# TRANSFORMING GROWTH FACTOR-BETA SIGNALLING IN THE REGULATION OF EPSTEIN-BARR VIRUS INFECTION IN NASOPHARYNGEAL EPITHELIAL CELLS

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FACULTY OF DENTISTRY
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KUALA LUMPUR

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# TRANSFORMING GROWTH FACTOR-BETA SIGNALLING IN THE REGULATION OF EPSTEIN-BARR VIRUS INFECTION IN NASOPHARYNGEAL EPITHELIAL CELLS

## ABSTRACT

Undifferentiated nasopharyngeal carcinoma (NPC) is consistently associated with Epstein-Barr virus (EBV) infection. However, the molecular events that regulate the establishment of stable EBV infection in nasopharyngeal epithelial (NPE) cells remain largely undefined. It is now recognised that persistent EBV latent infection in NPE cells is dependent on specific pre-existing genetic changes. There is evidence to show that Transforming growth factor-beta (TGF-β) signalling is de-regulated in NPC. TGF-β signalling regulates a variety of cellular processes and functions as a tumour suppressor in the early stage of epithelial carcinogenesis by inhibiting cell proliferation and promoting differentiation, apoptosis and senescence. Our preliminary data showed that epithelial cells that are no longer responsive to TGF-β1-induced growth inhibition are more amenable to stable EBV infection, while epithelial cells harbouring an intact TGF-β signalling pathway are not able to sustain EBV genomes, suggesting that de-regulation of TGF-β signalling is a prerequisite for the establishment of stable EBV infection. To test this hypothesis, two different approaches were used to disrupt TGF-β signalling in three immortalised NPE cell lines (NP361hTert, NP460hTert and NP550hTert): (1) inhibiting the type 1 TGF-β receptor (TGFR-1) kinase with a chemical inhibitor, SB431542 and (2) overexpression of a kinase-deficient form (dominant negative) of the type 2 TGF-β receptor (*DnTGFBR2*). Functional disruption of the TGF-β signalling pathway was confirmed by a reduced growth inhibitory response to TGF-β1, inhibition of TGF-β1-induced phosphorylation of Smad2 and a loss of Smad2/3-dependent transcription activity following TGF-β1 stimulation. These

cells were then infected with a GFP-tagged recombinant EBV (Akata strain) and FACS used to determine the percentages of GFP-positive cells over a period of 21 days post-infection. Compared to the respective controls, both TGFR-1 kinase inhibition and overexpression of *DnTGFBR2* resulted in higher numbers of cells carrying the EBV genomes in all three cell lines. Given that EBV infection is associated with growth inhibition and senescence, as well as entry into the lytic cycle in a manner that is linked to epithelial cell differentiation, the influence of TGF-β signalling disruption on cellular differentiation, EBV-induced senescence and induction of the EBV lytic cycle, was investigated. The results showed that both the inhibition of TGFR-1 kinase and overexpression of *DnTGFBR2* suppressed the differentiation of NPE cells in response to serum and calcium as well as TGF-β1, as shown by reduced involucrin expression. Following EBV infection of these cells, the expression of SIRT1 was readily detected while p16 and p21 levels were significantly decreased, indicating that the cells were resistant to EBV-induced senescence and growth inhibition. Further, the expression of EBV-encoded *BZLF1* was reduced, suggesting that disruption of TGF-β signalling suppressed EBV lytic cycle induction. Lastly, to conclusively demonstrate an essential role for TGF-β signalling in sustaining EBV infection in NPE cells, *TGFBR2* was knocked out using CRISPR/Cas9. Similarly, knockout of *TGFBR2* resulted in a more persistent EBV infection in NP460hTert, and these effects were reversed following expression of a wild-type *TGFBR2*. Collectively, the results of this study demonstrate that disruption of TGF-β signalling supports stable EBV infection in NPE cells, possibly by suppressing cellular differentiation, EBV-induced senescence and EBV lytic cycle induction.

Keywords: Nasopharyngeal carcinoma, Epstein-Barr virus, Transforming growth factor-beta, Differentiation, Senescence, Lytic cycle

**TRANSFORMING GROWTH FACTOR-BETA SIGNALLING IN THE REGULATION OF EPSTEIN-BARR VIRUS INFECTION IN NASOPHARYNGEAL EPITHELIAL CELLS**

## ABSTRAK

Karsinoma nasofarinks (NPC) tidak berdiferensiasi sering dikaitkan dengan jangkitan virus Epstein-Barr (EBV). Namun begitu, proses molekul yang meregulasi pemantapan jangkitan EBV yang stabil dalam sel-sel epitelium nasofarinks (NPE) masih tidak jelas. Jangkitan EBV laten yang berterusan dalam sel-sel NPE kini dikenalpasti bahawa ia bergantung kepada perubahan genetik spesifik yang sedia ada. Terdapat bukti menunjukkan bahawa pengisyaratan 'Transforming growth factor-beta' (TGF-β) deregulasi dalam NPC. Pengisyaratan TGF-β meregulasi pelbagai proses di peringkat sel dan berfungsi sebagai penindas tumor pada tahap awal karsinogenesis epitelium dengan merencat proliferasi sel dan menggalak diferensiasi, apoptosis dan penuaan. Data awal kami menunjukkan bahawa sel-sel epitelium yang tidak lagi responsif terhadap perencatan pertumbuhan yang diaruhkan oleh TGF-β1 adalah lebih cenderung kepada jangkitan EBV yang stabil, sedangkan sel-sel epitelium yang mempunyai pengisyaratan TGF-β1 yang lengkap tidak dapat mengekalkan genom EBV, justeru deregulasi pengisyaratan TGF-β dicadangkan sebagai prasyarat dalam pemantapan jangkitan EBV yang stabil. Untuk menguji hipotesis ini, dua pendekatan berbeza digunakan untuk menggendalakan pengisyaratan TGF-β dalam tiga jenis sel NPE yang 'immortalised' (NP361hTert, NP460hTert dan NP550hTert): (1) merencat reseptor TGF-β jenis 1 (TGFR-1) 'kinase'dengan perencat kimia, SB431542 dan (2) pengekspresan melampau reseptor TGF-β jenis 2-tanpa 'kinase' (*DnTGFBR2*). Penggendalaan fungsi pada laluan pengisyaratan TGF-β telah dibuktikan dengan penurunan respons perencatan pertumbuhan terhadap TGF-β1, perencatan fosforilasi Smad2 yang diaruh oleh TGF-β1 dan kehilangan aktiviti transkripsi yang bergantung kepada Smad2/3 berikutan rangsangan TGF-β1. Sel-sel ini kemudiannya dijangkiti dengan EBV rekombinan strain Akata yang ditanda dengan GFP dan peratusan

sel-sel GFP-positif ditentukan dengan menggunakan FACS dalam tempoh masa 21 hari selepas jangkitan EBV. Apabila dibandingkan dengan kawalan masing-masing, kedua-dua perencatan TGFR-1 'kinase' dan pengekspresan melampau *DnTGFBR2* menunjukkan peratusan sel-sel yang membawa genom EBV adalah lebih tinggi dalam ketiga-tiga sel NPE. Memandangkan jangkitan EBV dikaitkan dengan perencatan pertumbuhan, penuaan, dan juga kemasukannya ke dalam kitaran litik dengan kaitan diferensiasi sel epitelium, pengaruh terhadap penggendalaan pengisyaratan TGF-β dalam diferensiasi sel, induksi penuaan yang diaruhkan oleh EBV dan induksi kitar litik telah dikaji. Keputusan menunjukkan bahawa kedua-dua perencatan TGRF-1 'kinase' dan pengekspresan melampau *DnTGFBR2* menindas diferensiasi sel-sel NPE yang dirangsang dengan serum dan kalsium serta TGF-β1, seperti ditunjukkan oleh pengurangan pengekspresan 'involucrin'. Berikutan jangkitan EBV ke atas sel-sel ini, pengekspresan SIRT1 sedia dikesan, manakala paras p16 dan p21 mengurang secara signifikan, lantas menunjukkan bahawa sel-sel ini adalah resistan terhadap induksi penuaan dan perencatan pertumbuhan berikutan jangkitan EBV. Seterusnya, pengekspresan 'EBV-encoded' *BZLF1* yang menurun mencadangkan bahawa penggendalaan pengisyaratan TGF-β menindas induksi kitar litik EBV. Akhir sekali, 'knockout' *TGFBR2* dengan CRISPR/Cas9 diguna untuk .menunjukkan kepentingan pengisyaratan TGF-β terhadap pengekalan jangkitan EBV dalam sel-sel NPE. 'Knockout' *TGFBR2* juga menunjukkan pemantapan jangkitan EBV yang lebih berterusan dalam sel NP460hTert dan kesan ini boleh berbalik selepas pengekspresan *TGFBR2* 'wild-type'. Secara keseluruhannya, keputusan kajian ini menunjukkan penggendalaan pengisyaratan TGF-β menyokong jangkitan EBV yang stabil dalam sel-sel NPE, kemungkinan berkaitan dengan perencatan dalam selular diferensiasi, penuaan yang diaruh oleh EBV dan induksi kitar litik EBV.

Kata kunci: Karsinoma nasofarinks, Virus Epstein-Barr, 'Transforming growth factor-beta', diferensiasi, Penuaan, Kitaran litik.

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| AKT | : | Protein kinase B |
| ASR | : | Aged-standardized rate |
| BARF1 | : | BamHI-A fragment rightward reading frame 1 |
| BARTs | : | BamHI-A rightward transcripts |
| BHRF1 | : | BamHI fragment H rightward open reading frame 1 |
| BL | : | Burkitt's lymphoma |
| BSA | : | Bovine serum albumin |
| CR2 | : | Complement receptor 2 |
| CTAR1 | : | C-terminal activation region 1 |
| CTAR2 | : | C-terminal activation region 2 |
| dnRNA | : | Double-stranded RNA |
| EA | : | Early antigen |
| EBER | : | EBV-encoded RNA |
| EBNA | : | Epstein-Barr virus nuclear antigen |
| EBV | : | Epstein-Barr virus |
| ECL | : | enhanced chemiluminescene |
| EGF | : | Epidermal growth factor |
| EMT | : | Epithelial-mesenchymal transition |
| ERK | : | Extracellular signal-regulated kinase |
| FBS | : | Fetal bovine serum |
| GWAS | : | Genome-wide association studies |
| HLA | : | Human leukocyte antigen |
| HRP | : | Horseradish peroxidase |
| hTert | : | Human telomerase reverse transcriptase |

| | | |
|---|---|---|
| IFN | : | Interferon |
| Ig | : | Immunoglobulin |
| IGF | : | Insulin-like growth factor |
| IL | : | Interleukin |
| IM | : | Infectious mononucleosis |
| JNK | : | c-Jun N-terminal kinase |
| kb | : | Kilobase pair |
| LCL | : | Lymphoblastoid cell line |
| LMP | : | Latent membrane protein |
| LOH | : | Loss of heterozygosity |
| MAPK | : | Mitogen-activated protein kinase |
| MHC | : | Major histocompatibility |
| miRNA | : | MicroRNA |
| mRNA | : | Messenger RNA |
| NF-κB | : | Nuclear factor-kappa B |
| NGS | : | Next-generation sequencing |
| NK | : | Natural killer |
| NMU | : | N-nitroso-N-methylurea |
| NPC | : | Nasopharyngeal carcinoma |
| NPE | : | Nasopharyngeal epithelial cells |
| PI3K | : | Phosphatidylinositol-3-kinase |
| PKR | : | Protein kinase R |
| PML | : | Promyeloctic leukaemia |
| PVDF | : | Polyvinylidene difluoride |
| Qp | : | Q promoter |
| QpCR | : | Quantitative polymerase chain reaction |

| | | |
|---|---|---|
| RIG-1 | : | Retinoic acid-inducible gene |
| RNA | : | Ribonucleic acid |
| SARA | : | Smad anchor for receptor activation |
| SDS-PAGE | : | SDS-polyacrylamide gel electrophoresis |
| SNP | : | Single nucleotide polymorphism |
| TBS | : | Tris buffered saline |
| TBST | : | Tris buffered saline tween |
| TGF-β | : | Transforming growth factor-beta |
| *TGFBR1* | : | Transforming growth factor-beta receptor 1 |
| *TGFBR2* | : | Transforming growth factor-beta receptor 2 |
| TNF | : | Tumour necrosis factor |
| TRAF2 | : | TNF receptor-associated factor 2 |
| VCA | : | Viral capsid antigen |
| VEGF | : | Vascular endothelial growth factor |
| WES | : | Whole exome sequencing |
| WHO | : | World Health Organization |
| Wp | : | W promoter |

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# CHAPTER 1: INTRODUCTION

## 1.1 General Introduction

Nasopharyngeal carcinoma (NPC) is a distinct type of head and neck cancer that arises from the epithelial lining of the nasopharynx (Sham et al., 1990). Globally, NPC is a rare malignancy; however, it is very prominent in the Southern part of China and Southeast Asia with an incidence rate of approximately 20-50 per 100,000 population per year (Cao et al., 2015; Wei et al., 2014). According to the IARC Globocan 2012 (http://globocan.iarc.fr), the aged-standardized rate (ASR) of NPC in Malaysia was 7.2 per 100,000 population, representing the highest ASR in the world.

NPC is classified into two categories, namely keratinizing squamous cell carcinoma and non-keratinizing carcinoma which is further sub-divided into differentiated and non-differentiated carcinoma (Shanmugaratnam & Sobin, 1991). Non-keratinizing NPC is more common in the endemic regions and is consistently associated with Epstein-Barr virus (EBV) infection (Niedobitek et al., 1991a). EBV has two distinct life cycles, namely the latent cycle during persistent infection and the lytic cycle during the production of mature infectious virions. NPC exhibits EBV latency II in which the expression of EBV genes is restricted to Epstein-Barr nuclear antigens (EBNA1), latent membrane proteins (LMP1 and LMP2), non-coding Epstein-Barr virus-encoded RNAs (EBER1 and EBER2), BamHI-A rightward transcripts (BARTs) and BamHI-A fragment rightward reading frame 1 (BARF1) (Young et al., 2016). It is now well-recognised that these EBV latent genes regulate various cellular signalling pathways that collectively contribute to the malignant transformation of nasopharyngeal epithelial (NPE) cells (Tsao et al., 2017).

Although a strong association between NPC and EBV has long been recognised, the mechanisms that regulate the maintenance of EBV genomes in epithelial cells are largely unknown. NPC cells carry monoclonal EBV genomes, indicating that EBV infection takes place before the expansion of the malignant cells. Although EBV replication has been found within the epithelial cells in the oropharynx and salivary glands (Niedobitek et al., 1991b), it has been a challenge to establish persistent EBV latent infection in epithelial cells in an experimental setting. However, in 2012 a study showed that inactivation of *p16* or overexpression of cyclin D1 (*CCND1*), two characteristic features of NPC tumours, contributed to the stable EBV infection in immortalised NPE cells (Tsang et al., 2012). Significantly, these data demonstrate that epithelial cells displaying pre-malignant genetic alterations are susceptible to EBV latent infection.

Interestingly, we noted that undifferentiated NPC cell lines (e.g. HONE1) that were not growth inhibited by TGF-β1 were susceptible to EBV infection, whereas differentiation-competent squamous epithelial cells (e.g. immortalised oral keratinocytes, OKF6) which were unable to sustain EBV infection were responsive to the cytostatic effects of TGF-β1. These observations suggested that the loss of TGF-β signalling may be a key determinant in maintaining EBV genomes in epithelial cells. There is some evidence to show that TGF-β signalling is de-regulated in NPC. Mutations of the *TGFBR2* gene were initially reported in a subset of primary NPC tissues, and subsequently, a gene expression microarray study showed that the TGF-β signalling pathway was de-regulated in EBV-positive NPC tissues compared to normal NP tissues (Harn et al., 2002; Sriuranpong et al., 2004). Further, the expression of both *TGFBR1* and *TGFBR2* mRNA and protein were found to be frequently down-regulated in primary NPC tissues and cell lines (Fang et al., 2008; Lyu et al., 2014; Zhang et al., 2012). Notably, the only EBV-positive NPC cell line amenable to culture *in vitro*,

C666.1, lacked expression of TGFR-2 (Wood et al., 2007). Importantly, low levels of TGFR-1 and TGFR-2 were correlated with increased cancer aggressiveness, as well as poor overall survival rates in NPC patients (Zhang et al., 2012).

