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糖皮質素受體及其共同活化因子核受體交互作用蛋白質在人類子宮頸瘤病毒十六型轉錄調控區域之角色

The Roles of Glucocorticoid Receptor and Its Coactivator NRIP in the Regulation of Human Papillomavirus Type 16 Long Control Region

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糖皮質素受體及其共同活化因子核受體交互作用蛋白質在人類子宮頸瘤病毒十六型轉錄調控區域之角色

The Roles of Glucocorticoid Receptor and Its Coactivator NRIP in the Regulation of Human Papillomavirus Type 16 Long Control Region

本論文係呂培瑜君（學號 R95445110）在國立臺灣大學微生物學所完成之碩士學位論文，於民國 97 年 7 月 18 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

人類乳突瘤病毒十六型 (HPV-16) 感染是造成子宮頸癌的主因，而且此癌症的發生與體內的糖皮質激素濃度相關。在前人的研究中指出糖皮質素 (glucocorticoid) 會藉由位於 HPV-16 之 long control region (LCR) 中的三個糖皮質素反應序列 (glucocorticoid response element, GRE) 來增強 HPV-16 的轉錄能力，以提升其下游的主要致癌蛋白質 E6 及 E7 之表現，因而促成細胞轉形及癌症發展。此外，GR 屬於細胞核受體家族的一員，具有轉錄因子的功能，在受到配體刺激的情況之下，會結合至目標基因上游的 GRE，並招募其他共同活化因子或共同抑制因子前來進行調控，顯示 GR 的轉錄共同因子在 HPV-16 的調控中，必定也扮演重要角色。其中，本實驗室先前發現之細胞核受體交互作用蛋白質 (nuclear receptor interaction protein, NRIP) 已被證明能夠增強 GR 的轉錄活性，可作為 GR 之共同活化因子。因此，本論文針對 GR 及其共同活化因子 NRIP 在 HPV-16 LCR 之調控中所扮演的角色進行研究。首先，我們以染色質免疫沉澱法 (chromatin immunoprecipitation, ChIP) 證明 GR 在受到配體 dexamethosome (Dex) 的刺激後，會結合至 HPV-16 LCR 上的 GRE。啟動子活性分析的結果除了證實 HPV-16 LCR 上的三個已知 GREs 都能被 GR 所增進，序列刪除突變株的結果更指出第四個具有功能的 GRE 之存在。在細胞受到配體刺激的情形下，我們以 ChIP 證實共同活化因子 NRIP 會藉由 GR 共同結合至 HPV-16 LCR。以核酸干擾技術降低 NRIP 的表現量後，HPV-16 的 E6 及 E7 的 mRNA 表現量也隨之降低，子宮頸癌細胞株受 GR 配體促進而提升的生長速率也隨之減緩。

本篇結果顯示 GR 藉由結合 HPV-16 LCR 上的四個 GREs 來促進其轉錄活性，而共同活化因子 NRIP 又對 GR 的調控功能扮演著舉足輕重的角色。

關鍵字：子宮頸癌、人類乳突瘤病毒、糖皮質素受體、核受體交互作用蛋白質、轉錄調控

Abstract

The major cause of cervical cancer is infection of human papillomavirus type 16 (HPV-16), which pathogenicity and oncogenicity are correlated with the hormone glucocorticoid. Previous studies have shown that glucocorticoid upregulates the transcription activity through the three glucocorticoid response elements (GREs) in HPV-16 long control region (LCR), enhancing the expression of the two major oncogenes E6 and E7 downstream, resulting in cell transformation and oncogenesis.

Moreover, GR belongs to Type I nuclear receptor family and serves as transcription factor. When treated with ligand, GR binds to the GREs upstream of its target genes and recruits other coregulators to exert its function, implicating that the cofactors of GR may play roles in the regulation of HPV-16. Previously, our lab found a novel cofactor of GR, named NRIP, which was demonstrated to upregulate the transactivity of GR. Hence, we investigate the roles of GR and its coactivator NRIP in the regulation of HPV-16. First, we demonstrated that GR bound to the GRE(s) in HPV-16 LCR upon ligand dexamethosome (Dex) treatment by chromatin immunoprecipitation (ChIP). The results of luciferase activity assays not only proved the three unknown GREs were separately functional, but also revealed the existence of the fourth GRE. We also proved that NRIP bound HPV-16 LCR through GR in the

presence of Dex. Knockdown of NRIP downregulated the mRNA expression of HPV-16 E6 and E7, and also decreased the cell proliferation promoted by Dex.

These results indicate that GR enhances the transcription activity of HPV-16 LCR by binding to the four GREs, and cofactor NRIP also plays an important role in GR-mediated regulation.

Keywords: cervical cancer, human papillomavirus type 16 LCR (HPV-16 LCR), glucocorticoid receptor (GR), nuclear receptor interaction protein (NRIP)

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1. Introduction

1.1 HPV and cervical cancer risk

Cervical cancer is the second most women cancer worldwide and the most common in Taiwan (Pisani et al, 2002; Wang, 2007). The major cause of cervical cancer has been identified to be certain types of human papillomaviruses (HPVs) (zur Hausen, 1989; zur Hausen, 1991), which DNA has been identified in greater than 95% of all cervical tumors (Walboomers et al, 1999).

Human papillomaviruses (HPVs) are a group of small double-stranded DNA viruses that specifically infect and induce proliferative lesions of mucosal and cutaneous epithelium (PM, 1996). Over 100 types of HPV have been identified, with roughly one-third infecting the genital epithelium (Hebner & Laimins, 2006; zur Hausen, 2002). HPVs can be classified into low risk or high risk categories based on the potential for induction of malignant transformation. Low-risk HPV types, most frequently HPV6 and 11, typically cause benign warts and are rarely associated with malignancy. In contrast, high-risk types, most commonly HPV16, 18, 31 and 45 have been linked to the development of cervical carcinoma as well as other genital malignancies (Lorincz et al, 1992; zur Hausen, 2002).

1.1.1 HPV genome

The circular genomes of HPVs encode eight ORFs that are transcribed as polycistronic mRNAs, which are processed through alternative splicing and then translated. There are two promoters in the genomes of high-risk HPVs. One is the early promoter named P97, from which transcripts encoding E6, E7, E1 and E2 are transcribed at the beginning of infection. The other is the differentiation-dependent late promoter, from which E1, E2, E4, E5 and the capsid proteins L1and L2 are transcribed during the productive phase of the viral life cycle (Hebner & Laimins, 2006).

1.1.2 The regulation of HPV transcription

Early viral transcription is regulated by elements located in a non-coding region proximal to the E6 ORF called the upstream regulatory region (URR), or the long control region (LCR). The LCR is about 1 kb in size and contains a 400-bp cell type specific enhancer spanning the DNA region from 7454 to 7854 bp (Gloss et al, 1987). Various transcription factors have been demonstrated to regulate HPV genome. The enhancer region contains binding sites for a variety of cellular transcriptional factors, including AP-1, cEBP, NF-1, oct-1, TEF-1, TEF-2, Sp-1, and YY1 (Bernard & Apt, 1994; Chong et al, 1991; Gloss et al, 1989; Ishiji et al, 1992; Kanaya et al, 1997;

Morris et al, 1993a; Morris et al, 1993b; Tan et al, 1994).

Nuclear receptor factor I (NFI) binds the palindromic sequence TTGGCNNNNNNCCAA but also has reduced affinity to half-palindromes, i.e., TTGGC or GCCAA. The enhancers of all HPV types are activated in epithelial cells by three to seven half-palindromic NFI binding sites (Chong et al, 1991).

TEF-1 contains variations in its binding motif GCACAT. Four TEF-1 sites play an important role in the enhancer of HPV-16. TEF-1 is not epithelial specific, but it functions depend on a cofactor which is present in epithelial cells (Ishiji et al, 1992).

Viral early protein E2 is another transcription factor of HPV genome, which binds to the ACCN6GGT motif in the LCR (Androphy et al, 1987). E2 also associates with nuclear receptors to stimulate nuclear receptor- and E2-dependent transcriptional activations (Wu et al, 2007a; Wu et al, 2007b).

1.1.3 HPV E6 and E7 oncoproteins

The E6 and E7 proteins are the main determining factors in the development of HPV-induced cervical carcinoma. The best-known function of E6 is to interfere with p53-mediated cell-cycle arrest or apoptosis. E6 binds and facilitate the rapid turnover of p53, allowing viral replication (Scheffner et al, 1990). In the meanwhile, one of the

main functions of E7 is to bind and degrade hypophosphorylated Rb, thereby constitutively releasing E2F, activating transcription in S phase (Boyer et al, 1996).

Both E6 and E7 proteins are essential to induce and maintain cellular transformation, due to their interference with cell cycle control and apoptosis. Genomic instability is thought to be an essential part of the cellular transformation and it has been shown that E6 and E7 together cause polyploidy soon after they are introduced into cells. This appears to result from deregulation of Plk1 by the loss of p53 through E6, and pRb family members by E7, overcoming the safeguard arrest response (Incassati et al, 2006). Acute loss of pRb family members by E7 has also shown to induce centrosome amplification and aneuploidy (Iovino et al, 2006), In addition, E6 and E7 cause deregulation of cellular genes controlling the G2/M phase transition and progression through mitosis, such as the genes controlling centrosome homeostasis (Patel et al, 2004; Walboomers et al, 1999).

1.2 Glucocorticoid receptor and cervical cancer risk

Although HPV has been identified as the major causative agent of cervical cancer, HPV infection alone is not sufficient for cervical cancer progression. Various agents have been reported either as an association or causation of cervical cancer, one of

which is steroid hormones.

The linkage between steroid hormones and cervical cancer was first perceived in long term steroid oral contraceptive users, who had increased risk of progressing from cervical dysplasia to cancer *in situ* (Fortney et al, 1985; Stern et al, 1977). At the same time, HPV replication was reported to be activated in the lower genital tract of pregnant women (Schneider et al, 1987). Under these two situations, the progesterone concentrations are increased.

Among the various nuclear steroid hormone receptors, progesterone receptor (PR) and glucocorticoid receptor (GR) share the same characteristics. They both belong to type I nuclear hormone receptor superfamily, which enter nucleus to bind specific DNA sequence and serve as transcription factors upon the stimulus of ligand.

1.2.1 Glucocorticoid receptor response elements (GREs) on HPV-16 promoter

In addition to the clinical phenomena, the regulation mechanism of glucocorticoid on HPV has been investigated. Oncogenic transformation by HPV-16 DNA is glucocorticoid hormone dependent (Pater et al, 1988).

GR exerts its function by binding consensus partial palindromic DNA sequences, called GR response elements (GREs). The LCR of HPV16 genome was found to

contain at least three GREs, including 7640 bp to 7655 bp (Chan et al, 1989; Gloss et al, 1987), 7385 bp to 7400 bp and 7474 bp to 7489 bp (Mittal et al, 1993b), here designated GRE3, GRE1, and GRE2 for short (**Figure 1**).

The binding between GR protein and GREs was demonstrated by DNase I footprinting (Gloss et al, 1987) and mobility shift assays (Mittal et al, 1993a). The three GREs were activated when treated with synthetic glucocorticoid dexamethasone (Dex) or progesterone, thereby increasing its transcription activity, viral gene expression, immortalization, and transformation activity. However, these activities were inhibited when treated by the anti-progestin RU486 (Chan et al, 1989; Mittal et al, 1993b), which is the antagonist for both dexamethasone and progesterone (Strahle et al, 1987).

