The same negative results between Kaiso-HDAC1 and Kaiso-HDAC2 were obtained for an interaction between Kaiso and mSIN3A in both these cell lines. In Figures 8C Kaiso was not detected in the anti-mSIN3A (lane 5) immunoprecipitation. This absence of interaction is corroborated by a reciprocal study presented in Figure 9. There was no mSIN3A detected in lane 1 where Kaiso was the target for an immunoprecipitation. Taken together neither Kaiso nor mSIN3A coimmunoprecipitate each other under the conditions used in this study.

In summary, based on these results, Kaiso does not coimmunoprecipitate HDAC1, HDAC2 or mSIN3A in any of the eight cell lines studied. The data is

consistent in all cell lines studied but the possibility of a cell line specific effect remains for untested cell lines.

4.3 ANALYSIS OF THE TRANSCRIPTIONAL ACTIVITY OF KAISO

4.3.1 OPTIMIZING EXPERIMENTAL CONDITIONS

4.3.1.1 OPTIMAL CELL LINE FOR EXOGENOUS KAISO EXPRESSION

Before Kaiso could be assayed as a transcription factor, an appropriate cell line was needed for reporter gene analysis. To assess this, three cell lines, MCF-7, HCT116 and TIB73 (mouse hepatocytes), were transiently transfected with a truncated form of Kaiso, Δ*poz*Δ*zf* (MW ~60 kDa) and assayed for expression (Figure 10). These cell lines were chosen simply based on availability. The first three lanes depict each cell line that was transfected with the exogenous DNA, while the last three lanes are untransfected controls. Based on qualitative assessment of the results, HCT116 cells gave the most prominent signal for the ΔPOZΔZF exogenous protein and therefore were selected for future transfection assays. The endogenous 111kDa Kaiso is visible in this same figure.

4.3.1.2 OPTIMAL CELL LINE FOR REPORTER GENE EXPRESSION

In order to confirm HCT116 as the optimal cell line for reporter gene analysis, the same three cell lines (HCT116, MCF-7 and TIB73) were assayed for reporter gene expression. Figure 11 is a graphical representation of reporter gene expression in each of the three cell lines. Since the highest reporter gene

expression was observed in HCT116 (black bars), this cell line was chosen for subsequent studies.

4.3.1.3 OPTIMAL POST TRANSFECTION ASSAY TIME

Cochran and Stiles in 1983 and Iyer *et al.* in 1999 both demonstrated the temporal dependence of gene expression (14, 46). Their data clearly indicates that not all genes are expressed at the same time relative to growth factor stimulation. Therefore, some genes are expressed immediately, and are considered primary response genes, while other genes are expressed later, and are considered secondary response genes. This concept of temporal dependent gene expression became one of the crucial factors to consider in deciding optimal reporter expression conditions.

Before conducting a reporter gene assay for the effects of Kaiso on four copies of the Kaiso Binding sequence (KBS) upstream of an SV40 promoter-enhancer in pGL3 (4xKBS-pGL3), optimal transfection conditions were assessed. Figure 12-14 depicts the results of three separate assays where post-transfection time varied. In Figure 12, reporter gene assays were conducted 8 hours post transfection, while in Figures 13 and 14 the assays were conducted after 24 and 48 hours, respectively. Within each experiment, effector concentration was also titred. Three concentrations of effector (150 ng, 500 ng and 750 ng) were utilized to observe the effect on reporter gene expression. The pcDNA3 backbone vector was used as a control to contrast the effects of either full length Kaiso or the negative control ΔPOZ Kaiso. Since expression levels (y-

axis scale) was the deciding criterion for optimal post transfection time, the highest level of reporter expression occurred 24 hours post-transfection and therefore this time point was used for subsequent studies.

4.3.1.4 OPTIMAL REPORTER DNA CONCENTRATION

Reporter concentration was the final parameter that was titred. Using 150 ng and 500 ng of 4xKBS-pGL3, *luciferase* expression was determined. The results from these experiments are depicted in Figures 15 and 16. A trend was more readily discernable using 500 ng of reporter DNA rather than 150 ng. As a result, 500 ng was used for subsequent assays.

4.3.2 KAISO ACTIVITY IS INSENSITIVE TO TRICHOSTATIN A

To further characterize Kaiso as a transcriptional repressor (52, 80), reporter gene expression was assayed in the presence of Trichostatin A (TSA), a known inhibitor of HDAC-mediated transcriptional repression. Since HDAC recruitment is a common mechanism of repression, it was hypothesized that Kaiso may utilize this mechanism when repressing its target genes. Alleviation of repression by TSA would indicate that repression is occurring through an HDAC-dependent complex. Although I failed to detect an interaction between Kaiso and several components of the HDAC complex, this experiment was conducted to account for the possibility of a Kaiso interaction with other HDAC family proteins thus still exhibiting TSA sensitivity. The results of this study, which utilized the 4xKBS-pGL3 system, are presented in Figure 17. Since there is no variation in reporter

gene expression in the presence or absence of TSA, this inhibitor does not appear to have any effect on reporter gene expression.

4.3.3 KAISO CAN UPREGULATE A REPORTER GENE CONTAINING THE 4xKBS ELEMENT

The second objective of this study was to assay the effect of Kaiso on a novel binding site, the KBS. Since this sequence varied from the published methylation-dependent sequence (80), either repression or activation was possible. Therefore, the 4xKBS-pGL3 system was used where both an activator or a repressor could be detected. Using the optimal conditions previously determined (Section 3.1.1-3.1.4), the effect of Kaiso on 4xKBS-pGL3 was assayed. The results from the study are presented in Appendix 1-29 and summarized in Figures 18-21. For these studies, 800 ng of reporter DNA and 200 ng of effector DNA were used and the experiments conducted 24 hours post transfection. Although these values deviated from the 500 ng (reporter) and 150 ng (effector) established in this data, 800 ng and 200 ng where more suited to bring the final transfection DNA concentration to 1 µg without any additional 'filler' DNA. Also, the values of 800 ng and 200 ng were used for reporter studies of HIC-1 (19).

4.3.3.1 KAISO ACTIVATES EXPRESSION OF THE 4XKBS REPORTER IN HCT116 CELLS

Using HCT116 cells, the effect of Kaiso on a 4XKBS *cis*-element was assayed in eleven independent trials. These trials are presented in Appendices 1-11. These experiments were all conducted under the conditions previously

outlined. The effect of Kaiso is always presented in the 2nd bar of all the figures as fold expression relative to the basal expression, which is standardized to a value of 1 (1st bar). From all these trials a range of 1-5 fold expression was observed for Kaiso, while an unexpected range of 0.5-7 fold was observed for the POZ domain of Kaiso. An average of all the independent trials is presented in Figure 18. From this figure, a mean activation value of 1.7 fold expression was observed for Kaiso and a 1.2 fold expression for the POZ domain.

4.3.3.2 KAISO ACTIVATES EXPRESSION OF THE 4xKBS REPORTER IN HCA-7 CELLS

Since the previous experiment demonstrated the unexpected results of activation the study was repeated in another cell line. In order to account for a cell line specific activation effect of Kaiso on the 4XKBS *cis*-element, other cell lines were used for these same studies. The second cell line of choice was the human colon carcinoma cell line, HCA-7. The results of five independent trials, in this cell line, are presented in Appendices 12-16. A range of 0.9-6 fold is observed for Kaiso while 0.7-2.1 fold was observed for the POZ domain. Interestingly, as observed in Figure 19, pooled results from all five trials present an average activation of 2.1 fold expression for Kaiso and 0.9 fold expression for the POZ domain.

4.3.3.3 KAISO ACTIVATES EXPRESSION OF THE 4xKBS REPORTER IN 293 CELLS

The results obtained from both HCT116 and HCA-7, although consistent, were surprising in that they contradicted the published results of Kaiso repressing

gene expression in reporter gene assays (80). Therefore, 293 cells were another cell line selected for the 4XKBS-pGL3 reporter gene expression studies. All the conditions were the same as the previous, HCT116 and HCA-7, cell lines. Eight trials were conducted in 293 cells and are presented in Appendices 17-24. In these Appendices, Kaiso ranges from 1.3-4 fold expression relative to basal levels. The POZ domain has a different range of 0.4-2 fold expression. The averaged effect of Kaiso and POZ are presented in Figure 20. In this pooled data, Kaiso has a mean activation of 1.6 fold expression while the POZ domain has only a 0.6 fold mean expression.

4.3.3.4 KAISO HAS NO ACTIVITY ON THE 4XKBS-pGL3 REPORTER IN MDCK CELLS

The last cell line selected for these studies were nontransformed Madin-Darby Canine Kidney (MDCK) cells. These cells were chosen to eliminate the possible affects of a transformed phenotype on gene expression. The results representing five independent trials are presented in Appendices 25-29. Although the range for Kaiso in the independent trials is from 0.7-1.5, the averaged result, presented in Figure 21, is only 1.1 fold expression. The same trend is observed for the POZ domain. The independent results present a range of 0.5-1.2, but the mean result is only 1.1 fold expression.

All these results taken together, indicate a minimum of 1.5 fold activation by full length Kaiso in HCT116, HCA-7 and 293 cells but only a 1.1 fold expression in MDCK cells. The POZ domain demonstrates inactivity by ranging from 0.6-1.2 in the pooled results for all the cell lines tested.

FIGURE 1: Schematic Diagram of Kaiso and Kaiso Derivatives. Schematic representation of Kaiso showing the amino terminal POZ domain, the central acidic domains and the carboxy terminal zinc fingers. Below the diagram are four truncated variations of Kaiso used to assess the function of Kaiso. Full length (Kaiso), ΔPOZ (Del), ΔPOZΔAR and POZ.

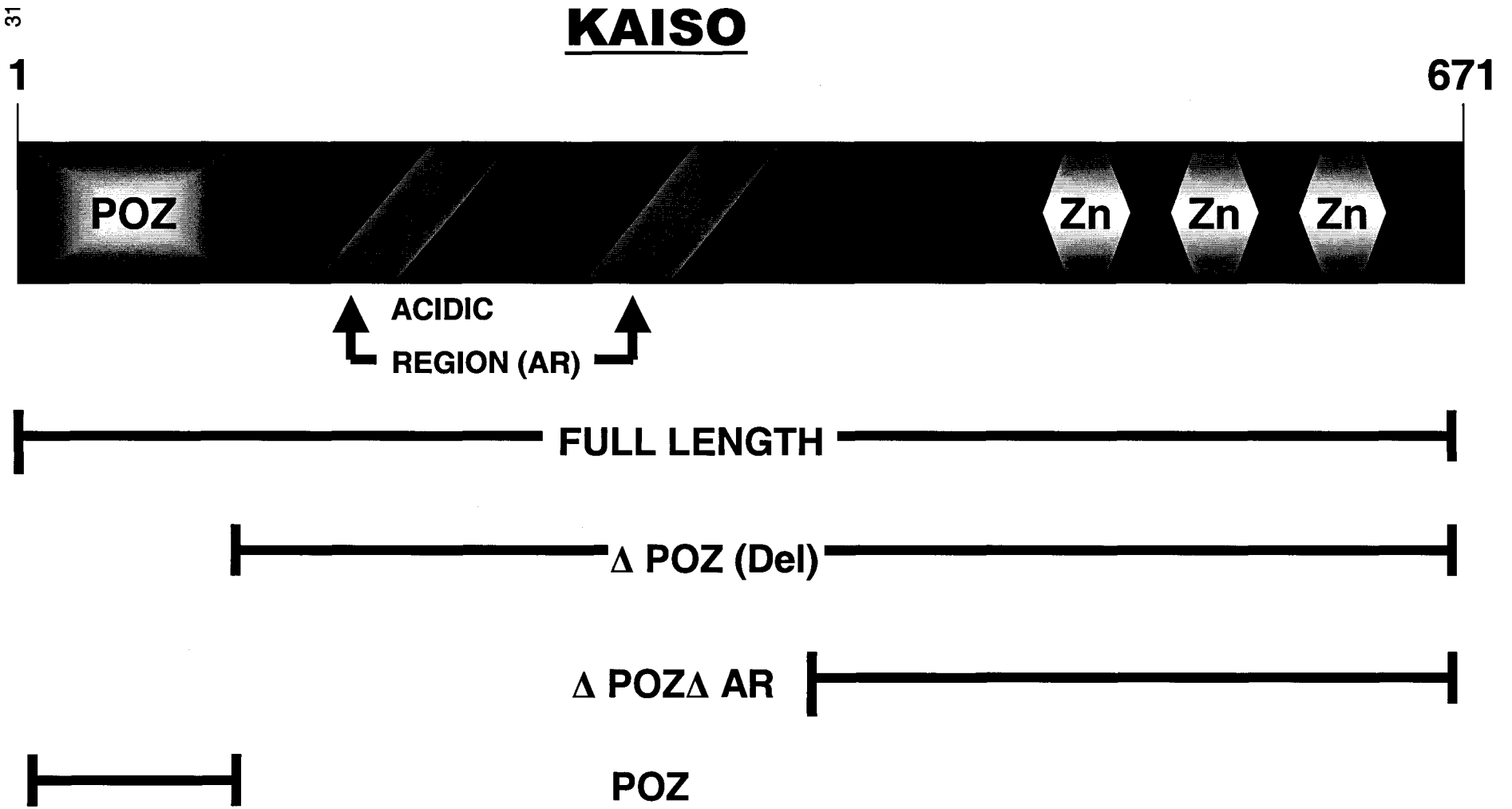

KAISO
1
671
POZ
Zn
Zn
Zn
ACIDIC
REGION (AR)
FULL LENGTH
Δ POZ (Del)
Δ POZΔ AR
POZ

FIGURE 2: Vector Map of 4xKBS-pGL3 Construct. Vector map of the 4xKBS cloned into a pGL3 (Promega) backbone vector. This vector was constructed for reporter gene studies. There are four copies of the KBS cloned upstream of an SV40 early promoter followed by the Firefly *luciferase* reporter gene and an SV40 enhancer.

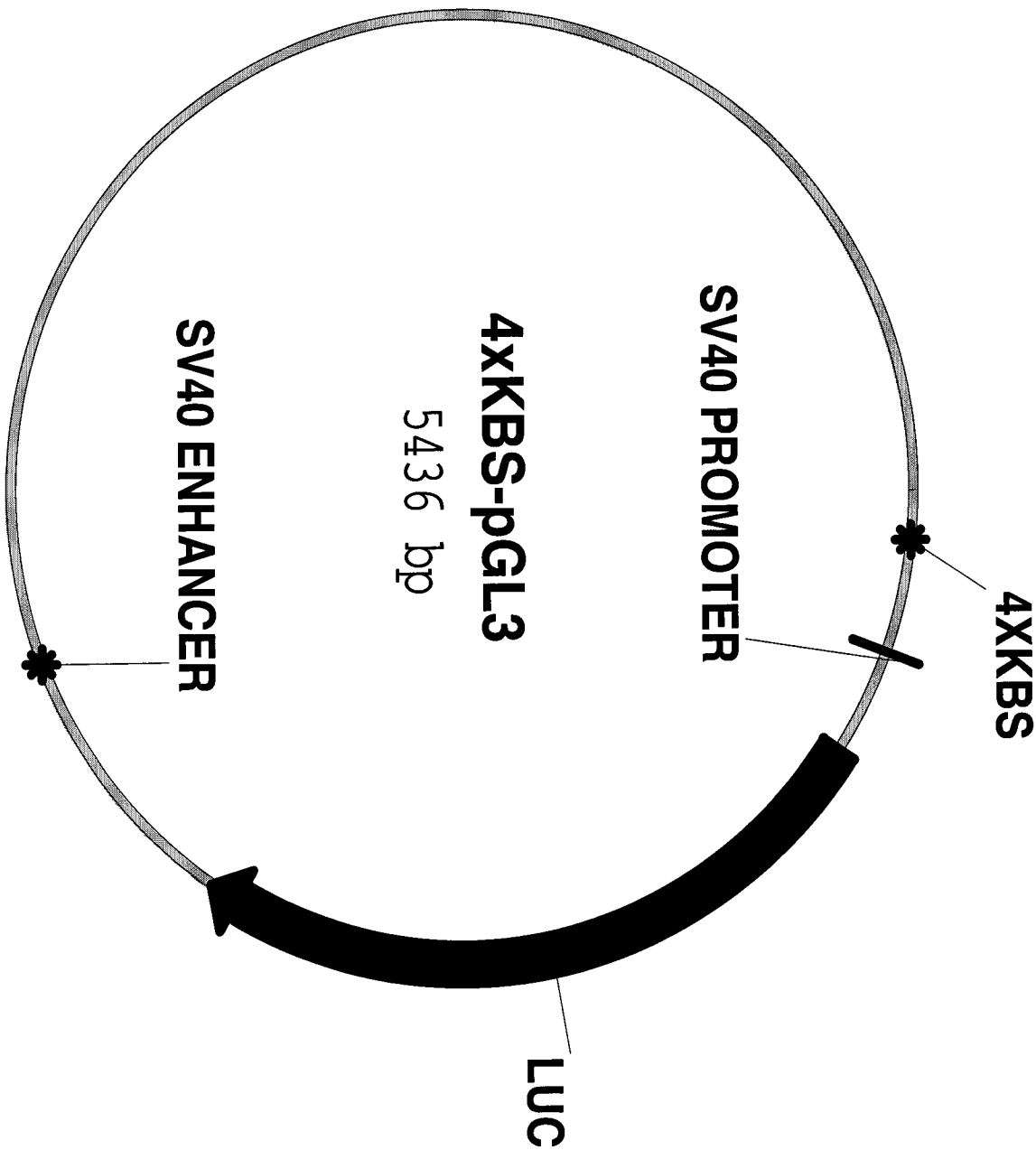

4XKBS
SV40 PROMOTER
4xKBS-pGL3
5436 bp
LUC
SV40 ENHANCER

FIGURE 3: HDAC1 Does Not Interact with Kaiso in Epithelial Cells. Whole cell lysates were immunoprecipitated with antibodies to Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC1 (lane 3), mSIN3A (lane 4) and 12CA5 (lane 5) and subjected to SDS-PAGE and then detection of HDAC1 from indicated cell lines. The target proteins were immunoprecipitated from 100% confluent, 100 mm plates of indicated cell line. HDAC1 protein is only visible in lanes 3 and 4, corresponding to a direct immunoprecipitation (IP) of HDAC1 and coimmunoprecipitation with mSIN3A, respectively. No HDAC1 protein was detected in the Kaiso immunoprecipitation.

ANTI-HDAC 1 WESTERN BLOTS

CELL LINE:

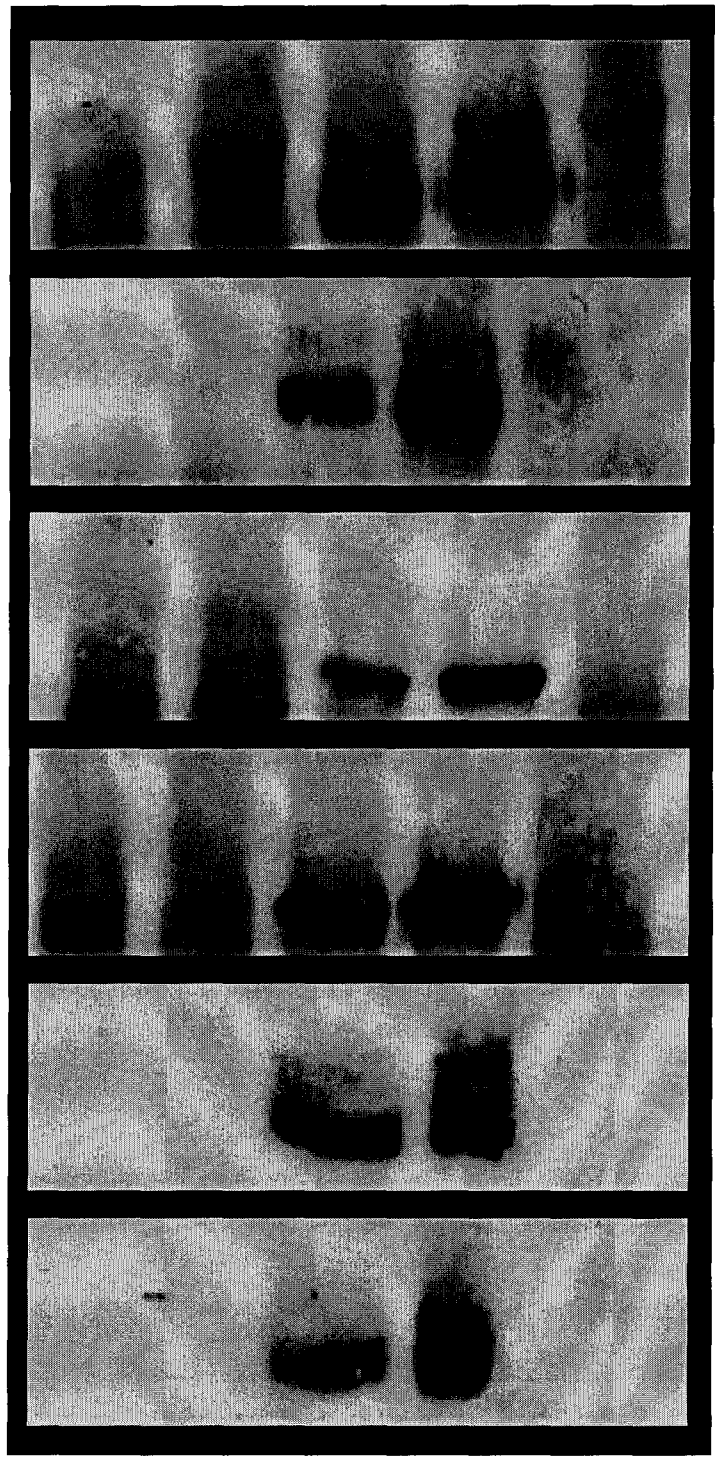


FIGURE 4: Kaiso Does Not Interact with HDAC1 in Epithelial Cells. Whole cell lysates were immunoprecipitated with antibodies to Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC1 (lane 3), mSIN3A (lane 4) and 12CA5 (lane 5) and subjected to SDS-PAGE and then detection of Kaiso from indicated cell lines. The target proteins were immunoprecipitated from 100% confluent, 100 mm plates of indicated cell line. Kaiso protein is only visible in lanes 1 and 2, corresponding to a direct immunoprecipitation (IP) of Kaiso and coimmunoprecipitation with $p120^{ctn}$, respectively. No Kaiso protein was detected in either the HDAC1 or mSIN3A immunoprecipitations. A nonspecific band that was only detected in the HCA-7 cell line is visible at a higher molecular weight in lane 2. This band may be due to cell line specific cross-reactivity of the polyclonal anti-Kaiso antibody.

ANTI-KAISO WESTERN BLOTS

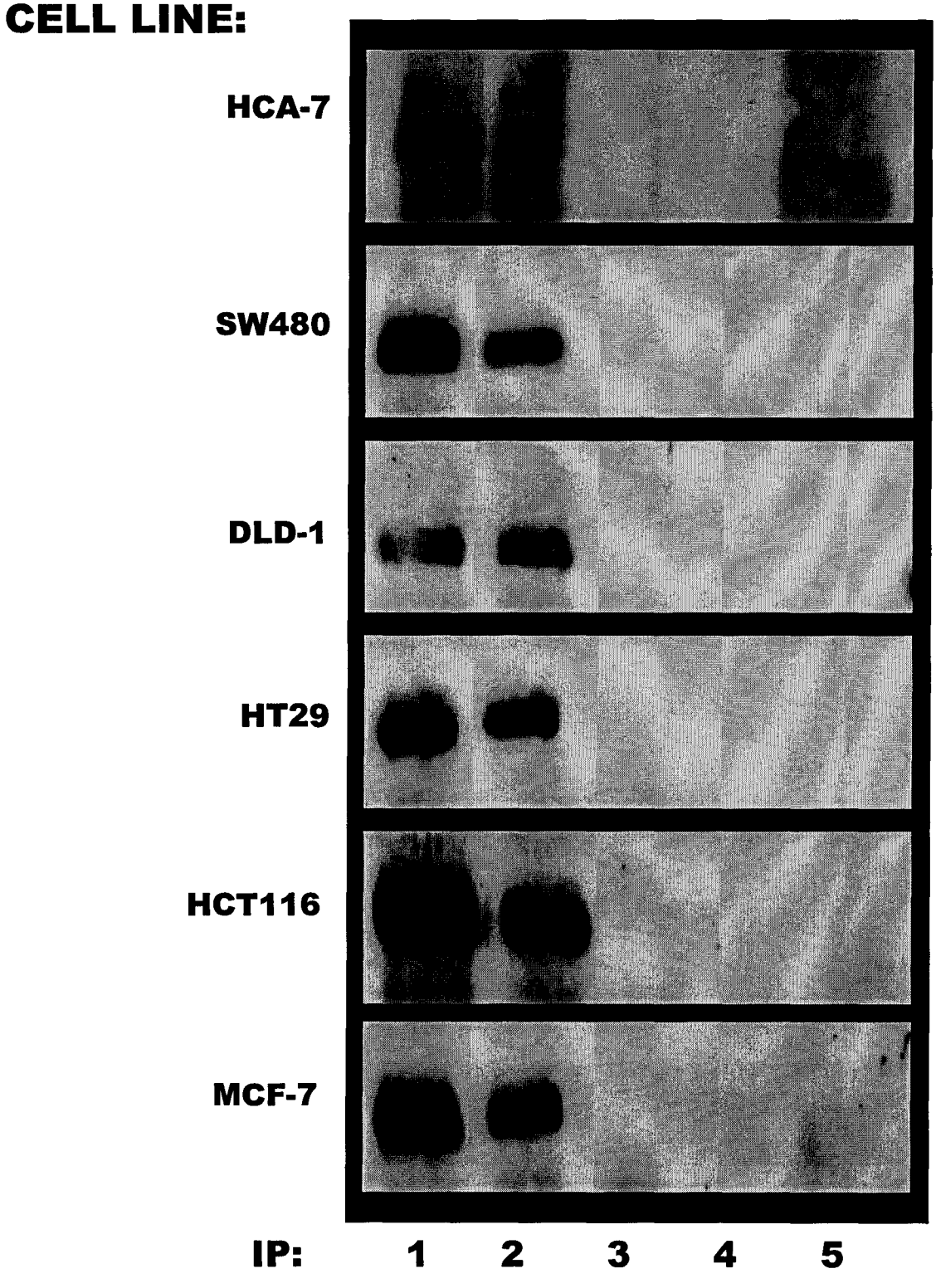


FIGURE 5: HDAC2 Does Not Interact with Kaiso in Epithelial Cells. Whole cell lysates were immunoprecipitated with antibodies to Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC2 (lane 3), mSIN3A (lane 4) and 12CA5 (lane 5) and subjected to SDS-PAGE and then detection of HDAC2 from indicated cell lines. The target proteins were immunoprecipitated from 100% confluent, 100 mm plates of indicated cell line. HDAC2 protein is only visible in lanes 3 and 4, corresponding to a direct immunoprecipitation (IP) of HDAC2 and coimmunoprecipitation with mSIN3A, respectively. No HDAC2 protein was detected in the Kaiso immunoprecipitation.