TGF-β signalling plays an important role in regulating numerous biological processes such as proliferation, apoptosis, cell differentiation, senescence, angiogenesis, epithelial-mesenchyme transition (EMT) and immune suppression (Derynck et al., 2001; Siegel & Massague, 2003). In terms of cancer development, TGF-β signalling has been shown to exert both tumour suppressive and tumour promoting functions depending on the cancer stage (Lebrun, 2012; Siegel & Massague, 2003). TGF-β1 acts as a potent growth inhibitor for many tumour types of epithelial origin through cell cycle G1/S arrest. As tumours progress, cancer cells often develop genetic abnormalities within the TGF-β signalling pathway components that result in loss of responsiveness to TGF-β signalling, thereby blocking the growth inhibitory effect of TGF-β1 on cancer cell growth (Pickup et al., 2013). At some point during malignant progression, TGF-β1 switches to act as a tumour promoting factor by stimulating the proliferation of mesenchymal cells, increasing extracellular matrix (ECM) production and accelerating migration. Indeed, elevated levels of TGF-β1 were found in sera from NPC patients compared to healthy individuals (Sun et al., 2007; Xu et al., 1999). Importantly, it has been shown that treatment of NPC cells with exogenous TGF-β1 led to the activation of TGF-β signalling, but the cells were not growth inhibited in response to exogenous ligand (Xiao et al., 2010). Although a role for TGF-β signalling in promoting NPC tumorigenesis has been demonstrated, the biological significance of this pathway in regulating EBV latent infection has not been investigated. The present study aimed to examine the influence of TGF-β signalling disruption on the outcome of EBV maintenance in NPE cells.

## 1.2 General Aims

The impact of EBV infection on the development of NPC is thought to be a consequence of the aberrant establishment of virus latency in epithelial cells harboring pre-malignant genetic changes. The present study was initiated to test the hypothesis that epithelial cells fostering defects in TGF-β signalling are more amenable to persistence EBV infection. The first part of this study generated NPE cells with disrupted TGF-β signalling by inhibiting TGFR-1 kinase using a chemical inhibitor (SB431542) or by the overexpression of a dominant negative *TGFBR2* (*DnTGFBR2*) gene. After determining the consequence of TGF-β signalling disruption on the maintenance of EBV genomes in the NPE cell lines, subsequent experiments were aimed to investigate the possible mechanisms that were responsible for these effects. Given that EBV infection is associated with growth inhibition and senescence, as well as entry into lytic cycle in a manner that is linked to epithelial cell differentiation, this study examined the effects of TGF-β signalling disruption on the expression of senescence-related markers (SIRT1, p16 and p21), a differentiation marker (involucrin) and the EBV gene that controls the switch from latent to lytic cycle (*BZLF1*).

Lastly, to conclusively demonstrate the role of TGF-β signalling in facilitating EBV maintenance in NPE cells, *TGFBR2* was knocked out in NP460hTert cells using the CRISPR/Cas9 system and the effects on EBV persistence examined. Rescue experiments were then performed by re-expressing a wild-type *TGFBR2* gene in the CRISPR9/NP460hTert cells.

## 1.3 Objectives

The objectives of present study are as follows:

I. To study the influence of TGF-β signalling disruption on the outcome of EBV infection in NPE cells

II. To examine the effects of TGF-β signalling disruption on cellular differentiation, EBV-induced senescence and the induction of EBV lytic cycle

III. To study the outcome of CRISPR/Cas9-mediated *TGFBR2* knockout on the maintenance of EBV genomes in NP460hTert cells

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Cancer

Cancer is a global health problem affecting men and women. It is one of the leading causes of mortality and morbidity worldwide (Ferlay et al., 2015). Globally, there were 14.1 million new cancer cases reported in 2012 with a higher incidence rate in males compared to females (Ferlay et al., 2015). In Malaysia, a total number of 103, 507 new cancer cases were reported between 2007 and 2011 (Azizah et al., 2015).

Cancer can be defined as a disease in which a group of abnormal cells proliferate beyond their usual boundaries and invade nearby tissues (Hejmadi, 2010). Normal cells invariably respond to signals or stimuli such as growth factors that dictate whether the cells should divide, differentiate into another cell type or die. In contrast, cancer cells employ different mechanisms to achieve immortalisation (Hejmadi, 2010).

In general, all cancers are caused by a combination of both external and internal risk factors (Parsa, 2012). There are three main external risk factors which can contribute to the development of cancer: firstly, chemical or non-chemical carcinogens such as N-nitroso-N-methylurea (NMU), ultraviolet and ionizing radiation (Cadet et al., 2005; Gruijl et al., 2001); secondly, lifestyle factors including diet, such as consumption of alcohol and tobacco smoking (Key et al., 2004); lastly, biological carcinogens, such as infection with certain types of bacteria or virus (Vedham et al., 2014).

Once the transformation process is initiated, it takes multiple additional steps for cancer to form. In 2000, six hallmarks or common traits that govern the transformation of normal cells to cancer cells were proposed; namely, sustaining proliferative signalling, evading growth suppression, resisting cell death, tissues invasion and

metastasis, inducing angiogenesis and enabling replicative immortality (Hanahan & Weinberg, 2000). Two additional hallmarks of cancer were proposed in 2011, which are de-regulating cellular energy metabolism and evasion of immune destruction (Hanahan & Weinberg, 2011). The acquisition of these eight hallmarks of cancer was suggested to be facilitated by two important characteristics, which are genomic instability and mutation, as well as tumour-promoting inflammation (Hanahan & Weinberg, 2011).

## 2.2 Nasopharyngeal carcinoma (NPC)

### 2.2.1 Epidemiology

Globally, NPC is a rare malignancy affecting 1 per 100,000 people annually. However, it is very prominent in the Southern part of China and Southeast Asia with the incidence rate of approximately 20-50 per 100,000 per year especially in Southern Chinese those of Cantonese origin (Cao et al., 2015; Chang & Adami, 2006; Jia et al., 2006; Parkin et al., 2005; Wei et al., 2014). In Malaysia, NPC is the fifth most common cancer and fourth leading cancer among males (8.1% of total male cancers) (Azizah et al., 2015). Further, in 2012, it was estimated that Malaysia had the highest aged-standardized rate (ASR) of NPC in the world with an ASR of 7.2 per 100, 000 people (http://globocan.iarc.fr). NPC is also closely related to ethnicity. Studies conducted in multi-cultural countries like Malaysia and Singapore demonstrated that the Chinese populations have a higher incidence rate of NPC compared to Malay or Indian populations (Azizah et al., 2015; Chang & Adami, 2006; Seow et al., 2004). Interestingly, an early study conducted in year 2004, has shown that one of the indigenous groups in Sarawak (a state in East Malaysia), Bidayuh, had the highest incidence rate of NPC in the world (Devi et al., 2004).

### 2.2.2 Histopathology

NPC is a distinct type of head and neck cancer that arises from the epithelial lining of the nasopharynx. In 1991, NPC was classified into two categories; keratinizing squamous cell carcinoma and non-keratinizing carcinoma, which is sub-divided into differentiated and undifferentiated carcinoma (Shanmugaratnam & Sobin, 1991). In general, obvious squamous differentiation features including the presence of intercellular bridges, keratinisation and epithelial pearl formation characterize keratinizing squamous cell carcinoma. In contrast, non-keratinizing carcinoma characterized by sheets of epithelial cells show syncytial architecture with lymphocytes intimately associated with the neoplastic cells and absence of keratinization. The undifferentiated NPC is also referred as lymphoepithelioma of the nasopharynx due to the high frequency of reactive lymphocytes in the tumour microenvironment (Thompson, 2007).

Keratinizing NPC is more common in low incidence areas, for example, approximately 78% of reported NPC cases in the United States are keratinizing NPC (Marks et al., 1998). Patients with keratinizing NPC have a higher frequency of locally advanced tumours and they are less responsive to treatment that results in a poor survival rate (Reddy et al., 1995). In contrast, non-keratinizing NPC is more common in endemic regions, such as Hong Kong (Lo et al., 2004b; Marcus & Tishler, 2010). Patients with non-keratinizing NPC have higher tendency to develop distant metastases, but they are more responsive to treatment, and have a better prognosis (Marks et al., 1998; Reddy et al., 1995).

### 2.2.3 Aetiology

There are three main risk factors for NPC, namely environmental factors, genetic susceptibility and EBV infection.

#### 2.2.3.1 Environmental factors

Based on case-control studies conducted in different populations, it is proven that high consumption of food with a large amount of nitrite and nitrosamines (such as salted fish, preserved or processed foods), particularly during childhood, correlates with the development of NPC (Jia et al., 2010; Ning et al., 1990; Ward et al., 2000; Yu et al., 1988; Yuan et al., 2000). In contrast, frequent consumption of vegetables and/or fresh fruits is associated with a lower risk of NPC (Jia et al., 2010; Liu et al., 2012; Polesel et al., 2013; Yuan et al., 2000).

In addition, multiple studies have demonstrated that cigarette smoking is significantly associated with an increased risk for NPC and may also increase the risk of mortality in NPC patients (Ekburanawat et al., 2010; Hsu et al., 2009; Lin et al., 2015a; Xie et al., 2015). Studies examining the association between alcohol consumption and NPC are still not conclusive. High alcohol consumption has been reported to correlate with the risk for NPC in White Americans (Nam et al., 1992; Vaughan et al., 1996); however, studies conducted in Chinese populations failed to show similar findings (Ji et al., 2011). The discrepancy in findings may be due in part to study populations. Several case-control studies have also shown that occupational exposure to chemical carcinogens such as wood dust, formaldehyde, cotton dust, acids caustics and long duration of working in dyeing and printing factories increased the risk of developing NPC (Hildesheim et al., 2001; Li et al., 2006b; Vaughan et al., 2000).

#### 2.2.3.2 Genetic susceptibility

The importance of genetic susceptibility in the development of NPC derived from the observation that the second and third generations of Chinese who emigrated to low endemic areas had a higher incidence of NPC than the local Caucasians (Buell, 1974). Familial aggregations of NPC and the occurrence of multiple cases of the disease in first-degree relatives have subsequently been reported in Southern Chinese populations (Jia et al., 2004). Moreover, epidemiologic studies have shown that the risk of NPC was higher among individuals with a first-degree relative with NPC, compared with those without a family history (Chen & Huang, 1997; Hsu et al., 2011; Jia et al., 2004).

It is now well documented that genetic variations caused by mutations or single nucleotide polymorphisms (SNPs) contribute to the development of NPC. A number of genome-wide association studies (GWAS) have revealed several variants or SNPs of major histocompatibility (MHC) class 1 genes on chromosome 6p21.3 were strongly associated with NPC risks (Bei et al., 2010; Tang et al., 2012; Tse et al., 2009). The MHC class 1 genes, including human leukocyte antigen *(HLA)-A, HLA-B,* and *HLA-C*, encode proteins that play important roles in triggering host immune responses by identifying and presenting foreign antigens (such as EBV-encoded peptides) to the cytotoxic T cells (Hansen & Bouvier, 2009). An increased risk of NPC is found in individuals with *HLA-A*02:07, A*33:03* and *B*38:02* alleles, whereas those with *HLA-A*11:01, HLA-A*31:01, B*13:01* and *B*55:02* alleles have a decreased risk of NPC (Bei et al., 2012; Goldsmith et al., 2002; Tang et al., 2012; Tian et al., 2015). Additionally, variations in other genes that are located within the MHC regions such as *GABBR1*, *NLRC5*, *B2M*, *HCG9* and *CIITA* were also shown to increase the risk of developing NPC (Cui et al., 2016a; Li et al., 2017; Tang et al., 2012; Tse et al., 2009).

In addition to the MHC region, GWAS studies have identified other genetic susceptibility loci for NPC, including *CDKN2A/CDKN2B* (9p21), *CLPTMIL/TERT* (5p15.3), *MECOM* (3q26) and *TNFRS19* (13p12) (Bei et al., 2010; Bei et al., 2016; Cui et al., 2016a; Dai et al., 2016a). Further, a case-control GWAS study conducted in Malaysia identified genetic variations in the integrin-α 9 (*ITGA9*) gene which is located on chromosome 3p were associated with the increased susceptibility to NPC in Malaysian Chinese populations (Ng et al., 2009a). Additionally, genome-wide studies of copy number variations (CNV) showed that CNV of *MICA* and *HCP5* genes located on chromosome 6p21.3 were associated with increased risk of NPC (Tse et al., 2011). In addition to CNV on chromosome 6p21.3, CNV on chromosome 11q14.3 was also reported as an NPC susceptibility locus in Malaysian Chinese populations (Low et al., 2016). Further, whole exome sequencing (WES) of germline DNAs from three closely related family member of NPC revealed variants (T316S, S772L and Y816*) in the *MLL3* gene, suggesting a high penetrance inherited mutation predisposing to NPC (Sasaki et al., 2015). In a cohort of 161 NPC patients and 895 controls from Southern China, *MST1R* gene on chromosome 3p21.3 was identified as a novel susceptibility gene of NPC patients with early-age onset (age of ≤20y) (Dai et al., 2016b). Taken together, identification of NPC susceptibility loci and their biological function would greatly enhance the understanding of the genetic contributions to NPC tumourigenesis.

Polymorphisms in other genes including genes responsible for DNA repair (*hOGG1, RAD51L1, XRCC1*), interleukins (*IL1α, IL10, 1L16, IL18*), nitrosamine metabolism (*CYP2E1, CYP2A6*), detoxification of carcinogens 1 (*GSTM1*), cell cycle control (*MDM2 and TP53*), cell adhesion and migration (*MMP2*) have been shown to increase the risk of developing NPC (Cho et al., 2003; Cui et al., 2016b; Guo & Xia, 2013; He et al., 2007; Hildesheim et al., 1997; Hildesheim & Wang, 2012; Tiwawech et al., 2006; Yao et al., 2016). Lastly, there is increasing evidence to suggest that

polymorphisms in particular microRNAs (miRNAs), such as miR-146a, miR-196A2 and miR-423, may alter individual susceptibility to NPC (Binbin Li et al., 2017; Huang et al., 2014; Li et al., 2014; Lung et al., 2013).

#### 2.2.3.3 EBV infection

The link between EBV and NPC was initially identified in 1966 when elevated levels of antibodies against the EBV viral capsid antigen (VCA) and early antigen (EA) were found in the sera of NPC patients (Old, 1966). The association of EBV with NPC was later confirmed by the presence of EBV genomes in the biopsy samples of NPC patients (Nonoyama et al., 1973; Wolf et al., 1973). It is now well-recognised that NPC, particularly non-keratinising NPC, is invariably associated with EBV infection (Young et al., 2016). EBV genomes in NPC cells exist as monoclonal episomes, suggesting that NPC is derived from the clonal expansion of a single EBV-infected progenitor cell (Chen et al., 1993; Pathmanathan et al., 1995; Raab-Traub & Flynn, 1986). The contribution of EBV infection in NPC pathogenesis is further illustrated in section 2.3.

### 2.2.4 Clinical presentation, diagnosis and treatment

Due to non-specific symptoms, more than 70% of NPC patients are diagnosed at advanced stages (Zainal Ariffin & Nor Saleha, 2007). The most common presenting symptoms of NPC are neck lumps, nasal discharge, nasal blockage, mild hearing loss, mild blocked ears, unilateral facial numbness and unilateral headache (Chan et al., 2005; Khoo & Pua, 2013).