1.2.2 GR is a member of type I nuclear receptor family

The members of type I nuclear hormone receptor, also named steroid hormone receptors, includes estrogen receptor (ER), androgen receptor (AR), GR and PR, etc. The binding of their ligands induces receptor to translocate to nucleus, and then bind the cognate DNA sequences called hormone response elements (HREs) as homodimer.

Numbers of coregulators are recruited by transcription factors to the site of

regulation, involving in either transactivation or repression function. Since the nuclear receptors are associated with some cancers, their coregulators must play a role in the gene expression, transcription regulation and the genesis of cancer.

1.2.3 GR recruits cofactors to exert its function in transcription regulation

As a transcription factor, GR recruits a variety of cofactors to exert its function in either upregulation or downregulation of transcription. For example, steroid receptor coactivators (SRCs) enhance the transactivity of GR by causing chromatin remodeling and recruiting other common coactivators such as CBP/p300. On the other hand, corepressors NCoR and SMRT bind free form or antagonist-bound GR to repress transcription (Blanco et al, 1998; Heinlein & Chang, 2002a; Heinlein & Chang, 2002b; Hong et al, 1996; Kamei et al, 1996; Onate et al, 1995).

1.3 Nuclear receptor interaction protein (NRIP), a coactivator of GR

Previously, our lab identified a novel nuclear receptor interacting protein and named it nuclear receptor interaction protein (NRIP). NRIP enhances the transcription activity of glucocorticoid receptor (GR) and androgen receptor (AR) in a ligand-dependent manner (Tsai et al, 2005).

1.3.1 The discovery of NRIP

To study the protein-protein interactions between nuclear receptors and their potential coregulators, our lab used yeast two-hybrid assay to find novel factors. C-terminal domain (aa 595 to 918) of AR was used as bait, and a HeLa cDNA library was screened. Hence, NRIP was found, named (GenBank™ accession numbers AY766164 and AAX09330) and characterized.

RT-PCR analysis showed NRIP expressed in cervical cancer cell lines, including C33A, CaSki, HeLa, and SiHa, prostate cancer cell lines, including PC-3 and LNCaP, and kidney embryonic cells 293T; Northern blot analysis of normal tissue samples showed NRIP was relatively highly expressed in skeletal muscle and testis, and was expressed to a less degree in heart, prostate and adrenal gland (Tsai et al, 2005).

1.3.2 The effects of NRIP

NRIP has physically interactions with nuclear receptors GR and AR when treated with their ligands Dex and DHT respectively, either in the *in vivo* co-immunoprecipitation assay or the *in vitro* pulldown assay. NRIP enhances the transcription activity of GR on HPV-16, HPV-18 and MMTV promoters and the transcription activity of AR on PSA and MMTV promoters. The siRNA-mediated

NRIP gene silencing resulted in significantly diminished cell proliferation in prostate (LNCaP) and cervical (C33A) cancer cells (Tsai et al, 2005).

1.3.3 The structure of NRIP

NRIP contains 860 amino acids and has a molecular weight of 160 kDa. The most remarkable feature of this protein is that NRIP contains seven WD40 domains, five in the N terminus (aa 40-79, 82-124, 130-170, 184-220, and 238-281) and the other two in the C terminus (aa 706-747 and 750-789), which may serve as the protein-protein interaction domains. The other functional domains include one nuclear translocation signal (NLS) (aa 676-692), which may introduce NRIP into nucleus and one Myc leucine zipper dimerization domain (MycLZ) (690-721).

WD40 domain is a sequence motif containing a conserved core of approximately 40 amino acids, defined by the glycine-histidine (GH) dipeptide at 11-24 residues from the N terminus and the tryptophan-aspartic acid (WD) dipeptide at the end (Neer et al, 1994; Smith et al, 1999).

WD-repeat proteins belong to a large and fast-expanding conservative protein family, which are ubiquitously expressed in eukaryotes but not in prokaryotes. To date, 136 WD-repeat proteins were found in human, 98 in *Drosophila melanogaster*, 72 in

Caenorhabditis elegans, and 56 in *Saccharomyces cerevisiae*. WD-repeat proteins have versatile functions including signal transduction, transcription regulation and apoptosis (Li & Roberts, 2001).

The Myc functions as a transcription regulator in cells by homodimerizing or heterodimerizing with Max via a basic region/helix-loop-helix/leucine zipper (bHLHZip) domain(Luscher & Larsson, 1999), designated MycLZ, implicating that NRIP might dimerize through its MycLZ domain.

1.3.4 NRIP-B, an alternatively spliced isoform of NRIP

During discovery of NRIP, an alternative spliced isoform NRIP-B was also obtained. The 860-aa full length NRIP is encoded from exon 1 to 11 and 15 to 24 of *NRIP* gene, whereas NRIP-B is encoded from exon 14 to 24, lacking the N terminus of full length NRIP but containing a distinct seven amino acid sequence encoded by exon 14. The resulting 408-aa alternatively spliced isoform NRIP-B, having the same sequence as the N terminus of full length NRIP, contains one NLS motif (aa 224-240), two WD40 domains (aa 254-295 and 298-337), and one MycLZ domain (aa 238-269) (unpublished data).

Like the full length NRIP, NRIP-B can also interact with GR and AR. The results

of co-immunoprecipitation assays performed with domain deletion mutants of NRIP-B suggest that the MycLZ domain and the two WD40 domains are both important to its interaction with GR. More intriguingly, when NRIP is cotransfected with NRIP-B, the GR- or AR-mediated transcription activation of MMTV promoter is repressed (unpublished data). These findings showed that NRIP-B may serve as the dominant negative inhibitor of NRIP to regulate the activity of nuclear receptors.

1.4 Specific aims

Previous studies on the HPV transcription regulation by GR utilized promoter activity assay, transformation assay and *in situ hybridization* to investigate activity of HPV LCR, and used footprinting and mobility shift assay to investigate the interaction between GR and GREs *in vitro*.

In this study, our aim is to further understand the role of GR in HPV transcription regulation *in vivo*. First, we examine the binding between GR and the GREs of HPV-16 by ChIP assay and analyze the function of each GRE by luciferase activity assay. Second, we want to know if NRIP enhances GR-mediated HPV-16 gene expression through binding HPV-16 LCR GREs via GR. Third, we examine the effect of NRIP on GR-mediated upregulation by knockdown of NRIP.

On the other hand, to study the possible regulation mechanisms of NRIP, we examine the interaction between NRIP and its alternatively spliced isoform NRIP-B by performing co-immunoprecipitation with domain deletion mutants of NRIP-B.

2. Materials and Methods

2.1 Cell culture

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, and 100 U/ ml of Penicillin-100 μg/ ml Streptomycin (Gibco). Cells were incubated at 37°C in 5% CO_2 and passaged using standard cell culture techniques. For hormone treatment, cells were incubated in phenol red- free DMEM containing 5% charcoal/dextran treated FBS (CDS) (HyClone).

For co-immunoprecipitation experiments, 293 and 293T cells were used to over-express indicated proteins.

SiHa and C33A cells are both cervical carcinoma derived cell lines. SiHa cells, containing integrated HPV-16 genome and GR, were used to investigate the interaction between endogenous GR and exogenously expressed HPV-16 promoter. On the other hand, C33A cells are negative for HPV DNA and RNA and were used as the counterpart of SiHa cells. For endogenous GR expression test, HaCaT, a human immortalized keratinocytes cell line, HeLa, a HPV-18 genome integrated cervical carcinoma cell line, and CaSki, a HPV-16 genome integrated cervical carcinoma cell line were also used.

2.2 Plasmid construction

All primers used in this study were listed in **Table 1**. The expression plasmids were constructed as described below.

2.2.1 Serial deleted mutagenesis of HPV-16 promoter

The promoter sequence HPV-16-LCR-832 was originally cloned by PCR amplifying the long control region (LCR) from 7155 bp to 83 bp of HPV-16 genome (a kind gift from Dr. zur Hausen H., Deutsches Krebsforschungzentrum, Heidelberg, Germany). The full length 832-bp promoter was amplified using *Kpn*I-LCR7155-F and *Xho*I-HPV-16-LCR-R. The amplified fragments were inserted into reporter vector pGL4.10-basic after digested with restriction enzymes *Kpn*I and *Xho*I, and the plasmid was named pGL4-HPV16LCR-832.

To construct the serial deleted reporter plasmids, reverse primer XhoI-HPV-16-LCR-R and forward primers *Kpn*I-LCR7401-F, *Kpn*I-LCR7490-F, *Kpn*I-LCR7656-F, and *Kpn*I-LCR7711-F were used respectively. The amplified promoter sequences HPV-16-LCR-586, -497, -331, and -276 were inserted into pGL4.10-basic after digested with restriction enzymes *Kpn*I and *Xho*I, and the resulting plasmids were named pGL4-HPV16LCR-586, -497, 331, and -276,

respectively.

2.2.2 Site-directed mutagenesis of HPV-16 promoter

The mutagenic primers containing desired mutations are listed in **Table 1**. The PCR condition is listed below.

	Volume (μl)	Final concentration
plasmid	1	< 1 μg
2.5 mM dNTP	2.5	400 M each
10X Reaction buffer	2.5	1X
10 μM primer	1	1 μM
Pfu ultra DNA polymerase	1	2.5 U
Double distilled H_2O	17	-
Total volume	25	-

To construct the site-directed mutants of HPV-16-LCR-832, a series of mutagenic primers with mutation in GRE1, 2, 3 or predicted GRE4 were used. First, template pGL4-HPV-16-LCR-832 and primers HPV16LCR-mGRE1, -mGRE2, and -mGRE3 were used to generate the single GRE mutants pGL4-HPV16-LCR832-mGRE1, -mGRE2, and -mGRE3 respectively. Next, the single GRE mutants were used as templates to generate the double GRE mutants pGL4-HPV16-LCR832-mGRE1+2,

-mGRE2+3, and -mGRE1+3. Finally, the triple mutants -mGRE1+2+3, -mGRE1+2+4a, -mGRE2+3+4a, -mGRE1+3+4a and all-mutant -mGRE1+2+3+4a were generated so on.

The mutagenesis PCR program: 95°C, 5 min for denaturation; 95°C, 30 sec, 55°C, 30 sec, 72°C, 7 min for 35 cycles; 72°C 10 min and paused at 4°C. 1 μl of restriction enzyme *Dpn*I was added into each PCR product and then digested for 2 hours at 37°C. The digested PCR product was transformed into *E. coli* DH5 α to select mutants.

2.3 Transfection

2.3.1 Calcium phosphate method

293 or 293T cells were seeded on 6-cm dishes and grown overnight. When the cells reached about 60% confluency, replaced fresh medium 30 minutes before transfection. For each sample, expression plasmids were diluted in 180 μl sterilized water, and 20 μl 2.5 mM $CaCl_2$ was gently added into the diluted DNA. And the solution was mixed by vortex at level 8 for 20 seconds. Next, 200 μl HBS was dropped into the Ca^{2+}-DNA solution while vortexing at the same time and then mixed by vortex for another 30 seconds. The DNA solution was dropped into dish and then swirl to mix.