ANTI-HDAC 2 WESTERN BLOTS

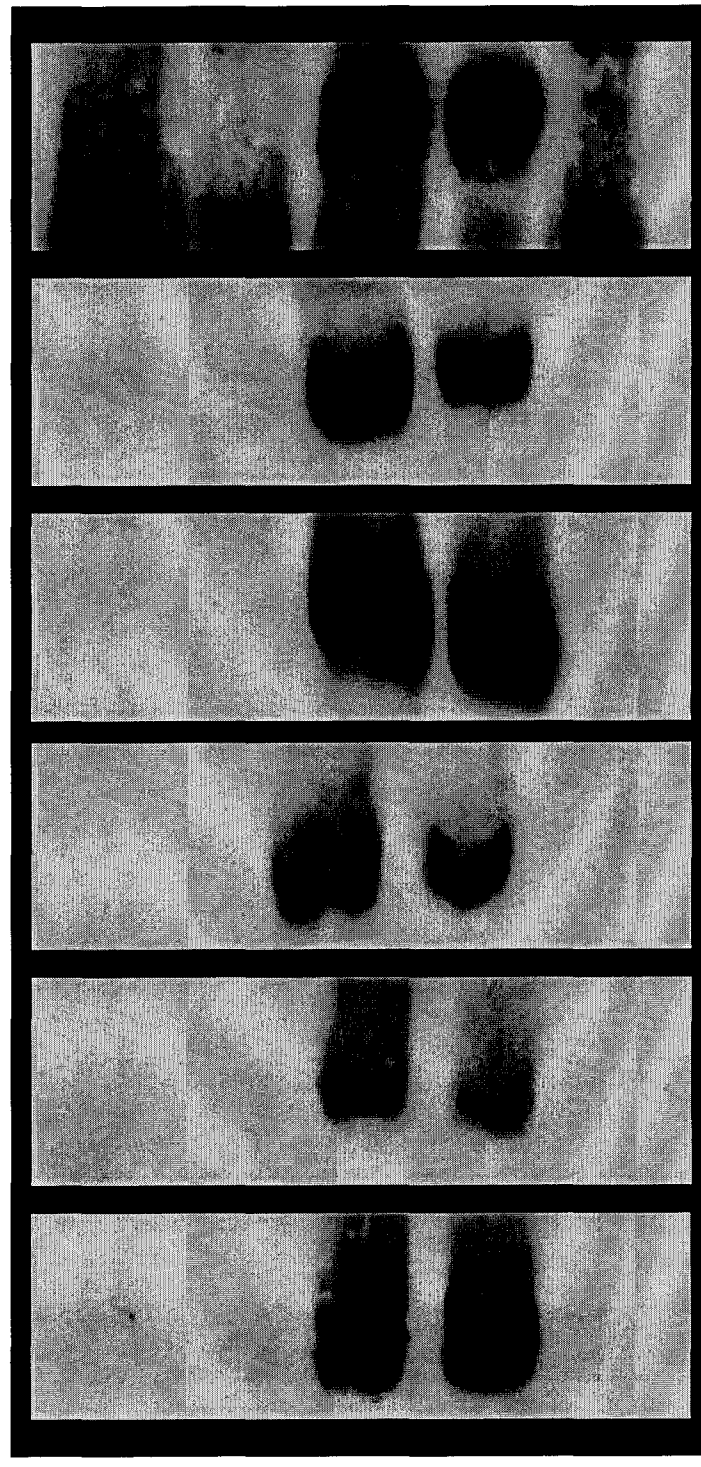


FIGURE 6: Kaiso Does Not Interact with HDAC2 in Epithelial Cells. Whole cell lysates were immunoprecipitated with antibodies to Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC2 (lane 3), mSIN3A (lane 4) and 12CA5 (lane 5) and subjected to SDS-PAGE and then detection of Kaiso from indicated cell lines. The target proteins were immunoprecipitated from 100% confluent, 100 mm plates of indicated cell line. Kaiso protein is only visible in lanes 3 and 4, corresponding to a direct immunoprecipitation (IP) of Kaiso and coimmunoprecipitation with $p120^{ctn}$, respectively. No Kaiso protein was detected in either the HDAC2 or mSIN3A immunoprecipitations.

ANTI-KAISO WESTERN BLOTS

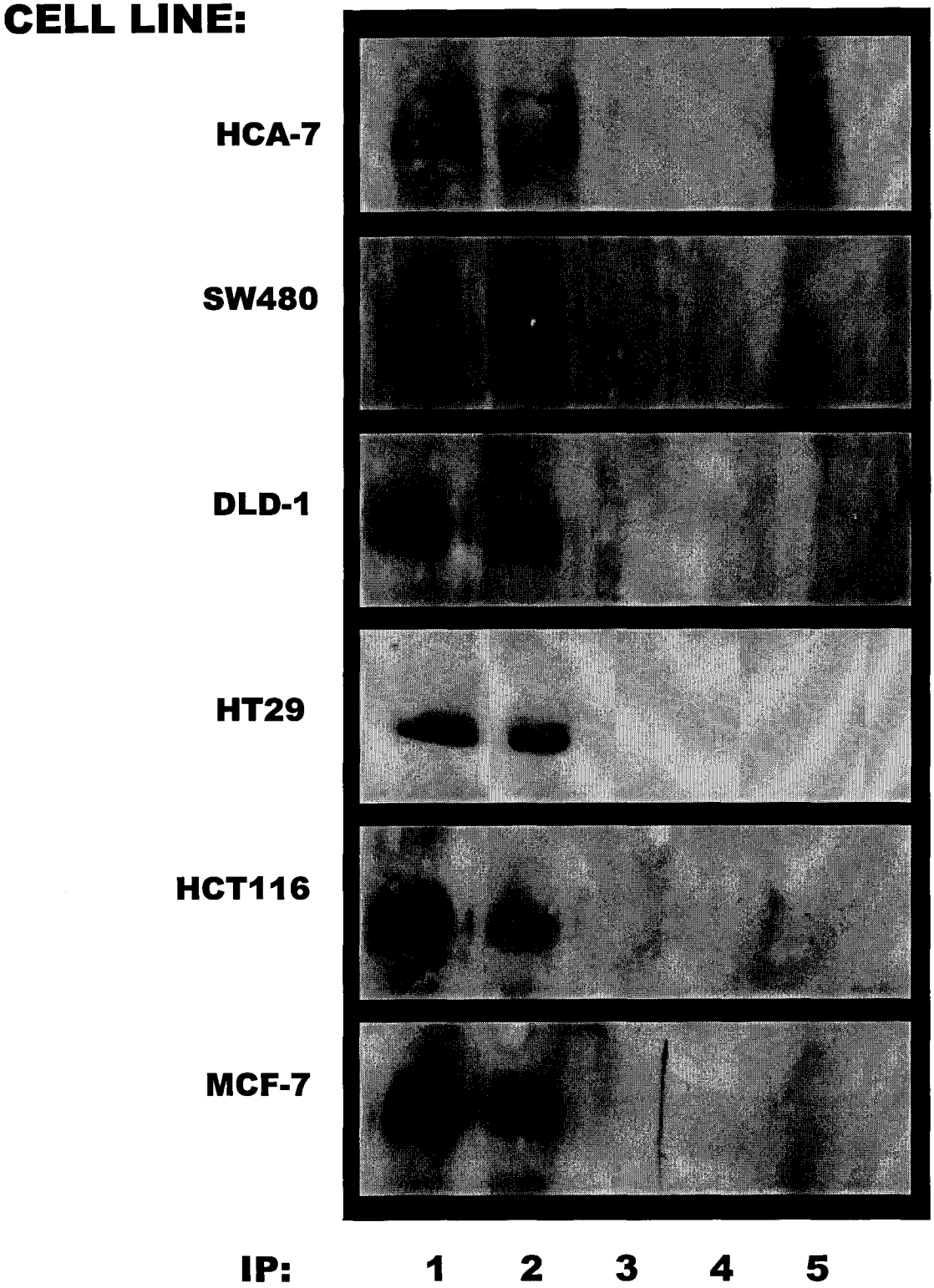


FIGURE 7: mSIN3A Does Not Interact with Kaiso in Epithelial Cells. Immunoprecipitation of Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC1 or HDAC2 (lane 3), mSIN3A (lane 4) and 12CA5 (lane 5) and Western blot detection of mSIN3A from indicated cell lines. These results are from stripped nitrocellulose membranes that were reprobed for mSIN3A. The 4 μg of mSIN3A antibody used in the immunoprecipitation gave a high background and therefore the mSIN3A lane (4) was darker than expected. A nonspecific interaction is detected in all lanes from HCA-7, DLD-1, HT29 and HCT116 cell lysates. mSIN3A protein corresponds to the lower molecular weight band detected in both HDAC and mSIN3A immunoprecipitations, indicated (arrow). SW480 cells were not used in this study as they were not available. mSIN3A protein is only visible in lanes 3 and 4, corresponding to HDAC1/HDAC2 and mSIN3A, respectively. No mSIN3A protein is visible in lane 1 where Kaiso was the target of the immunoprecipitation.

ANTI mSIN3A WESTERN BLOTS

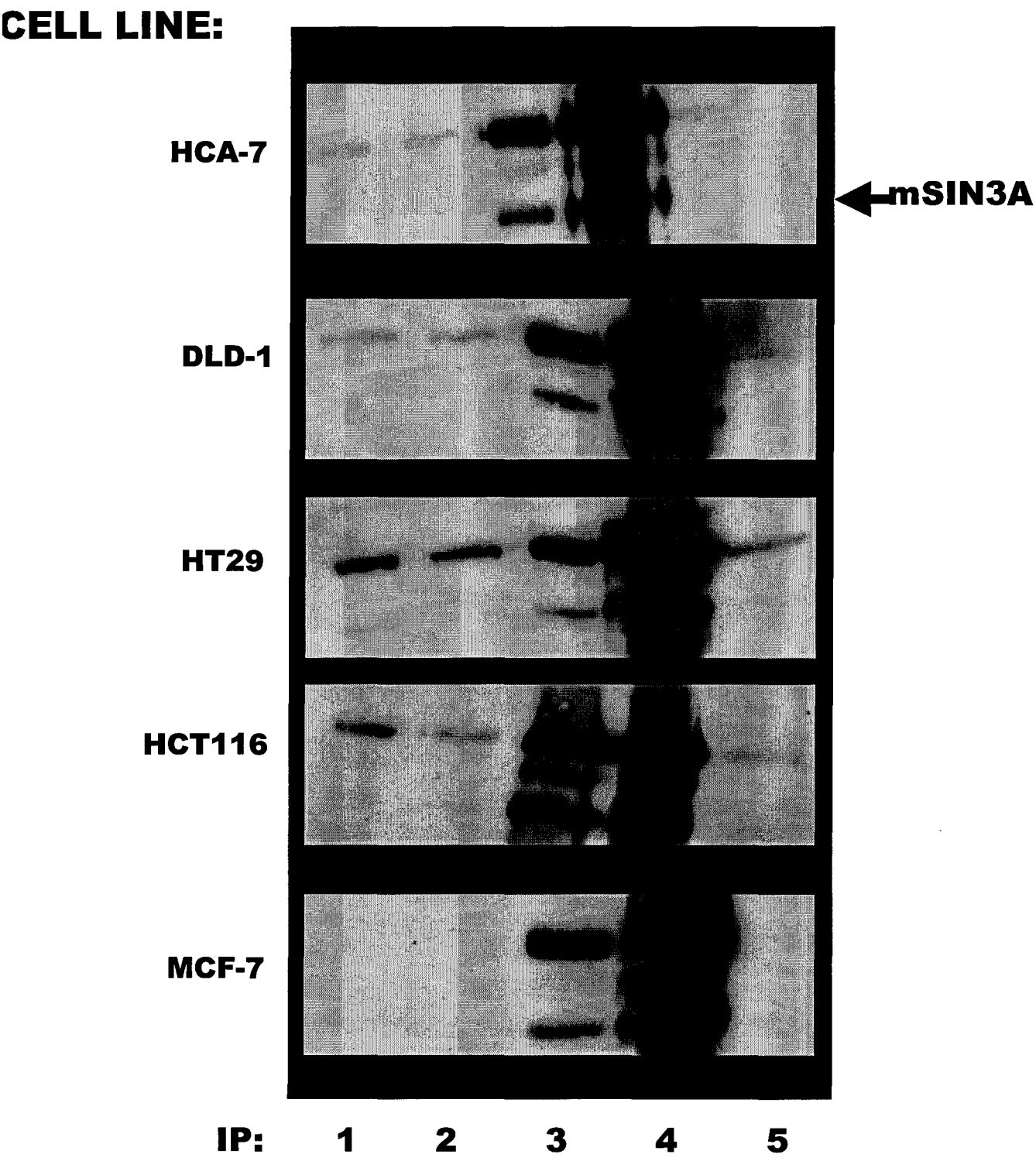
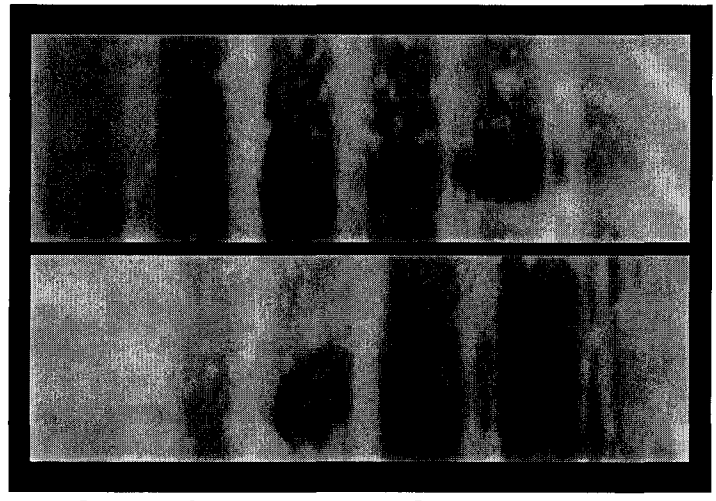


FIGURE 8: Kaiso Does Not Interact with HDAC1 or HDAC2 in Fibroblast Cells. Immunoprecipitation of Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC1 (lane 3), HDAC2 (lane 4), mSIN3A (lane 5) and 12CA5 (lane 6) and Western blot detection of HDAC1 (A), HDAC2 (B) and Kaiso (C) from indicated cell lines. The target proteins were immunoprecipitated from 100% confluent, 100 mm plates of the indicated cell line. The proteins were electrophoresed on 10% (HDAC1 and HDAC2) and 7% (Kaiso) polyacrylamide gels. A) HDAC1 protein is only visible in lanes 3, 4 and 5, corresponding to HDAC1, HDAC2 and mSIN3A, respectively. No HDAC1 protein is visible in lane 1 where Kaiso was the target of the immunoprecipitation. B) HDAC2 protein is only visible in lanes 4 and 5, corresponding to HDAC2 and mSIN3A, respectively. No HDAC2 protein is visible in lane 1 where Kaiso was the target of the immunoprecipitation. C) Kaiso protein is only visible in lanes 1 and 2, corresponding to Kaiso and $p120^{ctn}$, respectively. No Kaiso protein is visible in lanes 3, 4 and 5 where HDAC1, HDAC2 and mSIN3A, respectively, were targets of the immunoprecipitation.

KAISO-HDAC INTERACTION IN FIBROBLASTS

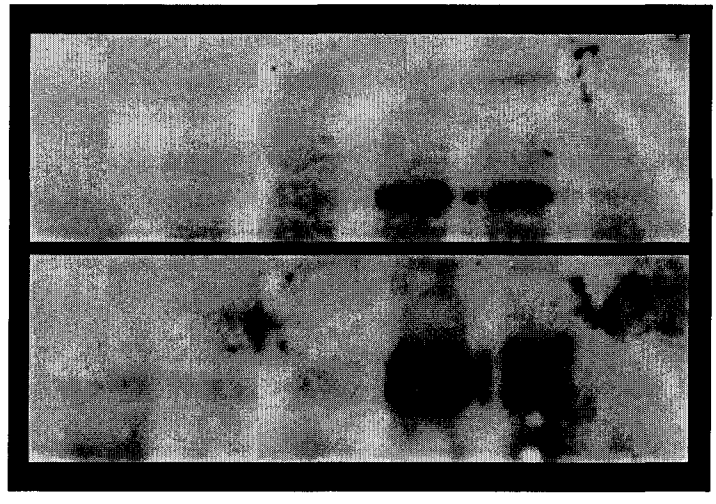
CELL LINE:



ANTI-HDAC 1

A

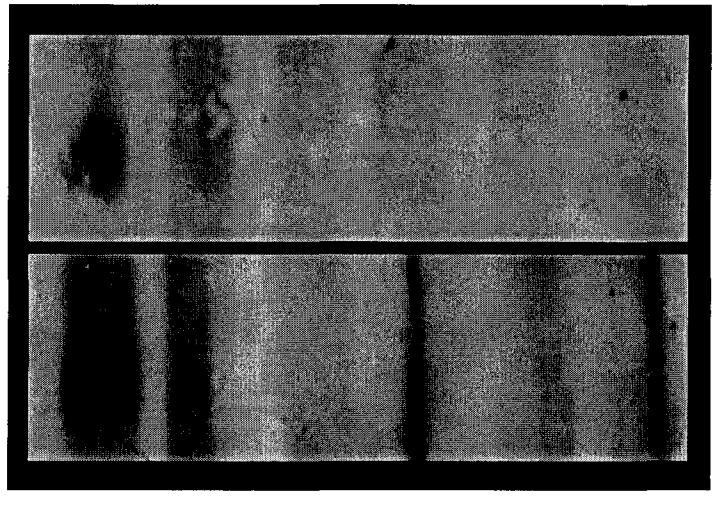
IP: 1 2 3 4 5 6



ANTI-HDAC 2

B

IP: 1 2 3 4 5 6



ANTI-KAISO

C

IP: 1 2 3 4 5 6

FIGURE 9: mSIN3A Does Not Interact with Kaiso in Fibroblast Cells. Whole cell lysates were immunoprecipitated with antibodies to Kaiso (lane 1), $p120^{ctn}$ (lane 2), HDAC1 (lane 3), mSIN3A (lane 4) and 12CA5 (lane 5) and subjected to SDS-PAGE and then detection of mSIN3A from indicated cell lines. The target proteins were immunoprecipitated from 100% confluent, 100 mm plates of the indicated cell line using 4 μg of specified antibody but only 1 μg of anti-mSIN3A antibody. This was to adjust for the high background obtained from 4 μg. The secondary antibody was used at a 1:1000 dilution instead of the usual 1:500. This then reduced the signal obtained from the HDAC1 immunoprecipitation (lane 3). mSIN3A protein is only visible in lanes 3 and 4, corresponding to a coimmunoprecipitation by HDAC1 and a direct precipitation of mSIN3A, respectively. No mSIN3A protein was detected in the Kaiso IP.

KAISO-mSIN3A INTERACTION IN FIBROBLASTS

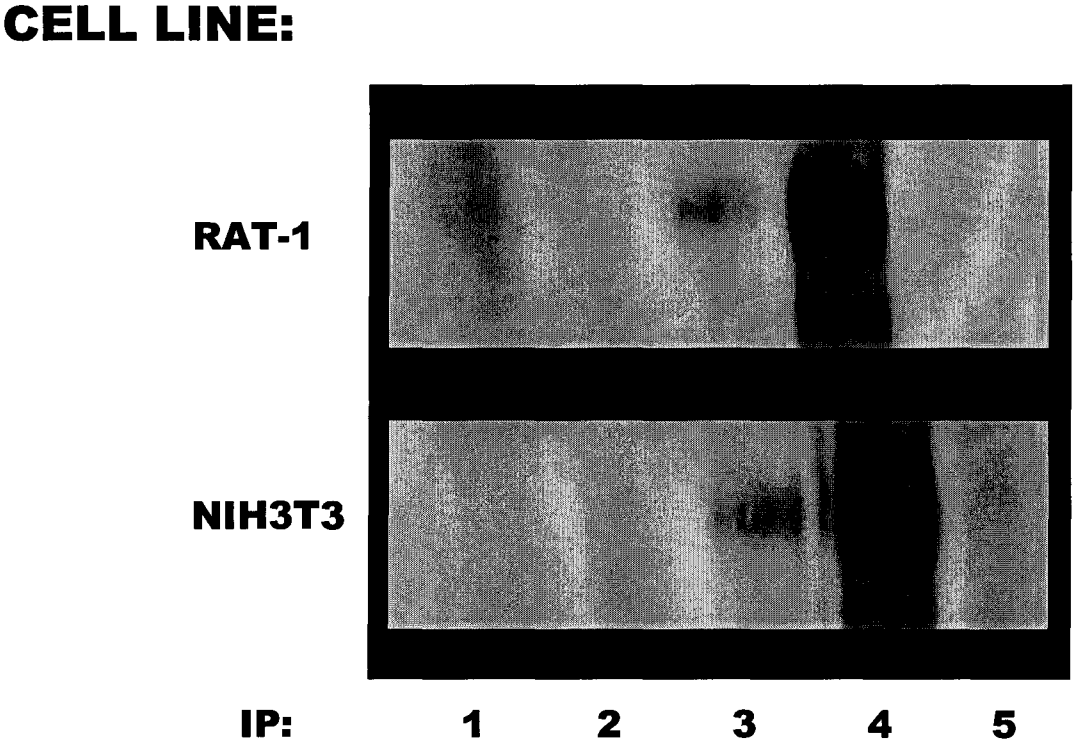


FIGURE 10: Anti-Kaiso Western Blot Detection of Transiently Expressed ΔPOZ-ΔZF. Immunoprecipitation of Kaiso and Western blot detection of Kaiso from ΔPOZ-ΔZF transiently transfected into 40%-60% confluent MCF-7 (lane 1), HCT116 (lane 2) and TIB73 (lane 3) cells. All cell lines were transfected with 10 μg of ΔPOZ-ΔZF DNA on 100mm plates. Lanes 1-3 are transfected while lanes 4-6 are untransfected controls. ΔPOZ ΔZF protein migrates at ~80 kDa indicated (arrow). The top arrow indicates endogenous Kaiso. The immunoprecipitations were conducted 24 hours post-transfection using 4 μg of 6F (anti-Kaiso) antibody. Qualitatively, the signal is most intense for HCT116 cells.

WESTERN BLOT DETECTION OF TRANSIENT EXPRESSION OF Δ POZΔ ZF IN VARIOUS CELL LINES

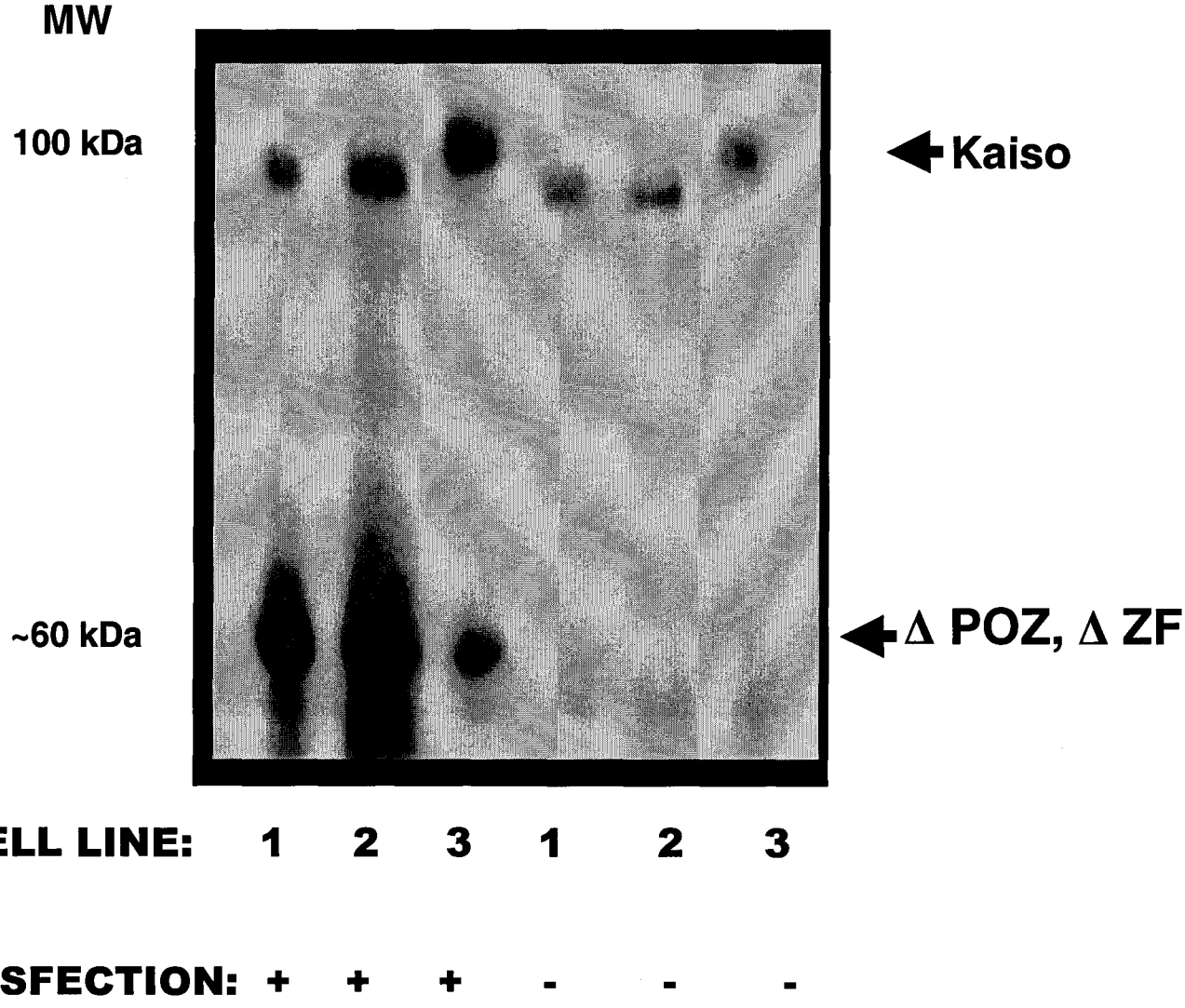


FIGURE 11: 4xKBS-pGL3 Reporter Expression in Three Cell Lines. 40%-60% confluent MCF-7 (grey), TIB73 (white) and HCT116 (black) cells were transiently cotransfected with 750 ng of 4xKBS-pGL3 reporter and 250 ng of various effector proteins. The first set of three bars have empty pcDNA3 as effector protein while the second and third set of three bars have Kaiso and POZ, respectively. The cells were then assayed for relative reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The first set of three bars is of most importance as only endogenous Kaiso is effecting reporter expression.