Diagnosis and staging of NPC are made based on clinical, histopathological and radiological examinations. Basic clinical examinations include a complete physical

examination of head and neck area (nose, mouth, throat, facial muscles and cervical lymph nodes) and a more detailed examination including nasal endoscopy of the nasopharynx to determine the presence of exophytic tumours (Abdullah et al., 2009). For histopathological examinations, tumour biopsy samples obtained from the nasopharyngeal mass are sent for microscopic examination to determine the presence of cancer cells and subtypes of NPC (Abdullah et al., 2009). Lastly, radiological examinations such as chest X-ray, bone scan, computerized tomography (CT) scan, integrated positron emission tomography (PET/CT) scan and magnetic resonance imaging (MRI) are used to assess tumour extension and disease stage (Ng et al., 2009b). Among these radiological techniques, MRI is more sensitive in detecting tumour involvement in the parapharyngeal space, skull base, intracranial area, sphenoid sinus and retropharyngeal nodes. Meanwhile, PET/CT scan is more superior in detecting local and distal metastases (Ng et al., 2009b). The combined use of MRI and PET/CT scan for diagnosis provides more accurate information on the disease staging which may help in designing treatment plans for NPC patients (Mao et al., 2008; Xu et al., 2017). The tumour, node, and metastasis (TNM) classification of the American Joint Committee on Cancers (AJCC) is used to determine the stage of the disease (Brennan, 2006). Although not routinely used in the clinics, other EBV-based methods, for example EBV serological examination and measurement of EBV DNA load in plasma, have been suggested as diagnostic tests for NPC and these examination may be useful for early detection of NPC in high-risk groups (Cao, 2017; Chan et al., 2013; Chen et al., 2015b; Tao & Chan, 2007).

Unlike other cancers of head and neck, radiotherapy (RT) instead of surgery is the mainstay of treatment for NPC due to the hypersensitivity of NPC tumours to radiation and location of the tumours in close proximity with many vital organs (Wei & Kwong, 2010). The commonly used RT techniques for NPC are two-dimensional RT

(2D-RT), three-dimensional conformal RT (3D-CRT) and intensity-modulated RT (IMRT). Among the above RT techniques, IMRT provides a better treatment option in treating all stages of NPC by ensuring high local tumour control, reduced toxicity rates and improved survival rates of NPC patients (Chen et al., 2016; Lai et al., 2011). Currently, concurrent chemo-radiotherapy is used to treat patients with locally advanced NPC, and the most common chemotherapy drugs are cisplatin and 5-fluorouracil (Chen et al., 2013; Paiar et al., 2012).

However, treatment failure using RT alone or concurrent chemo-radiotherapy in treating NPC patients with advanced and distant metastasis has become a major problem (Chen et al., 2016; Ma et al., 2016; Tan et al., 2016). This treatment often causes severe side effects due to the location of the tumour at the base of the skull, closely surrounded by vital organs such as the brain (Ma et al., 2016; Tan et al., 2016). The existence of EBV in almost all NPC cells provides an opportunities for the development of EBV-based treatment such as immunotherapies or inhibitors that may be useful in treating NPC patients in the future (Cao et al., 2014; Cao, 2017; Hutajulu et al., 2014; Lutzky et al., 2014; Smith et al., 2012).

### 2.2.5 Molecular basis of NPC

With the advances of genetic and cytogenetic techniques, a high frequency of chromosome abnormalities have been identified in NPC cell lines, xenografts and primary tissues (Lo et al., 2012; Lo & Huang, 2002). These data guided attempts to identify numerous oncogenes and tumour suppressor genes, which are involved in the pathogenesis of NPC. Frequent chromosome losses in NPC were detected on chromosome 3p, 9p and 14q which contain tumour suppressor genes such as *RASSF1A* (3p21.3), *CDKN2A/p16* (9p21.3), *TRAF* (14q32.3) and *NFKBIα* (14q13) (Cheng et al.,

2000; Cheung et al., 2009; Lo et al., 1995; Lo et al., 2000; Xiao et al., 2006; Yau et al., 2006; Zhou et al., 2009). Among these genes, inactivation of *CDKN2A/p16* by homozygous deletion and/or promoter hypermethylation has been reported in almost all NPC samples (Lo et al., 1996; Lo et al., 1995). *CDKN2A/p16* plays an important role in regulating G1/S phase of cell cycle progression and inactivation of this gene was shown to support persistent EBV infection in immortalised NPE cells (Serrano et al., 1993; Tsang et al., 2012). In addition, inactivation of *RASSF1A* has been detected in more than 80% of primary NPC tumours and inactivation of this gene was shown to accelerate mitotic progression that led to increased risks for chromosomal aberrations in EBV-positive NPC cell line, C666.1 (Lo et al., 2001). Further, inactivation of *CDKN2A/p16* and *RASSF1A* was consistently detected in pre-cancerous dysplastic lesions of nasopharynx, suggesting that inactivation of these genes is crucial in the initiation and progression of NPC (Chan et al., 2002; Chan et al., 2000).

Amplifications of several oncogenes such as *PIK3CA* (3q26.3), *cyclin D1/CCND1* (11q13) and *LTBR* (12p13) have been reported in NPC (Hui et al., 2002; Hui et al., 2005; Or et al., 2010), suggesting the involvement of activated nuclear factor-kappa B (NF-κB) and phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) in the progression of NPC. In addition, two oncogenic fusion genes, E3 ubiquitin-protein ligase component n-recognin 5 (*UBR5*)-zinc finger protein 423 (*ZNF423*) and fibroblast growth factor receptor 3 (*FGRF3*)-transforming acidic coiled-coil-containing protein 3 (*TACC3*), were identified in NPC primary tumours (Chung et al., 2013; Zheng et al., 2016).

In 2014, Lin and colleagues were the first to describe a comprehensive mutational landscape of NPC genomes using a combination of WES, target deep sequencing and SNP array analysis. A number of distinct genetic alterations, such as

deletion and/or mutations of genes affecting important cellular processes and pathways including chromatin modification (*ARID1A, MLL2, BAP1, MLL3, TET*), ERBB-PI3K signalling (*PIK3CA, ERBB2, ERBB3, AKT2, PTEN*) and autophagy machinery (*ATG2A, ATG7, ATG13*) were identified in the study (Lin et al., 2014a). More recently, loss-of-function mutations of multiple NF-κB pathway negative regulators (*NFKBIα, CYLD, TNFAIP3, NLRC5*) were reported in NPC using WES analyses, demonstrating that activation of NF-κB signalling is crucial in the pathogenesis of NPC (Li et al., 2017; Zheng et al., 2016). Notably, expression of EBV-encoded LMP1 appears to be mutually exclusive with the presence of the mutated NF-κB regulators (Li et al., 2017), suggesting that occurrence of mutation in the negative regulators of NF-κB pathway supplants the needs for LMP1-mediated NF-κB activation during the progression of NPC. Collectively, these observations suggest that the NF-κB pathway is constitutively activated in NPC and its activation is mediated by either LMP1 or somatic mutations to confer an inflammatory response that is crucial in NPC tumorigenesis. It is of interest that mutations in *TP53* and alipoprotien B mRNA editing enzyme catalytic polypeptide-like (APOBEC)-mediated signatures genes (*APOBEC3B, APOBEC3A*) were also identified in NPC (Lin et al., 2014a; Zheng et al., 2016). Collectively, these data have led to a genetic progression model being proposed for NPC (Figure 2.1).

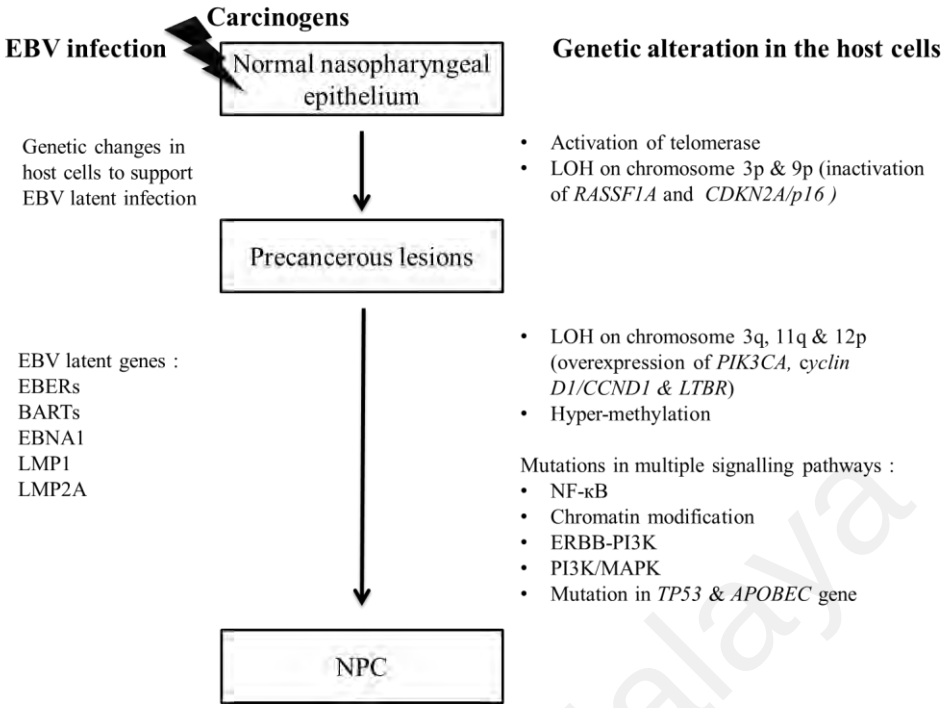



**Figure 2.1. Model of NPC Pathogenesis.** Long-term exposure of the NPE cells to environmental carcinogens (e.g. salted food and preserved food) induces various genetic alterations in the NPE cells, including activation of telomerase activity and inactivation of *RASSF1A* and *CDKN2A/p16* on chromosome 3p and 9p, which facilitate immortalisation and genome instability. Further de-regulation of cellular signalling of host cells (inactivation of *CDKN2A/p16* and/or overexpression of *cyclin D1/CCND1*) promotes persistent EBV infection in NPE cells. Subsequently, the expression of latency II genes including EBERs, BARTs, EBNA1, LMP1 and LMP2A alters multiple cellular pathways and modulates host's microenvironments that encourage tumour formation. Importantly, EBV facilitates global hyper-methylation, which inactivates various tumour suppressive genes. Further, the occurrence of acquired mutations in multiple signalling pathways including NF-κB, chromatin modification, ERBB/PI3K and PI3K-MAPK alters the activities of various cancer-related genes to enhance tumour heterogeneity. NF-κB pathway is constitutively activated in NPC and its activation is mediated by either LMP1 or somatic mutations to confer an inflammatory response that is crucial in NPC tumorigenesis. Further, somatic mutations of *TP53, APOBEC* and other genes may also drive the tumour progression. Figure modified from Tsao et.,al 2017.

## 2.3 Epstein - Barr virus (EBV)

EBV was discovered in 1964 as the first human tumour virus (Epstein et al., 1964) and is now classified as a Group I carcinogen. A number of malignancies are associated with EBV infection, including Burkitt lymphoma (BL), Hodgkin lymphoma (HL), post-transplant Lymphoproliferative disorders (PTLD), NPC and Gastric carcinoma (GC) (Young et al., 2016).

### 2.3.1 EBV genome and sequence variations

EBV was identified in a Burkitt's lymphoma biopsy by Anthony Epstein's group (Epstein et al., 1964). EBV belongs to the *γ-herpesvirinae* subfamily and its genome is composed of linear double-stranded DNA of approximately 184 kilobases (kb) that encodes for more than 85 genes. It is an enveloped virus that contains a DNA core surrounded by an icosahedral nucleocapsid and a tegumen. The viral genome has a series of 0.5kb terminal direct repeats (Trs) at each terminus that divide the genome into short and long unique sequences (Baer et al., 1984; Bankier et al., 1983; Kintner & Sugden, 1979). When EBV infects a cell, the Trs join to form a covalently closed circular episome. The joining of the TRs regions occurs randomly and, as a result, each circularized episome has a unique but variable number of Trs; therefore, the number of Trs can be used to determine EBV clonality in the infected cells (Bankier et al., 1983).

Two main subtypes of EBV have been identified, namely EBV-1 (type 1) and EBV-2 (type 2). These two types differ in the sequences of EBV nuclear antigen (EBNA)-2 and EBNA-3 genes (Dambaugh et al., 1984; Rowe et al., 1989; Sample et al., 1990). Compared to type 2, type 1 is more efficient in transforming B-lymphocytes and the infected cells grow significantly faster (Rickinson et al., 1987). Further, type 1 is

more predominantly observed in NPC and is the most common strain identified worldwide, while type 2 is more common in some areas of Africa (Rickinson et al., 1987; Zimber et al., 1986).

EBV strain variations are postulated to contribute to different EBV-associated diseases in different geographical locations. It was not until 1984 that the first complete EBV genome of the B95.8 strain was sequenced by Sanger sequencing (GenBank accession no. NC_007605) (Baer et al., 1984). In 2003, a "wild-type" EBV genome of 171kb was constructed using B95.8 as a backbone whilst a 12kb deleted segment was provided by the Raji sequences (de Jesus et al., 2003). With the advancement of next-generation sequencing (NGS) technology, almost 100 EBV strains from all over the world have been sequenced and published, including EBV strains isolated from NPC (GD1, GD2, HKNPC1-9 and M81) and BL (AG876, Mutu and Akata) biopsies (Dolan et al., 2006; Kwok et al., 2012; Kwok et al., 2014; Lin et al., 2013; Palser et al., 2015; Santpere et al., 2014; Tsai et al., 2013; Zeng et al., 2005).

Analysis of the EBV genomes demonstrated that the differences in EBNA2, EBNA3A, 3B and 3C gene sequences remain the major variations among different strains (Palser et al., 2015). Recently, studies have shown that different EBV strains possess different transforming abilities and there is a correlation between the cell tropism and the lineage of the tumours they induce. For example, the M81 EBV strain (isolated from an NPC) infected primary epithelial cells more efficiently than, B95.8 (isolated from an IM patient) and Akata (isolated from a BL) EBV strains, which displayed a stronger tropism for B cells (Tsai et al., 2017; Tsai et al., 2013).

Variations in some EBV latent genes might also result in functional differences, and LMP1 is of particular interest. An LMP1 variant containing a 10-amino acid (30bp) deletion at a region upstream of C-terminal activating region (CTAR)-2 (amino acids 346-355) was commonly found in NPC from the endemic regions (Cheung et al., 1998; Cheung et al., 1996; Hu et al., 1991; Miller et al., 1994). LMP1 has been detected in EBV isolates from various parts of the world, however from NGS analysis, sequences variations have been identified in LMP1 isolated from NPC tumours (Kwok et al., 2012; Kwok et al., 2014; Palser et al., 2015). Compared to the prototype B95.8-LMP1, LMP1 isolated from NPC tumours (NPC-LMP1) has an increased transforming ability *in vivo* and *in vitro* (Hu et al., 1993; Lo et al., 2004a). Further, NPC-LMP1 has been shown to activate NF-κB signalling more efficiently (Lo et al., 2004a; Miller et al., 1998; Rothenberger et al., 1997). Notably, sequence variations of EBNA1, EBNA2 and EBNA3 and there is no substantial evidence of disease association with these sequence variations (Kwok et al., 2012; Kwok et al., 2014; Palser et al., 2015; Wu et al., 2012).

### 2.3.2 Dual tropism of EBV infection

More than 95% of the adult population worldwide is infected with EBV (Young et al., 2016). In most developing countries, primary EBV infection in healthy individuals occurs within the first few years of life and is generally asymptomatic. In developed countries, primary infection is often delayed until late adolescence or adulthood and is often accompanied by infectious mononucleosis (IM). IM is a self-limiting lymphoproliferative disease that is described as acute glandular fever and is characterized by expansion and proliferation of B-cells (Evans et al., 1968).