2.3.2 Lipofectamine method

SiHa cells were seeded at 3 x 10^4 cells per well in 24-well plate using phenol red-free DMEM containing 5% CDS and grown overnight. For each well, 1 μg of plasmid was diluted in 250 μl of phenol red-free DMEM without 5% CDS. Besides, 3 μl of Lipofectamine 2000 (Invitrogen) was diluted in another 250 μl of phenol red-free DMEM without 5% CDS. After incubated for 5 minutes at room temperature, the diluted DNA was combined with diluted Lipofectamine 2000, mixed gently by pipeting and incubated for 20 minutes at room temperature. The 500 μl complexes were added to each well and mixed gently by rocking the plate back and forth. The cells were then incubated at 37°C and changed with fresh medium 4 hours post-transfection.

2.3.3 FuGENE HD method

SiHa cells were seeded at 3 x 10^4 cells per well in 24-well plate using phenol red-free DMEM containing 5% CDS and grown overnight. For each well, 1 μg of plasmid was diluted in 500 μl of phenol red-free DMEM without 5% CDS and 4μl of FuGENE HD reagent was added. After incubated for 20 minutes at room temperature, the 500 μl complexes were added to each well and mixed gently by rocking the plate

back and forth. The cells were then incubated at 37°C and changed with fresh medium overnight.

2.4 Semi-quantitative RT-PCR

For endogenous GR expression test, C33A, HaCaT, CaSki, HeLa, and SiHa cells were seeded on 6-well plate and incubated for two days. For testing the effect of dexamethosome (Dex) on E7 expression, SiHa cells were seeded on 6-well plate in phenol red-free DMEM containing 5% CDS and incubated overnight, and then treated with Dex at indicated concentration for 16 hours. For testing the effect of siNRIP on HPV-16 E6 and E7 expression, SiHa cells were seeded on 6-well plate in phenol red-free DMEM containing 5% CDS and incubated overnight, transfected with pSUPER-siGFP 646 (a kind gift from Dr. Shin-Lian Doong) or pSUPER-siNRIP (Tsai et al, 2005) by Fugene HD method, and treated with Dex (10 nM) or EtOH for 16 hours. Total RNA was extracted with TRIzol reagent as follows.

2.4.1 RNA extraction

To lyse and homogenize cells, medium was aspirated and 1 ml of TRIzol reagent (Invitrogen) was added to each well, and then pipeted to mix. The homogenized

samples were transferred to microfuge tubes and incubated at room temperature for 5 minutes.

To separate the RNA phase, 0.2 ml of chloroform was added. Tubes were capped securely and shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase, in which RNA remained exclusively.

To precipitate the RNA, the aqueous phase (about 0.5 ml) was transferred to a fresh tube and mixed with 0.5 ml of isopropanol. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate then formed a gel-like pellet on the side and bottom of the tube.

To wash the RNA, the supernatant was removed and 1 ml of 75% ethanol was added. The pellet was gently flicked and centrifuge at 7,500 x g for 5 minutes at 4°C, briefly air-dried for 5 to 10 minutes. Finally, 50 μl of DEPC-treated water was added to dissolve the RNA pellet by gently pipetting.

To quantify the RNA sample by spectrophotometry, its absorbance at a wavelength of 260 nm was measured. The RNA samples were stored in -70°C for later

use.

2.4.2 First-strand cDNA synthesis

Reverse transcription was performed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

For each sample, 2 μg of total RNA was used to synthesize cDNA. First, RNA was mixed with 1 μl of 50 μM oligo$(dT)_{20}$, 1 μl of 10 mM dNTP mix, and DEPC-treated water to 10 μl. The RNA/primer mixture was incubated at 65°C for 5 min and then placed on ice for at least 1 min. Next, 10 μl of the cDNA synthesis mix, prepared in the indicated order: 2 μl of 10X RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT (40 U/ μl), and 1 μl of SuperScript III reverse transcriptase (200 U/ μl) was added. The mixture was mixed gently, collected by brief centrifugation and incubated at 50°C for 50 minutes. The reaction was terminated at 85°C for 5 min, chilled on ice and then collected by brief centrifugation. 1 μl of RNase H was added and incubated at 37°C for 20 minutes. cDNA samples were stored at -20°C or used for PCR immediately.

2.4.3 Polymerase chain reaction (PCR)

2 μl of each cDNA was used as template and mixed with 6 μl of 10X reaction buffer, 5 μl of 2.5 mM dNTP, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 1 μl of Taq DNA polymerase (Genemark), and double distilled H_2O to 60 μl. Each PCR mixture was separated into 3 aliquots for indicated extension cycles of PCR. The PCR products were resolved on 2% agarose gel to compare the expression level of target genes.

For endogenous GR expression test, primers GR-F and GR-R were used to detect the mRNA level of GR, and the internal control β-actin was amplified by primers BA-3 and BA-4.

For testing the effect of siNRIP on HPV-16 E6 and E7 expression, primer pairs HPV16-E6-F and HPV16-E6-R, HPV16-E7-F and HPV16-E7-R, NRIP-F and NRIP-R, GR-F and GR-R, BA-3 and BA-4 were used to amplify the fragments of E6, E7, NRIP, GR and β-actin.

2.5 Chromatin immunoprecipitation assay (ChIP)

SiHa cells were used to investigate the binding between endogenous GR and the putative GREs on HPV-16 promoter *in vivo*, while C33A cells were used to

overexpress GR, NRIP and HPV-16 promoter plasmids to detect their interactions.

6 x 10^6 of SiHa cells were seeded on 10-cm dish in 5% phenol-red free CDS DMEM and grew for two days. Changed fresh medium containing 10 nM Dex or vehicle control EtOH five minutes before performing ChIP assay.

To crosslink proteins and DNA, 270 μl of 37% formaldehyde was added to 10 ml of growth media and then gently swirled dish to mix. After incubated at room temperature for 15 minutes, 1ml of 1.25 M glycine was added to quench unreacted formaldehyde for 5 minutes.

To harvest the chromatin-protein complexes, dishes were placed on ice, medium was aspirated, and cells were washed with cold 1X PBS for 3 times. Next, 1 ml of cold PBS containing 1mM PMSF and 1mM DTT was added to dish. Cells were scraped from each dish into a microfuge tube and pelleted by spinning at 700 x g at 4°C for 5 minutes. And then supernatant was removed and pellet was resuspended in 250 μl of SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris, pH 8.1) containing 1mM PMSF and 1mM DTT.

To shear DNA, cell lysates were sonicated on wet ice using the Misonix sonicator 3000 for 20 rounds of 5 seconds sonication followed by 20 seconds rest at level 5.5 power output. Insoluble material was removed by spinning at 15,000 x g at 4°C for 10

minutes.

To immunoprecipitate crosslinked protein-DNA complexes, supernatant was removed to fresh microfuge tubes in 100 μl aliquots and 900 μl of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167mM NaCl) containing 1mM PMSF and 1mM DTT was added into each tube. And then the immunoprecipitating antibody was added. 10 μl (i.e. 1%) of diluted lysate was removed as input and saved at 4°C. Added 2.0 μg of GR antibody (Santa Cruz) and its negative control (normal rabbit IgG) respectively. The IPs were incubated overnight at 4°C with rotation. 30 μl of protein G sepharose (Upstate) was added and incubated for 2 hours at 4°C with rotation to collect the antibody/ antigen/ DNA complexes and then briefly centrifuged at 5000 x g for 1 minute to remove the supernatant fraction. The protein G agarose-antibody/chromatin complex was washed with 1 ml of low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl) for 3 times, high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl) for 2 times, LiCl wash buffer (0.25M LiCl, 1% NP-40, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1) for 1 time, and TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) for 1 time.

To elute the protein/ DNA complexes, added 100 μl of elution buffer (1% SDS,

0.1M $NaHCO_3$) to all IP tubes as well as all input tubes and mixed by flicking tube gently. After incubated at room temperature for 15 minutes, agarose was centrifuged briefly at 5000 x g for 1 minute and supernatant was collected into new microfuge tubes. The above elution steps were repeated again and eluates were combined.

To reverse crosslinks of protein/ DNA complexes, 8 μl of 5M NaCl was added to all tubes and incubated at 65°C overnight. Next, added 20 μg of RNase A and incubated for 30 minutes at 37°C and then added 4 μl 0.5 M EDTA, 8 μl 1 M Tris-HCl and 20 μg Proteinase K and incubated at 45°C for 2 hours. DNA was purified by gel extraction kit (Geneaid) and eluted with 50 μl Tris-HCl (pH 7.5).

To perform PCR, made 60 μl cocktail containing 4 μl eluted DNA, 5 μl 2.5 mM dNTP, 6μl 10X Reaction buffer, 1 μl 10 μM forward primer, 1 μl 10 μM reverse primer, 1 μl Taq DNA polymerase, and 42 μl double distilled H_2O. Each PCR mixture was separated into 3 aliquots for indicated extension cycles of PCR. The PCR program was: 95°C, 5 min for denaturation; 95°C, 30 sec, 62°C, 30 sec, 72°C, 30 sec for 26, 28 or 30 cycles; 72°C 15 min and paused at 4°C. The PCR products were resolved on 2% agarose gel to compare the quantity.

2.6 Luciferase assay

For the detection of the response of HPV-16 promoter mutants toward glucocorticoid, SiHa cells were seeded at 1 x 10^5 cells per well on 24-well plate in phenol red-free DMEM containing 5% CDS and grown overnight. 0.5 μg of luciferase reporter plasmid was then transfected by Fugene HD (Roche) along with 1 ng of internal control pGL4.74 (HSV-TK promoter driven Relina luciferase gene). For deletion mutants test, luciferase reporter plasmids pGL4-HPV-16-LCR-832, -586, -497, -331, -276 and vector pGL4.10-basic were transfected, respectively. For site-directed mutants, luciferase reporter plasmids pGL4-HPV-16LCR-mGRE1+2+3, -mGRE1+2+4a, -mGRE2+3+4a, -mGRE1+3+4a, all-mutant -mGRE1+2+3+4a and vector pGL4.10-basic were transfected, respectively.

Cells were treated with Dex (10 nM) or EtOH 24 hours post-transfection and then subjected to luciferase assay using The Dual-Glo Luciferase Assay System (Promega) after another 18 hours.

2.7 Co-immunoprecipitation and Western blot

2.7.1 Co-immunoprecipitation

293T cells were plated at 1x10^6 on 6-cm dish one day before transfection.

For the interaction between NRIP and NRIP-B, pEGFP-c1-NRIP and pCMV-HA-NRIP-B were co-transfected by Calcium phosphate method. Cell lysates were collected by NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH7.4) containing protease inhibitor two days post-transfection and quantified by BCA Protein Assay (Thermo). Lysates were stored at -20°C or used for immunoprecipitation immediately.

For each immunoprecipitation, 300 μg of cell lysate was diluted in 500 μl of NP-40 buffer containing protease inhibitor and immunoprecipitated with 1 μg of anti-FLAG (Sigma) or anti-HA (Roche) antibody at 4°C overnight. Immunoprecipitation products were pulled down by 20 μl of Protein G sepharose beads (Amersham Biosciences) and washed 3 times with NP-40 buffer. After centrifugation at 6,000 x g for 1 minute, supernatant was carefully removed and 20 μl of 2X protein sample buffer was added to elute the proteins. The sample was boiled at 95°C for 3 minutes and then subjected to Western blot.