4xKBS-pGL3 REPORTER EXPRESSION IN VARIOUS CELL LINES

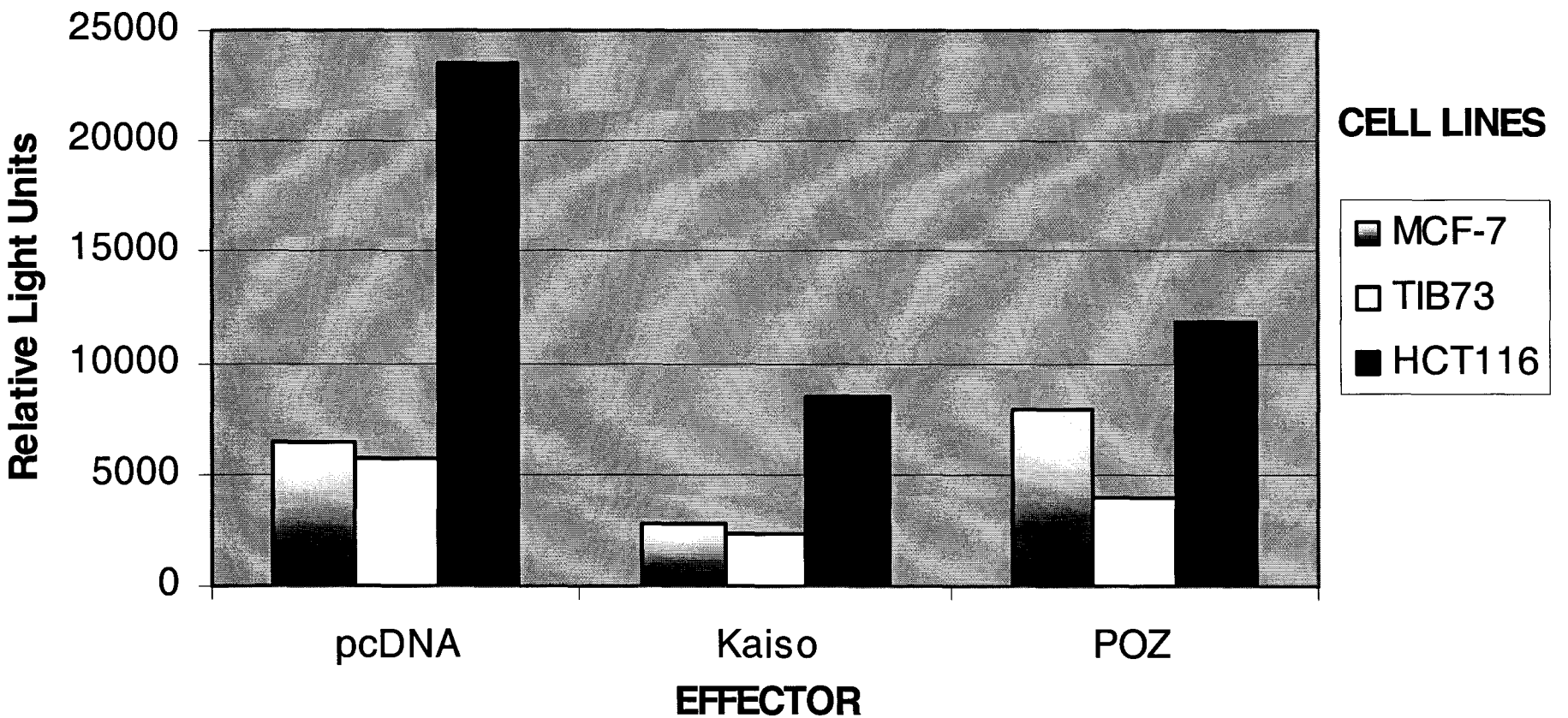


FIGURE 12: 4xKBS-pGL3 Reporter Expression 8 Hours Post-Transfection in HCT116 Cells. 40%-60% confluent HCT116 cells on a 6-well plate were transiently cotransfected in triplicate with 150 ng of 4xKBS-pGL3 reporter and with 150 ng (black), 500 ng (white) or 750 ng (grey) of effector proteins. The effector proteins, as indicated on the x-axis, are empty pcDNA3, Kaiso or ΔPOZ. The cells were then assayed for relative reporter expression 8 hours post-transfection using a Luciferase Assay Kit (Promega). Standard deviation is depicted by the error bars. Reporter expression ranges from 0-2.00 RLU 8 hours post-transfection.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER 8 HOURS POST-TRANSFECTION

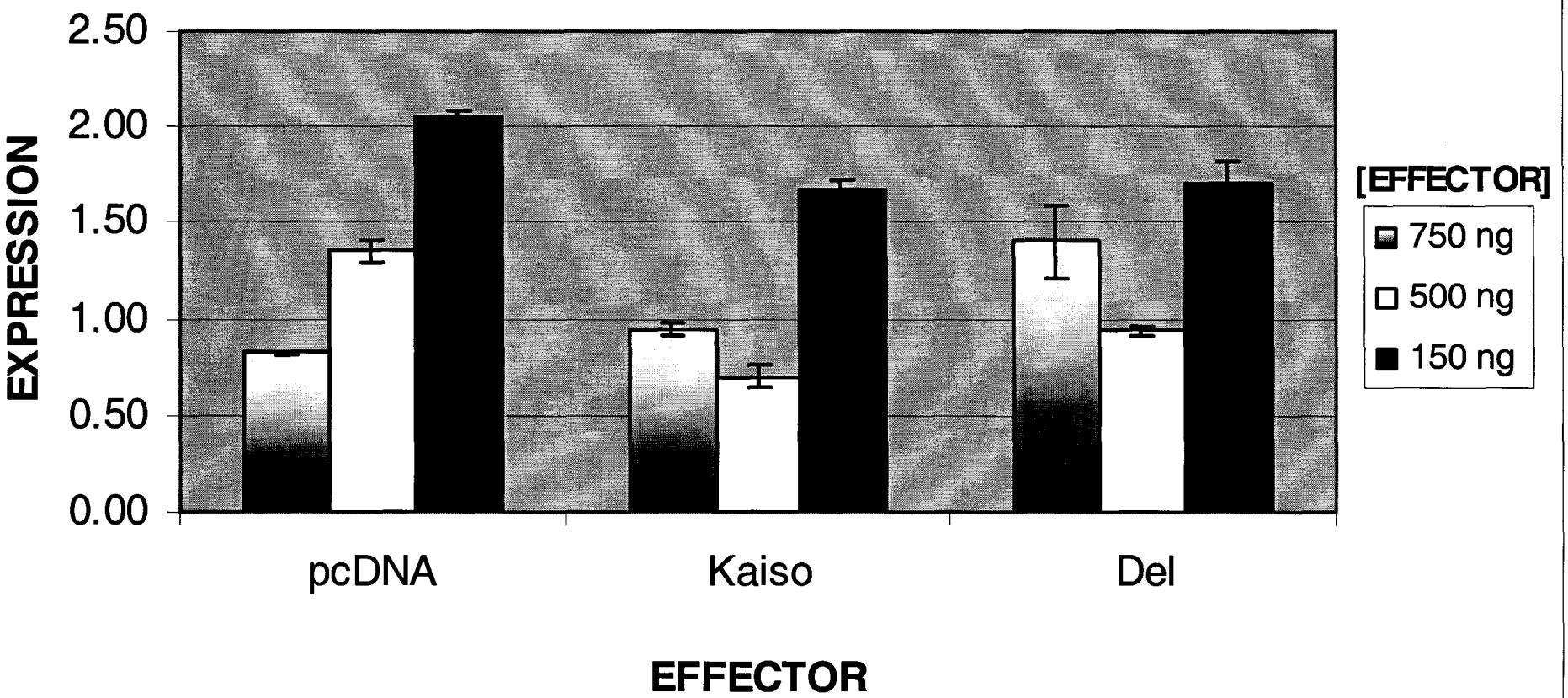


FIGURE 13: 4xKBS-pGL3 Reporter Expression 24 Hours Post-Transfection in HCT116 Cells. 40%-60% confluent HCT116 cells on a 6-well plate were transiently cotransfected in triplicate with 150 ng of 4xKBS-pGL3 reporter and with 150 ng (black), 500 ng (white) or 750 ng (grey) of effector proteins. The effector proteins, as indicated on the x-axis, are empty pcDNA3, Kaiso or ΔPOZ. The cells were then assayed for relative reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). Standard deviation is depicted by the error bars. Reporter expression ranges from 0-22 RLU 24 hours post-transfection.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER 24 HOURS POST TRANSFECTION

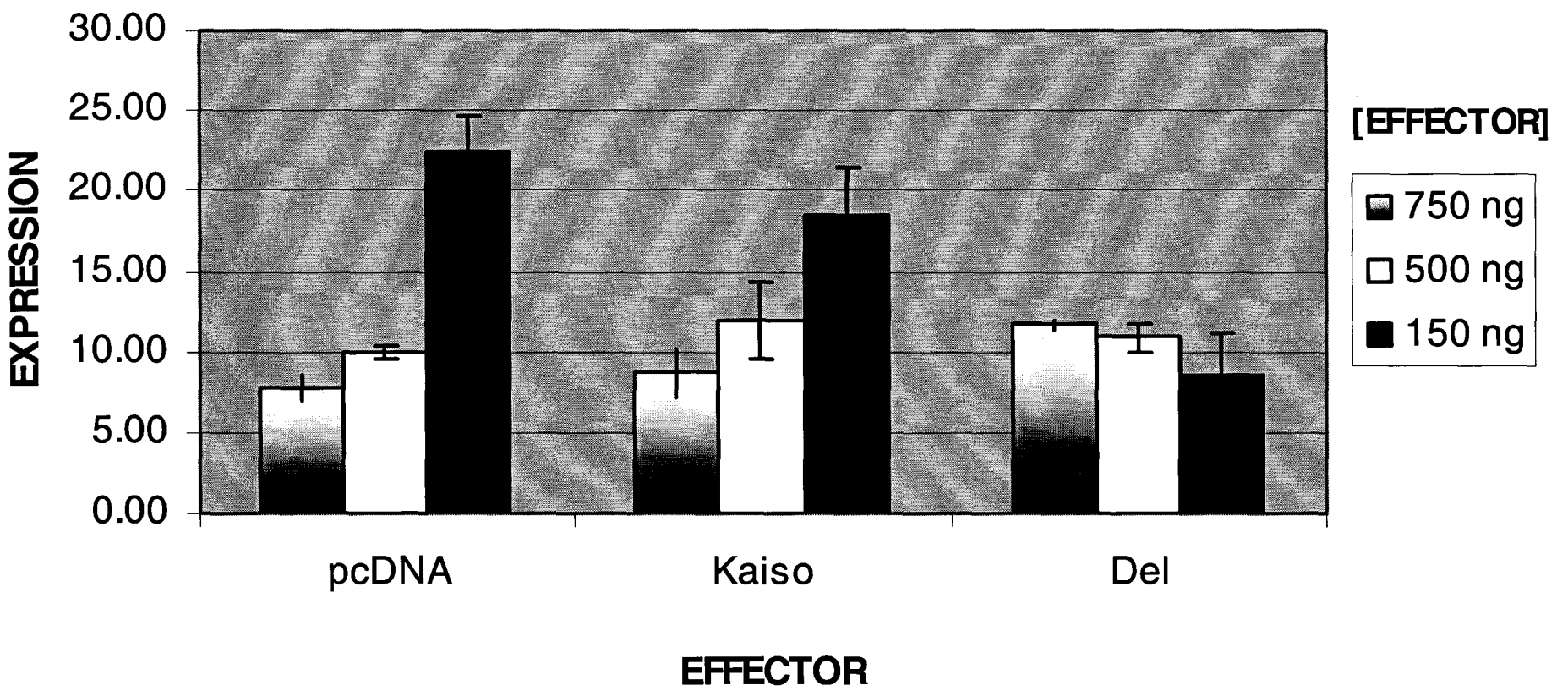


FIGURE 14: 4xKBS-pGL3 Reporter Expression 48 Hours Post-Transfection in HCT116 Cells. 40%-60% confluent HCT116 cells on a 6-well plate were transiently cotransfected in triplicate with 150 ng of 4xKBS-pGL3 reporter and with 150 ng (black), 500 ng (white) or 750 ng (grey) of effector proteins. The effector proteins, as indicated on the x-axis, are empty pcDNA3, Kaiso or ΔPOZ. The cells were then assayed for relative reporter expression 48 hours post-transfection using a Luciferase Assay Kit (Promega). Standard deviation is depicted by the error bars. Reporter expression ranges from 0-22 RLU 48 hours post-transfection.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER 48 POST TRANSFECTION

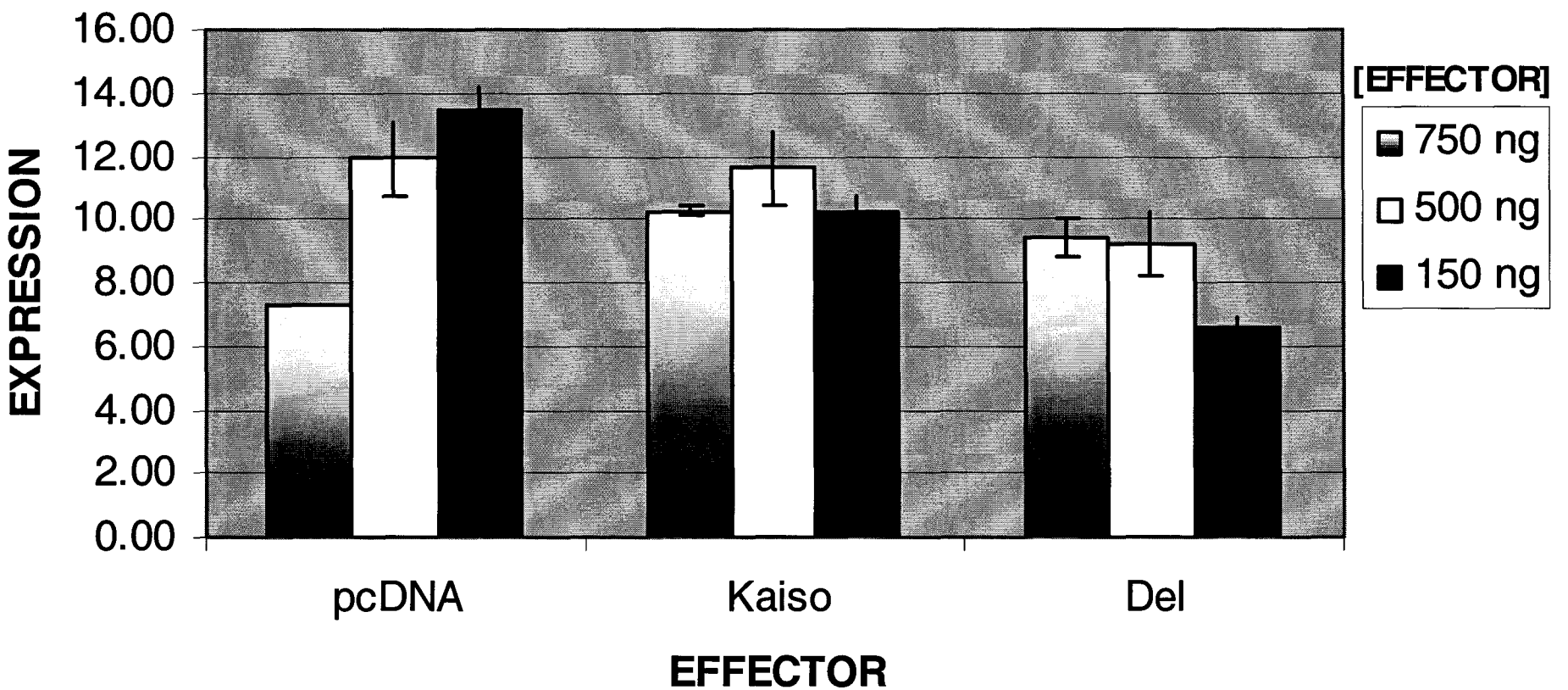


FIGURE 15: Effect of Kaiso on 150 ng of 4XKBS-pGL3 Reporter Expression in HCT116 Cells. 40%-60% confluent HCT116 cells on a 6-well plate were transiently cotransfected in triplicate with 150 ng of 4xKBS-pGL3 reporter and 750 ng of either empty *pcDNA3, kaiso* or d*el* effector proteins. Empty pcDNA3 was used for the remaining 100 ng of DNA for a total of 1 μg. The cells were then assayed for reporter expression relative to basal expression (pcDNA3) 24 post–transfection using a Luciferase Assay Kit (Promega). Standard deviation is depicted by the error bars. No discernable trend is apparent.

EFFECT OF KAISO ON 150 ng OF 4XKBS-pGL3 REPORTER EXPRESSION IN HCT116 CELLS

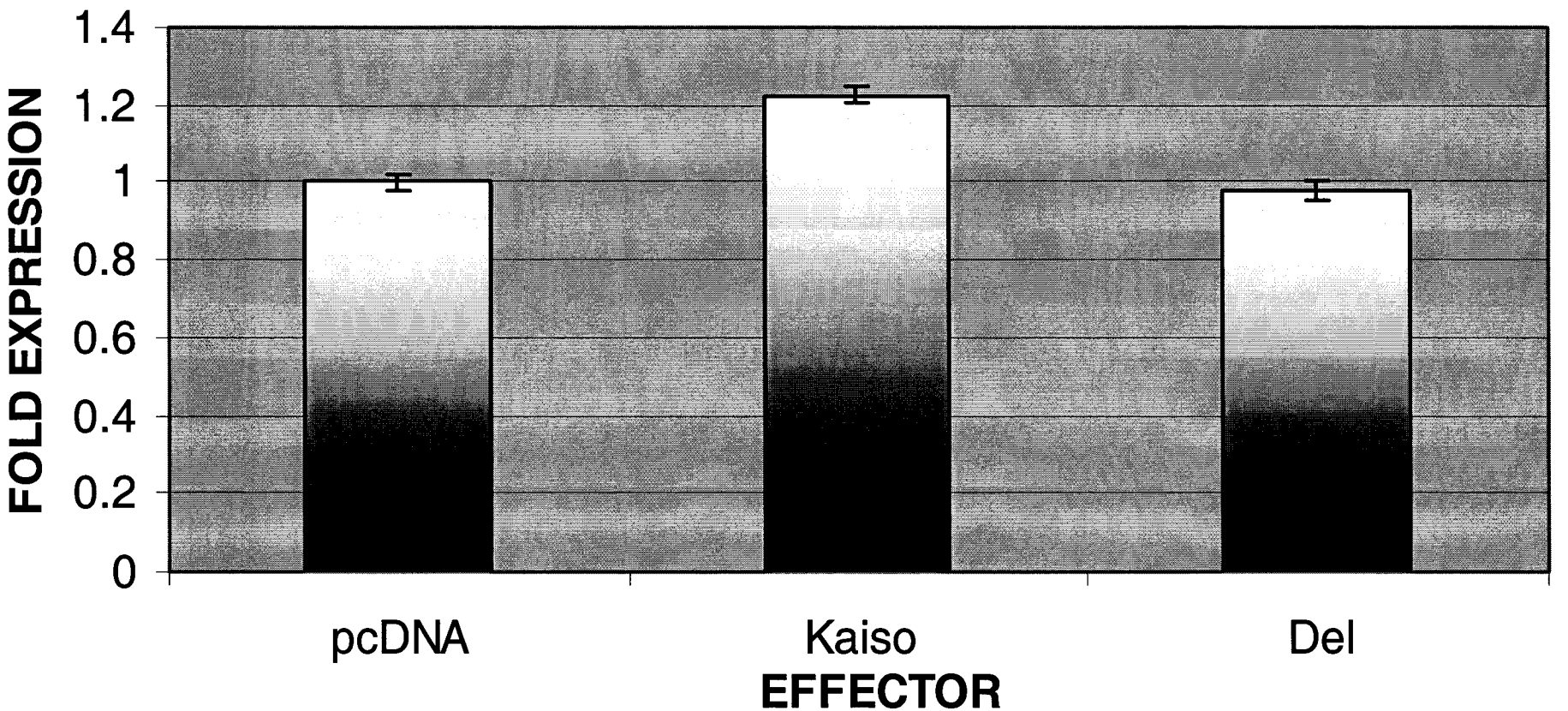


FIGURE 16: Effect of Kaiso on 500 ng of 4XKBS-pGL3 Reporter Expression in HCT116 Cells. 40%-60% confluent HCT116 cells on a 6-well plate were transiently cotransfected in triplicate with 500 ng of 4xKBS-pGL3 reporter and 250 ng of either empty *pCDNA3, kaiso* or d*el* effector proteins. Empty pcDNA3 was used for the remaining 250 ng of DNA for a total of 1 μg. The cells were then assayed for reporter expression relative to basal expression (pcDNA3) 24 post–transfection using a Luciferase Assay Kit (Promega). Standard deviation is depicted by the error bars. A more discernable trend is apparent than Figure 13.

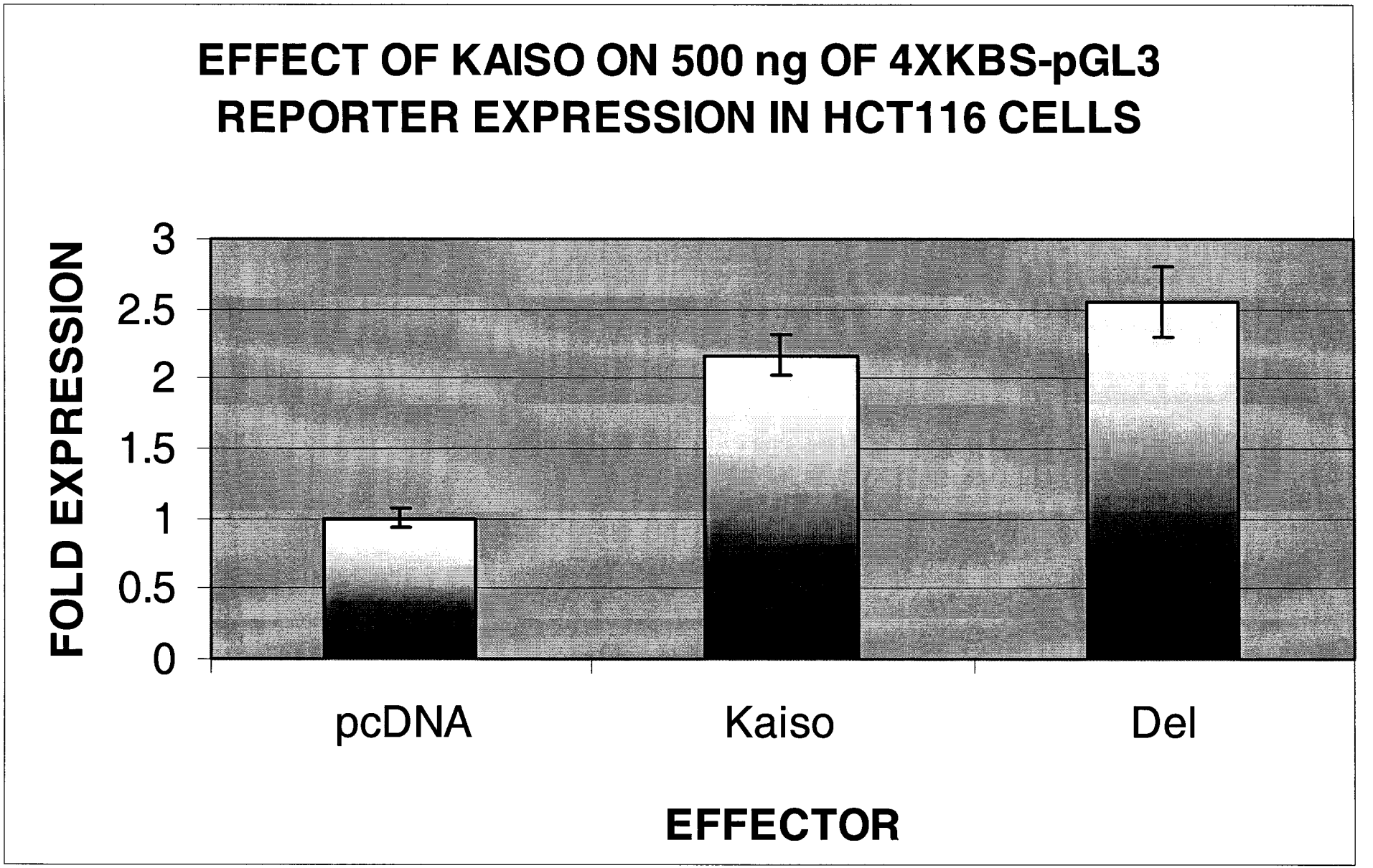

EFFECT OF KAISO ON 500 ng OF 4XKBS-pGL3
REPORTER EXPRESSION IN HCT116 CELLS
FOLD EXPRESSION
3
2.5
2
1.5
1
0.5
0
pcDNA
Kaiso
Del
EFFECTOR

FIGURE 17: Effect of Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells in the Presence of Trichostatin A (TSA). 40%-60% confluent HCT116 cells on a 6-well plate were transiently cotransfected in triplicate with 750 ng of 4xKBS-pGL3 reporter and 250 ng of either pcDNA3, Kaiso or Del effector proteins. 300 mM TSA or 300 mM ethanol was added 24 hours post-transfection. The reporter expression was then assayed + (white)/- (grey) TSA 48-hours post-transfection using a Luciferase Assay Kit (Promega). TSA appeared to have no effect on Kaiso activity in this assay.

EFFECT OF KAISO ON REPORTER EXPRESSION USING 4XKBS-pGL3 IN HCT116 CELLS IN THE PRESENCE OF TRICHOSTATIN A

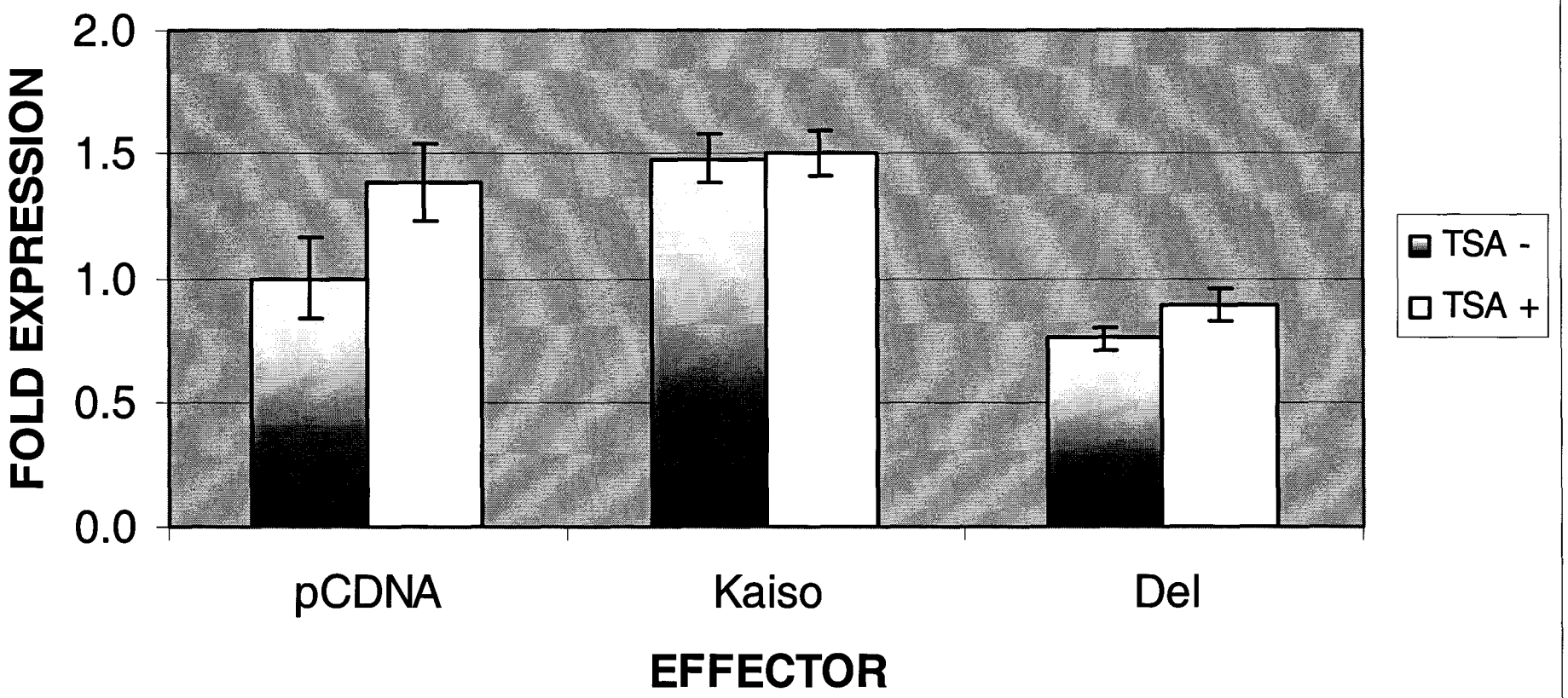


FIGURE 18: Mean Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Pooled Data). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* effector proteins. The reporter expression was then assayed 24 hours post-transfection using a Luciferase Assay Kit (Promega). The pcDNA3 represents background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars. This graph represents the mean value from eleven independent trials depicted in Appendix 1-11.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (POOLED DATA)

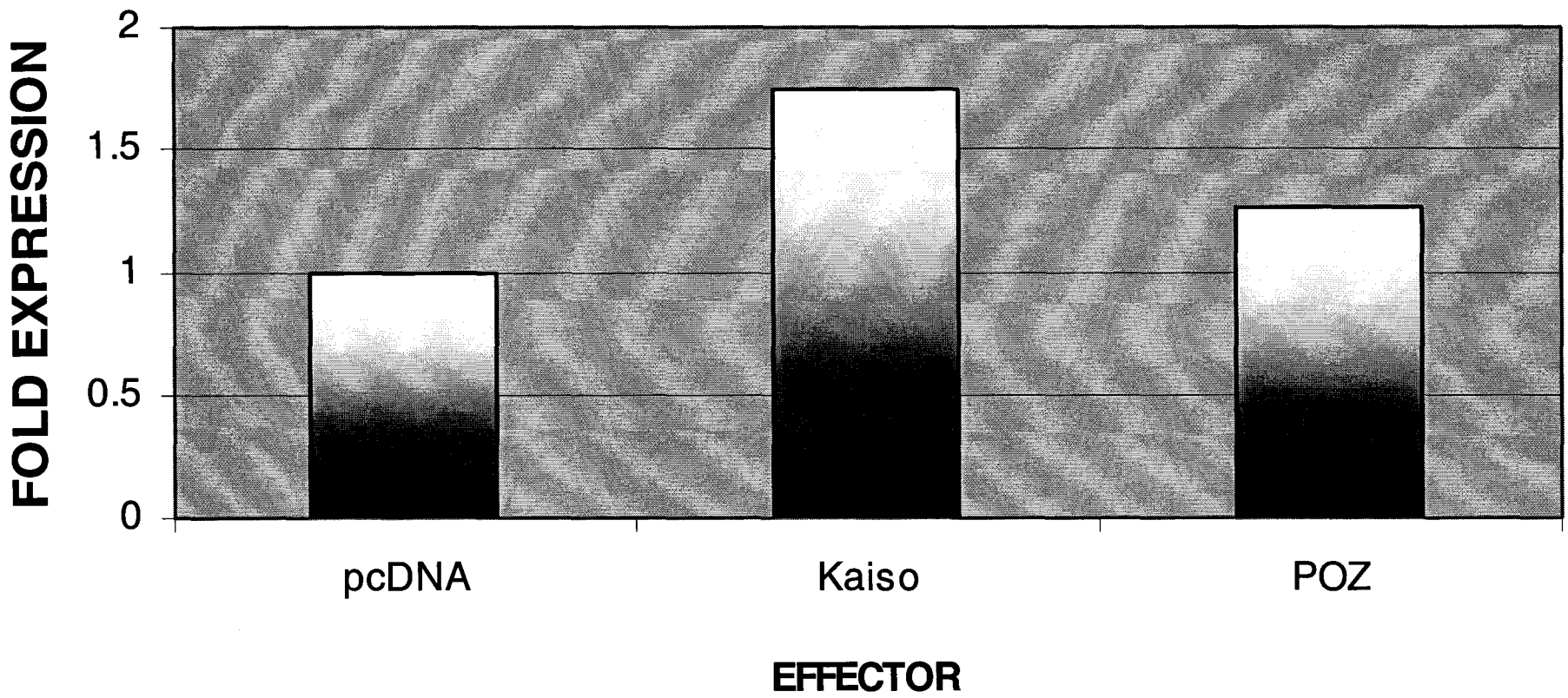


FIGURE 19: Mean Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCA-7 Cells (Pooled Data). 40%-60% confluent HCA-7 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* effector proteins. The reporter expression was then assayed 24 hours post-transfection using a Luciferase Assay Kit (Promega). The pcDNA3 represents background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars. This graph represents the mean value from five independent trials depicted in Appendix 12-16.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCA-7 CELLS USING 4XKBS-pGL3 REPORTER (POOLED DATA)