Primary EBV infection is believed to be initiated by the virus crossing the epithelium of the oropharynx, infecting naïve B cells in the tonsil and driving their proliferation to become activated B blasts (Figure 2.2). Some infected B cells escape cytotoxic T cell responses and transit through a germinal center reaction. Through a series of viral latency programs, the infected B cells are eventually driven into resting memory B cells and a life-long infection is established (Rickinson, 2014; Thorley-Lawson & Gross, 2004). The differentiation of memory B cells into plasma cells trigger EBV lytic cycle to release new virions for spreading to new hosts (Laichalk & Thorley-Lawson, 2005). The oropharyngeal epithelium is believed to be a major site for viral replication, supported by observations showing that EBV replication was found in the epithelial cells of oral 'hairy' leukoplakia in patients with human immunodeficiency virus (HIV) (Niedobitek et al., 1991b). Furthermore, a study has shown that EBV can establish a productive infection in the suprabasal layers of stratified epithelium in an organotypic oral keratinocyte culture system, further pointing a role for epithelial cells in the replication and spreading of EBV virions (Temple et al., 2014). This dual tropism of EBV infection appears to be crucial for the virus to maintain a persistent infection in humans.

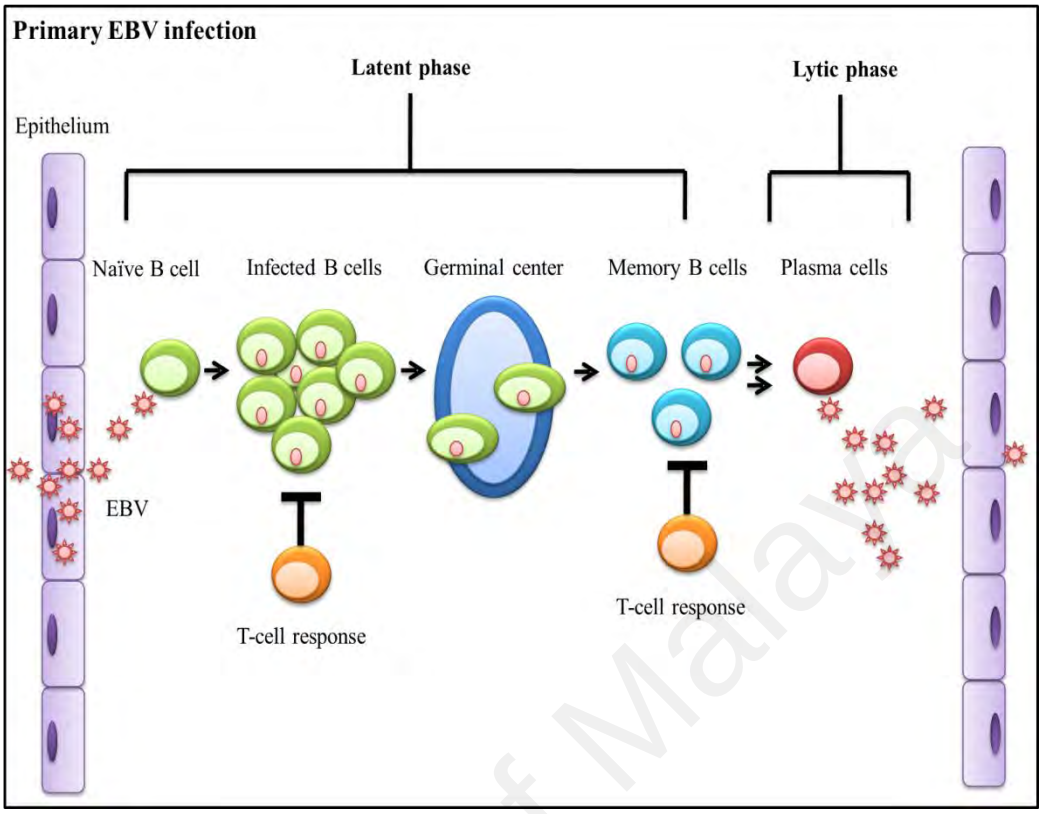



**Figure 2.2. Primary EBV infection.** Primary EBV infection begins in the epithelium of the oropharynx. EBV infects naïve B cells in the tonsil and drives their proliferation to become activated B blasts. Infected B cells, which have escaped the cytotoxic T cell response, enter into a germinal center. Through a series of viral latency programs, the infected B cells are eventually driven into resting memory B cells to achieve a life-long infection. The differentiation of the infected memory B cells into plasma cells triggers EBV lytic cycle to release new virions for spreading to new host. Figure modified from Rickinson, 2014.

### 2.3.3 EBV entry mechanisms

EBV infection of B cells is initiated by a receptor-mediated process, whereby the EBV envelope protein, gp350, binds the CR2 (CD21) receptor on the surface of B cells (Nemerow et al., 1987). The fusion of the EBV virions into B cells is subsequently triggered by the interaction between another viral envelope glycoprotein, gp42, with the HLA class II molecule on the B cell surface. Gp42 also binds directly to another viral glycoprotein, gH, a component of the viral "core fusion machinery" that consists of a homotrimer gB and a heterodimer gHgL. These interactions trigger the fusion of the virion envelope with the host cell membrane to facilitate EBV entry (McShane et al., 2003).

Compared to B cells, EBV infection of human epithelial cells is relatively less well studied. Neither CR2 nor HLA class II molecules are expressed on the surface of epithelial cells (Imai et al., 1998; Shapiro & Volsky, 1983). Consequently, EBV infection of epithelial cells *in vitro* is not as efficient as that of B-cells (Tsang et al., 2014) and the infection rate can be enhanced by direct co-culturing of the epithelial cells with IgG-treated EBV-producing Akata cells (Daibata et al., 1990; Imai et al., 1998; Takada et al., 1991). Although EBV glycoprotein gp42 is essential for EBV entry into B cells, it impedes the entry of EBV into epithelial cells by forming a ternary complex with gHgL glycoprotein (Sathiyamoorthy et al., 2016; Wang et al., 1998). Indeed, EBV particles released from B cells lack gp42 which facilitate an efficient infection of epithelial cells (Borza & Hutt-Fletcher, 2002). It has been shown that fusion of EBV with epithelial cells was mediated by the direct interaction between EBV glycoproteins gHgL and the host integrin's (αvβ5, αvβ6 and αvβ8) (Chesnokova & Hutt-Fletcher, 2011; Chesnokova et al., 2009; Tugizov et al., 2003; Wang et al., 2015). On polarized epithelial cells, which resemble more closely the *in vivo* environment, an interaction

between the viral membrane protein BMRF-2 and the host β1 and/or αv integrin's has been demonstrated to mediate the entry of EBV virions through basolateral membranes (Tugizov et al., 2003; Xiao et al., 2008; Xiao et al., 2007). Another mechanism of EBV entry into polarised epithelial cells via the basolateral surface was described as a process of 'transfer infection' whereby EBV-loaded CD11b+ B cells form conjugates with epithelial cells by binding to the CD44v3 and lymphocyte-endothelial-epithelial cell adhesion molecule (LEEP-CAM) (Shannon-Lowe et al., 2006; Shannon-Lowe & Rowe, 2011). Further, neuropilin 1 and non-muscle myosin heavy chain IIA, have been found to mediated EBV entry into epithelial cells by interacting with the gHgL complex (Wang et al., 2015; Xiong et al., 2015). Of note, a novel "in-cell infection" mechanism for EBV infection in an immortalised NPE cell line (NPEC1-Bmi1) and an EBV-negative NPC cell line (CNE2) has been described. This process occurs through the penetration of EBV infected B-cells into epithelial cells by forming cell-in-cell structures, resulting in the release of virions into epithelial cells (Ni et al., 2015). Very recently, a study has shown that EBV entry into epithelial cells is mediated by the binding of the viral gHgL complex to the cellular protein, Ephrin receptor tyrosine kinase A2 (EphA2) (Chen et al., 2018).

### 2.3.4 EBV infection cycle

EBV displays two distinct lifecycles, namely lytic and latent cycles. The lytic cycle is associated with viral replication in which new virions are produced while latent cycle is a state of persistent infection (Tsurumi et al., 2005).

#### 2.3.4.1 EBV lytic infection

The lytic form of EBV infection is important for virus spreading and transmission from host to host. EBV lytic infection can be induced *in vitro* efficiently in latently infected B cells using various chemicals such as phorbol 12-mysistate 13-acetate (TPA), sodium butyrate, trichostatin A and valproic acid (Countryman et al., 2009) or by cross-linking the cell surface receptor of B cells with anti-human IgG antibody (Sinclair et al., 1991). While the precise *in vivo* mechanisms responsible for triggering the EBV lytic cycle are not fully understood, this process is intimately associated with the differentiation of the host cells (Becker et al., 1991; Laichalk & Thorley-Lawson, 2005; Nawandar et al., 2015; Young et al., 1991). Following reactivation, the lytic genes of EBV are expressed in a temporally regulated manner and more than 80 EBV lytic genes have been identified so far (Murata, 2014; Young et al., 2007). The viral immediate-early genes, *BZLF1* and *BRLF1*, which encode the transcription factors, Zta and Rta respectively, are the earliest viral genes, which are expressed upon lytic reactivation. These genes trigger the activation of lytic cycle by activating the transcription of EBV early genes which result in the replication of the EBV DNA and finally leading to the expression of EBV late proteins resulting in the production of infectious virions (Binne et al., 2002; Kolman et al., 1996; Le Roux et al., 1996; Ragoczy et al., 1998; Schelcher et al., 2005). It is recognised that expression of *BZLF1* alone is sufficient to disrupt EBV latency, thereby activating EBV lytic genes including *BRLF1*. Compared to *BZLF1*, expression of *BRLF1* alone is less potent to disrupt EBV latency, suggesting that *BZLF1* and *BRLF1* may act in synergy to activate the viral lytic cycle (Kolman et al., 1996; Le Roux et al., 1996; Ragoczy et al., 1998) (Schelcher et al., 2005). A study has shown that expression of both the *BZLF1* and *BRLF1* is more effective in activating EBV lytic cycle compared that either gene expressed (Liu et al., 1997). *BZLF1* and *BRLF1* synergistically induce the expression of

multiple early lytic genes, including six viral protein required to assemble the replication machinery; BALF5 (the DNA polymerase), BALF2 (the single-stranded DNA-binding protein homologue), BMRF1 (the DNA polymerase processivity factor), BSLF1 (the primase homologue), BBLF4 (the helicase homologue) and BBLF2/3 (formed from the splicing of *BBLF2* and *BBLF3* open reading frames; a potential homologue of the third component of the helicase–primase complex) (Young et al., 2016). An abortive lytic cycle has been proposed to be the initial mode of primary EBV infection of both B cells and epithelial cells that is considered essential for the transformation process (Cochet et al., 1993; Ersing et al., 2017; Klein et al., 1974; Lin & Raab-Traub, 1987; Martel-Renoir et al., 1995). Evidence has emerged to show that lytic genes play important roles in the pathogenesis of EBV-associated malignancies, for examples, several lytic genes encode immune evasion proteins, including BNLF2a (inhibits the transporter of antigen processing), BILF1 (induces MHC class I internalization and degradation) and BGLF5 (mediates host shut-off) (Young et al., 2016).

#### 2.3.4.2 EBV latency programs

A limited set of EBV genes is expressed during latent infection. EBV transforms resting B cells into immortalised lymphoblastoid cell lines (LCLs) in which the full spectrum of EBV latent genes - is expressed (latency III), including six Epstein-Barr nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and LP), three latent membrane proteins (LMP1, LMP2A and LMP2B), two non-coding Epstein-Barr virus-encoded RNAs (EBER1 and EBER2), BamHI-A rightward transcripts (BARTs), miRNAs of BARTs and BamHI fragment H rightward open reading frame 1 (BHRF1). During various stages of B cell differentiation *in vivo*, EBV expresses either the latency III or a more restricted form of latency known as latency II (expression of EBNA1, LMPs, EBERs,

BARTs, miR-BARTs) or latency 1 (only EBNA1, EBERs, BARTs and miR-BARTs are expressed) (Rowe et al., 1992). NPC expresses a latency II programs with an additional latent protein called BamHI-A fragment rightward reading frame 1 (BARF1). During latency III, all the EBNAs are transcribed from either the C promoter (Cp) or W promoter (Wp). In contrast, EBNA1 is transcribed from the Q promoter (Qp) during latency I and latency II (Figure 2.3) (Lear et al., 1992; Li & Minarovits, 2003; Salamon et al., 2001; Tao et al., 1998). EBV-associated B cell malignancies express either latency I, II or III, while EBV-associated epithelial cancers express a latency II pattern (Table 2.1).

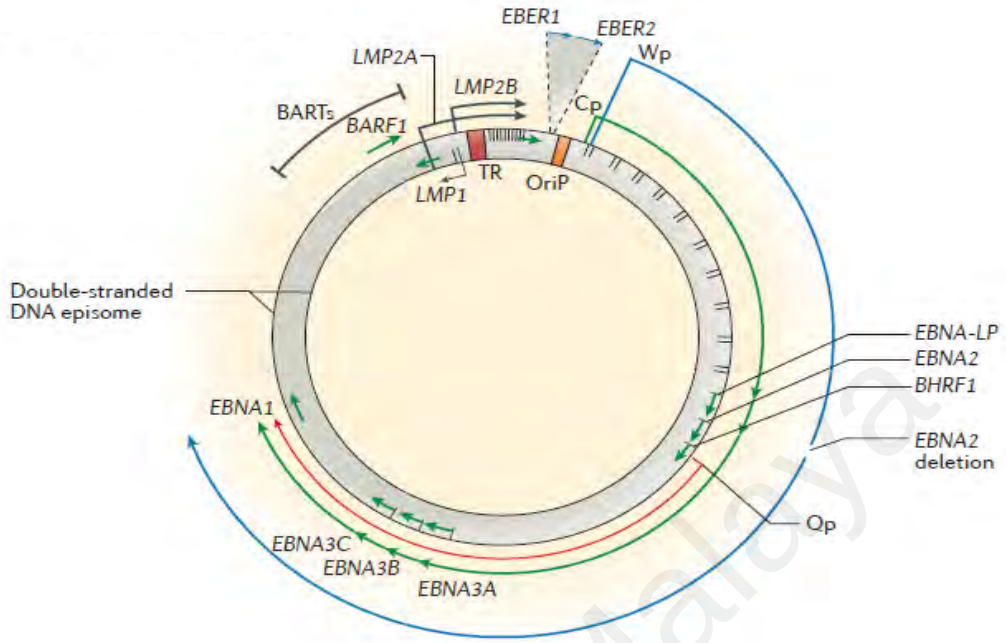



**Figure 2.3. The EBV genome.** The origin of plasmid replication (OriP) is shown in orange. The short thick green arrows represent exons encoding latent proteins: six Epstein-Barr nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and LP), three latent membrane proteins (LMP1, LMP2A and LMP2B), BamHI fragment H rightward open reading frame 1 (BHRF1) and BamHI-A fragment rightward reading frame 1 (BARF1). The short blue arrows at the top represent the highly transcribed non-polyadenylated EBV-encoded RNAs (EBER1 and EBER2). The middle long green arrow represents EBV transcription during latency III, in which all the EBNAs are transcribed from either the Cp or Wp promoter. The inner red arrow represents the EBNAs transcript, which originated from Qp promoter during latency I and II. Taken from Young et al., 2016.