2.7.2 Western blot

Protein samples were separated on 8% SDS-PAGE, and transferred to PVDF membrane using Semi-Dry Transfer Unit (GE Healthcare Life Sciences). Membrane

was blocked with 5% skimmed milk-TBST and then blotted with anti-FLAG, anti-HA or anti-β-actin primary antibodies. Anti-mouse Ig-HRP (Amersham Biosciences) was added after 3 washes by TBS-T and signals were detected by ECL Western blotting detection system (Amersham Biosciences) and captured by X-ray film (Fuji film).

2.8 Cell growth assay

SiHa cells were seeded at 3 x 10^4 cells per well in 24-well plate using phenol red-free DMEM containing 5% CDS and grown overnight. 1 μg of pSUPER-siGFP or pSUPER-siNRIP plasmid was transfected using Lipofectamine 2000.

Medium was changed to phenol red-free DMEM containing 5% CDS and Dex (10 nM) or EtOH 4 hours post-transfection and changed every other day. Cells were trypsinized and counted by trypan blue exclusion method at 4, 28, 52, 76, 100 hours post-transfection.

3. Results

3.1 The regulation mechanisms of GR on HPV-16 promoter

3.1.1 To search for the proper cell line as research model

To unravel the regulation mechanism of GR on HPV-16 gene expression *in vivo*, we had to choose a proper cell line which expresses GR and HPV-16 genome, in addition, a cell line not expressing GR and HPV-16 genome had to be used as a negative control. So, first of all, we compared the GR expression levels in different cervix derived cell lines by RT-PCR (**Figure 3**). Among all the HPV-positive cell lines tested, SiHa, containing an integrated HPV-16 genome, had the highest expression level of GR, whereas CaSki, containing about 600 copies of HPV-16 genome, had lower expression level than HeLa, containing HPV-18 sequences. In the case of cell lines negative for HPV DNA or RNA, cervical carcinoma cell line C-33 A had no expression of GR, and human immortalized keratinocyte HaCaT expressed a medium level of GR. So we decided to use SiHa cells to perform the following experiments and use C33A cells as its negative counterpart.

3.1.2 To test the effect of glucocorticoid on endogenous HPV-16 E7 expression

After being incubated in phenol red-free DMEM supplemented with 5% CDS,

SiHa cells were treated with increasing dose of dexamethosome (Dex) for 16 hours and then subjected to RT-PCR. Compared to vehicle control EtOH, the E7 mRNA level was higher in the presence of 0.1 nM Dex and rose with increasing Dex concentration (**Figure 4**). By the point of 100 nM Dex, it showed no obvious addition than 10 nM. On the other hand, the mRNA level of GR did not change with Dex treatment. Hence, 10 nM Dex treatment was used for the following experiments.

3.1.3 To investigate the interaction between GR and the putative GREs on HPV-16 promoter

For detecting the *in vivo* interaction between endogenous GR and HPV-16 promoter, SiHa cells were used to perform chromatin immunoprecipitation (ChIP) assay. After treated with Dex for 30 minutes, endogenous GR could bind HPV-16 promoter inserted in the SiHa chromosome (**Figure 5**).

3.1.4 To elucidate which site is important for GR-mediated HPV gene expression

We transfected the HPV-16-LCR serial deletion mutants into SiHa cells to examine their responses toward Dex stimulation. Comparing the responses of LCR-832, -586 and -497, which contained the sequences from 7155, 7401 or 7490 bp

to 82 bp of HPV-16 genome, respectively, the relative luciferase activities were not decreased with the sequences. However, LCR-331, started from 7656 bp, dropped to only about one sixth of the activity of LCR-497; moreover, LCR-276, started from 7711 bp, lost about half the activity of LCR-331 (**Figure 6**).

On the other hand, the luciferase activity of the full length promoter LCR-832, which has three previously verified GREs, had a 1.49-fold increase upon Dex treatment compared to EtOH treatment, but LCR-586, -497, and -331, despite the loss of GREs, had no lower ratio of Dex enhancement. Here the notable fact was that, even though the three previously identified GREs was deleted, Dex could still enhance the luciferase activity of LCR-331 to 1.98 fold compared to EtOH, which was even higher than the 1.49-fold increase of wild type LCR-832, and the enhancement was barely seen in LCR-276, giving us a hint that there might be a novel GRE on HPV-16 promoter.

3.1.5 To search for the novel GRE on HPV-16 promoter

We blasted the sequence of HPV-16 LCR on two transcription element search websites TESS and TFBIND and found that, agreeing with our expectation, there is a novel putative GRE on HPV-16 LCR. In addition to the three known GREs, this novel

GRE was predicted to be located at 7695 to 7710 bp on HPV-16 LCR (**Figure 7**) and was designated as GRE4.

In order to further confirm the function of GRE4 and to understand the distinctive response toward Dex of each GRE, site-directed mutants against GRE1 to 4 were generated and reporter assay was performed. GRE4 not only showed the highest luciferase activity among all the mutants having only one GRE, but also responded to Dex stimulation like the other three GREs did (**Figure 9**). Moreover, when GRE1, 2, 3, and 4 were all mutated, Dex could no longer enhance HPV-16 LCR, which was coincided with the result of LCR-276, the deletion mutant lacking four GREs.

3.2 The effect of coregulator NRIP on GR-mediated HPV-16 gene expression

3.2.1 To investigate whether NRIP enhances GR through binding to HPV-16 promoter

The endogenous NRIP was pulled down by NRIP antibody along with HPV-16 promoter integrated into SiHa chromosome after 30 minutes of Dex treatment (**Figure 10** (a)). Furthermore, we immunoprecipitated the DNA-protein complex by GR antibody following by a second-round ChIP (re-ChIP) by NRIP antibody, and the

HPV-16 promoter could still be detected, proving that the endogenous NRIP colocalized with endogenous GR on HPV-16 promoter (**Figure 10** (b)).

3.2.2 To investigate whether NRIP binds to HPV-16 promoter through GR

To further confirm that NRIP exert its coregulator function through the binding of GR, endogenous and exogenous experiments were performed in SiHa and C33A cells, respectively. In SiHa cells, when endogenous GR was knockdown by shGR, the binding of NRIP and HPV-16 promoter was lost (**Figure 11** (a)). In C33A cells, NRIP could only bind HPV-16 when co-transfected with GR but not with GFP (**Figure 11** (b)).

3.2.3 The effect of siNRIP on GR-mediated E6 and E7 expression

SiHa cells were transfected with siNRIP or siGFP, treated with Dex or EtOH, and then subjected to RT-PCR (**Figure 12**). First, the effect of siNRIP was confirmed, no matter treated with Dex or not, the mRNA level of NRIP was decreasing in a dose-dependent manner, but the mRNA level of GR was not affected by siNRIP. Second, a drop of E7 expression was observed when treated with Dex, and the trend was coincided with the decrease of NRIP, but this phenomenon was not observed without

Dex treatment. Third, the expression of E6 was also slightly decreasing when treated with Dex.

3.2.4 The effect of siNRIP on GR-mediated cell proliferation

SiHa cells were transfected with siNRIP or siGFP, treated with Dex or EtOH, and then subjected to trypan blue exclusion test. First, the growth of cells transfected with siGFP, which was the control, was higher when treated Dex, the same result was seen in cells transfected with siNRIP (**Figure 13**). Second, siNRIP decreased the cell growth no matter treated with Dex or not. So we could see that the siGFP control treated with Dex had the highest growth rate. Third, comparing the differences between treated with Dex and EtOH, Dex could barely enhance the growth of cells transfected with siNRIP, suggesting that the GR-mediated cell proliferation was dependent on its cofactor NRIP.

3.3 The interaction between NRIP and its isoform NRIP-B

3.3.1 To test whether NRIP and NRIPB associate with each other

293 cells were co-transfected with p3XFLAG-NRIP (NRIP-FLAG) and HA-NRIP-B, and then lysates were subjected to co-immunoprecipitation test. NRIP-FLAG and HA-NRIP-B could be efficiently pulled down by FLAG and HA

antibodies, respectively (**Figure 14**). HA-NRIP-B could be co-immunoprecipitated with NRIP-FLAG with FLAG antibody, and in the reciprocal experiment NRIP-FLAG could also be co-immunoprecipitated with HA-NRIP-B by HA antibody. So the *in vivo* interaction between NRIP and its isoform NRIP-B was confirmed.

4. Discussion

4.1 The role of GR in HPV-16 LCR regulation

In the beginning of this study, we tested the GR expression levels of various cervical carcinoma cell lines and found that SiHa cell line, with HPV-16 genome in its chromosome, had relatively high expression of GR, and its expression of HPV-16 oncogenes E6 and E7 were also elevated upon Dex treatment. Thus, SiHa was used to study the role of GR in HPV-16 LCR regulation. First, we proved the *in vivo* interaction of GR and HPV-16 LCR by ChIP assay. Next, to investigate the importance of each GRE, serial deletion mutants of HPV-16 LCR were cloned into pGL4.10 vector and subjected to luciferase activity assay. The responses of serial deletion mutants not only confirmed that the three GREs were individually functional as previous reports (Chan et al, 1989; Gloss et al, 1987; Mittal et al, 1993b), but also, to our surprise, revealed that there might be a novel GRE on HPV-16 LCR. The fourth GRE was predicted to be located at 7695 to 7710 bp; to further verify its existence, we generated a series of site-directed mutants retained only one GRE out of the three verified GREs and this predicted one. The results of luciferase activity assay, again, proved the existence of the fourth GRE, which also functioned independently of the other three GREs. Taken together, we proved that GR bound to the GRE(s) on HPV-16 LCR and

enhanced the transcription activity of HPV-16 through GRE1 to 3, along with the newly discovered GRE4.

4.1.1 The binding between GR and the four GREs on HPV-16 LCR

In this study, we used ChIP assay to demonstrate the binding between GR and the GRE(s) on HPV-16 LCR *in vivo*. However, the binding of each GRE could not be discriminated by examining the interaction between endogenous GR and HPV-16 LCR integrated in SiHa chromosome because of the proximity of their positions. The chromatin DNA sheared by sonication mostly became fragments raging form 500 to 1000 bp in length, while these GREs located within a mere 400-bp region, which were not able to be separated even by the specific primer pairs flanking their sequences. Therefore, in order to testify the individual binding of each GRE and GR *in vivo*, providing physical evidence for the newly found GRE4, an exogenous overexpression strategy must be employed. By comparing the binding between GR and exogenously overexpressed HPV-16 LCR site-directed mutants retaining only one of four GREs and mutant losing all four GREs, we might further understood the action of GR on HPV-16 LCR.

In fact, the interactions of the three known GREs on HPV-16 LCR were proved *in*

vitro back at the time when they were discovered. The Bernard group, found the first GRE (GRE3) on HPV-16 LCR, revealed its binding by DNaseI footprinting and DMS protection assays using partially purified GR from rat liver (Gloss et al, 1987). Later on, the Pater group identified GRE1 and GRE2 by more *in vitro* interaction assays, including mobility shift assays done with HeLa whole cell extracts and oligonucleotide probes, UV cross-linking assays, and Southwestern blot analysis (Mittal et al, 1993a).