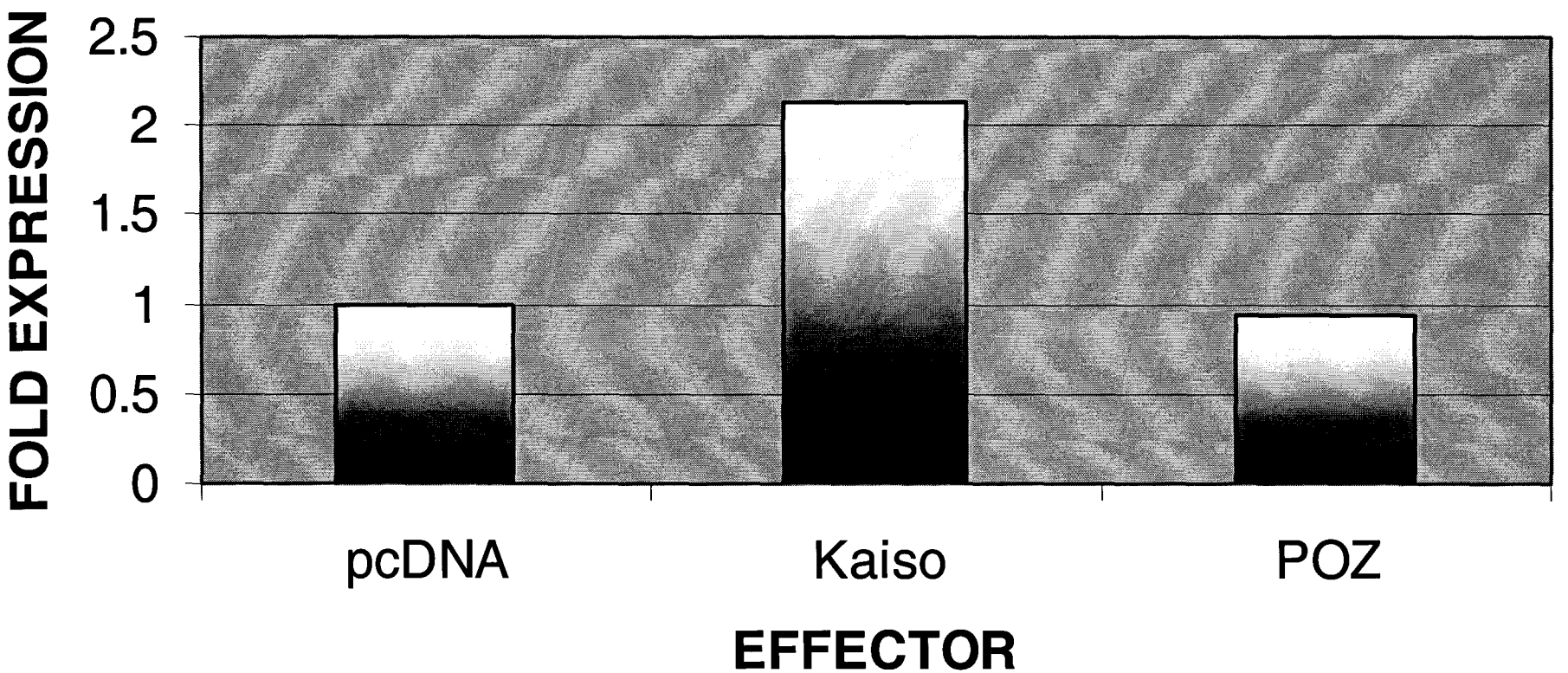


FIGURE 20: Mean Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Pooled Data). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* effector proteins. The reporter expression was then assayed 24 hours post-transfection using a Luciferase Assay Kit (Promega). The pcDNA3 represents background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars. This graph represents the mean value from eight independent trials depicted in Appendix 17-24.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (POOLED DATA)

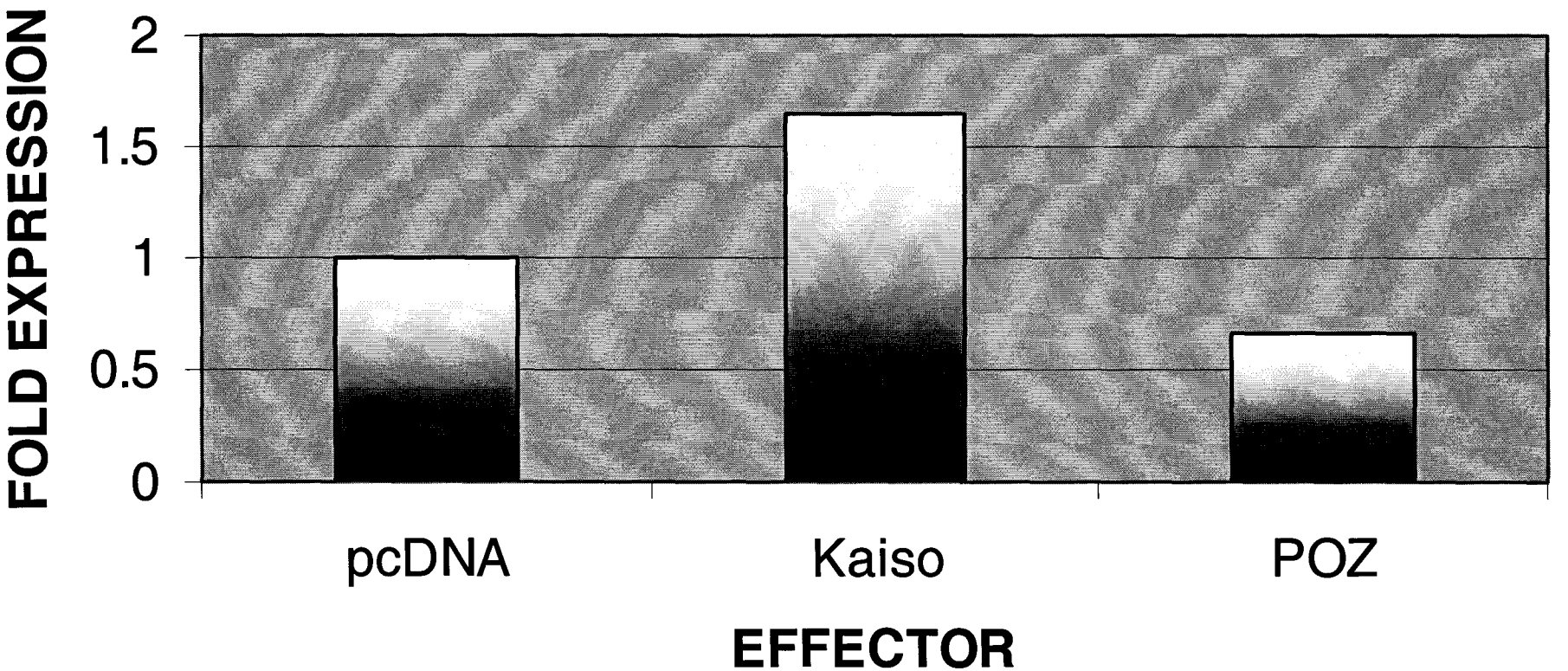
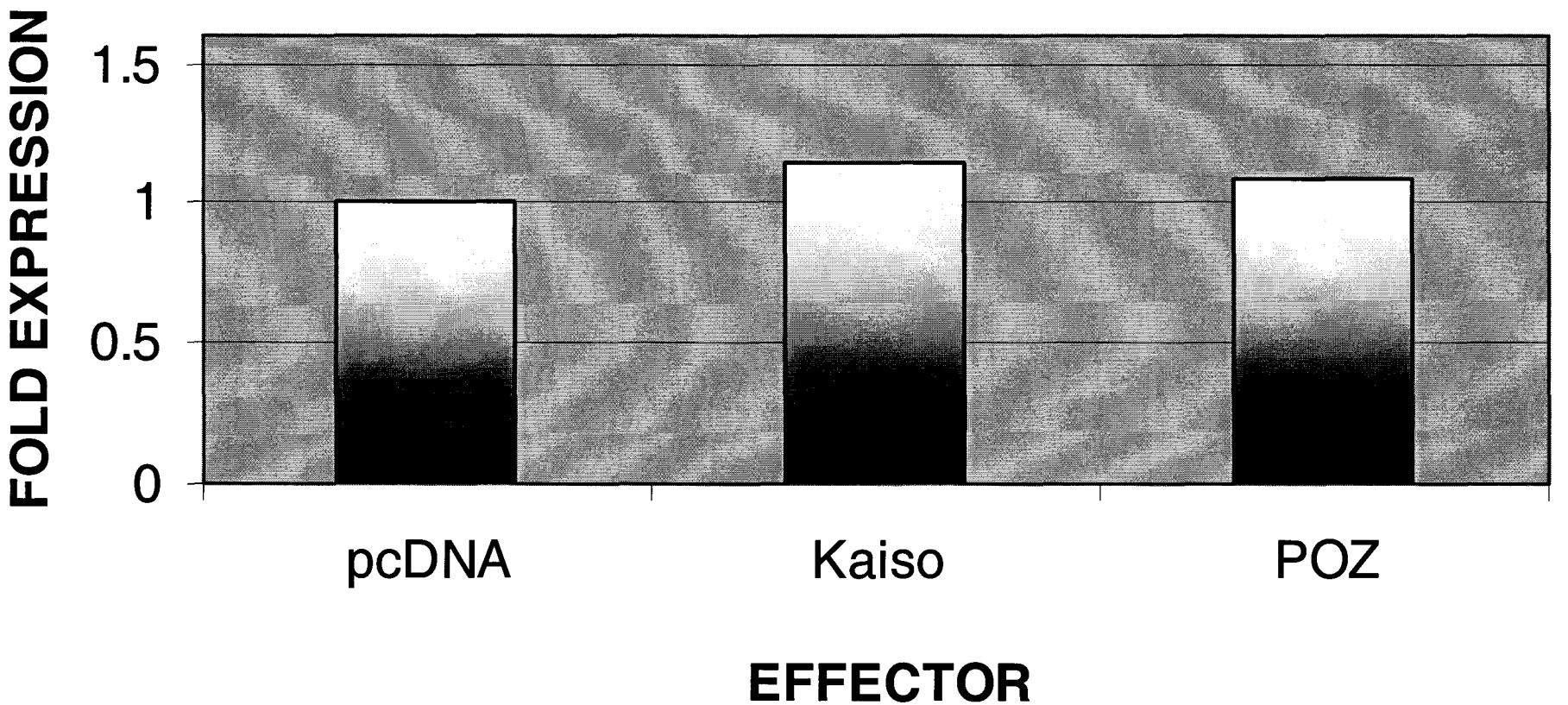


FIGURE 21: Mean Effect by Kaiso on 4XKBS-pGL3 Reporter Expression in MDCK Cells (Pooled Data). 40%-60% confluent MDCK cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* effector proteins. The reporter expression was then assayed 24 hours post-transfection using a Luciferase Assay Kit (Promega). The pcDNA3 represents background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars. This graph represents the mean value from five independent trials depicted in Appendix 25-29.

EFFECT OF KAISO ON REPORTER EXPRESSION IN MDCK CELLS USING 4XKBS-pGL3 REPORTER (POOLED DATA)



DISCUSSION

5.1 KAISO AND HDAC-MEDIATED REPRESSION

This project was conducted to address two main objectives about the function of Kaiso which has recently been identified, by two independent groups, as a transcriptional repressor (52, 80). The first objective in this study pertained to identifying the mechanism of repression used by Kaiso to downregulate gene expression. Since Kaiso falls into the POZ-ZF family of proteins, there was a strong possibility that it would use the same mechanism recruited by other POZ-ZF proteins. PLZF and BCL-6 are the most characterized POZ-ZF proteins and both utilize the mSIN3A/HDAC1 mechanism of repression. Since the recruitment of the Histone Deacetylase Complex is common and based on the structural similarities between Kaiso, BCL-6 and PLZF, it was hypothesized that Kaiso also interacts with members of this complex.

5.1.1 KAISO DOES NOT RECRUIT NuRD OR SIN3 COMPLEXES

HDAC1, HDAC2 and mSIN3A were selected as potential corepressors in order to elucidate the exact mechanism of repression used by Kaiso. HDAC1 and HDAC2 are common to two well-characterized repression complexes, SIN3 and NuRD while mSIN3A is exclusive to SIN3 (55). Therefore, it was expected that Kaiso would interact with at least one of these two proteins to recruit either one of these repression complexes.

Recent evidence demonstrates that Kaiso is a member of the Methyl-CpG binding complex (MeCP1) that is composed of the NuRD complex (30). Since

Kaiso is a member of this macromolecular multi-protein complex (80), it is surprising that no interaction with HDAC1 or HDAC2 was observed. Since Kaiso does not interact with HDAC1 or HDAC2, it is unlikely that Kaiso directly recruits the NuRD complex to repress transcription.

An alternate mechanism of repression assayed was SIN3. Although failure to detect a Kaiso-HDAC1/HDAC2 interaction implicated a Kaiso-mSIN3A interaction, no interaction was observed (Figures 4, 7, 8C and 9). However, the SIN3 proteins are not found in the MeCP1 complex and therefore the absence of a Kaiso-mSIN3A interaction was less surprising. Therefore, the lack of Kaiso-HDAC1/HDAC2/mSIN3A interaction, evident from Figures 2-9, eliminates NuRD and SIN3 mediated repression as mechanisms recruited by Kaiso.

5.1.2 C-TERMINAL BINDING PROTEINS AND GROUCHO: UNLIKELY ALTERNATE MECHANISMS OF REPRESSION

The SIN3 and NuRD complexes are not the only mechanisms of repression, they are simply the most characterized. Two other emerging mechanisms of repression are C-Terminal Binding Proteins (CtBP) and Groucho (79). A recently published study has demonstrated that HIC-1 can repress transcription through a direct interaction with CtBP. This interaction does not occur at the POZ domain, but instead at a GLDLSKK motif located between the POZ and Zinc Finger domains (20). Although not novel, these results have identified another increasingly significant repression mechanism, CtBP, recruited by POZ-ZF proteins. HIC-1 is similar to Kaiso in that both proteins displayed insensitivity towards TSA (Figure 17). However, recruitment of CtBP proteins renders HIC-1

activity TSA sensitive (20). This discovery of another repression domain on HIC-1 was therefore interesting because of the identification of another potential repression mechanism. Unfortunately, CtBP binding is specific for a P/GLDLSLKK/R motif (94). After extensive analysis of the Kaiso translated sequence, this motif was not found and therefore Kaiso cannot directly recruit CtBP as a mechanism for repression.

To a lesser extent, possible recruitment of the Groucho-mediated repression complex was also assessed. The essential WRPW motif, required for complex binding (79, 105), was not found in the Kaiso translated sequence. Therefore, it is unlikely that Kaiso recruits this complex for repression.

5.1.3 POTENTIAL INTERACTION BETWEEN KAISO AND OTHER HDAC PROTEINS

Although there is no interaction with HDAC1 or HDAC2, Kaiso may still exhibit HDAC-mediated repression through other members. There are still other HDAC proteins that may interact with Kaiso to mediate transcriptional repression. If Kaiso interacts with any of these remaining HDAC family members (HDAC3-10) then there is the possibility that repression occurs in a non-NuRD/SIN3 manner. For example, if Kaiso is involved in CtBP-mediated repression it would be through intermediary proteins such as HDACs. This is because some HDAC proteins (HDAC4, 5 and 7) are capable of recruiting CtBP proteins (64, 94). If Kaiso interacts with any one of these three HDAC proteins, then CtBP-mediated repression remains a possibility. Interestingly, HDAC5 does not possess the defining PXDLS motif but can still recruit CtBP proteins (94). This observation

allows for the possibility of CtBP recruitment by Kaiso. Based on this possibility, a general conclusion cannot be made between Kaiso and HDAC proteins until the remaining proteins (HDAC3-10) are studied for an interaction. Therefore, the exact mechanism used by Kaiso to exhibit transcriptional repression remains to be elucidated, but other possible corepressors involved (HDAC3-10) remain to be tested.

5.1.3.1 FUTURE EXPERIMENTS TO ASSESS KAISO-HDAC INTERACTION

The experimental approach used in this project to detect an interaction between Kaiso and HDAC1/HDAC2 was ideal to detect an *in vivo* interaction. This same approach should be pursued further to determine if any of the remaining HDAC proteins (HDAC3-10) are capable of interacting with Kaiso. This technique of coimmunoprecipitation and Western blot analysis is of course restricted to known proteins. In order to identify an unknown binding partner, a yeast-two hybrid approach would be more suited, but since there is potential for a known binding partner (HDAC3-10), a coimmunoprecipitation and Western blot approach is an ideal starting point. Once an interaction is established, other techniques may be implemented to confirm the interaction. Two approaches that would assist in confirming an interaction would be a mammalian two-hybrid assay and a GST pulldown assay.

An indirect assay to demonstrate interaction is a GST pulldown assay. In this assay a Glutathione S Transferase-fusion protein is incubated with whole cell lysate and then precipitated out of solution. Through Western Blot analysis, a

known/suspected partner can be identified. This approach should be performed first out of the three approaches because it is not concentration dependent. A high concentration of GST-fused protein can be added to whole cell lysates and circumvent any protein concentration limitations.

Another assay used to demonstrate protein-protein interaction is the mammalian two-hybrid assay. This assay is based on interacting proteins forming an artificial transcription factor that activates transcription of a reporter gene downstream of a Gal4 upstream activation sequence. This approach should be conducted second in order to determine if the protein-protein interactions can occur within a cell.

The final approach should be the coimmunoprecipitation and Western blot analysis. This approach is both physiologically relevant and *in vivo*. Since this intracellular content is not altered in this approach, this would give the most accurate intracellular representation.

5.1.4 TRICHOSTATIN A INSENSITIVITY AND HDAC-INDEPENDENT ACTIVITY: IS KAISO A TRANSCRIPTIONAL ACTIVATOR?

Although HDAC1, HDAC2 and mSIN3A interaction with Kaiso was not observed in this study, a general conclusion about HDAC recruitment cannot be made without further evidence. Preliminary evidence against HDAC recruitment is demonstrated by Kaiso insensitivity to Trichostatin A (TSA), Figure 17. This experiment was conducted using the 4XKBS *cis*-element to test Kaiso sensitivity to the Histone Deacetylase (HDAC) inhibiting drug, TSA. Since an interaction between Kaiso and the remaining HDACs was not assayed, Kaiso activity was

tested in the presence of TSA and found to be unaffected. This data lends further support to the results depicted in Figures 3-9 where Kaiso did not interact with members of the HDAC complex. TSA insensitivity complements my previous findings against a Kaiso-HDAC component interaction. These results also contradict the published findings which implicate TSA sensitivity because HDAC1/HDAC2 are subcomponents of the MeCP1 complex that contains Kaiso (30, 80). Although my results are quite evident, a conclusion eliminating any HDAC recruitment is premature. This is demonstrated by HIC-1 displaying insensitivity to TSA, but through CtBP interactions becomes TSA sensitive. Likewise, Kaiso may display TSA sensitivity only in the presence of a yet to be identified binding partner. Based on my results, I conclude that Kaiso activity on the 4xKBS *cis*-element is insensitive to TSA. The observation that Kaiso does not interact with members of the HDAC complex and Kaiso activity is insensitive to TSA, led to the possibility that Kaiso may be functioning as a transcriptional activator on the 4XKBS element.

5.2 KAISO CAN ACTIVATE TRANSCRIPTION

The failure of Kaiso to interact with HDAC complex proteins and insensitivity to TSA suggested that Kaiso may function as a transcriptional activator. This hypothesis was tested in the second half of this project that dealt with Kaiso functioning as a transcription factor. The objective here was to assess how Kaiso regulates gene expression through studies of the 4xKBS element.

Specifically, is the 4xKBS a physiological target that is transcriptionally regulated by Kaiso?

Although the literature proves that Kaiso is a transcriptional repressor on methylated target DNA (80), this study pertained specifically to the effect of mouse Kaiso on nonmethylated DNA. Until now, this effect was unknown. The reported studies demonstrating Kaiso as a repressor used Gal4 reporter assays or a methylated sequence (52, 80). These experiments used the empirically determined unmethylated KBS (15). Reporter gene studies were then conducted to assay the activation potential of Kaiso.

HCT116 and HCA-7 cells were ideal for use because of both high (HCT116) and low (HCA-7) endogenous Kaiso expression, but these are transformed cell lines. Since the activation data established in Figures 18-20 was unexpected, it was possible their transformed phenotype may be a contributing factor to activation. To address this, nontransformed epithelial (MDCK) cells were also used in reporter gene studies. The results from these experiments are depicted in Appendices 25-29 and Figure 21. Based on these data, it appears that Kaiso had no effect on the same *cis*-acting element (4XKBS) in a non-transformed cell line. Although attributing transformation to Kaiso activation is premature, it is clear that Kaiso does activate and this may be a cell line specific phenomenon. Although MDCK cells are nontransformed epithelial cells, the selection of this cell line in these studies may not have been ideal. The reason for this is that it was difficult to demonstrate the $p120^{ctn}$-Kaiso interaction in this cell line. This is unexpected because the original studies to characterize Kaiso were conducted in

MDCK cells (15). It was therefore hypothesized that this difficulty may be attributed to an aberrant clone of MDCK cells that affected these studies.

The averaged results in HCT116, HCA-7 and 293 cells all demonstrate that Kaiso activates expression of a reporter gene that contains the 4xKBS as a *cis*-element (Figures 18-20). This indicates that the 4xKBS is a physiological target that is regulated by Kaiso activity. Since the transactivation data presented in these studies provides strong evidence for KBS regulation by Kaiso in certain cell lines, potential target genes can be elucidated that contain the core KBS sequence (TCCTGCNA, where N is any nucleotide). Some promoters that contain this sequence are *gsk3* (5 copies), *e-cadherin* (2 copies each; human and mouse), *apc* (3 copies) and *matrilysin* (2 copies mouse, 3 copies human). Further evidence for *matrilysin* as a target gene have been provided by *in vitro* studies in our lab. Through EMSA analysis, both human and mouse *matrilysin* have demonstrated stringent binding by Kaiso (15). Whether these potential target genes are *bone fide* remains to be elucidated, but can readily be resolved using the same reporter gene assays used in these studies. Therefore, the presence of the KBS sequence in the promoters of these genes makes them good candidates for target genes regulated by Kaiso.

An important factor affecting target gene regulation by Kaiso is cell signaling. The nuclear function of Kaiso is indirectly linked to cell adhesion and any alterations in cell-cell contact may influence gene expression through Kaiso. A further understanding of the transcriptional activity of Kaiso will provide a bridge between cell surface signaling and gene expression. For example, if a break in

cell-cell contact results in the release of p120^{ctn} from E-cadherin then p120^{ctn} is free to interact with Kaiso. Since the p120^{ctn} binding site overlaps with the DNA binding site then Kaiso may be inhibited from regulating gene expression. Although further studies are required to elucidate what signals would activate Kaiso, some potential signals may be hypothesized. For example, since p120^{ctn} is a Src kinase target, a Src–activating pathway may also activate Kaiso through the same signaling molecule (29). Some of these p120^{ctn}-phosphorylation inducing mitogens include: Platelet-Derived Growth Factor (PDGF), Colony–Stimulating Factor 1 (CSF-1) and Epidermal Growth Factor (EGF) (29).

Involvement in cell adhesion is evidence for a role of Kaiso in tumourigenesis. Not only are the other components of this cell adhesion pathway, E-cadherin and p120^{ctn}, known to be involved in tumourigenesis, but other POZ-ZF proteins (PLZF, BCL-6 and HIC-1) as well. Yap previously found that 50% of metastatic carcinomas are a result of a disruption in cell-cell adhesion involving E-cadherin (102), while p120^{ctn} is implicated in a variety of carcinomas including breast (26), prostate (48), gastric (50) and bladder (89). The POZ-ZF proteins PLZF and BCL-6 are involved in lymphomagenesis (27, 76) while HIC-1 is involved in various carcinomas (96). The data presented in this project provides more insight to the oncogenic potential of Kaiso. Through the recruitment of mSIN3A/HDAC1 by both PLZF and BCL-6, David *et al.* (18) purposed that Leukemias are specific to this form of regulation. The data here clearly demonstrates that Kaiso does not interact with either mSIN3A or HDAC1. This may eliminate the involvement of Kaiso in Leukemias, but allows for a role in

latter point is supported by a Kaiso-p120^{ctn} interaction only observed in epithelial cells (15) and the involvement of both p120^{ctn} and E-cadherin in epithelial transformations (48, 50, 102). The cell adhesion pathway and Kaiso activity both affect epithelial cells. Therefore, it is likely that aberrant Kaiso functioning will affect this cell type. Though the exact role of Kaiso in tumourigenesis remains to be elucidated, these findings provide a strong foundation to further research into characterizing the transcriptional regulation of Kaiso. Once normal Kaiso function is established then abnormal Kaiso activity can easily understood.

5.2.1 POZ DOMAIN FUNCTION IS UNCLEAR

The POZ domain is hypothesized to play a role in transcriptional regulation for the POZ-ZF family. For example, the PLZF, BCL-6 and HIC-1 POZ domains alone are sufficient to mediate transcriptional repression in reporter gene studies (19, 24, 61). However, reporter analyses with the Kaiso POZ domain alone revealed no discernable trend in HCT116, HCA-7 and MDCK cells. However, I did detect transcriptional repression in 293 cells (Figure 20). This result appears to contradict that of full length Kaiso that displays 'weak' activation in the same cell line. This conclusion of repression for the POZ domain may be premature. The results presented here have an average of 0.6 fold activation (Figure 20) for the POZ domain, but the individual trials range from 0.4-2 fold activation (Appendices 17-24). This inconsistency in regulation makes any firm conclusion difficult. In fact, the inconsistencies point out the limitations of a reporter gene system. There are many variables in this approach and it is difficult to account

for them all. For example, a variation in the data can be attributed to cell confluency or the levels of effector expression. Both of these points would cause a change in the results obtained. This idea of broad fluctuation in the results does not apply to full length Kaiso because Kaiso did not fluctuate between both activator and repressor. Further studies of the POZ domain will have to be conducted before the true effects are elucidated. Based on these results a revision is required to the original model proposed in Appendix 30, Kaiso does not repress expression of the 4xKBS *cis*-element but may in fact activate expression (Appendix 31).

5.3 KAISO CAN ACTIVATE AND REPRESS TRANSCRIPTION

These results of no Kaiso-HDAC complex interaction, insensitivity to TSA and Kaiso activating gene expression, although complementary, contradict the current literature (80). Although this appears as a discrepancy there are a few factors that may account for the differing results and explain why the previous findings should not be expected. Firstly, reporter assays between the three studies were all conducted in different cell lines. Prokhortchouk *et al.* used HeLa and NIH3T3 cells (80) while Kim *et al.* conducted assays in early gastrula stage embryos (52). Secondly, the studies conducted by Kim *et al.* were using *Xenopus* Kaiso and not mouse Kaiso. Though these two proteins are orthologs, the possibility exists that the two proteins may not function the same especially since they are only 53% identical overall at the amino acid level (52). Thirdly, Kim et *al.* used an artificial Gal4 system (50). There are a few problems in using

the Gal4 system to assay Kaiso activity. First, the DNA binding site (Gal4 UAS) is not a physiological target of Kaiso. Therefore, DNA binding of Kaiso has been eliminated. This is not an ideal situation because Kaiso function may be determined by DNA target site sequence. By eliminating DNA binding, any site dependent effects of Kaiso could not occur. A second problem is the use of fusion proteins. The Gal4-Kaiso fusion protein may hinder the functioning of Kaiso. Deltour *et al.* suggest that large additions (Gal4 DBD or GST) to the N-terminal of HIC-1 impedes function. This was demonstrated by the failure of GST-HIC1 to interact with CtBP1, but HIC-1 successfully interacted with GST-CtBP1 (20). Therefore, the addition of a Gal4 domain to Kaiso may interfere with its function. Fourthly, Prokhortchouk *et al.* studied the effects of Kaiso on methylated DNA and found that Kaiso exhibits no effects on unmethylated DNA (80). This point deviates tremendously from the foundation of these studies. The 4xKBS sequence used in this project was not methylated and through EMSA analysis was confirmed as a stringent binding site for Kaiso (17). Finally, another key reason to account for the observed differences has to do with the methodology implemented. Kim *et al.* made the data physiologically relevant by using embryos, but did not use a Kaiso binding site to study transcriptional regulation (50). Prokhortchouk *et al.* used a methylation-dependent DNA binding sequence (MGMGTCTAGMG, where M represents a methylated cytosine residue) in reporter gene assays (78), but this sequence was not the same as the core KBS sequence (TAGCAGGA x 4) used in this project. The importance of the binding sequence is best displayed by another POZ-ZF protein ZF5.