**Table 2.1.** Latency programs in EBV-associated malignancies

| **Latency** | **Malignancies** |
|---|---|
| I | Burkitt's lymphomas |
| II | Gastric carcinoma, Extranodal natural killer (ENK)/T-cell lymphoma, Hodgkin's lymphomas, Nasopharyngeal carcinoma |
| III | Post-transplant lymphoproliferative disorders in immunodeficiency patients |

### 2.3.5 Function of EBV latent genes in NPC

#### 2.3.5.1 Epstein-Barr nuclear antigens (EBNA1)

EBNA1 is the only EBV-encoded protein that is expressed in all forms of latency. EBNA1 binds to the origin of replication (Orip) and enables the maintenance and replication of the EBV episomes in daughter cells during mitosis (Kanda et al., 2001). It also functions as a viral transcriptional transactivator to regulate its own expression and that of other EBV latency genes (EBNAs and LMP1) required for cell immortalisation (Altmann et al., 2006; Reisman & Sugden, 1986). A number of studies have described the ability of EBNA1 to contribute to the oncogenesis of NPC. Expression of EBNA1 in NPC cells, which were then inoculated into both nude and severe combined immunodeficiency mice, significantly enhanced tumour growth and metastasis (Sheu et al., 1996). EBNA1 has been shown to enhance the survival of epithelial cells with DNA damage by destabilizing p53 through the disruption of promyelocytic leukaemia (PML) nuclear bodies (Sivachandran et al., 2008). In addition, EBNA1 promotes genetic instability in NPC cells by inducing DNA damage and oxidative stress (Cao et al., 2012). EBNA1 was also shown to contribute to the pathogenesis of NPC by promoting epithelial-mesenchymal transition (EMT), metastasis and angiogenesis (Cao et al., 2012; O'Neil et al., 2008; Valentine et al., 2010; Wang et al., 2013b), partly through its ability to modulate various signalling pathways, including NF-κB, STAT, TGF-β1, AP-1 and IL-6 (O'Neil et al., 2008; Valentine et al., 2010; Wood et al., 2007). Notably, a dual role for EBNA1 in regulating EBV life cycle was demonstrated. EBNA1 was able to suppress spontaneous EBV lytic reactivation and facilitate lytic infection once the lytic cycle was induced (Sivachandran et al., 2012). Recent evidence also showed that EBNA1 maintained EBV latency by reducing

the levels of Dicer (a protein that promotes EBV reactivation) through the induction of host let-7 miRNAs (Mansouri et al., 2014).

#### 2.3.5.2 Latent membrane proteins (LMP1 and LMP2)

The transforming ability of LMP1 is well documented. LMP1 is a classical oncogene due to its ability to transform rodent fibroblasts *in vitro* and promote tumour formation in mice (Wang et al., 1985). Functionally, LMP1 mimics the tumour necrosis receptor (TNFR) family members, TNFR1 and TNFR superfamily member 5 (CD40), but signals in a ligand independent manner (Mosialos et al., 1995; Uchida et al., 1999). LMP1 activates multiple signalling pathways, including PI3K-AKT, ERK-MAPK, JNK-p38, JAK-STAT and NF-κB (Dawson et al., 2012), mainly through its C-terminal activation region 1-3 (CTAR 1-3) (Dawson et al., 2012; Tsao et al., 2002). LMP1 exerts an array of effects that favour epithelial tumorigenesis, such as promoting cell proliferation, EMT, angiogenesis, cell motility, invasion and metastasis as well as inhibiting apoptosis (Chen et al., 2014; Dawson et al., 2012; Gou et al., 2003; Guo et al., 2012; He et al., 2017; Lee et al., 2007a). Notably, NPC cells expressing LMP1 acquired stem cell/progenitor-like properties, possibly via activating the Hedgehog signalling pathway (Port et al., 2013). A number of studies also revealed the involvement of LMP1 in energy metabolism of epithelial cells by inhibiting the LKB1-AMPK pathway or promoting the aerobic glycolysis pathway (Lo et al., 2015; Lo et al., 2013; Xiao et al., 2014). In addition, LMP1 can stimulate the production of pro-inflammatory cytokines in NPC cells, for example, IL-1 and tumour necrosis factor (TNF), implying a potential role in modulating tumour microenvironment (Busson et al., 1987; Eliopoulos et al., 1997). In recent years, increasing evidence suggests that LMP1 can target the host miRNAs to promote carcinogenesis of both B cells and epithelial cells (Allaya et al., 2015; Chen et al., 2015a; Zhu et al., 2014).

The LMP2 gene encodes two proteins, LMP2A and LMP2B, that share 12 transmembrane domains and a short cytoplasmic C-terminus, but many functional effects of LMP2 proteins are attributed to the immunoreceptor tyrosine activation (ITAM) motif within the N-terminal cytoplasmic domain of LMP2A (Dawson et al., 2012; Tsao et al., 2015). Similar to LMP1, LMP2A exhibits its oncogenic functions through regulating multiple signalling pathways, particularly activation of PI3K/AKT signalling (Fukuda & Longnecker, 2007; Scholle et al., 2000). During epithelial carcinogenesis, LMP2A induces anchorage-independent growth, enhances cell adhesion and motility, inhibits cell differentiation and counteracts growth inhibition and apoptotic effects of TGF-β1 (Allen et al., 2005; Morrison & Raab-Traub, 2005; Scholle et al., 2000). In addition, LMP2A can induce EMT in NPC cells, which is associated with the acquisition of stem cell like properties and the increased expression of metastatic tumour antigen 1 (*MTA1*) through the mTOR pathway (Kong et al., 2010; Lin et al., 2014b). Importantly, LMP2A was shown to cooperate with LMP1 to modulate DNA damage signalling to provide survival advantage for NPC cells (Wasil et al., 2015). Apart from directly promoting tumour cell growth and survival, LMP2A has also been shown to contribute to immune evasion. Both LMP2A and LMP2B confer resistance to the antiviral effects of interferon α/β in epithelial cells (Shah et al., 2009), an effect that can be linked to the modulation of NF-κB and IL-6/STAT3 signalling by LMP2A (Stewart et al., 2004). LMP2A has also recently been shown to down-regulate MHC class I and class II expression and to impair the recognition of EBV-infected cells by cytotoxic T cells (Deb Pal & Banerjee, 2015; Lin et al., 2015b; Rancan et al., 2015).

#### 2.3.5.3 Epstein-Barr virus-encoded RNAs (EBER1 and EBER2)

EBERs are the most abundant viral transcripts in cells with latent EBV infection and serve as sensitive targets for detecting EBV infection in cells and tissues. EBERs exist as EBER1 and EBER2, which are non-polyadenylated (non-coding) RNAs that form double-stranded RNA (dsRNA)-like structures. In BL, EBERs induce the production of type 1 IFN through retinoic acid-inducible gene 1 (RIG-1) signalling or activation of toll-like receptor 3 (TLR3), contributing to the activation of innate immunity (Iwakiri et al., 2009; Samanta et al., 2006). In contrast, in epithelial cells, EBERs counteract the antiviral and anti-proliferative effects of IFNs by directly binding and inhibiting dsRNA-activated protein kinase R (PKR), protecting the EBV-infected cells from IFN-induced and Fas-mediated apoptosis (Nanbo et al., 2005). EBER1 or EBER2 also shown to stimulate the secretion of insulin-like growth factor 1 (IGF-1) which may act as an autocrine growth factor for NPC cells (Iwakiri et al., 2005).

#### 2.3.5.4 BamHI-A rightward transcripts (BARTs)

Until recently, the role of BARTs in NPC remained largely unknown. BART transcripts have been consistently detected in NPC and the discovery that the BARTs can encode miRNAs was a major advance in understanding its role in NPC pathogenesis. To date, 44 mature BART miRNAs have been verified (Chen et al., 2010; Wang et al., 2017). Evidence has emerged to show that BART miRNAs promotes NPC pathogenesis by targeting a variety of viral and cellular genes. BART miRNAs maintain and facilitate EBV latency by limiting the expression of several lytic genes, including viral DNA polymerase (*BALF5*) and immediate early genes (*BZLF1* and *BRLF1*) (Barth et al., 2008; Iizasa et al., 2010). Further, BART miRNAs can suppress the expression of LMP1 and LMP2A in NPC cells and reduce the sensitivity of LMP1-expressing cells to

cisplatin (Lo et al., 2007; Lung et al., 2009). One of the major functions of BART miRNAs is to prevent apoptosis of malignant epithelial cells. A number of pro-apoptotic proteins are targeted by BART miRNAs, including p53 up-regulated modulator of apoptosis (PUMA), Bcl-2 interacting mediator of cell death (BIM), translocase of the outer mitochondrial membrane 22 (TOMM22), caspase 3 and BH3-interacting domain death agonist (Bid) (Choy et al., 2008; Dolken et al., 2010; Marquitz et al., 2011; Shinozaki-Ushiku et al., 2015; Vereide et al., 2014). BART miRNAs also appear crucial in modulating host immune responses as they have been shown to suppress the expression of histocompatibility complex class I-related chain B (MICB), which is involved in eliminating infected cells (Nachmani et al., 2009). Other cellular targets of BART miRNAs include tumour suppressor genes, DICE1, PTEN and E-cadherin, all of which are involved in tumour growth and metastasis (Cai et al., 2015; Hsu et al., 2014; Lei et al., 2013).

#### 2.3.5.5 BamHI-A fragment rightward reading frame 1 (BARF1)

BARF1 was originally thought to be a lytic gene because it is expressed upon the induction of lytic cycle in BL cell lines (Zhang et al., 1988). However, subsequent studies in primary NPC and EBV-associated gastric cancer (EBVaGC) tissues confirmed that BARF1 is a latent gene (Seto et al., 2005). BARF1 is consider as an oncogene through its ability to induce malignant transformation of rodent fibroblasts (Sheng et al., 2001; Wei & Ooka, 1989) and immortalise primary monkey kidney epithelial cells (Wei et al., 1997), as well as enhance the tumorigenicity of EBV-negative BL and NPC cell lines (Seto et al., 2008; Sheng et al., 2001). BARF1 is a decoy receptor for macrophage colony-stimulating factor (M-CSF/CSF-1) that can block the CSF-1 signalling and interferes with the functions of macrophages, thereby showing an immune-evasive role of BARF1 in acute and persistent EBV infection

(Hoebe et al., 2012; Ohashi et al., 2012; Shim et al., 2012). In EBV-positive gastric cancer cells, BARF1 was shown to exerts an anti-apoptotic role by increasing Bcl-1 to Bax ratio and facilitates cell proliferation by up-regulating the expression of NF-κB RelA as well as NF-κB-dependent cyclin D1 proteins (Chang et al., 2013; Wang et al., 2006). Recently, BARF1 was shown to suppress Smad4 expression and inactivate TGF-β signalling through the induction of miR-146a expression in EBV-positive gastric cancer cells (Kim et al., 2016). The role of BARF1 in NPC is not well explored.

### 2.3.6 EBV latent infection in NPE cells

The ability of EBV to transform B cells into LCL *in vitro* has greatly facilitated the study of EBV infection in B cell tumours. However, the inability of EBV to transform epithelial cells *in vitro* and the difficulties in generating EBV-positive cell lines have hampered progress in elucidating the mechanisms regulating EBV latent infection in epithelial cells (Tsao et al., 2017; Young et al., 2016).

EBV infection of B cells leads to an increase in the proliferation of the infected cells. In contrast, EBV infection of epithelial cells has been shown to cause growth inhibition or cellular senescence that eventually results in the loss of EBV genomes (Tsang et al., 2010). These growth-inhibitory effects of EBV infection can be overridden in cells displaying *p16* inactivation and/or overexpressing *CCND1* (Tsang et al., 2012). Given that EBV infection is closely associated with a number of undifferentiated carcinomas, the undifferentiated properties of epithelial cells may be a prerequisite for establishing persistent EBV latent infection. In this regard, overexpression of *CCND1* suppressed serum-induced differentiation, indicating that differentiation status of epithelial cells is important in the establishment of a stable EBV infection (Tsang et al., 2012).

Identification of monoclonal EBV genomes in preinvasive dysplastic lesions suggested that EBV infection of pre-malignant NPE cells is an early event in the pathogenesis of NPC (Pathmanathan et al., 1995). It is generally recognised that NPE cells with specific genetic abnormalities are susceptible to EBV latent infection, and once infected, the EBV latent genes provide survival benefits to drive the transformation process in these cells. However, currently only one study has shown that deletion of *p16* and/or overexpression of *CCND1* contributed to a persistent EBV infection in immortalised NPE cells (Tsang et al., 2012). A fuller understanding on genetic events that regulate EBV latent infection in NPE cells is crucial and the present study focused on the role of transforming growth factor-β (TGF-β) signalling pathway in this context.

## 2.4 Transforming growth factor (TGF)-β

The TGF-β superfamily is a group of multifunctional proteins comprising of more than 40 members that are clustered in several subfamilies, such as TGF-β itself, activins/inhibins, bone morphogenetic proteins (BMPs), nodal and growth differentiation factors (GDFs) (Derynck & Zhang, 2003; Siegel & Massague, 2003). TGF-β1 was the first member to be identified and was named due its ability to transform normal rat fibroblasts in soft agar assays (Roberts et al., 1980). TGF-β1 plays an important role in regulating numerous biological processes such as proliferation, apoptosis, cell differentiation, senescence, angiogenesis, epithelial mesenchyme transition (EMT) and immune suppression (Derynck et al., 2001; Siegel & Massague, 2003).

### 2.4.1 Synthesis and activation of TGF-β

There are three TGF-β isoforms ubiquitously expressed in mammals, namely TGF-β1, TGF-β2 and TGF-β3, each encoded by different genes (Assoian et al., 1983; Childs et al., 1982; Derynck et al., 1985; Derynck et al., 1988; Seyedin et al., 1987). These isoforms are highly similar and share around 70-80% homology within their sequences (Derynck et al., 1988; Segarini et al., 1987; Sporn et al., 1986). TGF-β1 was the first isoform to be characterized and studied (Assoian et al., 1983; Childs et al., 1982) and is considered as the prototype factor of the TGF-β superfamily (Lebrun, 2012). Most of the work describing the role of TGF-β in cancer has focused on TGF-β1 and, therefore, the following review will focus primarily on this isoform.

TGF-β1 is synthesized in a latent form as a large precursor protein that consists of 390 amino acids (Derynck et al., 1985). The precursor protein undergoes proteolytic digestion by the endopeptidase furin to produce two proteins, namely latency-associated peptide (LAP; 278 amino acids) and mature TGF-β1 (112 amino acids) (Dubois et al., 1995; Gentry et al., 1988). Despite the cleavage of the precursor protein, the LAP remains bound to the mature TGF-β1 making the TGF-β1 biologically inactive (Gentry & Nash, 1990). The activation of TGF-β1 can be mediated by several factors, such as changes in the cellular environment (for example extreme acidification or alkalinisation and high temperature), activation of proteases including plasmin and metalloprotease, release of thrombospondin-1 (TSP-1) in response to tissue injury, release of irradiation-induced reactive oxygen species (ROS) and induction of integrin $\alpha v\beta 6$ in response to inflammation (Annes et al., 2003; Barcellos-Hoff et al., 1994; Gentry & Nash, 1990; Jullien et al., 1989; Khalil, 1999; Munger et al., 1999; Sato & Rifkin, 1989; Yu & Stamenkovic, 2000).

### 2.4.2 TGF-β signalling pathway

#### 2.4.2.1 TGF-β receptors

Three types of TGF-β1 receptors have been identified, namely TGFR-1, TGFR-2 and TGFR-3 (Cheifetz et al., 1988; Derynck & Feng, 1997; Massague et al., 1982; Massague & Like, 1985). All the three receptors exist as homodimers at the cell surface and each receptor consists of a N-terminal extracellular domain, a transmembrane region and a C-terminal cytoplasmic domain (Ebner et al., 1993; Lin et al., 1992; Lopez-Casillas et al., 1991; Wang et al., 1991).