One thing about the binding between GR and its response elements is that, as a member of type I nuclear receptors, GR binds to similar sequences as progesterone receptor (PR); therefore, the specificity of the GREs is to be considered. In the HPV-16 LCR, the four GREs are GCTACATCCTGTTTT, GGCACAAAATGTGTT, TGTACATTGTGTCAT, and GGCACATATTTTTGG, respectively. The consensus GRE AGAACANNNTGTTCT and the consensus PRE GTTACAAACTGTTCT have the palindromic sequence ACANNNTGT in common. In fact, the three GREs found on HPV-16 LCR were demonstrated to be regulated by either glucocorticoid Dex or progesterone (Chan et al, 1989; Mittal et al, 1993b), but whether PR bound to the GREs was not investigated. A 15-bp sequence applicable to both GR and PR for gene regulation is not unusual, for example, similar GRE/PREs were at the promoter regions of tyrosine aminotransferase gene and the herpes simplex virus thymidine kinase gene

(Strahle et al, 1987).

4.1.2 The responses of GREs toward Dex treatment

By performing luciferase assays of HPV-16 LCR serial deleted and site-directed mutants, we demonstrated that GR upregulated the transcription activity of HPV-16 LCR through GRE1, 2, 3 and 4.

Compared with the full length LCR-832, the luciferase activities of LCR-586 and -497, with GRE1 and both GRE1 and 2 being deleted respectively, did not decrease correspondingly, but the luciferase activity of LCR-331greatly dropped, so did LCR-276. The inconsistent reduction of transcription activity could be attributed to the disruption of the 400-bp cell type specific enhancer spanning the DNA region from 7454 to 7854 bp (Gloss et al, 1987), which was further verified to be sufficient for full enhancer activity with a 232-bp fragment between 7524 and 7755 bp (Gloss et al, 1989). In our experiment, LCR-331 and LCR-276, started from 7656 and 7711 bp respectively, lost most of the enhancer activity.

In the luciferase activity assay of HPV-16 LCR GRE site-directed mutants, the respective responses of GRE1, 2, 3 and 4 toward Dex were 1.34 to 1.53-fold induced. Although the responses proved that each GRE was functional, the induction was not

very strong. As a matter of fact, even though the luciferase activity of wild type LCR-832, containing all four GREs, was 1.5 to 5 fold of the site-directed mutants, its induction upon Dex treatment was mere 1.66 fold.

Compared to the previous study done by the Pater group, in which single, double and triple GRE mutants on the HPV-16 LCR fragment spanning from 6850 to 7889 bp were generated and transfected into HeLa cells, and then choloramphenicol acetyltransferse (CAT) assays were performed. The full length wild type had a 3.5-fold induction in the presence of 100 nM Dex, while single of double mutants of GRE1, 2 and 3 could still give a 2.4 to 2.7-fold induction, but the triple mutant had only a 1.1-fold induction. When the oligonucleotides of GRE1 and 2 were cloned into vector separately, the constructs were induced about 2.5-fold (Mittal et al, 1993a), given that the three GREs were functional independently of the other factors in the enhancer.

4.2 The role of coregulator NRIP in GR-mediated HPV-16 LCR regulation

4.2.1 The binding of coregulator NRIP and HPV-16 LCR

In this study, we used ChIP assay to demonstrate that transcription coregulator NRIP bound the GRE(s) on HPV-16 LCR *in vivo* via GR. The binding of NRIP and

HPV-16 LCR was first confirmed by immunoprecipitating the protein-DNA complex by NRIP antibody. Furthermore, we used GR antibody to immunoprecipitate the GR protein-DNA complex, followed by a second round immunoprecipitation with NRIP antibody, and the HPV-16 GRE was still present in the re-ChIP complex, indicating that NRIP and GR colocated at the GRE on HPV LCR.

To further confirm that the coregulator NRIP was recruited by GR, we performed both knockdown and overexpression experiments. When endogenous GR of SiHa cells was silenced by shRNA-based knockdown, NRIP could no longer bind GRE sequence on HPV-16 LCR; on the other hand, in C33A cells, which lacked GR expression, the binding of NRIP and GRE was only seen when NRIP was cotransfected along with GR.

These results showed that NRIP exerted its transcription coactivity on HPV-16 LCR via binding to GRE through GR.

4.2.2 The effect of shNRIP on the expression of HPV-16 oncogenes E6 and E7

The transcription coactivity of NRIP was demonstrated on GR-regulated HPV-16 LCR, HPV-18 LCR and MMTV promoter by luciferase activity assays (unpublished data). In this study, we proved the effect of NRIP *in vivo* by NRIP knockdown.

Transfection of pSUPER-based shNRIP reduced the expression of NRIP, but the expression of GR was not affected. Compared with cells in the absence of Dex, SiHa cells transfected with shNRIP exhibited a decreased expression level of E6 and E7 when treated with Dex. Since the upregulation of HPV-16 LCR transcription activity by Dex was demonstrated previously, the decrease of E6 and E7 by shNRIP in the presence of Dex suggested that HPV-16 LCR transcription activity enhanced by GR could be mediated by NRIP.

4.2.3 The effect of shNRIP on GR-mediated cell proliferation

In this study, we observed the cell growth of SiHa cells transfected with shNRIP with or without Dex treatment to verify the role of NRIP in GR-mediated cell growth. First, cells grew faster in the presence of Dex no matter transfected with shNRIP or control shGFP, proving that GR upregulated the growth of SiHa cells, a HPV-16 genome contained cell line. When treated with Dex, the cell growth of control group was nearly two fold than treated with EtOH, while the increase caused by Dex was only about 1.25 fold in the shNRIP group. Second, shNRIP inhibited the cell growth of SiHa cell line, the cell growth was about half fast of control shGFP in the absence of Dex, while the cell growth in the presence of Dex was less than one third. In another

word, the inhibition of cell growth caused by shNRIP was more dramatic when treated with Dex than treated with EtOH. These results suggested that the GR-mediated cell proliferation was enhanced by the coactivator NRIP.

Although NRIP played a role in assisting GR-mediated cell growth, the effect of shNRIP on cell growth inhibition was more potent than the effect of Dex on promoting cell growth. A possible explanation might be that NRIP could employ its function through pathway other than GR and play more important roles in related with other cell transcription factors to modulate cell growth.

4.3 The interaction between NRIP and its alternative spliced isoform NRIP-B

NRIP was demonstrated in our lab to enhance the transcription activity of GR and AR, thus being regarded as coactivator, but its regulation mechanisms remained unclear. Previous study showed NRIP had an alternative spliced isoform called NRIP-B and might serve as antagonist of NRIP, inhibiting the transcription coactivity of NRIP on AR-mediated PSA promoter (unpublished data). In this study, we try to unravel the roles of NRIP and NRIP-B through their interaction. NRIP-B has the identical 401 amino acids to the C-terminus sequence of NRIP, whereas the initial 7

amino acids are distinct. The common functional domains of NRIP and NRIP-B include one NLS, one Myc-LZ dimerization domain, two WD40 repeat domains, and a Myb DNA binding domain.

To elucidate the respective role of NRIP and NRIP-B and the mutual effects on their target proteins, first of all, we demonstrated the *in vivo* interaction of exogenously overexpressed NRIP and NRIP-B by co-immunoprecipitation. Next, two function models were proposed. One was the competition model, which hypothesized that NRIP and NRIP-B competed for target protein such as AR through their common domain; the other proposal was the dominant negative model that NRIP-B bound to NRIP to form a nonfunctional dimer.

To testify the competition model, AR, NRIP, and increasing amounts of NRIP-B were cotransfected into 293 cells and co-IP was performed by AR antibody. However, the amount of NRIP binding to AR was not decreased with the increasing amount of NRIP-B (data not shown), implicating this model not feasible.

To verify the dominant negative model, NRIP had to be proved to function as homodimer firstly, but due to some experimental difficulties, this data was not applicable. So we generated the deletion mutants of NRIP-B, lacking two WD40 protein interaction domains or the Myc-LZ dimerization domain, to perform co-IP

experiment with NRIP. By comparing the binding ability with wild type NRIP-B, these deletion mutants might reveal the interaction domain between NRIP and NRIP-B, providing the solution to their regulation functions.

Figures

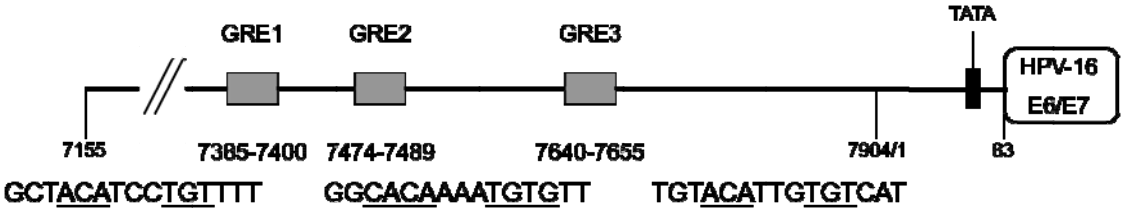

Figure 1 Schematic representation of GREs on HPV-16 LCR

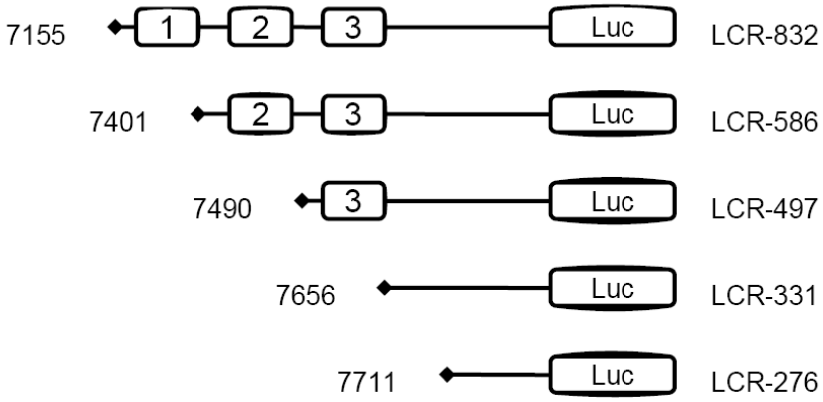

Figure 2 The construction of HPV-16 LCR serial deletion mutants

The HPV-16 LCR from 7155 to 82 bp was cloned into reporter vector pGL4.10 (Promega) at *Kpn*I/*Xho*I sites and named pGL4.10-HPV16-LCR832. The serial deletion mutants were constructed by PCR.

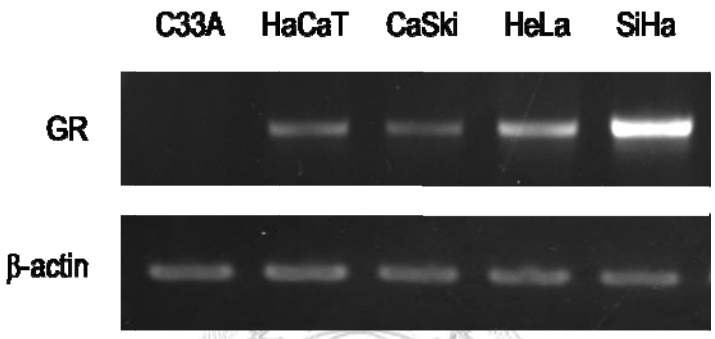

Figure 3 The mRNA expression level of GR in various cell lines

Cells were grown in medium supplemented with 10% FBS 48 hours before being subjected to RT-PCR experiment.