Transient cotransfection assays have revealed that ZF5 has the ability to both activate and repress transcription of target sites. ZF5 can activate HIV-1 LTR and repress the beta-actin promoter (49). Since ZF-5 has the highest homology to Kaiso, it is quite conceivable that Kaiso may also have a dual function that is target site dependent. This target site-dependent duality was confirmed for Kaiso based on these results and the current literature (78). Based on this dual functioning other important questions are raised including whether the signal that results in Kaiso activating target genes differs from a signal that results in Kaiso repressing methylated target sites and how is this dual activity of Kaiso regulated? Therefore, all the data on Kaiso taken together indicate that Kaiso is capable of both transcriptional activation and repression with many new questions remaining to be addressed.

Overall, this work has contributed to providing insight to the physiological functioning of Kaiso. Novel insight has been provided on the transcriptional activity of Kaiso on a DNA target site that is methylation-independent. This allows for a model of Kaiso activity to be elucidated because a physiological target sequence has been identified that requires Kaiso binding. This model (appendix 31) would outline transcriptional activation by Kaiso of a target site containing the KBS sequence. This model eliminates the possibility of transcriptional repression, mediated by an HDAC repression complex, on this target site. This model does open new hypotheses on Kaiso activity. Since repression and the recruitment of HDAC corepressors has been eliminated, there is the possibility now of coactivator recruitment. One hypothesis to be tested is

whether the coactivators recruited by Kaiso are the same as those with ZF5. Therefore, coactivators recruited by Kaiso remains to be elucidated, but with further research a more comprehensive model will be established.

The data presented in this thesis also provides preliminary insight into a signaling cascade that is initiated by E-cadherin and terminates at a target gene regulated by Kaiso. This discovery is novel because this signaling cascade involving E-cadherin does not involve β-catenin, but instead $p120^{ctn}$, therefore furthering the significance of cadherins in cell signaling. How this cascade is activated and if there is a simultaneous affect on the β-catenin pathway are just some of the questions remaining to be addressed. Overall, it is evident that cadherins are not exclusively involved in cell-cell adhesion, but also, through proteins like Kaiso, can affect cell signaling and gene transcription.

CONCLUSION

The transcriptional activity of Kaiso, previously demonstrated, is transcriptional repression (52, 80). This then led to the hypothesis for a potential Kaiso-HDAC component interaction. Due to the failure of a coimmunoprecipitation detection, Kaiso is concluded not to interact with HDAC1, HDAC2 or mSIN3A in the eight cell lines tested in this study. This trend did not correlate with a tumorigenic phenotype and was demonstrated to be consistent in both epithelial and fibroblast cell lines. Further support for this conclusion was provided by Kaiso activity displaying insensitivity to Trichostatin A (TSA), a known inhibitor of HDAC activity. TSA insensitivity clearly demonstrates an HDAC-independent mechanism of action. In fact, based on these studies, the mechanism of action is not repression, but instead, activation. Kaiso activates transcription of the 4xKBS *cis*-element in HCT116, HCA-7 and 293 cells, but has no activity on this element in MDCK cells. These observations of activation then confirm HDAC-independence. Therefore, Kaiso is a transcriptional activator of the KBS element and does not directly recruit the HDAC repression complex.

TABLE 1: ANTIBODIES USED IN IMMUNOPRECIPITATION ASSAYS

NAME	ANTIBODY	AMOUNT/mL
H51	Rabbit anti-HDAC1 polyclonal	4 μg
H54	Rabbit anti-HDAC2 polyclonal	4 μg
K20	Rabbit anti-mSIN3A polyclonal	4 μg
6F	Mouse anti-Kaiso monoclonal	4 μg
15D2	Mouse anti-p120^{ctn} monoclonal	4 μg
12CA5	Mouse anti-Hemaglutinnin monoclonal	4 μg

TABLE 2: ANTIBODIES USED FOR WESTERN BLOTS

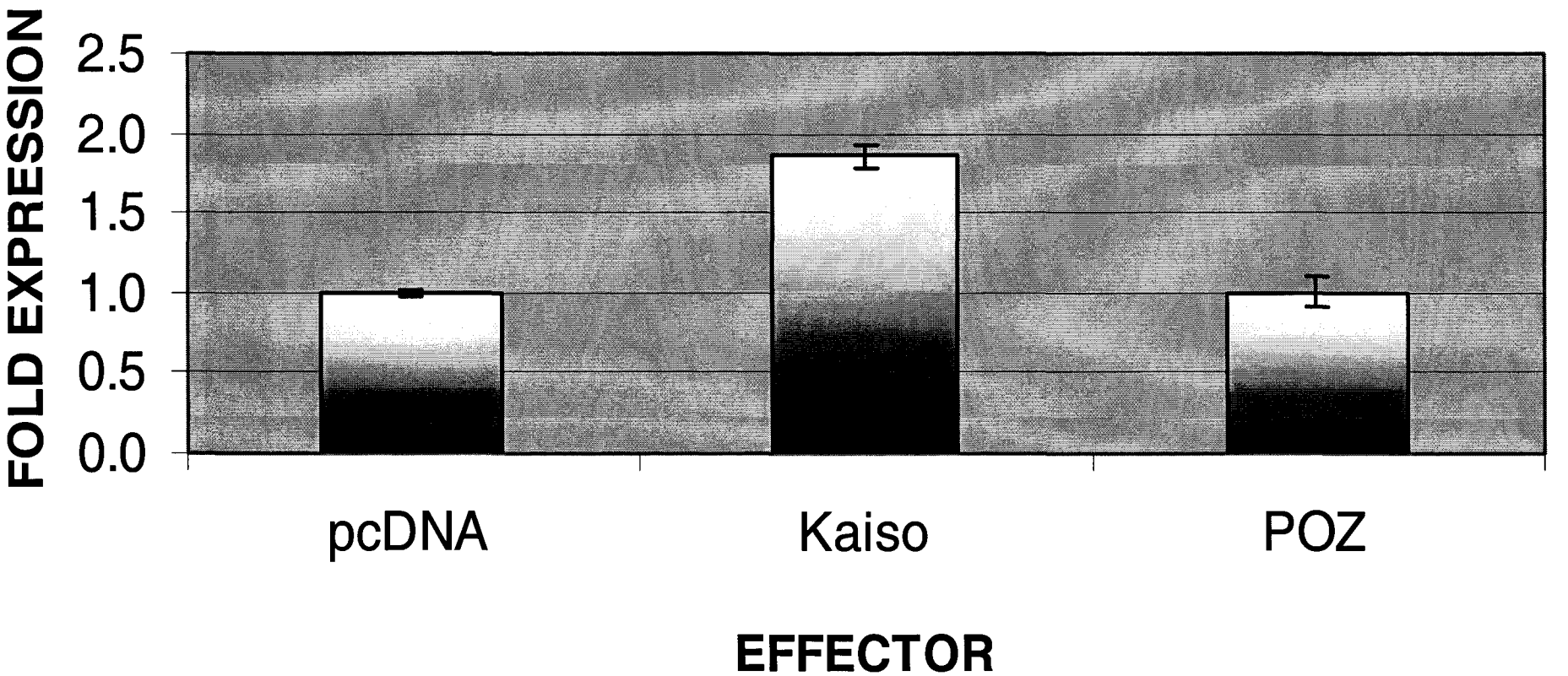
NAME	ANTIBODY	DILUTION
H51	Rabbit anti-HDAC1 polyclonal	1:500
H54	Rabbit anti-HDAC2 polyclonal	1:500
K20	Rabbit anti-mSIN3A polyclonal	1:500
Kaiso pAb	Rabbit anti-Kaiso polyclonal	1:12000

TABLE 3: PRIMERS USED FOR CLONING

PRIMER	TARGET VECTOR	USE
AB26015	5' pGL3 with additional KpnI restriction site	PCR
AB26016	3' pGL3 with additional SmaI restriction site	PCR
AB27010	5' pGL3	SEQUENCING

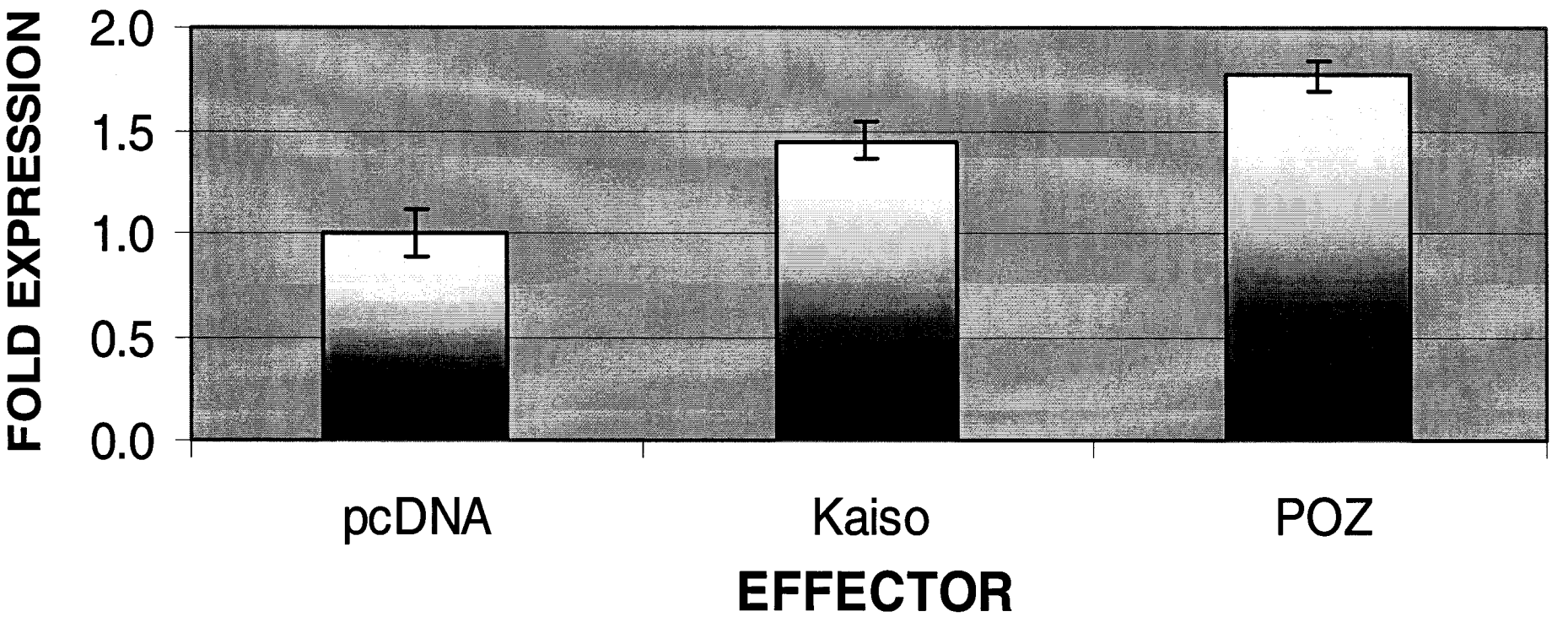
APPENDIX 1: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 1). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 USING 4XKBS-pGL3 REPORTER (TRIAL 1)



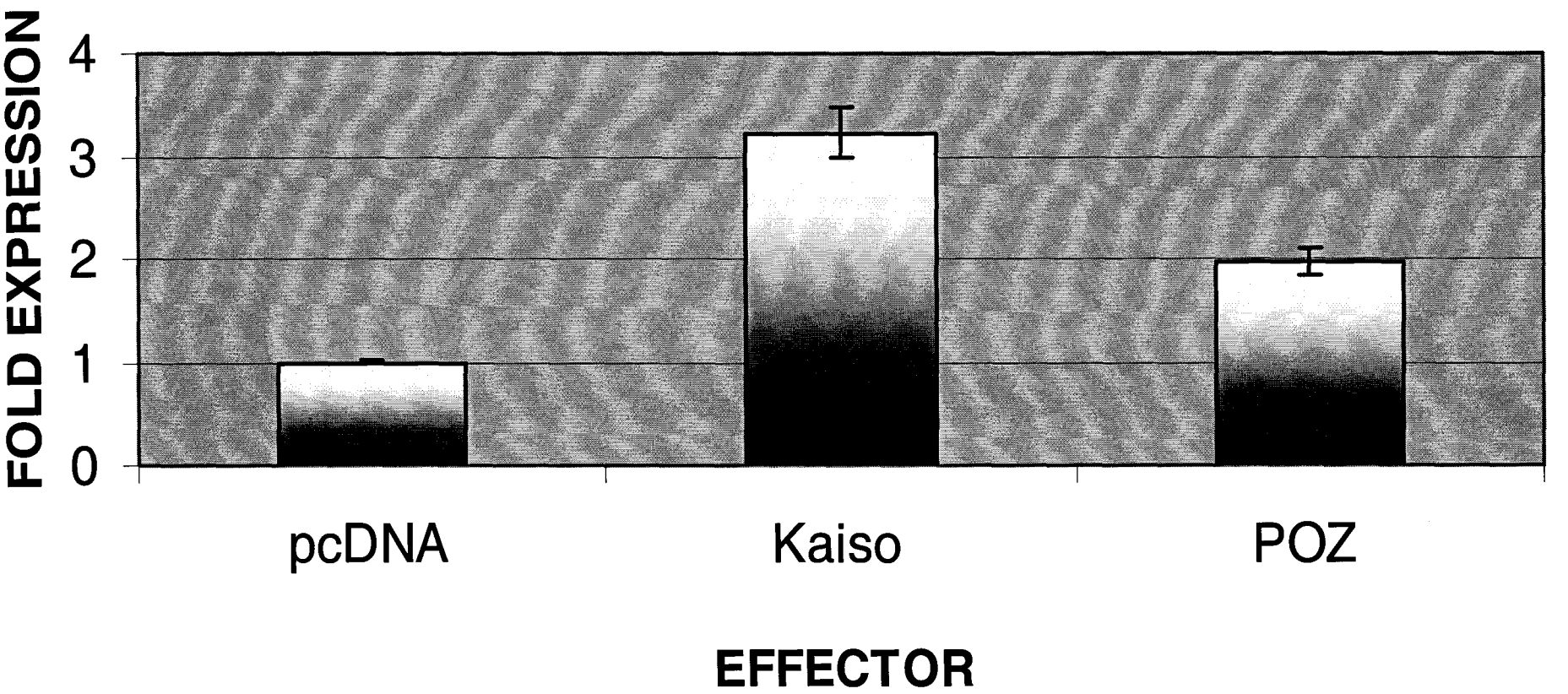
APPENDIX 2: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 2). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 2)



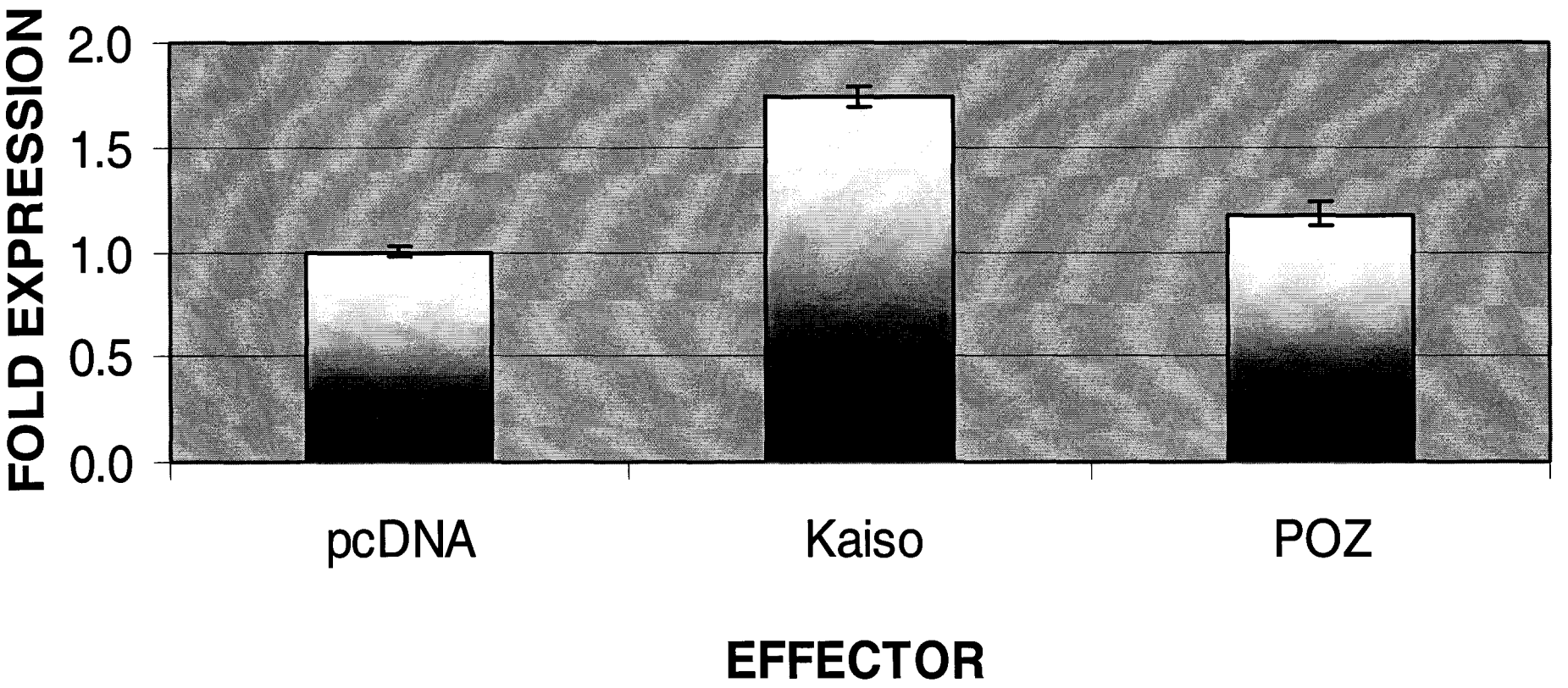
APPENDIX 3: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 3). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 3)



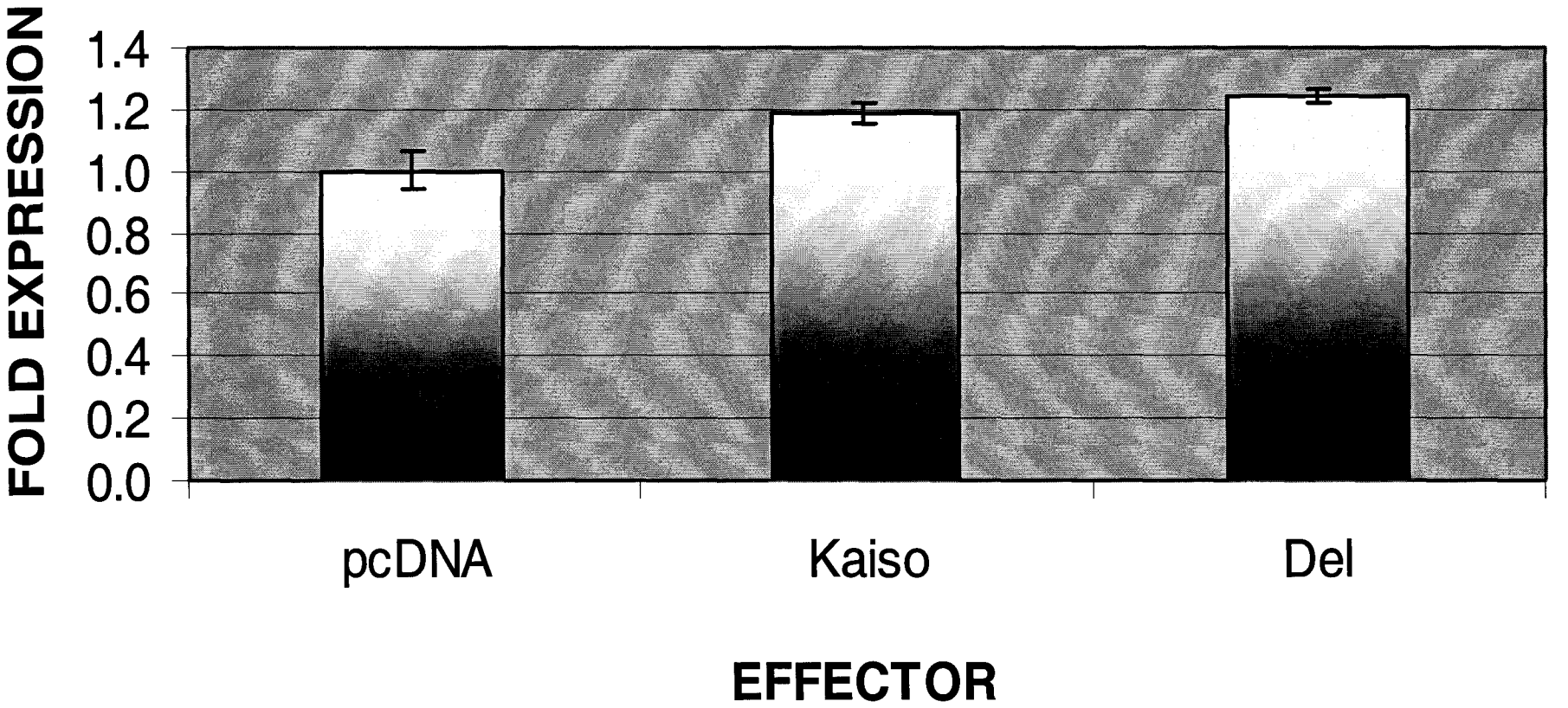
APPENDIX 4: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 4). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 4)



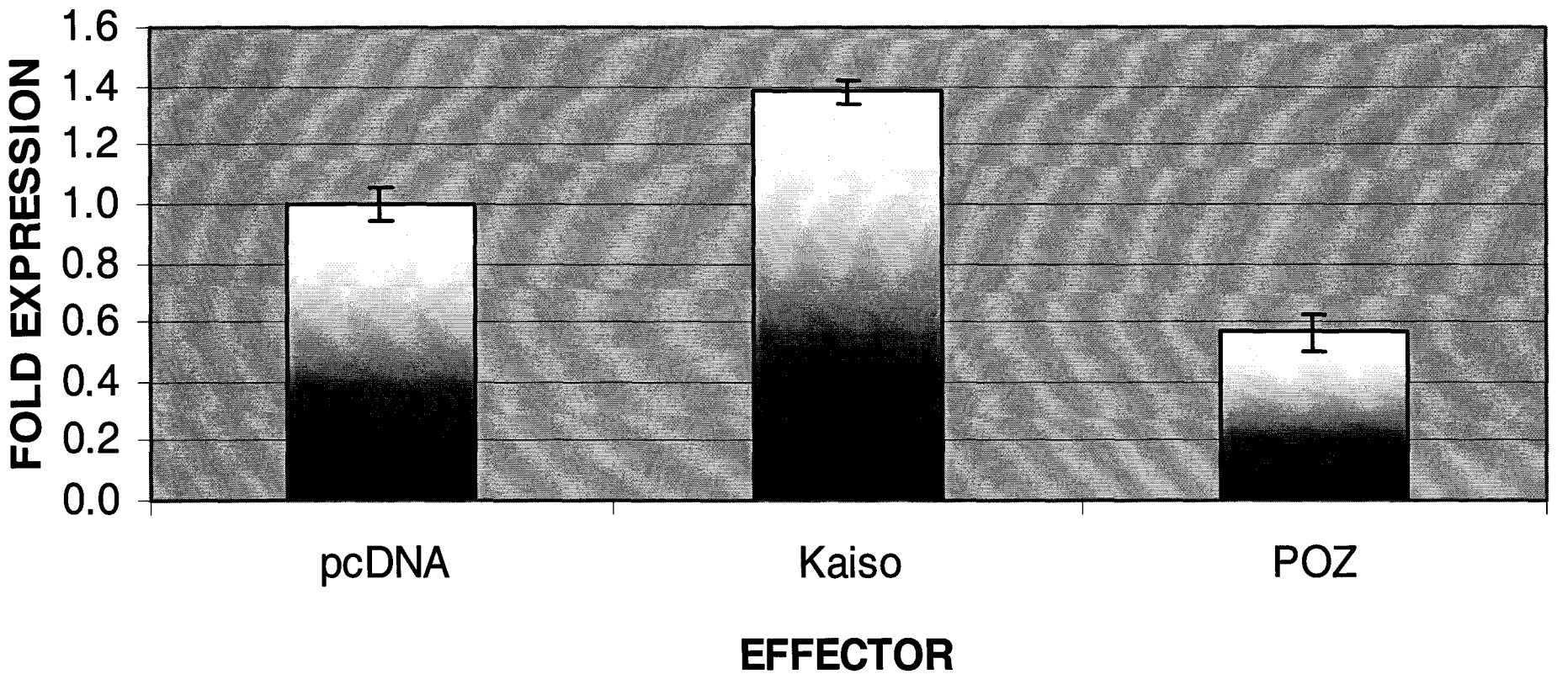
APPENDIX 5: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 5). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 5)



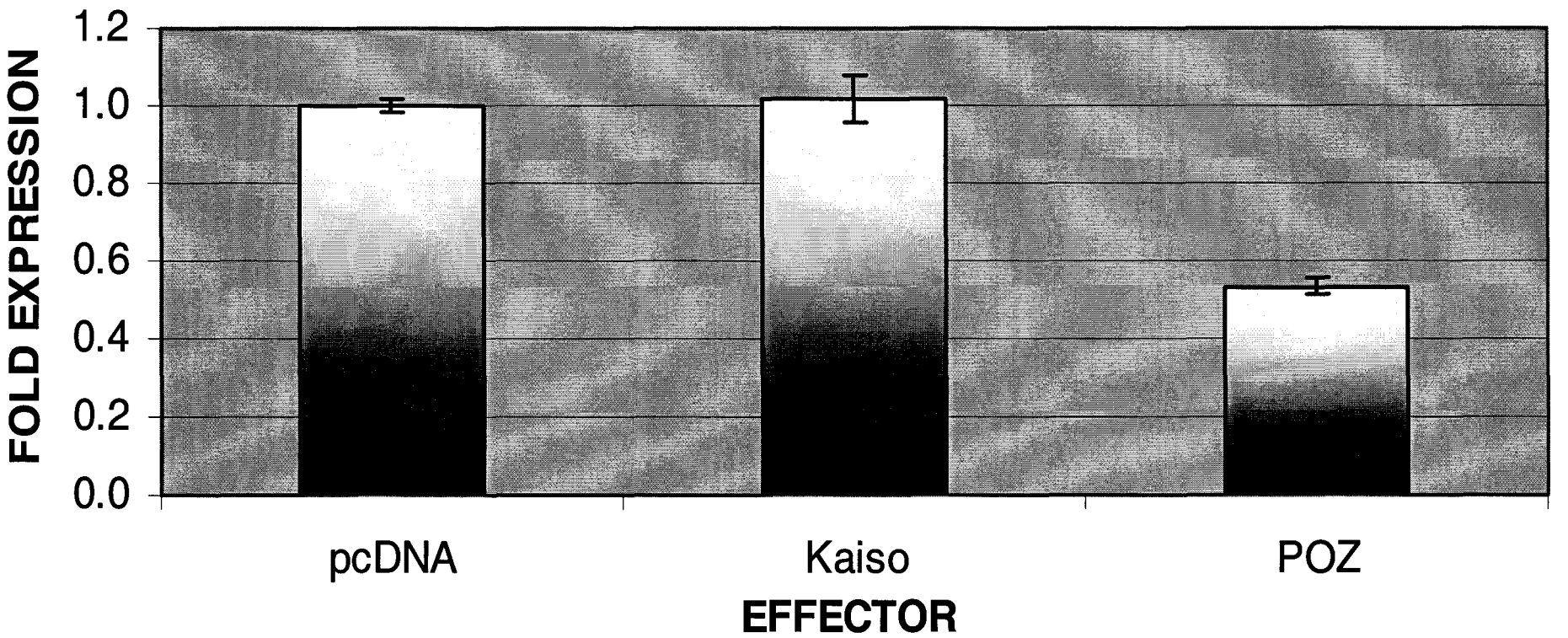
APPENDIX 6: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 6). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 6)