Both TGFR-1 and TGFR-2 are transmembrane serine/threonine kinase receptors (Ebner et al., 1993; Lin et al., 1992). TGFR-1 consists of 503 amino acids and contains a glycerine and serine (GS) rich domain (Ebner et al., 1993; Wieser et al., 1995). TGFR-2 consists of 567 amino acids and has a large number of cysteine residues in its extracellular domain (Lin et al., 1992; Suzuki et al., 1994). TGF-β signalling is activated upon binding one of the active TGF-β ligand (TGF-β1, TGF-β2 and TGF-β3) to TGFR-2 an event that triggers the phosphorylation of its serine/threonine kinase domain (Figure 2.4) (Franzen et al., 1993; Wrana et al., 1992). This leads to the recruitment and phosphorylation of serine/threonine residues in the GS rich domain of TGFR-1 (Franzen et al., 1993; Wrana et al., 1992; Yamashita et al., 1994). The activated heterotetramer TGFR-1/TGFR-2 complex subsequently triggers the canonical Smad-dependent signalling pathway, as well as non-canonical Smad-independent signals.

In contrast to TGFR-1 and TGFR-2, TGFR-3 is a membrane proteoglycan that consists of 853 amino acids (Cheifetz et al., 1988; Wang et al., 1991). TGFR-3 acts as a co-receptor of TGF-β signalling by presenting activated TGF-β ligands directly to

TGFR-2, thereby enhancing the binding of the activated ligands to the TGFR-2. This is particularly important for TGF-β2 because it has a low binding affinity towards TGFR-2 (Lopez-Casillas et al., 1993).

#### 2.4.2.2 The canonical TGF-β/Smad signalling pathway

Smad proteins were the first identified downstream signalling transducers of TGF-β1 (Zhang et al., 1996). The proteins of the SMAD family are the vertebrate homologs of the *Drosophila* mothers against decapentaplegic (MAD) protein and the *Caenorhabditis elegans* small body size (SMA) protein (Raftery et al., 1995; Sekelsky et al., 1995). These proteins are divided into three groups based on their functions; receptor-activated Smads (R-Smad; Smad2 and Smad3), common mediator Smad (Co-Smad; Smad4) and inhibitory Smads (I-Smads; Smad6 and Smad7) (Heldin et al., 1997; Moustakas et al., 2001; Savage et al., 1996). Structurally, the Smad contain two highly conserved Mad homology (MH) domains, MH1 and MH2, located at the N-terminal and C-terminal end, respectively (Shi et al., 1997; Shi et al., 1998). The MH1 and MH2 domains have an affinity for each other, therefore, in the basal state, the MH1 and MH2 domains interact with each other resulting in autoinhibition of Smad signalling (Shi et al., 1998).

Following TGF-β1 binding and receptor activation, the Smad anchor for receptor activation (SARA) protein binds TGFR-1 and the MH2 domains of Smad2 and/or Smad3 simultaneously, resulting in the phosphorylation of the Smad2 and/or Smad3 by TGFR-1 (Abdollah et al., 1997; Macias-Silva et al., 1996; Savage et al., 1996; Tsukazaki et al., 1998). Upon activation, Smad2 and/or Smad3 are released from the receptor complex and SARA, and oligomerize with Smad4 through their MH2 domains (Chacko et al., 2004; Tsukazaki et al., 1998; Wu et al., 2000; Wu et al., 2001).

The Smad2/Smad4 and/or Smad3/Smad4 complexes then translocate to the nucleus to activate various TGF-β1 target genes (Figure 2.4) (Nakao et al., 1997).

The activation of TGF-β signalling is tightly controlled by the two I-Smads, Smad6 and Smad7. Compared to Smad6, Smad7 has been shown to inhibit TGF-β signalling more efficiently through various mechanisms (Hanyu et al., 2001; Yan et al., 2009). These include inhibition of the phosphorylation of R-Smads by forming a complex with activated TGFR-1, degradation of the activated TGFR-1 by recruiting ubiquitin E3 ligases, such as Smurf1/2 or disruption of the formation of functional Smad-DNA complexes in the nucleus (Ebisawa et al., 2001; Hayashi et al., 1997; Kavsak et al., 2000; Zhang et al., 2007). Recent evidence also demonstrated that Smad7 may directly oligemerize with R-Smads and inhibit their activities (Yan et al., 2016).

#### 2.4.2.3 The non-canonical TGF-β/Smad-independent signalling pathway

Apart from the canonical TGF-β/Smad-dependent signalling pathway, there is also evidence to suggest that TGF-β1 can signal through non-canonical Smad-independent signalling pathways, including ERK-MAPK, p38-MAPK, PI3K-AKT and JNK (Figure 2.4) (Derynck & Zhang, 2003; Siegel & Massague, 2003). Previous studies have revealed different mechanisms employed by TGF-β1 to trigger these pathways. For example, the activation of ERK-MAPK pathway is mediated by the phosphorylation of TGFR-1, whereas the activation of both TGFR-1 and TGFR-2 are required for the induction of PI3K-AKT signalling pathway (Galliher & Schiemann, 2007; Lee et al., 2007b; Yi et al., 2005). Notably, activation of the canonical Smad-dependent and the non-canonical Smad-independent signalling pathways is not mutually exclusive (Kamaraju & Roberts, 2005; Ohshima & Shimotohno, 2003). For example in breast

cancer cells lines, both pathways act together to mediate TGF-β1-induced growth arrest (Kamaraju & Roberts, 2005).

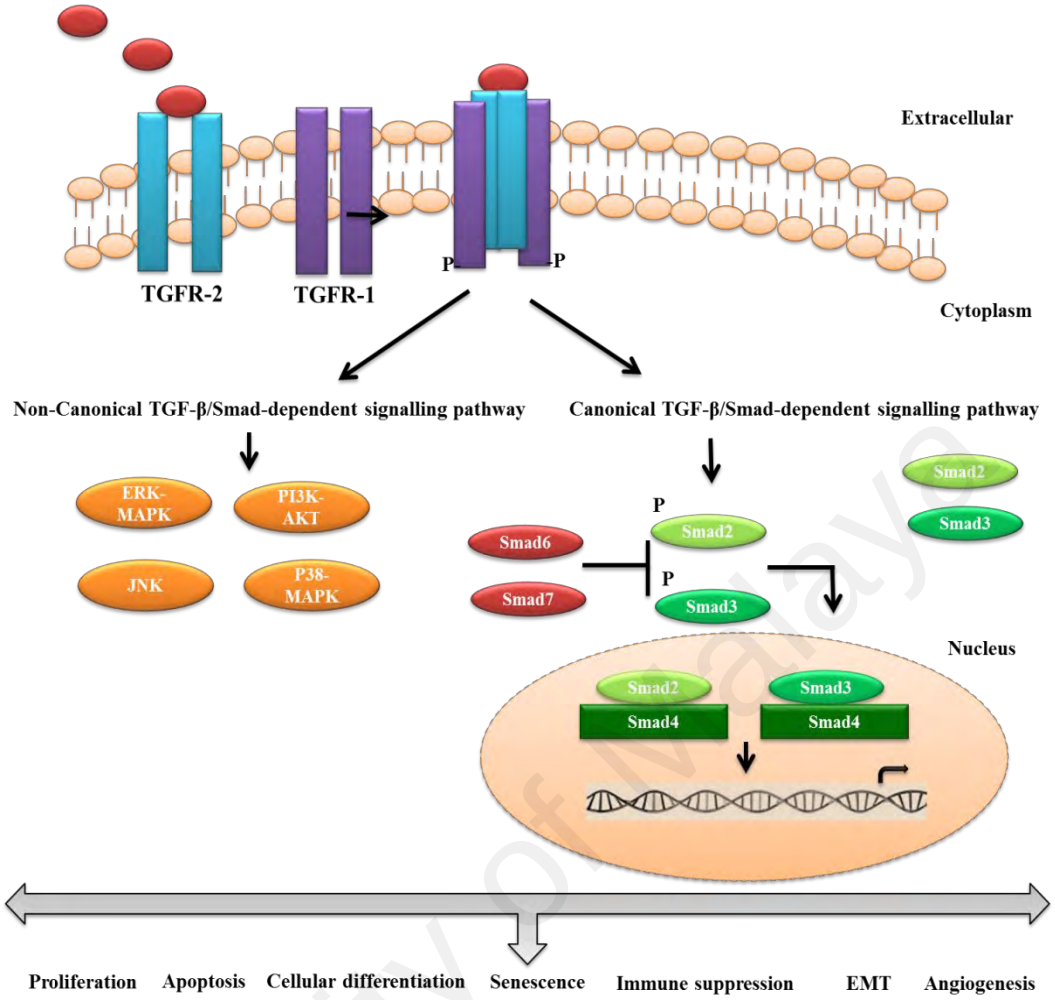



**Figure 2.4. TGF-β signalling pathway.** Binding of an activated TGF-β ligand to TGFR-2 recruits and activates TGFR-1. This, in turn, phosphorylates Smad2 and/or Smad3, which then form complexes with Smad4 and translocate into nucleus to regulate the transcription of various target genes. In addition to the canonical Smad-dependent signalling, activated TGF-β receptors can trigger other signalling pathways including ERK-MAPK, p38-MAPK, PI3K-AKT and JNK. TGF-β signalling regulates a wide range of biological processes including proliferation, apoptosis, cellular differentiation, senescence, immune suppression, EMT and angiogenesis. Figure modified from Siegel & Massague., 2003.

### 2.4.3 Dual role of TGF-β signalling in cancer progression

TGF-β signalling has been shown to exhibit both tumour suppressive and tumour promoting functions depending on the cancer stage (Lebrun, 2012; Siegel & Massague, 2003). In the early stages of cancer development, TGF-β signalling functions as a tumour suppressor by inhibiting cell cycle progression from G1 to S phase and inducing apoptosis, senescence and differentiation (Lebrun, 2012; Moses et al., 1987; Siegel & Massague, 2003; Yanagihara & Tsumuraya, 1992). Conversely in late stage diseases, it acts as a tumour promoter by inducing EMT, migration, invasion, metastasis, angiogenesis and immune suppression (Lebrun, 2012; Oft et al., 1998; Siegel & Massague, 2003; Torre-Amione et al., 1990; Ueki et al., 1992). Frequently, cancer cells become resistant to the tumour suppressive effects of TGF-β1, however functional TGF-β signalling still persists in these cells enabling TGF-β1-induced tumour promoting phenotypes (Chen et al., 2001; Lehmann et al., 2000; McEarchern et al., 2001; Zhang et al., 2004). For example, TGF-β1 directly contributed to the metastatic behavior of a mammary carcinoma cell line, yet these cells did not respond to TGF-β1-mediated growth inhibition (McEarchern et al., 2001).

#### 2.4.3.1 TGF-β1 as a tumour suppressor

TGF-β1 inhibits cell cycle progression from G1 to S phase in many normal and neoplastic epithelial cell lines and the effect is reversible, where the cells continue to proliferate in the absence of exogenous TGF-β1 (Moses et al., 1987; Shipley et al., 1986). The transition from G1 to S phase of the cell cycle is regulated by the cyclin-dependent kinase (CDK) complexes (Pines, 1995). Dimerization of cyclin D1 to CDK4 and/or CDK6 leads to the phosphorylation of retinoblastoma (Rb) protein, resulting in the release of transcription factors E2Fs from the phosphorylated Rb protein (Baldin et

al., 1993; Ewen et al., 1993a). The free E2Fs translocate into nucleus and trigger the transcription of cyclin E gene, leading to the formation of cyclin E-CDK2 complex, which drives further phosphorylation of Rb protein and results in its complete functional inactivation. This, in turn, allows the transcription of genes that usher the cells from G1 to S phase (Geng et al., 1996; Schulze et al., 1995). TGF-β1 inhibits the progression of the cell cycle from G1 to S phase by inducing the expression of CDK inhibitors, p15 and p21, thereby blocking the phosphorylation of Rb protein (Harper et al., 1993; Laiho et al., 1990; Reynisdottir et al., 1995). p15 binds to CDK4 and/or CDK6, blocking their association with cyclin D1 (Ewen et al., 1993b; Reynisdottir et al., 1995). In addition, the increase in p15 levels in response to TGF-β1 induces the release of p27 from CDK4 and/or CDK6 (Reynisdottir et al., 1995). p21 and the free p27 bind to CDK2, hence inhibit the formation of cyclin A-CDK2 and/or cyclin E-CDK2, thereby blocking the progression to S phase (Harper et al., 1993; Reynisdottir et al., 1995). Further, TGF-β1 suppresses the expression of the c-MYC protein, preventing c-MYC from inhibiting the expression of p15, p21 and p27 (Claassen & Hann, 2000; Gartel & Shchors, 2003; Seoane et al., 2001; Staller et al., 2001; Warner et al., 1999; Yang et al., 2001). This safeguards the induction of the CDK inhibitors and thereby leads to G1 cell cycle arrest (Claassen & Hann, 2000; Warner et al., 1999).

Additionally, TGF-β1 has been shown to induce apoptosis *in vivo* and *in vitro* (Bursch et al., 1993; Havrilesky et al., 1995; Hsing et al., 1996; Oberhammer et al., 1992; Sanchez et al., 1996). A role for TGF-β1 in apoptosis was initially identified from the finding that the TGF-β-inducible early gene (*TIEG*) is an early target gene of TGF-β1 (Tachibana et al., 1997). *TIEG* is a Sp1/Kruppel-like zinc finger transcription factor which was shown to trigger apoptosis in a human pancreatic cancer cell line (PANC1), the mink lung epithelial cell line (Mv1Lu) and hepatocytes (Hep 3B) (Chalaux et al., 1999; Ribeiro et al., 1999; Tachibana et al., 1997). *TIEG* induces apoptosis through the

generation of ROS, which leads to a decrease in Bcl-2 protein levels and subsequently results in the loss of mitochondrial membrane potential (Chalaux et al., 1999; Ribeiro et al., 1999). It is now recognised that TGF-β1 induces both the intrinsic and extrinsic apoptotic programs in a cell-type dependent manner (Siegel & Massague, 2003). In lymphoma, TGF-β1 induces the intrinsic apoptotic pathway by stimulating the expression of several pro-apoptotic Bcl-2 family members (such as Bmf, Bim and Bax), which in turns suppress the expression of anti-apoptotic proteins (Bcl-$X_L$ and Bcl-2) (Bakhshayesh et al., 2012). The ability of TGF-β1 in inducing the extrinsic apoptotic program was shown in liver and lung cancer cells in which the expression of death-associated protein kinase (DAPK) and Fas-mediated apoptosis was increased upon the exogenous addition of TGF-β1 respectively (Hagimoto et al., 2002; Jang et al., 2002; Perlman et al., 2001). Additionally, a study has revealed a synergistic action of TGF-β1 on cell cycle arrest and apoptosis. In a gastric cancer cell line (SNU-16), exogenous addition of TGF-β1 induced G1 cell cycle arrest in accordance with the activation of caspase-3. The activated caspase-3 subsequently cleaved p21, p27 and Rb proteins, resulting in the disruption of cell cycle arrest and aberrant CDK2 activation which subsequently leads to apoptosis (Kim et al., 2001).

Further, TGF-β1 is able to induce cellular senescence in cultures of normal fibroblasts and epithelial cells (Frippiat et al., 2001; Katakura et al., 1999; Kumar et al., 2017; Senturk et al., 2010; Wu et al., 2014; Yoon et al., 2005). It has been shown that TGF-β1 treatment induced senescence in the Mv1Lu, HCC cell lines and bone marrow-derived mesenchymal stem cells through the accumulation of ROS (Senturk et al., 2010; Wu et al., 2014; Yoon et al., 2005). Additionally, recent evidence has demonstrated crosstalk between TGF-β1 and NF-κB signalling in stimulating senescence in a human corneal epithelial cell line (Li et al., 2016). In human mammary epithelial cells, Ras-

induced senescence has been shown to be mediated through the activation of TGF-β signalling (Cipriano et al., 2011; Lin et al., 2012).