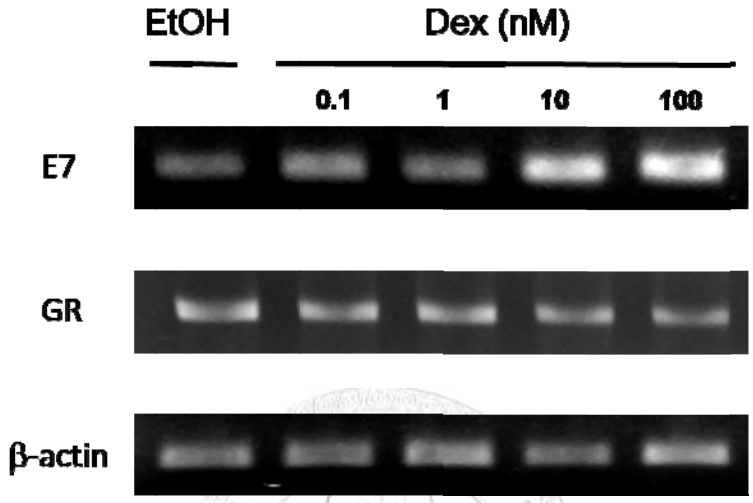

Figure 4 The mRNA level of endogenous HPV-16 oncogene E7 increased with Dex treatment. SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS 48 hours before being subjected to RT-PCR. Dex was added at indicated concentrations 16 hours before.

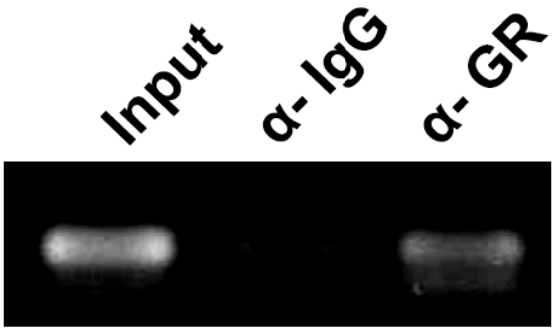

Figure 5 The binding of GR and the GREs on HPV-16 LCR

SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS for 48 hours and treated with 10 nM Dex 30 minutes before being subjected to ChIP assay. GR antibody (Santa Cruz) was used to immunoprecipitate the protein-DNA complex. Primers: GRE1-F and GRE1-R.

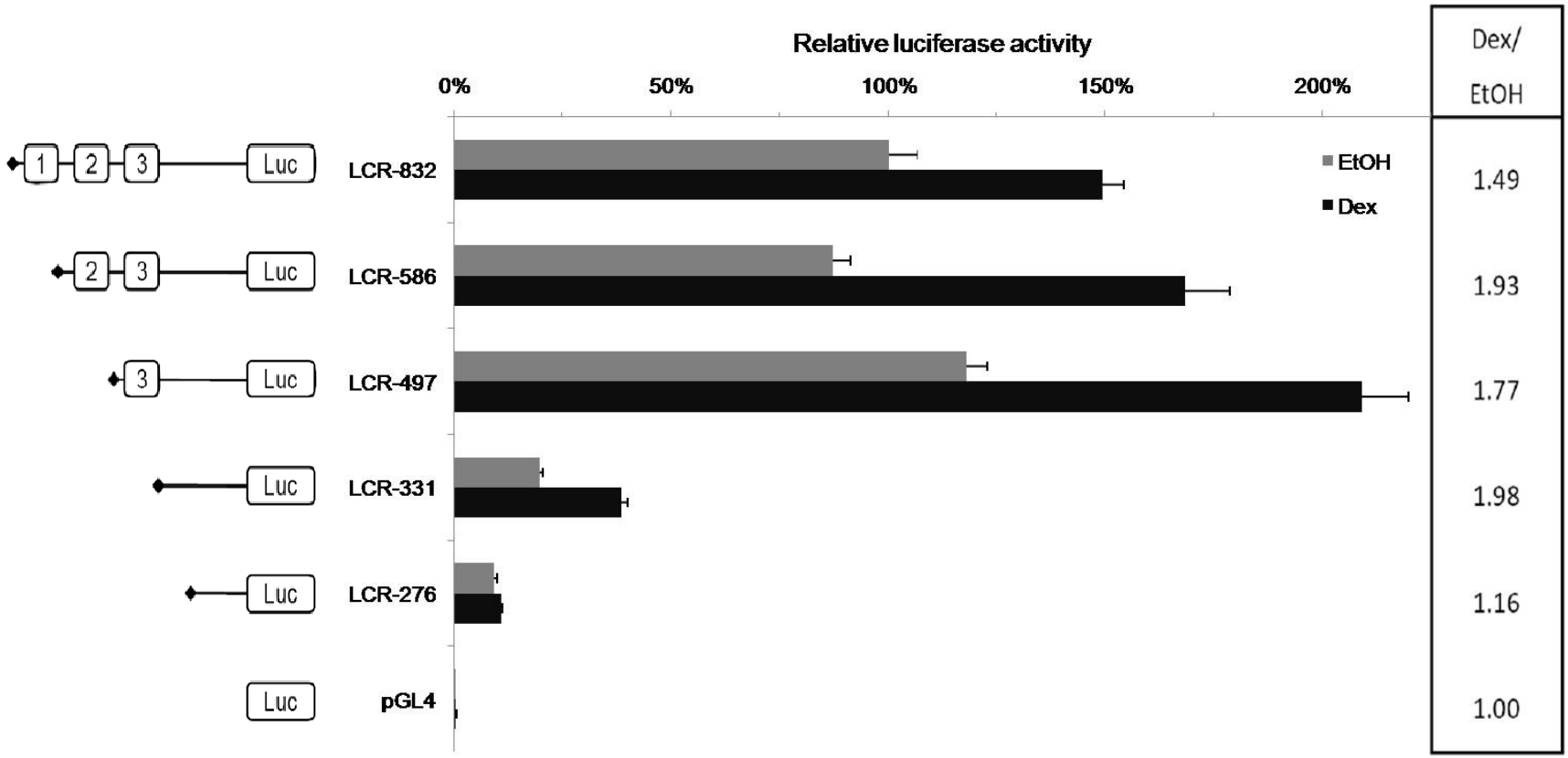

Figure 6 The responses of HPV-16 LCR deletion mutants toward Dex. SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS overnight, transfected with HPV-16 LCR deletion mutants and treated with 10 nM Dex 18 hours before being subjected to luciferase activity assay. Grey and black bars represent EtOH and Dex treatment, respectively.

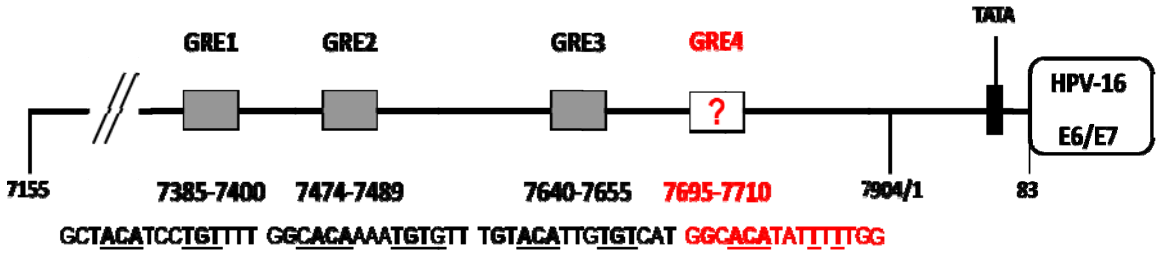

Figure 7 The novel putative GRE (GRE4) on HPV-16 LCR

Underlined: partially palindromic. Bold: consensus

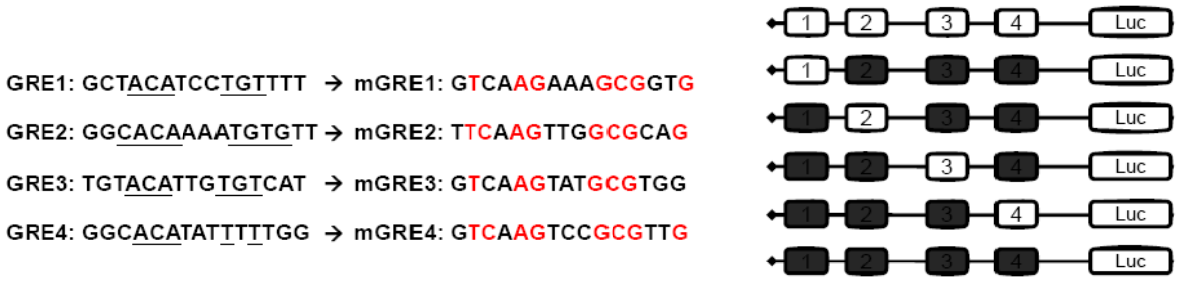

Figure 8 The construction of HPV-16 LCR site-directed mutants

Underlined: partially palindromic. Bold: consensus.

White box: wild type sequence. Black box: site-directed mutated sequence.