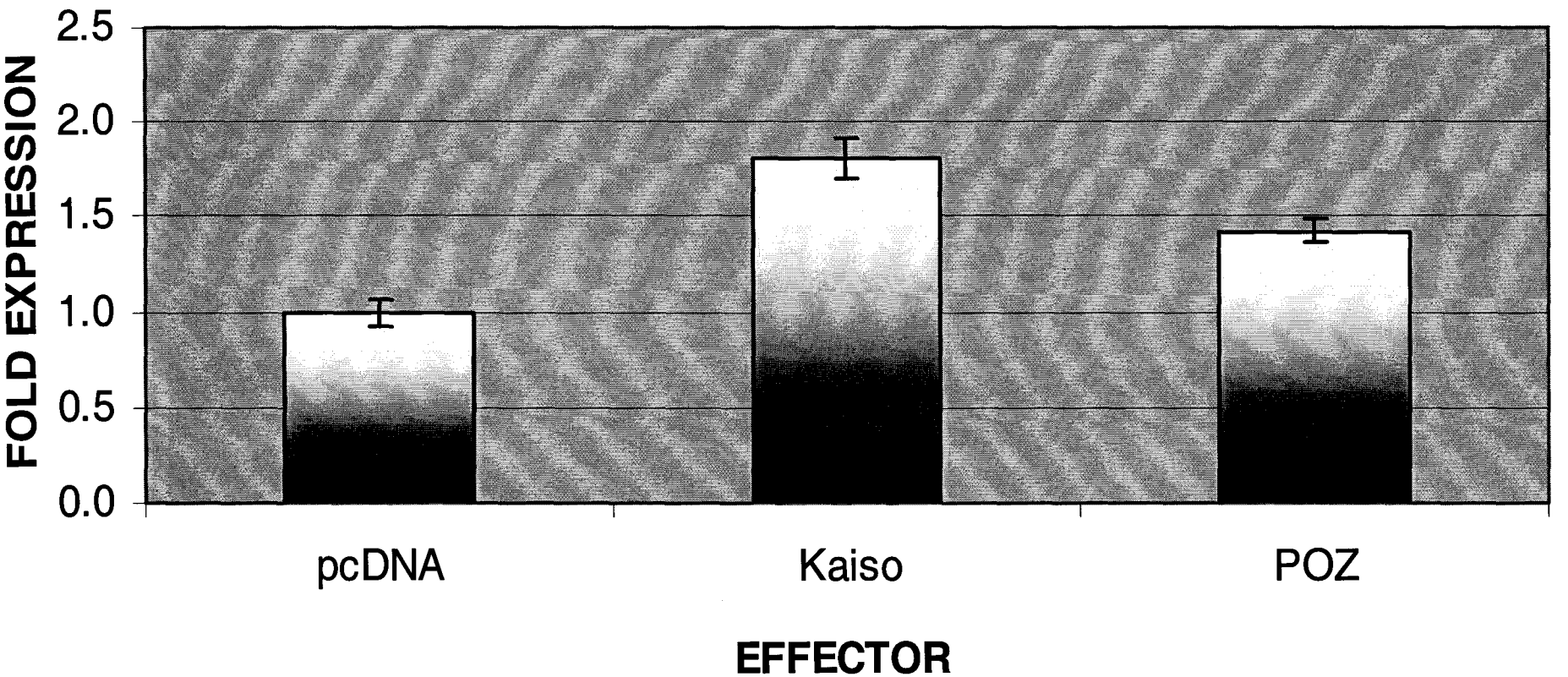
APPENDIX 7: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 7). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 7)



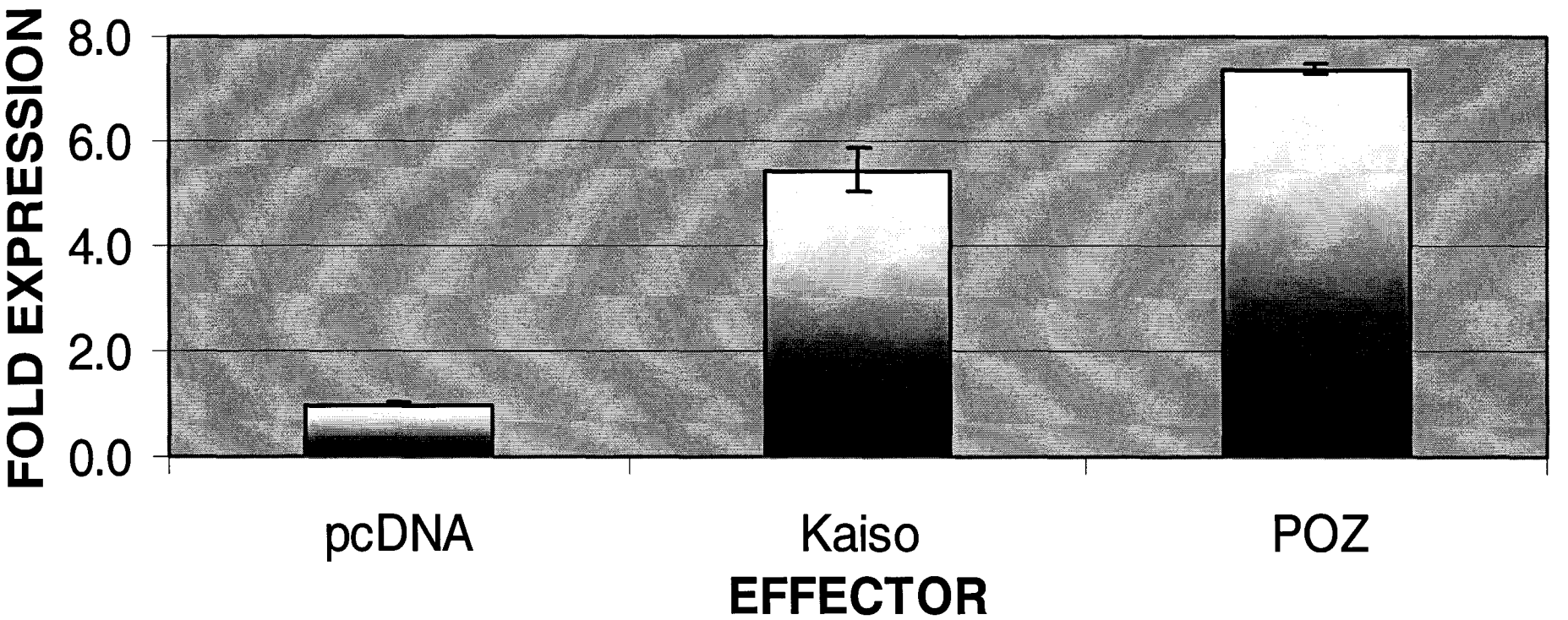
APPENDIX 8: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 8). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 8)



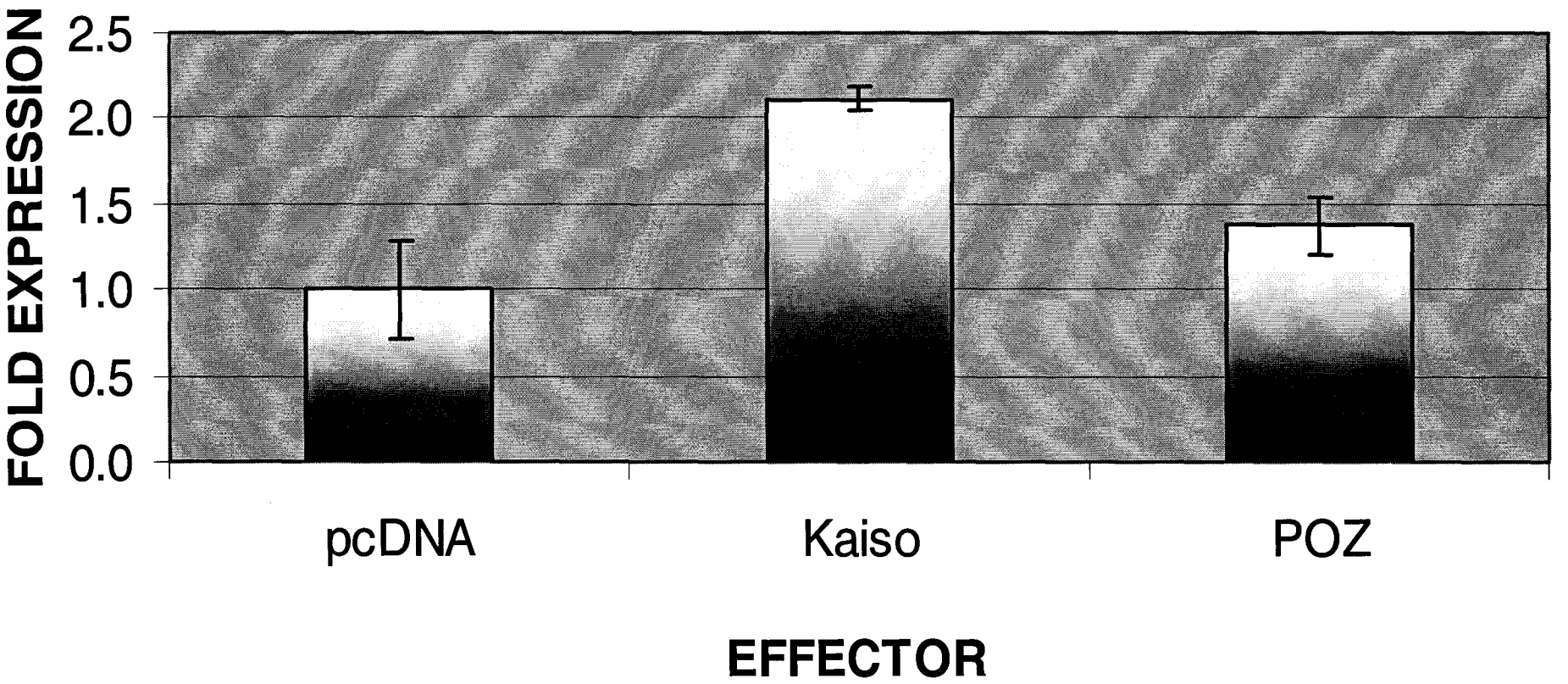
APPENDIX 9: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 9). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 9)



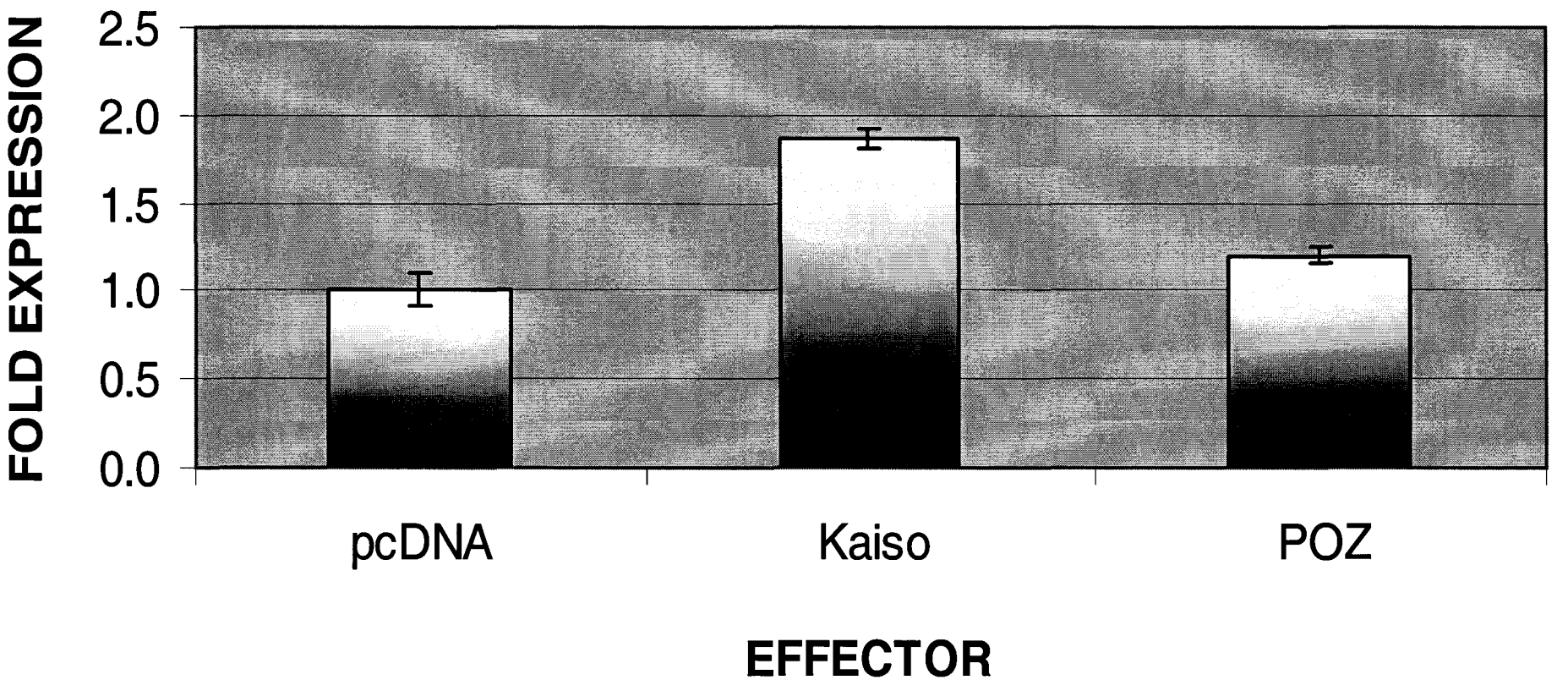
APPENDIX 10: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 10). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 10)



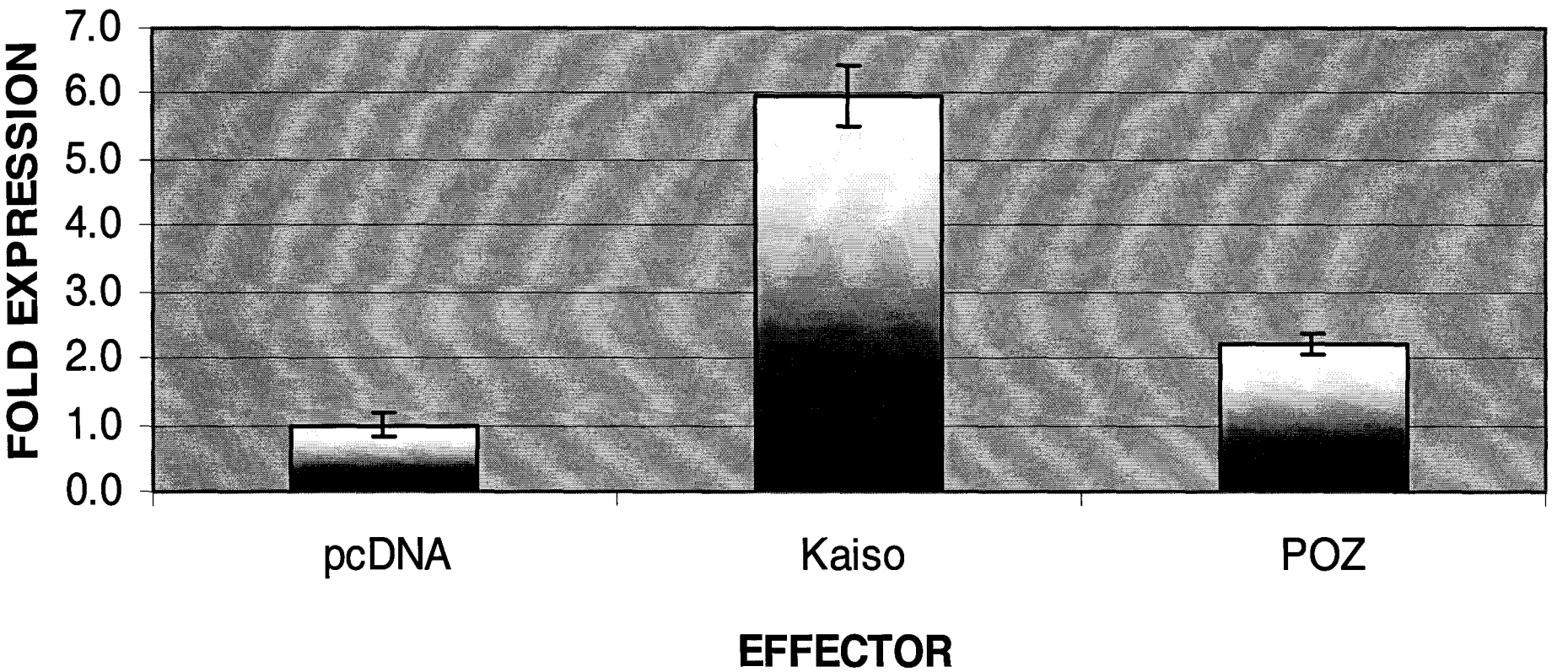
APPENDIX 11: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCT116 Cells (Trial 11). 40%-60% confluent HCT116 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCT116 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 11)



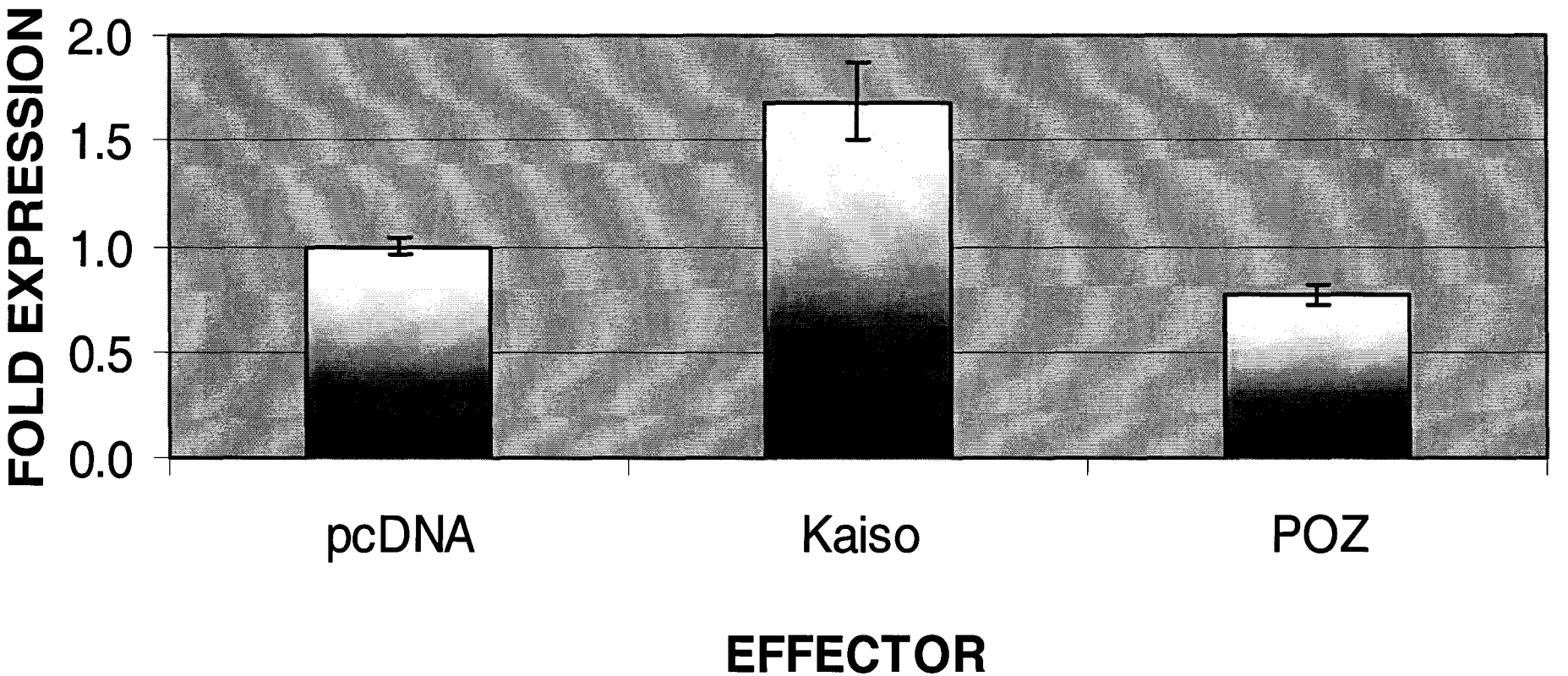
APPENDIX 12: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCA-7 Cells (Trial 1). 40%-60% confluent HCA-7 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCA-7 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 1)



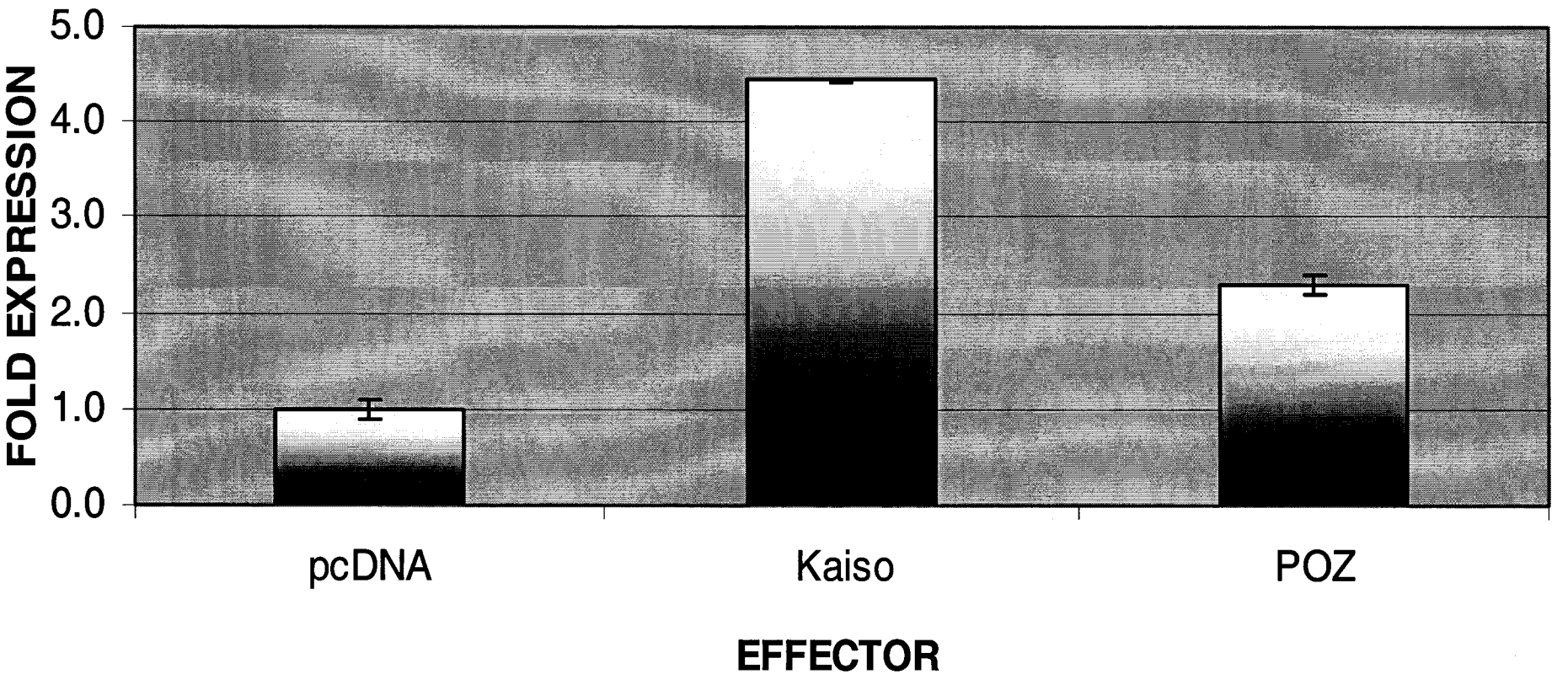
APPENDIX 13: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCA-7 Cells (Trial 2). 40%-60% confluent HCA-7 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCA-7 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 2)



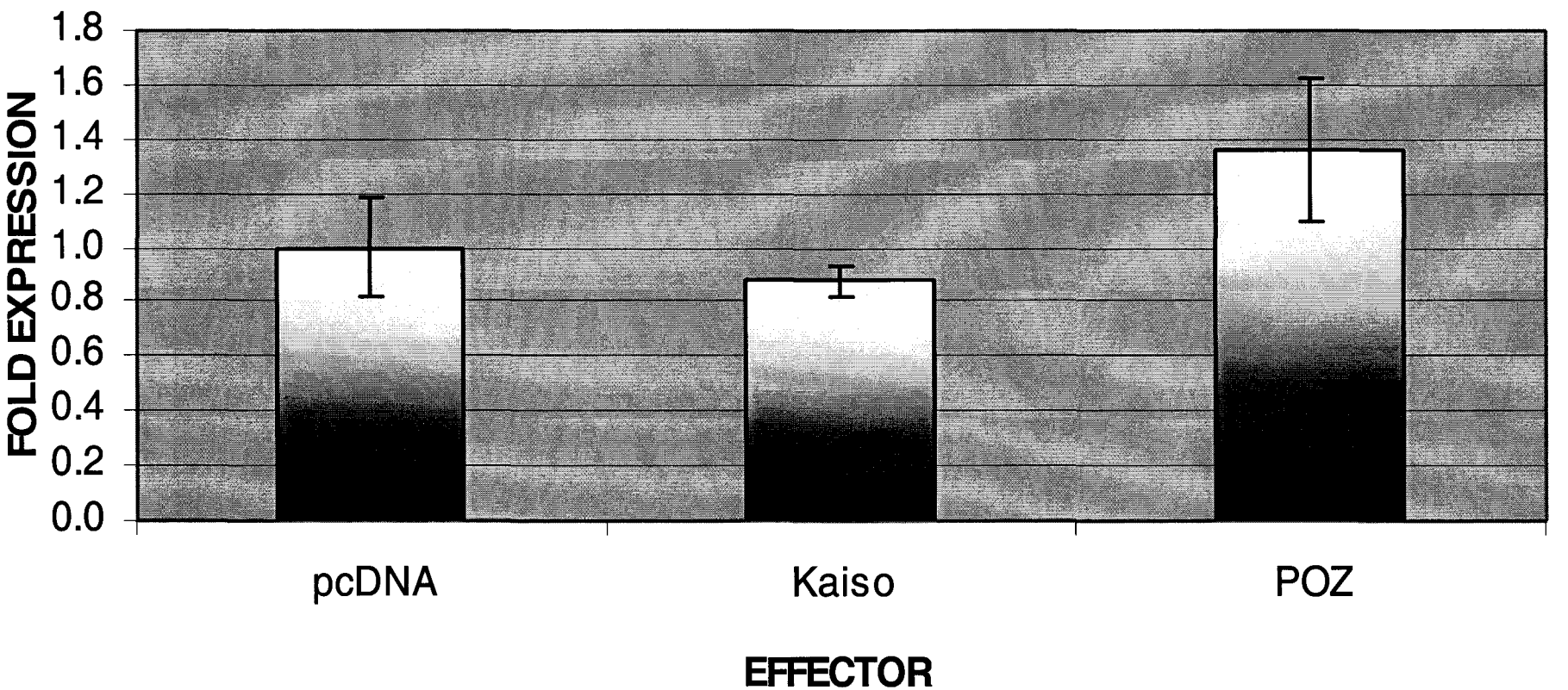
APPENDIX 14: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCA-7 Cells (Trial 3). 40%-60% confluent HCA-7 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCA-7 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 3)