There is increasing evidence to show that TGF-β1 signalling can regulate the differentiation of different cell types. A number of studies have shown that TGF-β1 can induce cellular differentiation of the immortalised human skin keratinocytes, HaCaT, through NF-κB signalling (Buschke et al., 2011; Cho et al., 2004; Descargues et al., 2008; Matsumoto et al., 1990). Further, *in vivo* studies have shown that tumours that formed from human oral keratinocytes and mouse epidermal keratinocytes carrying a dominant negative *TGFBR2* or *SMAD4* gene respectively was associated with the loss of cellular differentiation, data that demonstrated a role for TGF-β signalling in cellular differentiation (Huntley et al., 2004; Iglesias et al., 2000).

In summary, TGF-β1 exerts its tumour suppressive effects by inhibiting cell cycle progression from G1 to S phase and by inducing apoptosis, senescence and cellular differentiation. In order to evade TGF-β1-mediated tumour suppressive effects, cancer cells often develop genetic abnormalities on key molecules within the TGF-β signalling pathway, in particularly the canonical Smad-dependent signalling pathway (Table 2.2) (Paterson et al., 2001; Pring et al., 2006; Siegel & Massague, 2003; Tang et al., 1999; Yanagisawa et al., 1998; Yang et al., 2009).

Table 2.2. De-regulation of TGF-β signalling pathway components in cancers

| TGF-β signalling component | Cancer Types | References |
|---|---|---|
| Loss of TGFR-2 and Smad4 expression | Breast | Lynch et al., 2001; Stuelten et al., 2006 |
| Mutations of *TGFBR2* gene | Colon | Grady et al., 1999; Biswas et al., 2004 |
| Mutations of *TGFBR2, SMAD4* and *SMAD7* genes | Colorectal | Boulay et al., 2003; Fukushima et al., 2003 |
| Loss of Smad4 expression | Cervical | Baldus et al., 2005 |
| Hypermethylation of *TGFBR1* and mutations of *TGFBR2* | Gastric | Ohue et al., 1996; Pinto et al., 2003 |
| Loss of TGFR-2, TGFR-3 and Smad4 expression | Oral | Meng et al., 2011; Peng et al., 2006; X. M. Wang et al., 2009 |
| Mutations of *TGFBR2* gene | Ovarian | Lynch et al., 1998 |
| Mutations of *TGFBR1* gene | Prostate | Kim et al., 1996 |
| Mutations of *TGFBR2* and *SMAD4* genes | Pancreatic | Baldwin et al., 1996; Hahn et al., 1996 |

#### 2.4.3.2 TGF-β1 as a tumour promoter

One of the important functions of TGF-β1 as a tumour promoter is its ability to induce an EMT in both normal and cancer cells (Heldin et al., 2012; Miettinen et al., 1994; Oft et al., 1998; Piek et al., 1999). EMT is a naturally occurring process that takes place during embryogenesis and development, however, increasing evidence has suggested that EMT can be abnormally reactivated in adult tissues during pathological conditions especially in cancer (Heldin et al., 2012; Siegel & Massague, 2003). It is a process whereby highly organized and tightly connected epithelial cells transdifferentiate into disorganized and mobile mesenchyme cells (Heldin et al., 2012; Miettinen et al., 1994; Xu et al., 2009). Cells undergoing EMT are best exemplified by the loss of epithelial markers (such as E-cadherin, Zonula occludens 1, occludin, claudins, despmoplakin, β-catenin, cytokeratins 8, 18 and 19) and the induction of mesenchymal markers (such as N-cadherin, fibronectin and vimentin) (Miettinen et al., 1994; Oft et al., 1998; Piek et al., 1999; Xu et al., 2009). TGF-β1 induces EMT in cancer cells through various mechanisms, that include the up-regulation of the Snail

family of proteins (Snail and Slug), the basic helic-loop-helix protein (Twist), zinc-finger proteins (Zeb1 and Zeb2), the forkhead factor (FoxC3) (Dave et al., 2011; Fan et al., 2015; Peinado et al., 2003; Shirakihara et al., 2007) as well as down-regulation of miR-200 (Gregory et al., 2008; Gregory et al., 2011; Park et al., 2008). A number of *in vitro* studies have shown that exogenous addition of TGF-β1 promotes the migration and invasion of various epithelial cancer cells is through a mechanism involving activation of an EMT (Fischer et al., 2007; Oft et al., 1998; Pang et al., 2016). These observations were also confirmed *in vivo* where treatment of the skin of transgenic mice with TGF-β1 enhanced the conversion of benign skin tumours to carcinoma through the activation of EMT (Portella et al., 1998).

Importantly, several *in vivo* studies have also shown that TGF-β1 can directly contribute to a more metastatic phenotype of cancer cells independent of EMT. For example, TGF-β signalling enhanced the metastasis of breast cancer cells to bone by directly inducing the expression of parathyroid hormone-related protein (PTHrP), receptor activator of NF-κB ligand (RANKL), connective tissue growth factor (CTGF) and IL-11 (Kang et al., 2003; Thomas et al., 1999; Yin et al., 1999). Further, TGF-β signalling was shown to enhance the extravasation of breast cancer cells into lung parenchyma by inducing the expression of cyclooxygenase-2 (COX2), EGF and angiopoietin-like 4 (ANGPTL4) (Bos et al., 2009; Padua et al., 2008). Similar observations were also reported in a number of *in vitro* studies. TGF-β1 has been shown to promote the migration and invasion of different types of cancer cells through the modulation of miRNA expression (for example, miR-584 and miR-181) or the induction of various protein expression, such as CCAAT displacement protein (CUTL1), receptor tyrosine kinase (ErbB2), α3-integrin, *MDM2* and *NET1* (Araki et al., 2010; Fils-Aime et al., 2013; Giannelli et al., 2002; Michl et al., 2005; Seton-Rogers et al., 2004; Shen et al., 2001; Wang et al., 2010).

Angiogenesis is the formation of new capillaries from pre-existing blood vessels. This process is crucial to supply nutrients and oxygen to cancer cells as well as facilitating cancer cells to metastasize through the blood stream (Guerrero & McCarty, 2017; Nishida et al., 2006). TGF-β1 has been shown to stimulate tumour-associated angiogenesis, partly through the induction of vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) (Fang et al., 2012; Kang et al., 2003; Pertovaara et al., 1994; Shimo et al., 2001). In addition, *in vivo* study has shown that additional of TGF-β1 promote angiogenesis by induce the expression of miR-29a (Wang et al., 2013a).

TGF-β1 also promotes tumour progression by facilitating immune evasion (Miller et al., 1992; Strassmann et al., 1991; Yoshimura & Muto, 2011; Young et al., 1992). One of the early studies identified TGF-β1 as an immunosuppressor came from the observation that TGF-β1-expressing fibrosarcoma cells were resistant to cytotoxic T lymphocyte (CTL)-dependent killing (Torre-Amione et al., 1990). A number of studies subsequently showed that TGF-β1 inhibited the production of various cytolytic proteins such as IL-2, interferon-γ, Fas-ligand, caspase activating factors granzymes A and B and pore-forming protein perforin, which are collectively responsible for the killing of tumour cells by T-cells (Brabletz et al., 1993; Thomas & Massague, 2005). Further, TGF-β1 was shown to decrease the expression of MHC class II antigens on the surface of tumour cells, contributing to the inhibition of antigen recognition and presentation (Dong et al., 2001; Geiser et al., 1993; Wallick et al., 1990). TGF-β1 also directly impairs the function of immune cells by inhibiting the maturation of dendritic cells and suppressing the activity of natural killer (NK) cells (Geissmann et al., 1999; Strobl & Knapp, 1999).

In summary, TGF-β1 exerts its tumour promoting effects by inducing EMT, migration, invasion, metastasis, angiogenesis and immune suppression. High levels of TGF-β1 are commonly detected in many types of solid tumour and positively correlate with disease stage (Dalal et al., 1993; Friedman et al., 1995; Gorsch et al., 1992; Shim et al., 1999). TGF-β1 can be produced by the tumour cell itself or stromal cells in the tumour microenvironment including macrophages and platelets (Calon et al., 2012; Hawinkels et al., 2007). As tumours progress, most of the cancer cells develop genetic abnormalities within the pathway to escape the tumour suppressive effects of TGF-β signalling, therefore the produced TGF-β1 functions to promote tumour progression (Pickup et al., 2013). Although it is suggested that the tumour promoting effects of TGF-β1 are mainly mediated through the non-canonical Smad-independent signalling pathway (Bakin et al., 2000; Dumont et al., 2003; Han et al., 2005; Vinals & Pouyssegur, 2001), there is evidence to show the canonical Smad-dependent signalling pathway can also be responsible for these effects (Derynck et al., 2001; Derynck & Zhang, 2003; Siegel & Massague, 2003).

### 2.4.4 TGF-β signalling in NPC

There is some evidence to show that the TGF-β signalling pathway is de-regulated in NPC. Mutations of in *TGFBR2* gene were initially reported in a subset of primary NPC tissues and a gene expression microarray study subsequently showed that TGF-β signalling pathway was de-regulated in EBV-positive NPC tissues compared to normal NP tissues (Harn et al., 2002; Sriuranpong et al., 2004). These observations were further supported by data showing that the expression of *TGFBR1* and *TGFBR2* mRNA and protein were down-regulated in primary NPC tissues (Fang et al., 2008; Lyu et al., 2014; Zhang et al., 2012). Importantly, low levels of TGFR-1 and TGFR-2 were

correlated with increased cancer aggressiveness, as well as poor overall survival rates in NPC patients (Zhang et al., 2012).

Increasing evidence has shown that EBV infection contributes to the de-regulation of TGF-β signalling pathway in NPC. The EBV-positive NPC cell line, C666.1, lacks expression of TGFR-2 (Wood et al., 2007). Further, expression of EBV-encoded EBNA1 and EBV infection of NPC cells repressed TGF-β1-induced gene transcription via increased Smad2 turnover (Wood et al., 2007), pointing to a role for EBV in regulating TGF-β signalling in NPC. EBNA1 was also shown to decrease the expression of miR-200 and miR-200b, leading to the up-regulation of the EMT mediators, ZEB1 and ZEB2, through TGF-β-mediated signalling pathway (Wang et al., 2014). In addition, EBV-encoded miRNAs BART3 and BART5 were found to be up-regulated in NPC tissues and through pathway analyses, and these miRNAs target genes within the TGF-β signalling pathway to modulate apoptosis and transformation of NPC cells (Wan et al., 2015). Notably, the levels of TGFR-1 and TGFR-2 were negatively correlated with the expression of the EBER gene in NPC primary tissues (Zhang et al., 2012).

Evidence also exists to indicate that TGF-β1 can function as a tumour promoter in NPC. For example, elevated levels of active and total TGF-β1 were found in sera of NPC patients compared to healthy individuals and the levels of TGF-β1 were positively correlated with the cancer stage and local relapse rates, but were negatively associated with the overall survival rates in NPC patients (Sun et al., 2007; Xu et al., 1999). There are also some functional data to support this concept. For example, treatment of NPC cells with exogenous TGF-β1 led to the activation of the TGF-β signalling pathway in cells that were unresponsive to the growth inhibitory or pro-apoptotic apoptosis effects of the ligand (Poh et al., 2012; Xiao et al., 2010). Furthermore, it has been shown that

miR-93 reduced the expression of *TGFBR2* in two NPC cell lines (CNE1 and CNE2) and lead to the activation of PI3K/AKT signalling pathway to promote the NPC cell growth, invasion, metastasis and EMT (Lyu et al., 2014). Additionally, flotillin (FLOT)-1, a component of lipid raft, and high-mobility AT-hook2 (HMGA2), a member of high-mobility group family were overexpressed in primary NPC tissues and induced the secretion of TGF-β1. Overexpression of FLOT-1 and HMGA2 enhanced the migratory and invasive abilities of NPC cells *in vitro* and *in vivo* via TGF-β/Smad3-dependent signalling pathway mediated EMT induction (Cao et al., 2016; Xia et al., 2015). Further studies have shown that high levels of FLOT2 expression were positively correlated with cancer stage and associated with poorer overall survival in NPC patients (Liu et al., 2015; Zhao et al., 2015). Suppression of FLOT2 in NPC cells reversed the metastatic effect induced by TGF-β1 and inhibited TGF-β-induced Src phosphorylation (Zhao et al., 2015).

These data collectively show that the TGF-β signalling pathway is defective in NPC leading to escape from the various tumour suppressive functions of the ligand and that TGF-β1 can promote a more aggressive phenotype in NPC.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Cell lines

Three telomerase reverse transcriptase (hTert)-immortalised NPE cell lines, NP361hTert, NP460hTert and NP550hTert, were kindly provided by Prof. SW Tsao (University of Hong Kong, Hong Kong). The primary NPE cells (NP361, NP460 and NP550) were derived from nonmalignant nasopharyngeal biopsies and subsequently immortalized by over-expressing *hTert* gene to establish the hTert-immortalised NPE cell lines (Li et al., 2006a; Tsang et al., 2010). These cell lines were cultured in a 1:1 mixture of defined keratinocyte-serum free medium (Gibco Life technologies, NY) and Epilife® medium with growth supplements (Cascade Biologies™, NY). A simian virus 40 (SV40)-immortalised NPE cell line, NP69 (also a gift from Prof. SW Tsao) was cultured in keratinocyte-serum free medium (Gibco Life technologies, NY), containing 25µg/ml of bovine pituitary extract (BPE; Gibco Life technologies, NY), 0.2ng/ml of epidermal growth factor (EGF; Gibco Life technologies, NY) and 0.3mM of calcium chloride ($CaCl_2$, Sigma-Aldrich, USA). A BL cell line, Akata, carrying a green fluorescent protein (GFP)-tagged recombinant EBV was obtained from Dr Christopher Dawson (University of Birmingham, UK) and maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% Fetal Bovine Serum (FBS; Gibco Life technologies, USA) with 800ng/ml of Geneticin (G418; Biowest, France). Two NPC cell lines, HK1 and HONE1, were cultured in RPMI medium containing 10% FBS. HEK293T, a human embryonic kidney cell line, was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life technologies, USA) containing 10% FBS. All the cells were cultured at 37°C with 95% air and 5% $CO_2$ in a humidified chamber.

## 3.2 Materials

Human recombinant TGF-β1 (Miltenyi Biotec, USA) was dissolved in 4mM hydrochloric acid (HCI, Sigma-Aldrich, USA) containing 1mg/ml of bovine serum albumin (BSA, Sigma-Aldrich, USA) to yield a stock concentration of 20µg/ml and stored in 50µl aliquots at -20°C. For routine use, the stock solution was further diluted with the same diluent to 1ug/ml and stored in 4°C. A TGFR-1 kinase inhibitor (SB431542; Tocris, UK) was dissolved in 100% molecular grade ethanol (Merck, Germany) to achieve a stock concentration of 10mM and stored in -20°C in small aliquots. Human anti-IgG (Sigma-Aldrich, USA) was dissolved in 0.135M sodium chloride (Sigma-Aldrich, USA) and filtered through a 0.2µm filter to achieve a stock concentration of 1mg/ml and stored in 4°C. Puromycin dihyrochloride hydrate (Fisher Scientific, US) was dissolved in sterile water to obtain a stock concentration of 10mg/ml; the solution was filtered through a 0.2µm filter and stored in -20°C. Puromycin was further diluted in sterile water to achieve a working concentration of 1µg/ml and stored in 4°C. For FISH analyses the EBV probe was kindly provided by Professor B. Sugen (University of Wisconsin-Madison, Wisconsin).

## 3.3 Cell culture

### 3.3.1 Maintenance of cell lines

Adherent cells were grown in either 25cm$^2$ or 75cm$^2$ culture flasks (Corning, USA) and sub-cultured when they reached 80% confluency. The cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco Life technologies, USA) and subjected to cell dissociation using 0.25% Trypsin-EDTA (Gibco Life technologies, USA). For NP361hTert cells, TrypLE$^{TM}$ Express (Gibco Life technologies, USA) was

used as the dissociation agent. Media containing 10% FBS (complete growth medium) were added to neutralize the trypsin reactions and the cells pelleted by centrifugation at 112g for 8 minutes. The supernatants were discarded and the cells re-suspended in growth medium.