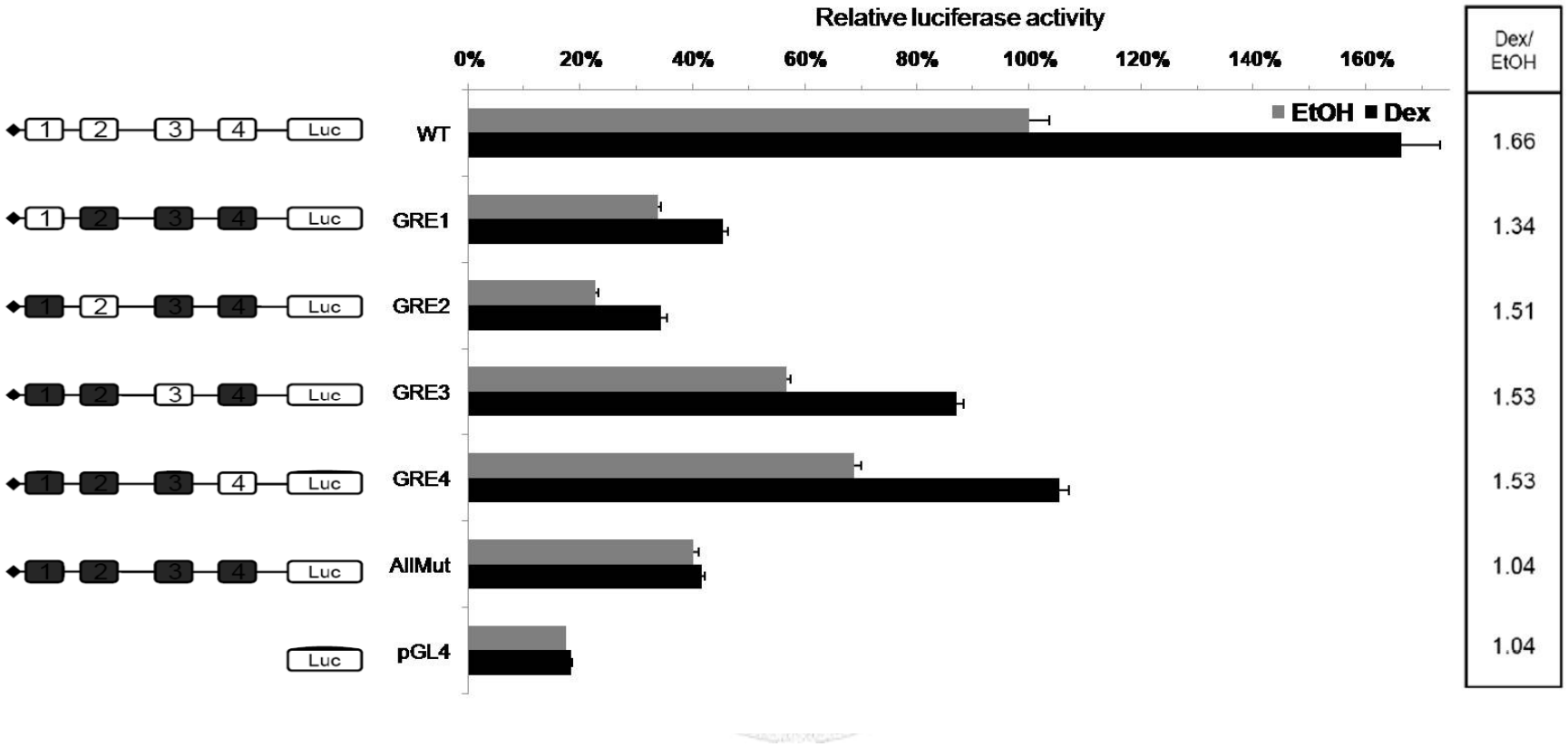

Figure 9 The respective responses of GRE1, 2, 3 and 4 toward Dex. SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS overnight, transfected with HPV-16 LCR site-directed mutants and treated with 10 nM Dex 18 hours before being subjected to luciferase activity assay. Grey and black bars represent EtOH and Dex treatment, respectively.

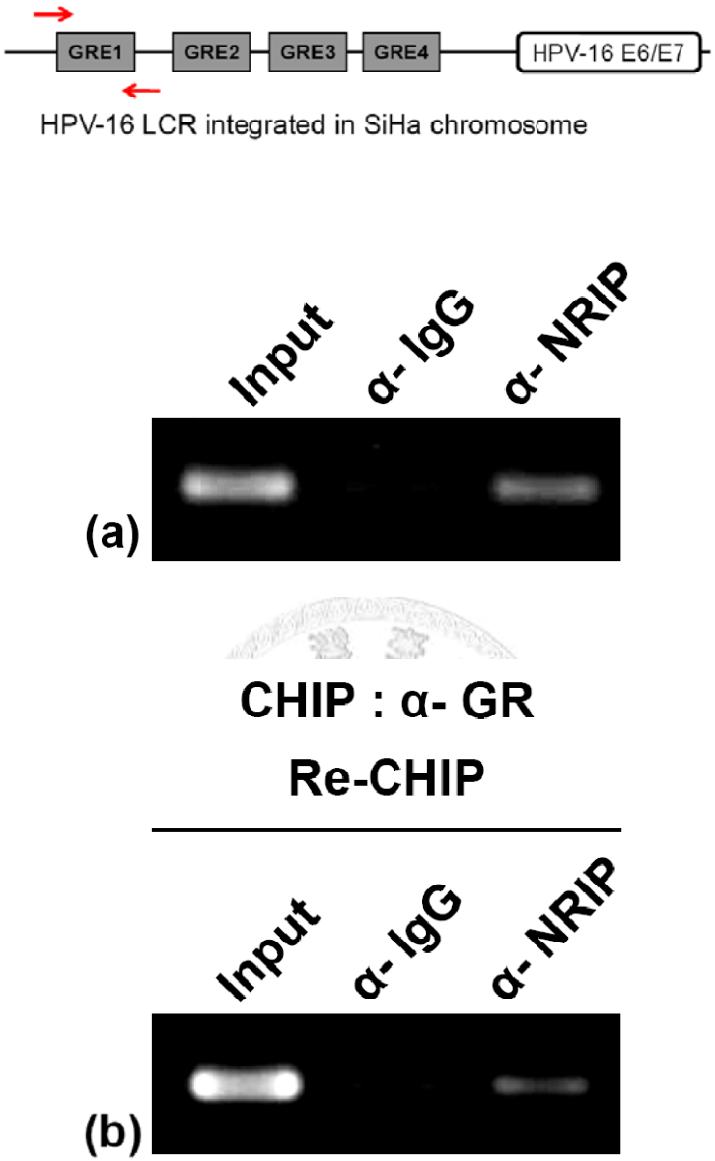

Figure 10 The binding of NRIP and the GREs on HPV-16 LCR

SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS for 48 hours and treated with 10 nM Dex 30 minutes before being subjected to ChIP assay. (a) GR antibody (Santa Cruz) was used to immunoprecipitate the protein-DNA complex.(b) NRIP antibody was used to perform re-ChIP. Primer pair: GRE1-F and GRE1-R.

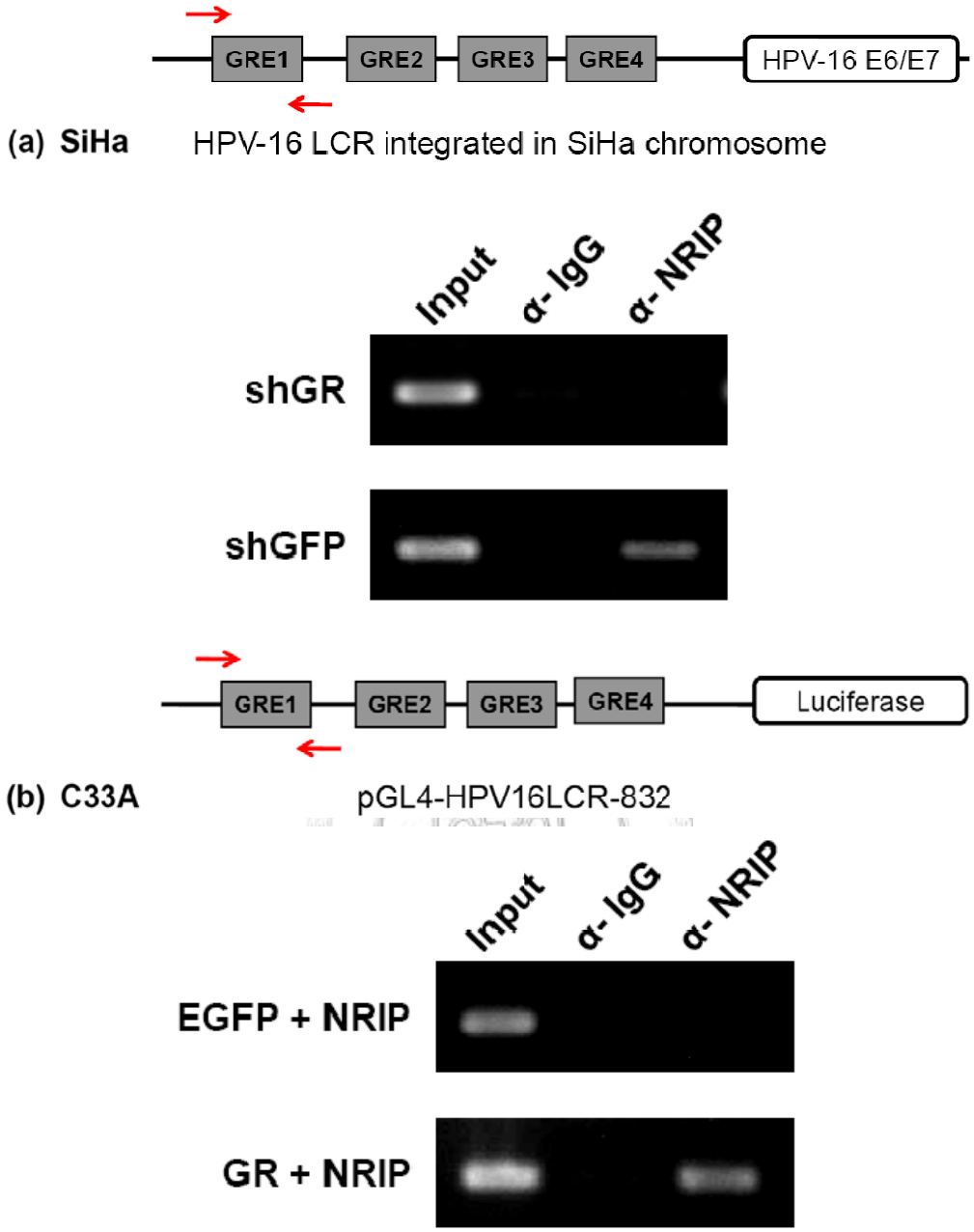

Figure 11 The GR-dependent binding of NRIP and the GREs on HPV-16 LCR

Cells were grown in phenol red-free DMEM supplemented with 5% CDS for 48 hours and treated with 10 nM Dex 30 minutes before being subjected to ChIP assay. NRIP antibody was used to immunoprecipitate the protein-DNA complex. (a) SiHa cells transfected with shGFP control or shGR (b) C33A cells cotransfected NRIP with EGFP control or GR. Primer pair: GRE1-F and GRE1-R.