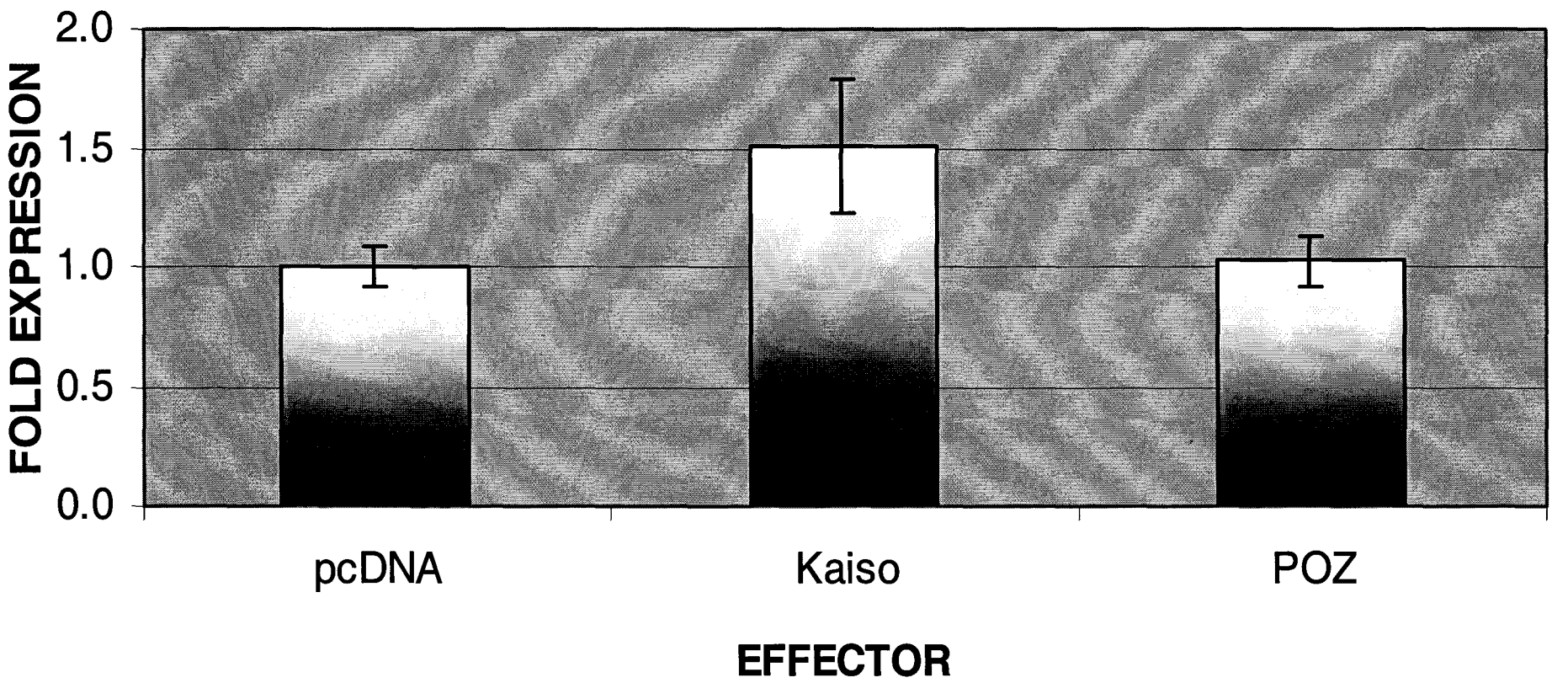
APPENDIX 15: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCA-7 Cells (Trial 4). 40%-60% confluent HCA-7 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCA-7 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 4)



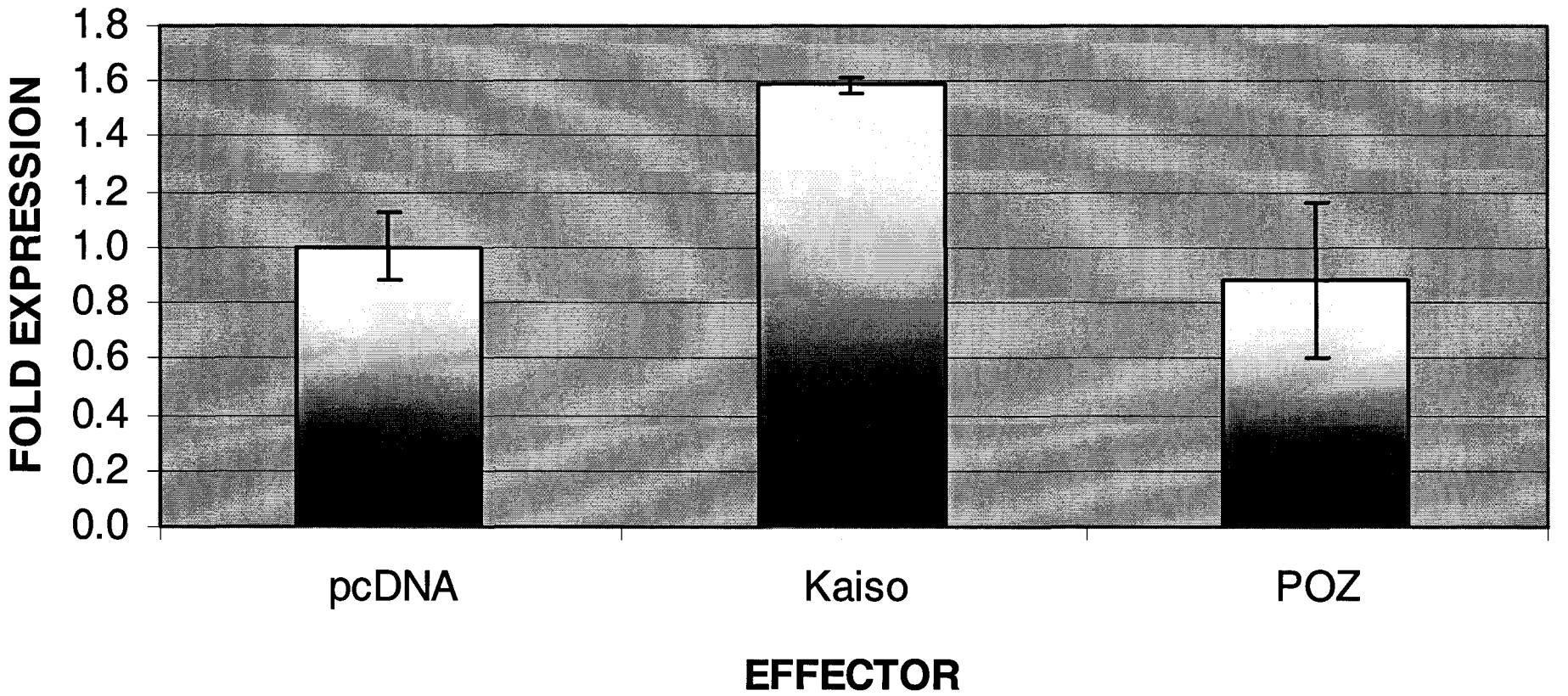
APPENDIX 16: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in HCA-7 Cells (Trial 5). 40%-60% confluent HCA-7 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN HCA-7 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 5)



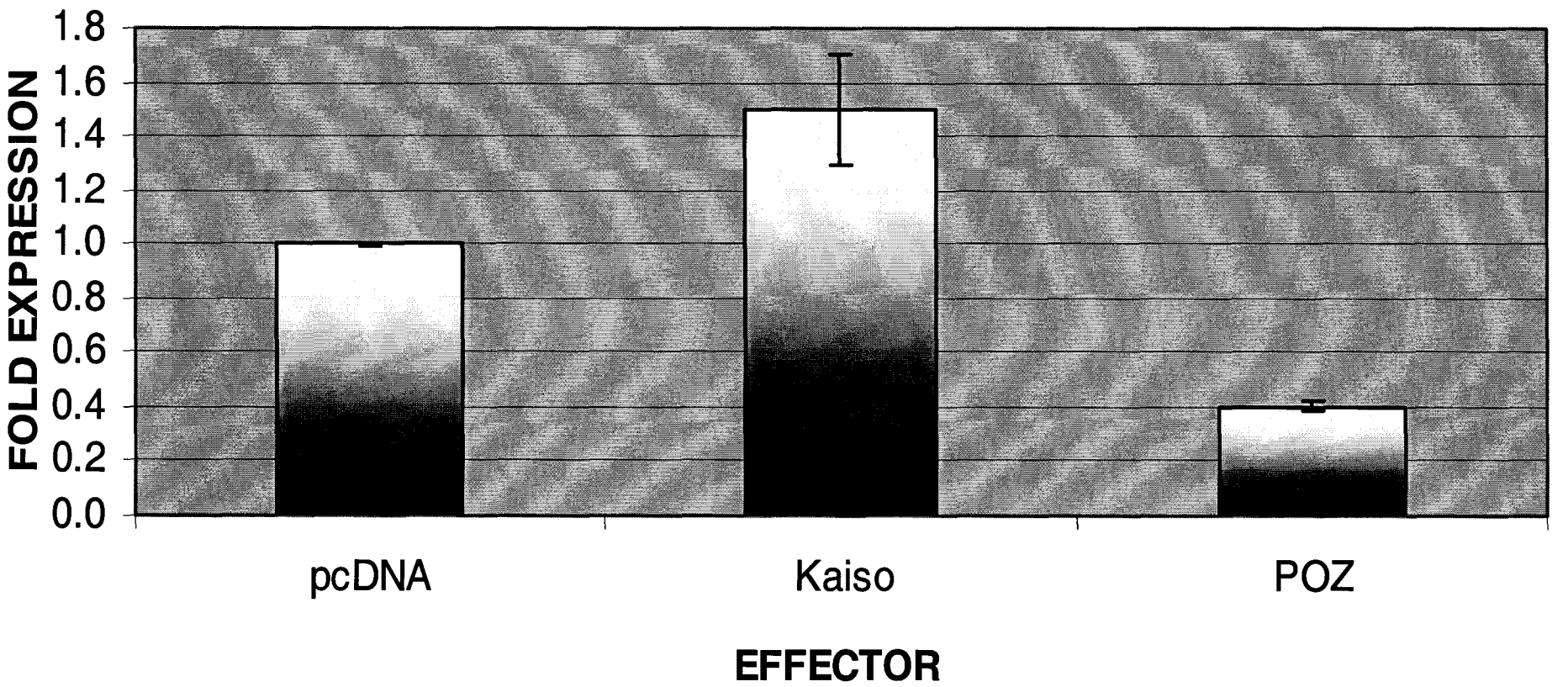
APPENDIX 17: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 1). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, Kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 1)



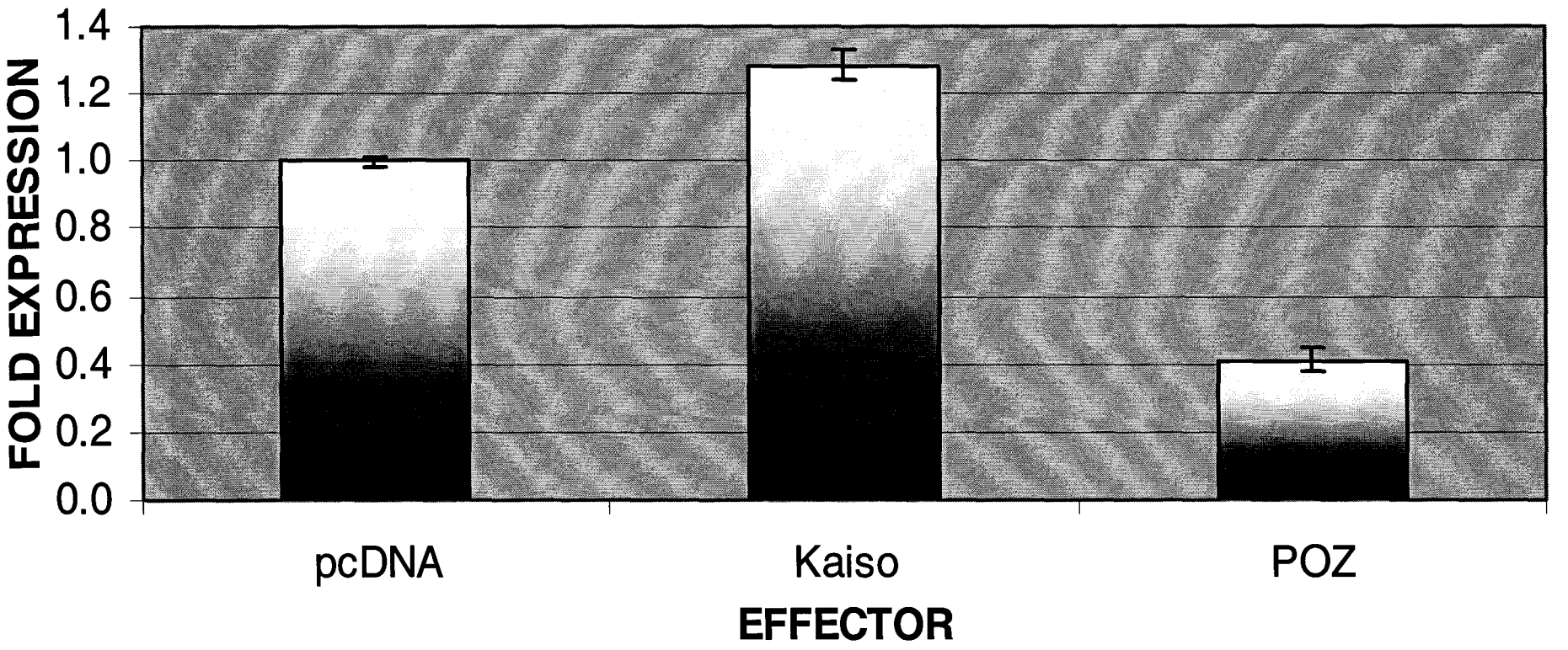
APPENDIX 18: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 2). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 2)



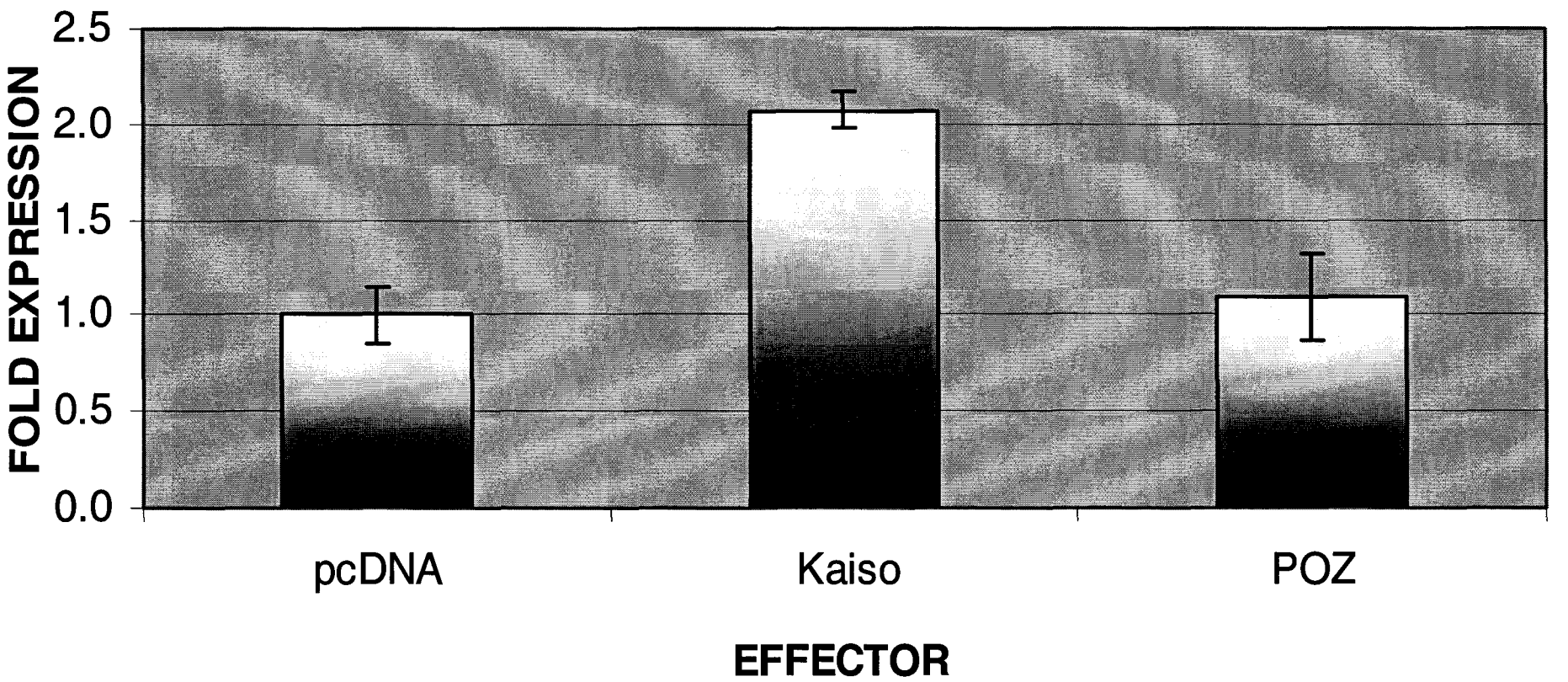
APPENDIX 19: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 3). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 3)



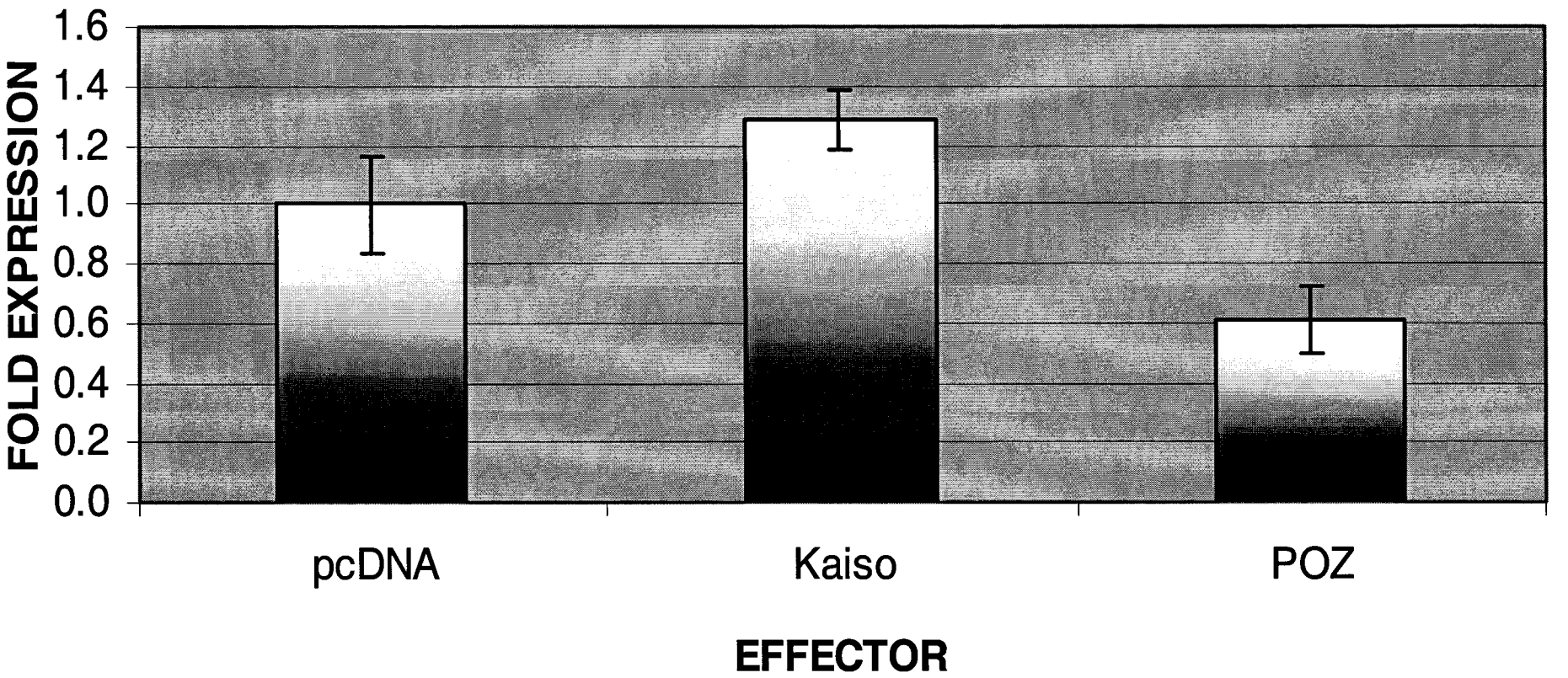
APPENDIX 20: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 4). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 4)



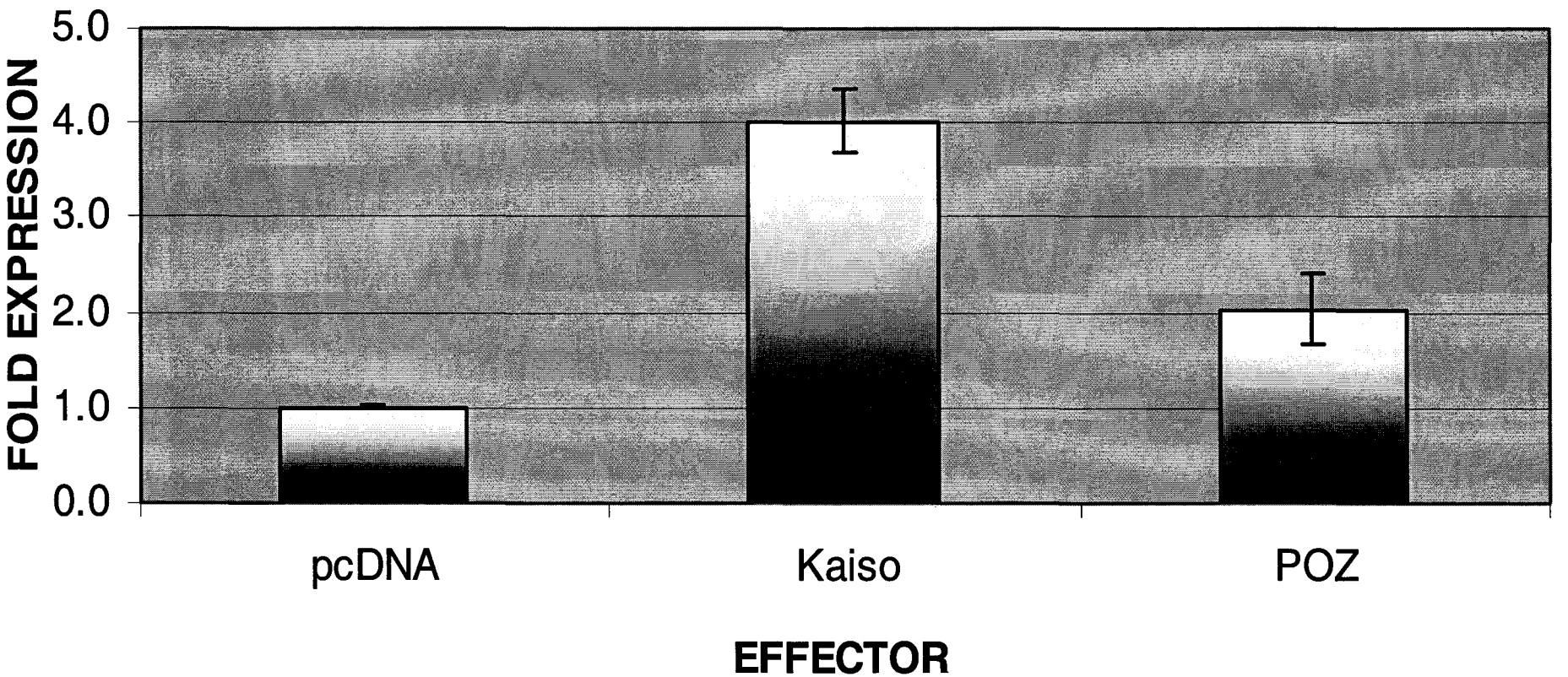
APPENDIX 21: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 5). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 5)



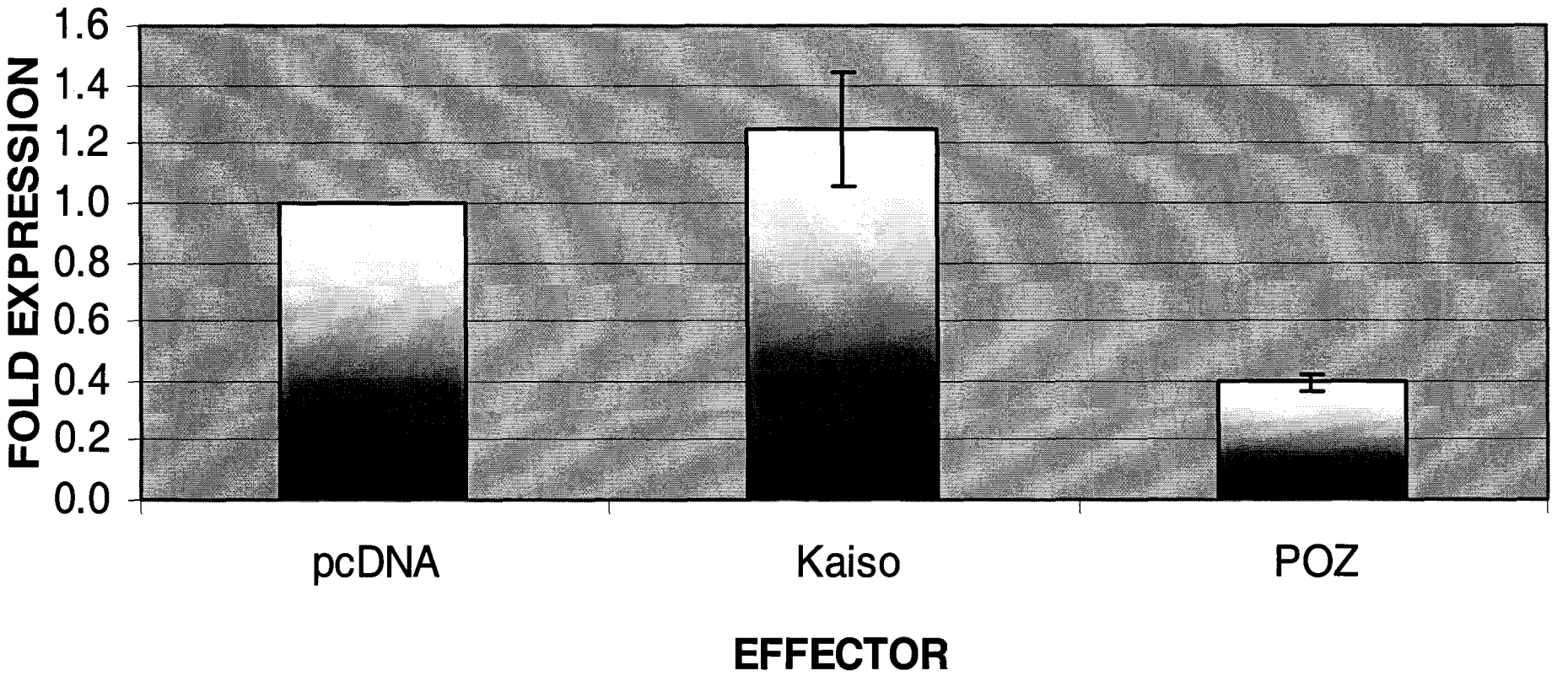
APPENDIX 22: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 6). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 6)



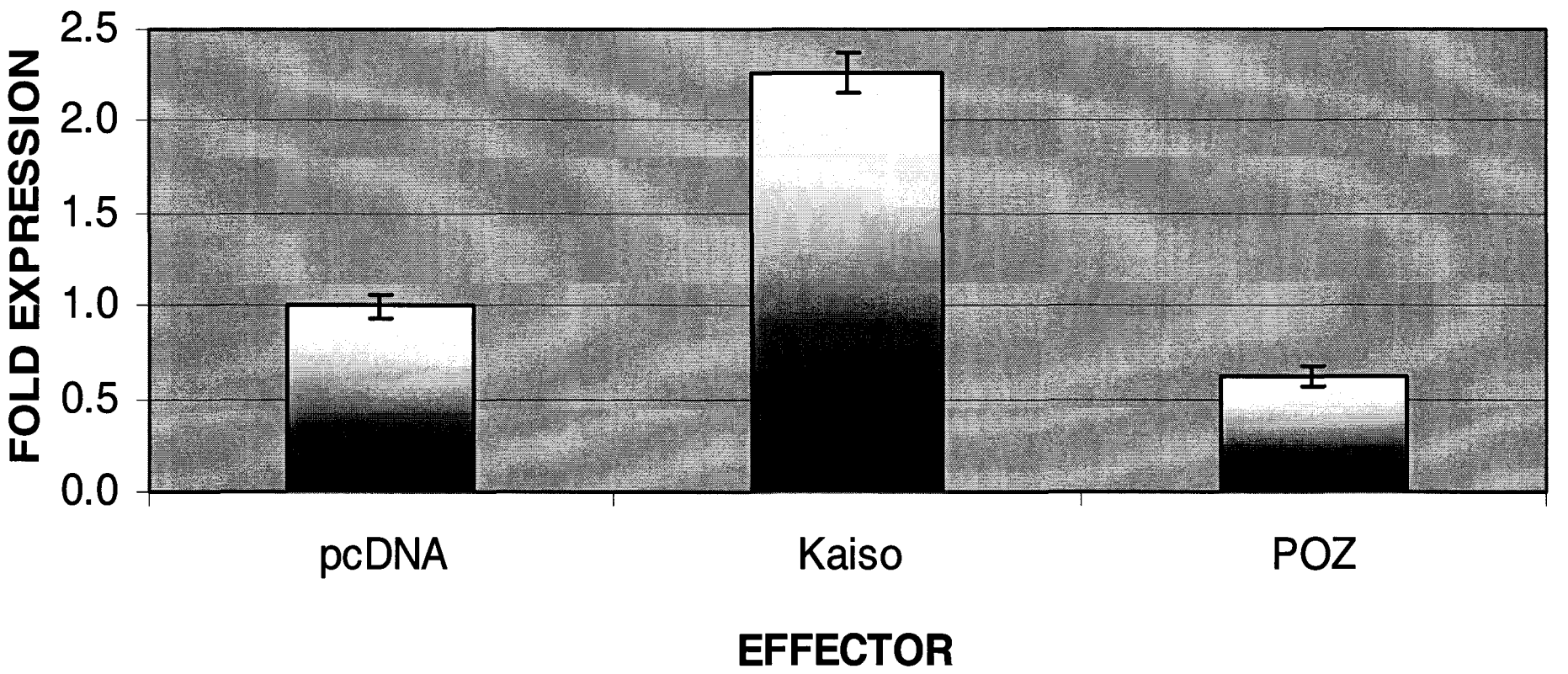
APPENDIX 23: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 7). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 7)



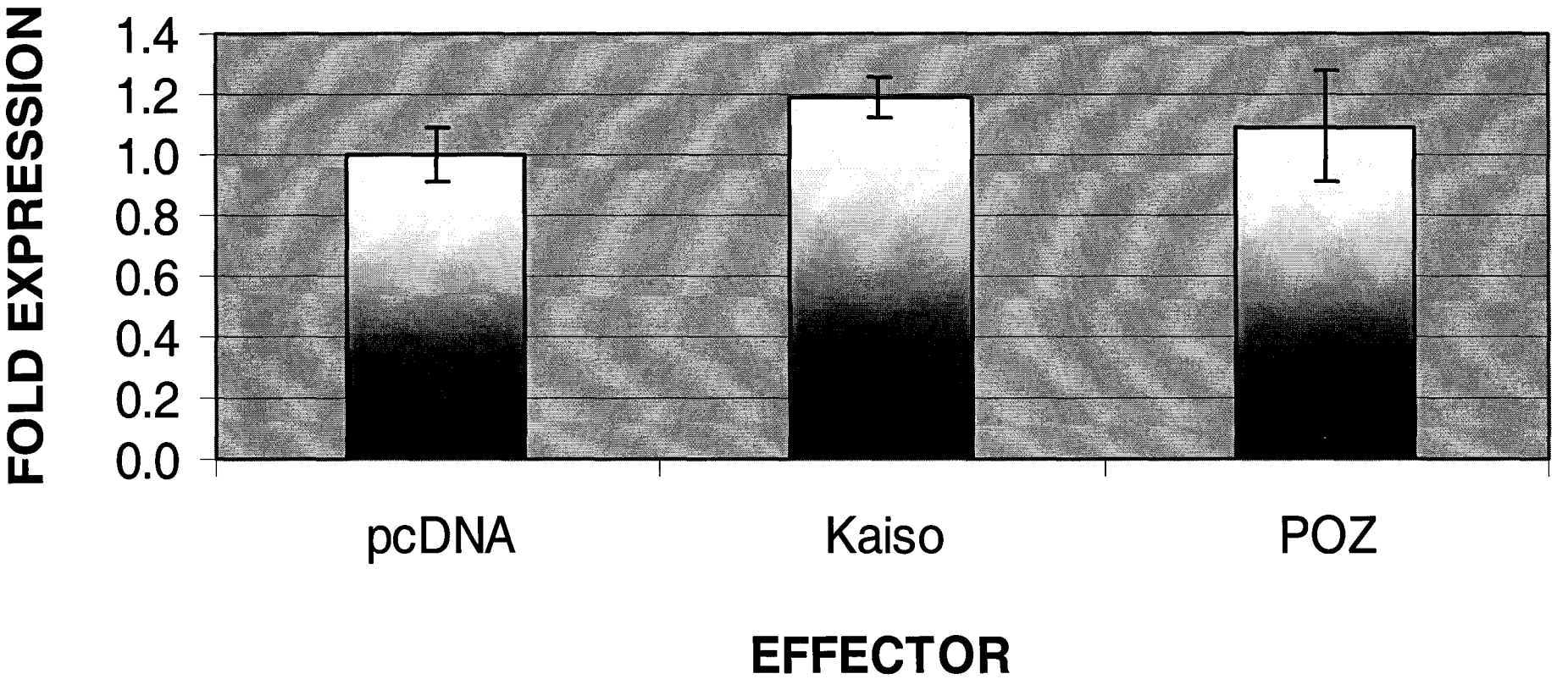
APPENDIX 24: Activation by Kaiso on 4XKBS-pGL3 Reporter Expression in 293 Cells (Trial 8). 40%-60% confluent 293 cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN 293 CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 8)



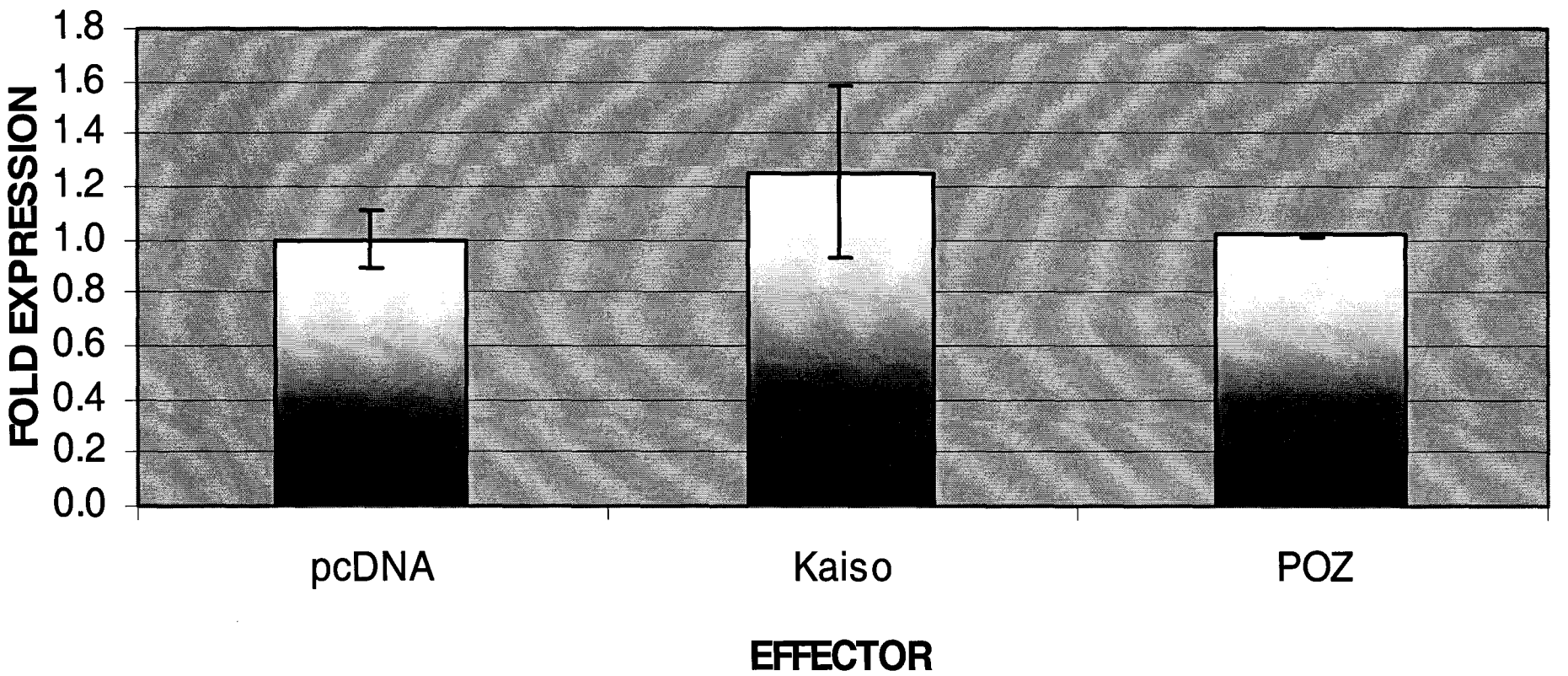
APPENDIX 25: No Effect by Kaiso on 4XKBS-pGL3 Reporter Expression in MDCK Cells (Trial 1). 40%-60% confluent MDCK cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN MDCK CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 1)



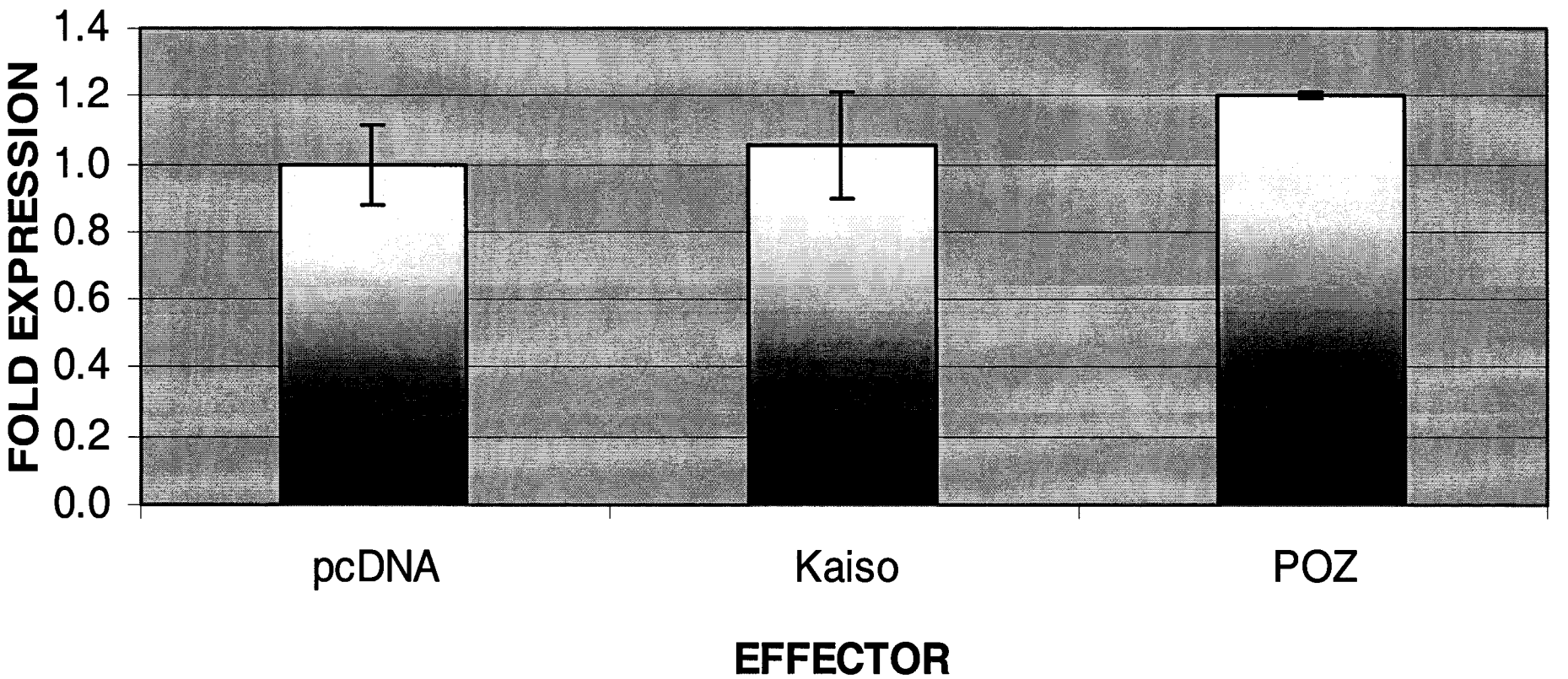
APPENDIX 26: No Effect by Kaiso on 4XKBS-pGL3 Reporter Expression in MDCK Cells (Trial 2). 40%-60% confluent MDCK cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN MDCK CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 2)



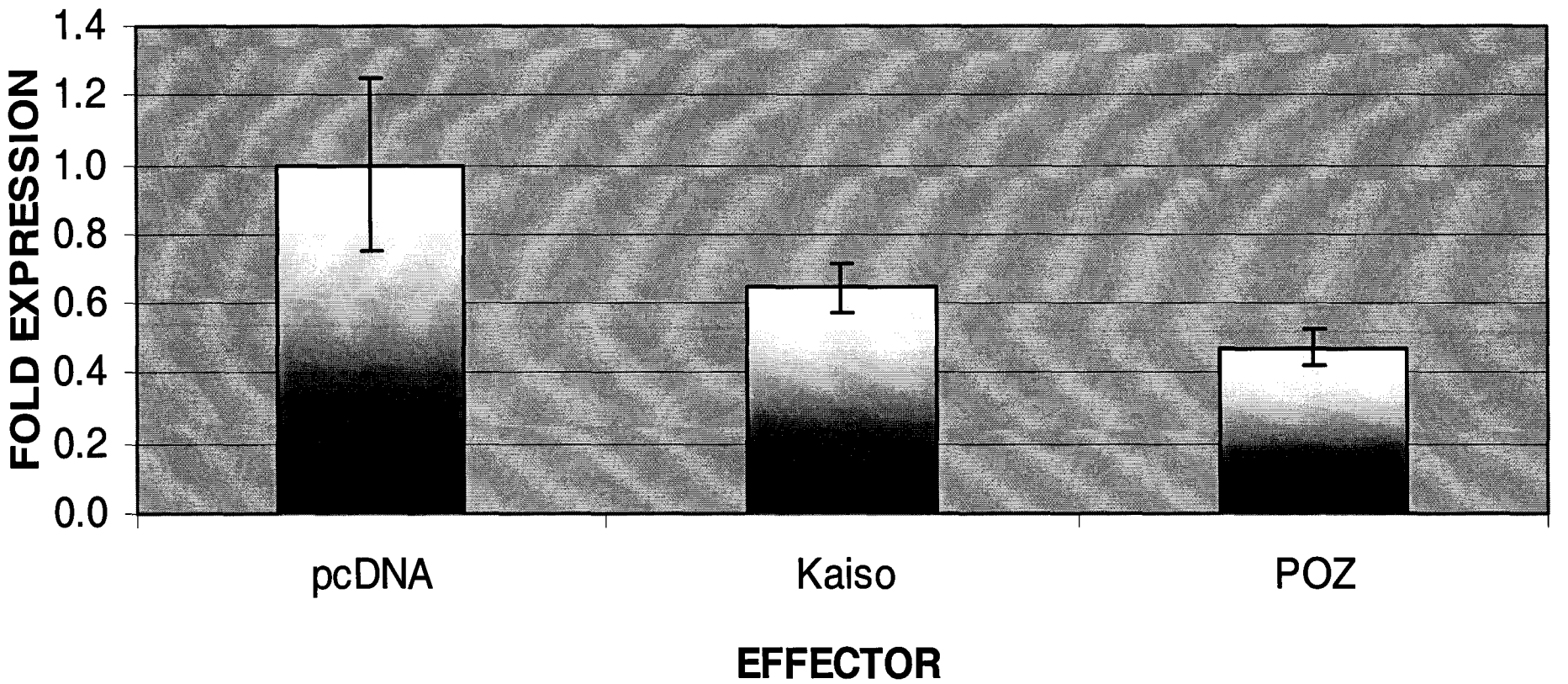
APPENDIX 27: No Effect by Kaiso on 4XKBS-pGL3 Reporter Expression in MDCK Cells (Trial 3). 40%-60% confluent MDCK cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN MDCK CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 3)



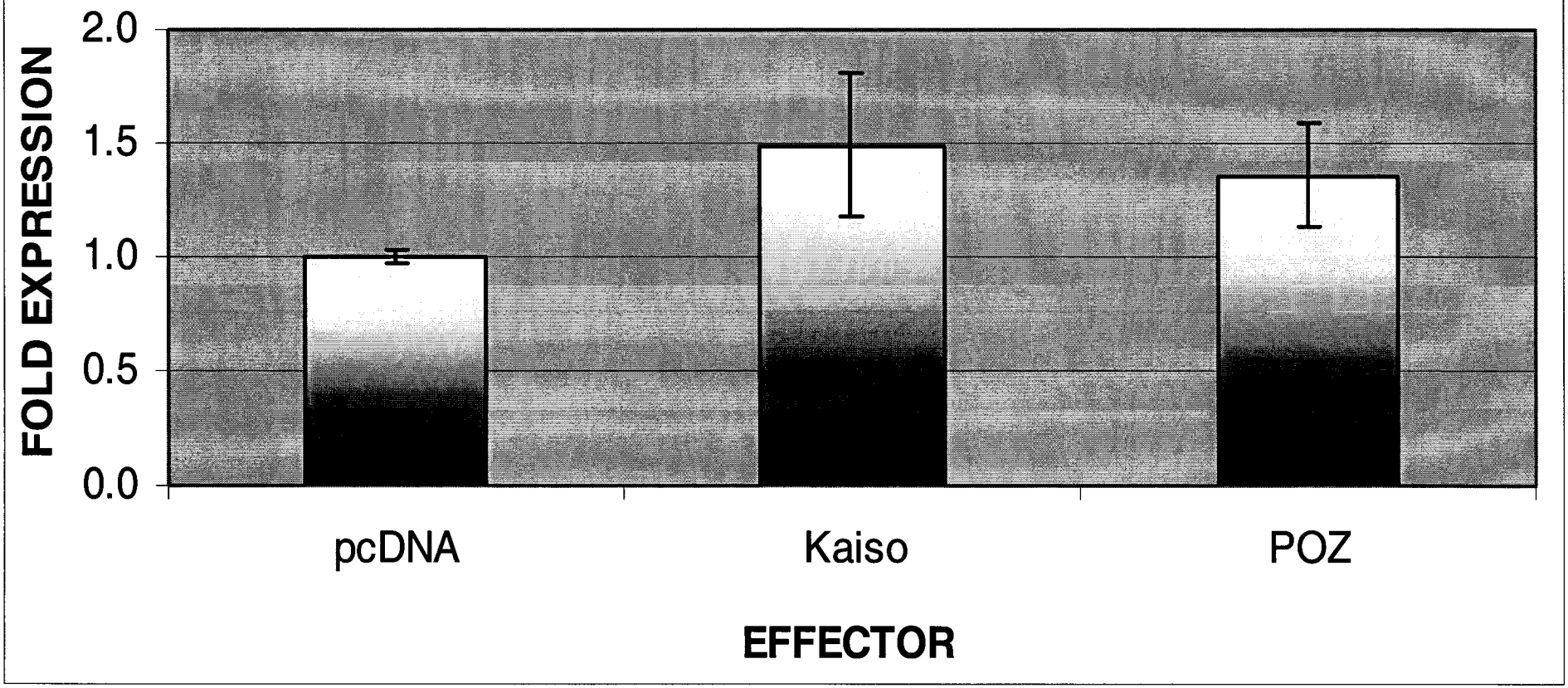
APPENDIX 28: Repression by Kaiso on 4XKBS-pGL3 Reporter Expression in MDCK Cells (Trial 4). 40%-60% confluent MDCK cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

EFFECT OF KAISO ON REPORTER EXPRESSION IN MDCK CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 4)

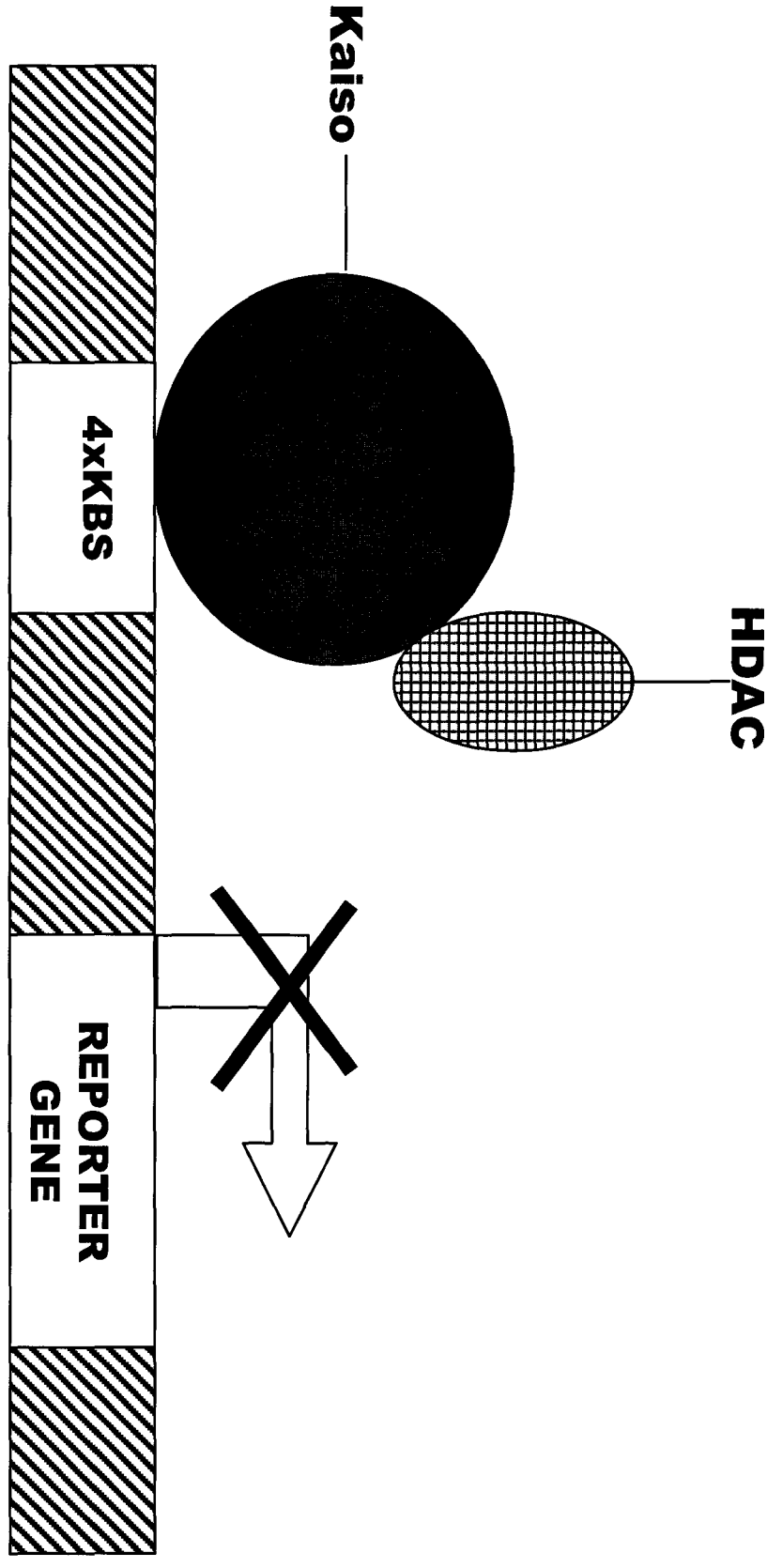


APPENDIX 29: No Effect by Kaiso on 4XKBS-pGL3 Reporter Expression in MDCK Cells (Trial 5). 40%-60% confluent MDCK cells were transiently cotransfected in triplicate with 800 ng 4xKBS-pGL3 reporter and 200 ng of either empty *pcDNA3, kaiso* or *poz* as indicated by the x-axis. The cells were then assayed for reporter expression 24 hours post-transfection using a Luciferase Assay Kit (Promega). The empty pcDNA3 (bar 1) represented background expression and was standardized to a value of 1. Standard deviation is depicted by the error bars.

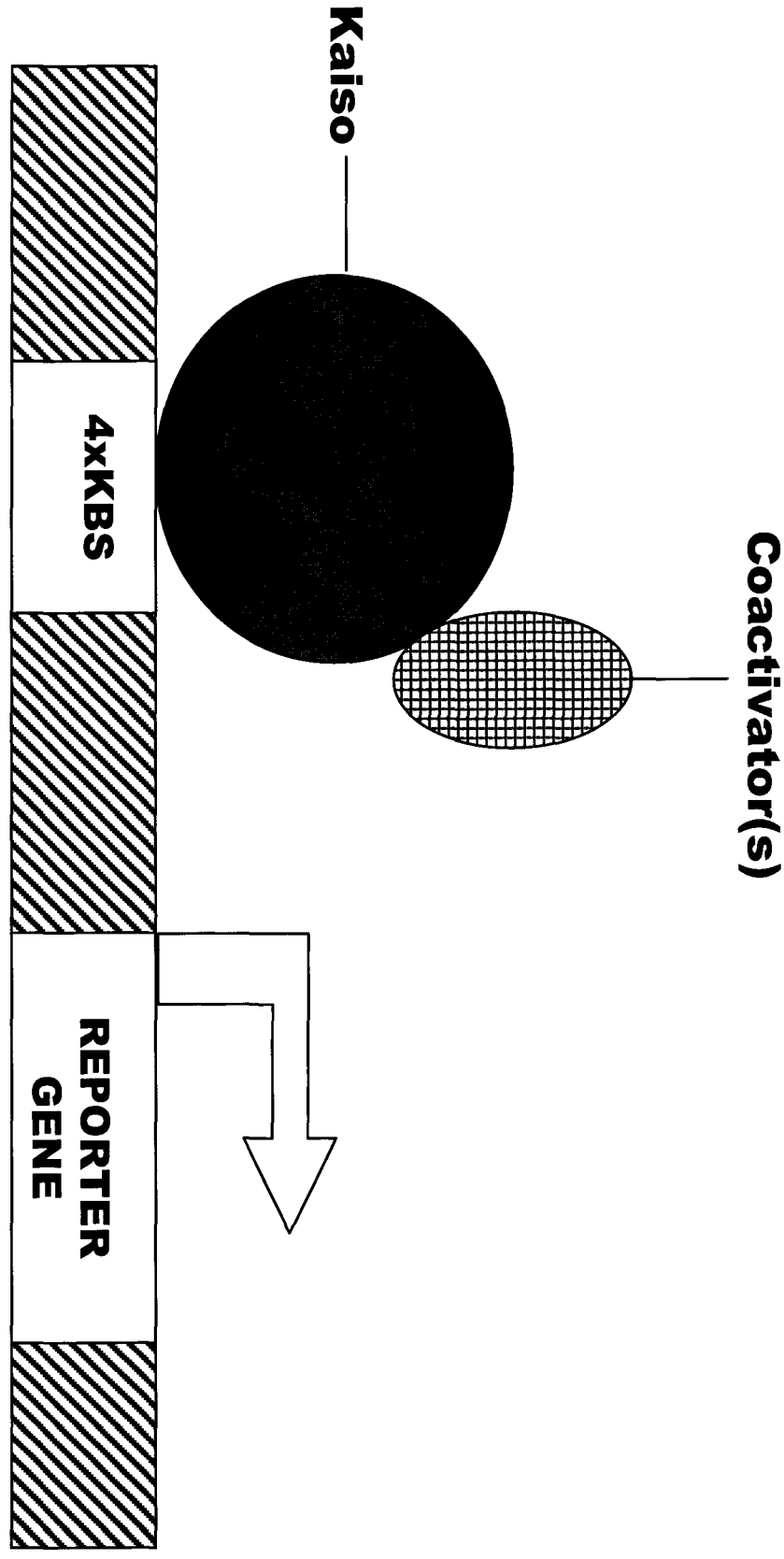
EFFECT OF KAISO ON REPORTER EXPRESSION IN MDCK CELLS USING 4XKBS-pGL3 REPORTER (TRIAL 5)



APPENDIX 30: Proposed Model For Kaiso Function. Based on the current literature and similarities to PLZF and BCL-6, Kaiso is proposed to be a transcriptional repressor that recruits the HDAC repression complex through an interaction with HDAC members.


Kaiso
HDAC
4xKBS
REPORTER GENE

APPENDIX 31: Revised Model For Kaiso Function. Based on the data established from these studies, Kaiso is proposed to be a transcriptional activator that may function by recruiting unidentified coactivators.


Kaiso
Coactivator(s)
4xKBS
REPORTER GENE

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