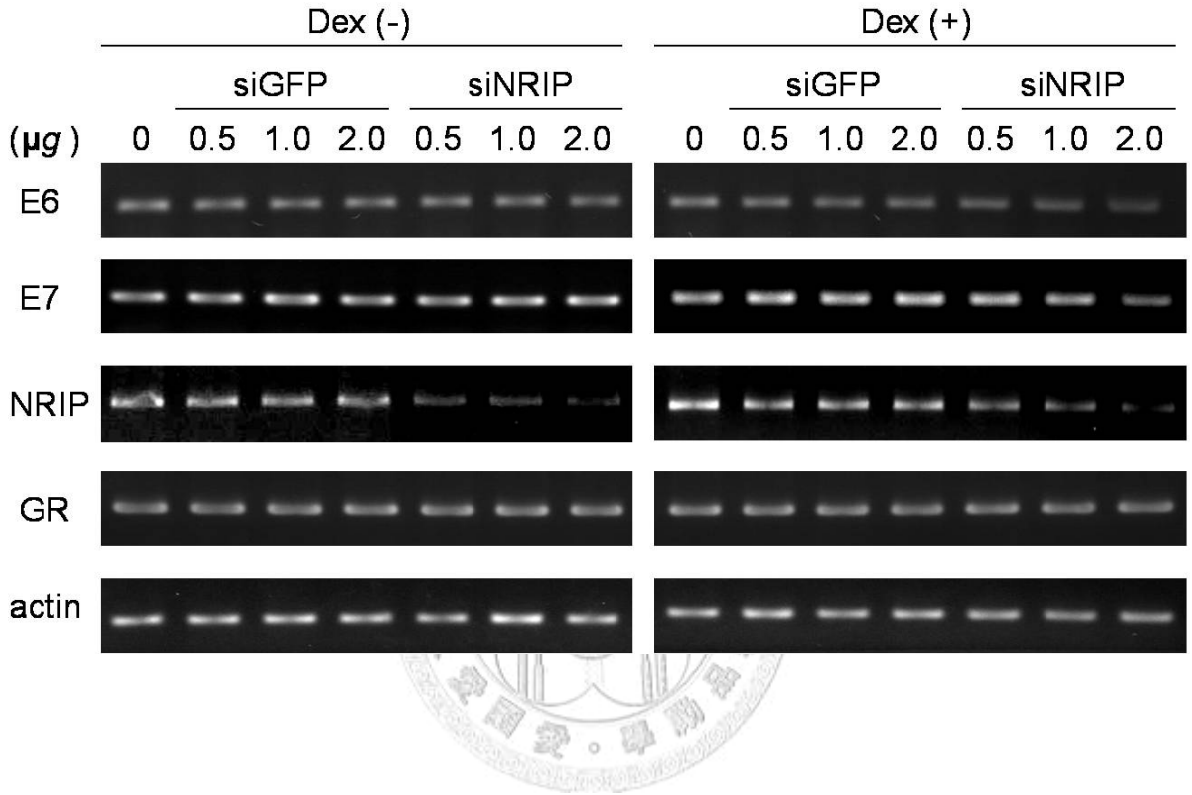

Figure 12 The effect of siNRIP on HPV-16 E6 and E7 expression *in vivo*

SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS for 48 hours and treated with EtOH or 10 nM Dex 30 minutes before being subjected to RT-PCR.

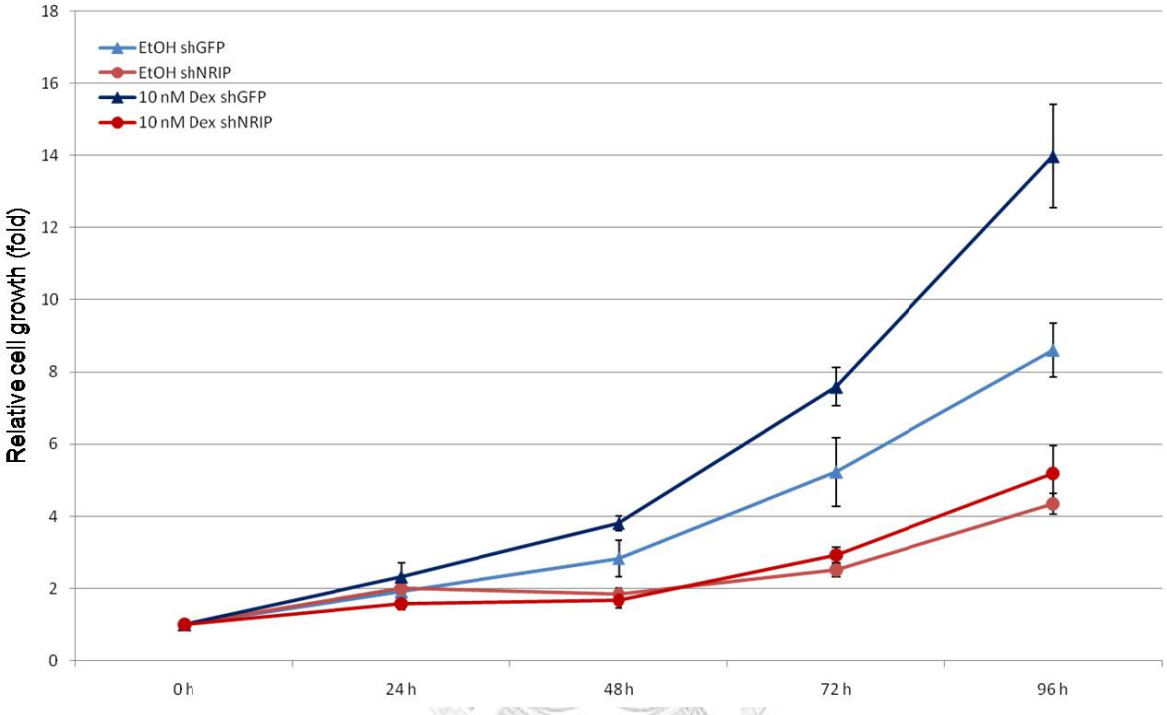

Figure 13 The effect of siNRIP on GR-mediated cell growth

SiHa cells were grown in phenol red-free DMEM supplemented with 5% CDS overnight, transfected with pSUPER-shNRIP or control –shGFP and then kept in the presence of EtOH or 10 nM Dex. Cell numbers were counted at indicated time point post transfection by trypan blue exclusion method.

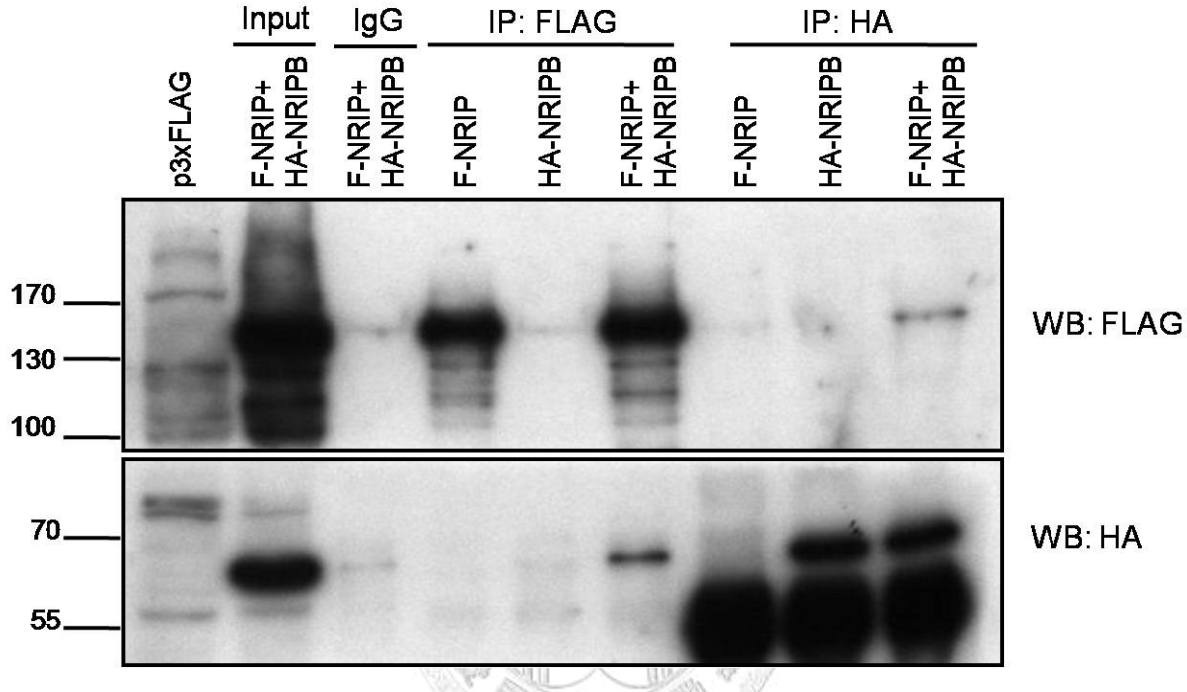

Figure 14 The *in vivo* interaction of NRIP and its alternative spliced isoform NRIP-B

293 cells were seeded in 6-cm dishes and grown in DMEM overnight, cotransfected with 6 μg of p3xFLAG-NRIP and pCMV-HA-NRIP-B, and lysates were collected 40 hours post transfection. 300 μg of each lysate was incubated with FLAG or HA antibody overnight in NP-40 buffer containing protease inhibitor, and the immunocomplexes were resolved by 8%SDS-PAGE.

Tables

Table 1 Primers

Name	Sequence	Description
*Xho*I-HPV-16-LCR-R	GCT**CTCGAG**AAAATGTCTGCTTTTATACTAACC	pGL4
*Kpn*I-LCR7155-F	GCT**GGTACC**GTATTGTATGTATGTTGAATTAGT	pGL4
*Kpn*I-LCR7401-F	TTTT**GGTACC**GTTTTATATATACTATATTTTGTAGCG	pGL4
*Kpn*I-LCR7490-F	TTTT**GGTACC**TTTTAAATAGTTCTATGTCAGCAAC	pGL4
*Kpn*I-LCR7656-F	TTTT**GGTACC**AAAATAAATCACTATGCGCCAAC	pGL4
*Kpn*I-LCR7711-F	TTTT**GGTACC**TGGCTTGTTTTAACTAACCTAATTG	pGL4
*Kpn*I-LCR7801-F	TTTT**GGTACC**GTGTAAGGTAGTCATACATTGTTC	pGL4
*Kpn*I-LCR7829-F	TTTT**GGTACC**TTGTAAAACTGCACATGGGT	pGL4
HPV16LCR-mGRE1	CATTGTATATAAACTATATTTGTCAAGTCCGCGTTGTGTTTTATATATACTATATTTTG	Mutagenic
HPV16LCR-mGRE2	GAATTCGGTTGCATGCTTTTTGTCAAGAAAGCGGTGTTTTTAAATAGTTCTATGTCAG	Mutagenic
HPV16LCR-mGRE3	GTGCAACTACTGAATCACTATTCAAGTTGGCGCAGATAAAATAAATCACTATGCGCC	Mutagenic

HPV16LCR-maGRE4	CGCCTTACATACCGCTGTTAGTCAAGTATGCGTGGCTTGTTTTAACTAACCTAATTGC	Mutagenic
*Kpn*I-GR-F	TTTT**GGTACC**ATGGACTCCAAAGAATCATTAACTC	pCMV-HA
*Not*I-GR-R	TTTT**GCGGCCGC**TCACTTTTGATGAAACAGAAGTTTTTTGA	pCMV-HA
C1-*Xho*I-NRIP-F	TTTT**CTCGAG**CTATGTCTCGGGGTGGCTCCTAC	pEGFP-c1
C1-*BamH*I-NRIP-R	TTTT**GGATCC**TTATTCCTCATCCTCATTTTCATTC	pEGFP-c1
NRIP-*Kpn*I	TTTT**GGTACC**TTATTCCTCATCCTCATTTTCATTCTC	pCMV-HA
*EcoR*I-NRIPB*2	TTTT**GAATTC**GGATGTCACTTGACGAGCAA	pCMV-HA
GR-F	CTCACTGGCTGTCGCTTCTCA	RT-PCR
GR-R	GTCTGATCTCCAAGGACTCTC	RT-PCR
NRIP-F	ATGTCTCGGGGTGGCTCCTACCCACAC	RT-PCR
NRIP-R	ACTGGTTCACCTGTCCCTGGTTTGG	RT-PCR
HPV-16E7-F	ATGCATGGAGATACACCTACATTG	RT-PCR
HPV-16E7-R	TTATGGTTTCTGAGAACAGATGG	RT-PCR
β-actin-F	ACCTTCAACACCCCAGCCATG	RT-PCR
β-actin-R	CTGGAAGAGTGCCTCAGGGCA	RT-PCR

Bold: restriction site.

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