Non-adherent cells were maintained in 75cm$^2$ culture flasks in 10ml of complete growth medium. Cells were passaged twice weekly by replacing 8 ml of the cell suspension with fresh complete growth medium. To remove dead cells, the cells were pelleted by centrifugation at 152g for 5 minutes and re-suspended in fresh complete growth medium every two weeks.

### 3.3.2 Cryopreservation and recovery of cell lines

One million cells were re-suspended in 1ml of freezing medium containing 90% FBS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) and added to cryovials (Nunc, USA). The cryovials were stored at -80°C for overnight in MrFrosty$^{TM}$ Cryo 1°C freezing container (Nalgene, USA) before transferring into liquid nitrogen for long term storage.

The cryopreserved cells were recovered by rapid thawing in 37°C water bath. The thawed cells were transferred into medium containing 10% FBS and pelleted by centrifugation at 112g for 8 minutes. The supernatants were discarded and the cell pellet re-suspended in growth medium and transferred to a 25cm$^2$ culture flask.

### 3.3.3 Inhibition of TGFR-1 kinase with chemical inhibitor, SB431542

NP361hTert, NP460hTert and NP550hTert cell lines were seeded in 100mm$^2$ dishes and cultured overnight to obtain a confluency of 50%. The cells were treated with a range of SB431542 concentrations (0, 2.5, 5 and 10μM) and incubated for 72 hours. The medium was removed and the cells were re-fed with fresh growth medium containing 1ng/ml TGF-β1 and incubated for additional one hour before harvesting for Western blotting. The concentration of SB431542 which showed the maximum reduction in phosphorylation of Smad2 was used for subsequent experiments.

### 3.3.4 Expression of wild type *TGFBR2* and dominant negative (Dn) *TGFBR2* in NPE cells

#### 3.3.4.1 Generating antibiotics selection killing curves

Cells were seeded in a 6-well plate one day before the experiments in order to obtain a confluency of 60%. The cells were treated with either puromycin (0.25, 0.5, 1, 2 and 4μg/ml) or blasticidin (1, 2.5 and 5μg/.ml) for three days. The minimum concentration of puromycin and blasticidin that killed 90% of the cells was used to select stable transfectants in subsequent experiments.

#### 3.3.4.2 Transfection of virus producing cells, HEK293T

HEK293T cells were transfected with expression plasmids (Table 3.1) together with lentiviral packaging plasmids to generate lentiviral particles. 12 x $10^6$ HEK293T cells were seeded in 150cm$^2$ culture flasks and cultured overnight to obtain a confluency of 80%. 40μg of expression plasmid and packaging plasmids (30μg psPAX and 10μg

pMD2.G) was added to 5ml of OptiMEM (Gibco Life technologies, USA) and filtered through a 0.2µm filter. 1µl of 10mM polyethylenimine (PEI) was added to another tube containing 5ml OptiMEM and filtered through a 0.2µm filter. The plasmid DNA and PEI solutions were mixed and incubated for 20 minutes at room temperature. After washing the HEK293T cells with OptiMEM, the plasmid/PEI mixture was added to the cells. The cells were incubated at 37°C for 4 hours and the medium then replaced with 15ml DMEM containing 10% FBS. The supernatant containing the lentiviruses was collected 48 hours later, filtered through a 0.45µm filter (Sartorius, Germany) and stored in 1ml aliquots at -80°C.

Table 3.1. Plasmid DNA used for transfection

| **Plasmid DNA** | **Controls** |
|---|---|
| pLVX-Puro/*DnTGFBR2* (*TGFBR2* with deletion in the cytoplasmic region) | pLVX-Puro |
| FUGW/*TGFBR2* (wild-type *TGFBR2*) | pFUGW |

#### 3.3.4.3 Transduction of NPE cells

Cells were seeded in 25 $cm^2$ culture flasks and cultured overnight to obtain a confluency of 60%. The lentiviral stock was reconstituted with fresh complete growth medium at 1:1 ratio and then polybrene (Sigma-Aldrich, USA) was added to give a final concentration of 8µg/ml. The growth medium was removed from the cells and the viral supernatant and polybrene mixture was then added to the cells and incubated at 37°C, 5% $CO_2$ overnight. The cells were cultured in fresh growth medium for an additional 48 hours before adding either 0.25µg/ml puromycin or 5µg/ml blasticidin to select stable transfectants.

### 3.3.5 Generation of *TGFBR2* knockout cells using the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) system

Two different guide RNAs (gRNAs), gRNA-1 (5'AGTGAGTCACTCGCGCGCA 3') and gRNA-2 (5'GAAGGAAAGTTCAGTTGCA3'), targeting specific regions in the *TGFBR2* gene were synthesized (Integrated DNA technologies, USA). The gRNAs were ligated separately into a pCas-guide plasmid, puro-PX459, to generate CRISPR9/*TGFBR2*_gRNA-1 and CRISPR9/*TGFBR2*_gRNA-2. 2 x $10^5$ NP460hTert cells per well were seeded in a 6-well plate and cultured overnight in order to obtain a confluency of 60%. A transfection complex was prepared by adding 2µg CRISPR9/*TGFBR2*_gRNA-1 and 2µg CRISPR9/*TGFBR2*_gRNA-2 plasmid DNA in 250µl OptiMEM before the addition of 12µl TransIT®-Keratinocyte transfection reagent (Mirus, USA). The transfection complex was incubated at room temperature for 15 minutes and cells were re-fed with fresh growth medium before adding the transfection complex in a drop-wise manner. 48 hours post-transfection, the cells were treated with 500ng/ml puromycin for 16 hours. The cells were then trypsinized and pelleted by centrifugation at 112g for 8 minutes, re-suspended in growth medium and seeded in 96-well plates at a predicted density of 3 cells per well. After 24 hours, the wells that contained only one cell (single clone) were selected and the media replaced every 48 hours. At 80% confluence, the cells were passage into 24-well plates and subsequently passaged again into 6-well plates when 80% confluent. DNA and protein were extracted from these monoclonal cells and were subjected to PCR and Western blotting, respectively. Cells which demonstrated knockout of *TGFBR2* gene were further propagated and designated as CRISPR9/NP460hTert.

### 3.4 Infection of epithelial cells with EBV

Epithelial cells, NP361hTert, NP460hTert, NP550hTert and their corresponding control cells were infected with a recombinant EBV (Akata strain) using an established protocol (Tsang et al., 2010). Briefly, 12 x $10^6$ Akata cells carrying a green fluorescent protein (GFP)-tagged recombinant EBV were cultured in 6ml complete growth medium. The cells were treated with 5µg/ml of human anti-IgG for 24 hours to stimulate the lytic production of infectious EBV virions. Epithelial cells were seeded in 100$mm^2$ dishes at 6 x $10^5$ cells per dish in 6ml growth medium. Following overnight incubation, 6ml of IgG-treated Akata cell suspension was added directly to the epithelial cells and co-cultured for 48 hours. The Akata cells were then removed by washing the culture three times thoroughly with DPBS. The infected epithelial cells were trypsinised and pelleted by centrifugation at 112g for 8 minutes. The supernatant was discarded and the cells were re-suspended in DPBS. The cells suspension was subjected to fluorescent-activated cell sorting (FACS) to count and isolate EBV-positive cells.

### 3.5 Fluorescent-activated cell sorting (FACS)

GFP expression was used as an indicator of EBV-positive cells. The EBV-infected cell suspension was passed through a BD-FACS tube with a cell strainer cap (BD biosciences, USA) to achieve a single cell suspension. The EBV-positive cells were counted and isolated using a BD FACSARIA III system (BD biosciences, USA). The isolated EBV-positive cells were then seeded into 6-well plates at a density of 1 x $10^4$ cells per well and cultured for 7, 14 or 21 days. The growth media were replaced every 48 hours. In experiments to inhibit TGFR-1 kinase, chemical inhibitor SB431542 was added to the fresh medium. At the desired time points, the cells were trypsinised and pelleted by centrifugation at 112g for 8 minutes. The cells were re-suspended in

DPBS and the percentage of EBV-positive cells was determined using the BD FACSARIA III system.

## 3.6 *In vitro* assays

### 3.6.1 Cell proliferation assays

#### 3.6.1.1 MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assays

Cells were seeded in 96-well plates and cultured overnight followed by treatment with a range of TGF-β1 concentrations (0.5, 1, 2.5, 5 and 10ng/ml) for 24, 48 and 72 hours. At the desired time points, 5mg/ml MTT in DPBS solution was added into each well and incubated for 4 hours. The culture medium containing MTT was then removed and 150µl DMSO added into each well to dissolve the purple formazan product. Spectrophotometric absorbance of the colored solution, which corresponds to the number of viable cells, was determined at 575nm in an Infinite 200 Pro NanoQuat microplate reader (Tecan, Switzerland) with a reference wavelength of 650nm. The growth response to TGF-β1 was determined by comparing the viabilities of the TGF-β1-treated cells and controls, diluent (4mM HCI containing 1mg/ml BSA)-treated cells.

#### 3.6.1.2 Cell count assays

To determine the growth response to TGF-β1 in the SB431542-treated and *DnTGFBR2*-expressing cells, cells were seeded in 6-well plates for overnight growth followed by treatment with 1ng/ml TGF-β1 for 48, 72 and 96 hours. At the desired time points, the cells were trypsinised and pelleted by centrifugation at 112g for 8 minutes.

The number of viable cells was counted using a Luna$^{TM}$ automated cell counter (Logos biosystems, USA).

### 3.6.2 Luciferase reporter assays

The *SMAD3*-dependent reporter plasmid $(CAGA)_{12}$-Luc which contains 12 repeats of the *SMAD*-binding element AGCCAGACA and the *SMAD2*-dependent reporter plasmid activin responsive element (ARE)-Luc containing part of the MIX.2 gene promoter were used to determine the activation of TGF-β/Smad signalling. Following TGF-β1 treatment, Smad2 and/or Smad3 bind to Smad4 forming complexes of Smad2/Smad4 and/or Smad3/Smad4, leading to translocation of the Smad complexes to nucleus. In the nucleus, Smad3 directly binds to the Smad-binding element (SBE) CAGA sequences, to activate TGF-β target genes (Dennler et al., 1998). In contrast, Smad2 lacks intrinsic DNA binding activity and does not bind to the SBE. Therefore, the cells are co-transfected with together with forkhead activin signal transducer (FAST-1), which has the ability to bind to the ARE, acting as a cofactor that interacts with Smad2/Smad4 complexes (Chen et al., 1997).

For luciferase reporter assays, cells were transfected with the *SMAD*-responsive reporter plasmids using a Neon® Transfection System (Invitrogen Life technologies, USA) according to the manufacturer's instructions. To determine Smad2-dependent transcription activation, a solution containing 7.2ug $ARE_3$-Luc plasmid DNA, 0.8µg XFast-1 plasmid DNA and 8µg *Renilla reniformis*-thymidine kinase (RL-TK)-Luc plasmid DNA was prepared. To determine Smad3-dependent transcription activation, a solution containing 8µg $(CAGA)_{12}$-Luc plasmid DNA and 8µg RL-TK-Luc plasmid DNA was prepared. 3.2 x $10^5$ cells were collected in an eppendorf tube and pelleted by centrifugation at 112g for 8 minutes. The supernatant was discarded and the cells re-

suspended in 50µl DPBS. The cell suspension was then mixed with the plasmid DNA and the final volume was adjusted to 60µl using DPBS prior to electroporation. For electroporation, 3 ml DPBS was added into the Neon® Tube before connecting to the Neon® Pipette Station. The mixture of plasmid DNA and cell suspension was aspirated using Neon® Tip. The Neon® Tip was then inserted into the Neon® Tube and the desired electroporation program was selected (Table 3.2). After electroporation, the transfected cells were immediately transferred into a new 1.5ml eppendorf tubes containing 940µl of growth medium. The cells were then seeded in 96-well plates with a seeding density of 1 x $10^4$ cells per well and incubated for overnight followed by treatment with 1ng/ml TGF-β1 for 24 hours. For experiments targeting *TGFBR1*, the cells were pre-treated with SB431542 for 30 minutes before the addition of TGF-β1.

Luciferase activity was measured using a Dual-Glo Luciferase assay kit (Promega, USA). 48 hours of post-transfection the culture medium was removed and 20µl of 1x Passive Lysis Buffer was added to the transfected cells. The cells were incubated at room temperature for 15 minutes with gentle vortex to achieve complete cell lysis and homogenization. Subsequently, 100µl of LARII reagent was added to the lysed cells and luciferase activity was immediately quantified using a Victor plate reader (Wallac, USA). RL-TK luciferase activity which acts as an internal control for the transfection efficiency was measured after adding 100µl of Stop & Glo reagent to the cells. Relative luciferase activity of the reporter plasmids was determined by normalizing the luciferase activity of the reporter plasmid against RL-TK activity.

Table 3.2. Electroporation programs used in the Neon® Transfection System

| Cell lines | Voltage | Width | Pulse |
|---|---|---|---|
| NP361hTert | 1100 | 20 | 3 |
| NP460hTert | 1200 | 20 | 4 |
| NP550hTert | 1200 | 20 | 2 |

### 3.6.3 Senescence associated–β-galactosidase (SA-β-Gal) assays

Cells were seeded in 6-well plates at 1 x $10^4$ cells per well and SA-β-Gal assays were performed at day 7 using a senescence detection kit (Biovision, USA) according to the manufacturer's protocol. Briefly, cells were washed once in DPBS and 1ml fixative solution was added to each well. After 15 minutes of incubation, the cells were washed twice in DPBS and 1ml solution containing 940µl staining solution, 10µl staining supplement and 50µl X-gal solution in DMSO (20mg/ml) was added to each well. The cells were incubated at 37°C, 5% $CO_2$ for overnight with the culture plates covered with aluminium foil. The cells were then washed twice with DPBS and senescent cells were stained blue around the perinuclear region. Phase-contrast photographs at 20x magnification were taken and the number of senescent cells was counted in five random fields.

### 3.6.4 Differentiation assays

1 x $10^4$ cells were seeded in a 100$mm^2$ dish 24 hours prior to the experiments in order to obtain a confluency of 20%. To induce differentiation, the cells were treated with growth medium containing 10% FBS and 2.8mM $CaCl_2$ with or without 1ng/ml TGF-β1. The culture medium was changed every 48 hours for 14 days. The cells were harvested for immunofluorescence staining and western-blotting analyses to examine the expression of the terminal differentiation marker, involucrin (ThermoFisher, USA).

For immunofluorescence staining, phase-contrast photographs at 20x magnification were taken.

## 3.7 Molecular biology techniques

### 3.7.1 DNA extraction

Cells were grown in 6-well plates and harvested when they were 80% confluent. DNA extraction was performed using a QIAamp DNA blood mini kit (Qiagen, Germany) according to the manufacturer's protocols. Briefly, cells were pelleted and re-suspended in 200µl DPBS in a 1.5ml eppendorf tube. To lyse the cells, 20µl proteinase K and 200µl AL buffer were added to the cells and incubated at 56°C for 10 minutes. The tube was briefly centrifuged and 200µl of 100% molecular grade ethanol was added to the lysate and mixed well. The mixture was loaded to a QiAamp mini spin column followed by centrifugation at 6,000g for 1 minute. The flow through was discarded and 500µl AW1 buffer added to the spin column. The spin column was centrifuged at 6,000g for 1 minute to wash the column. The flow through was discarded and the column was further washed twice with 500µl AW2 buffer. The spin column was then transferred to a new 1.5ml eppendorf tube and 100µl AE buffer was loaded directly onto the spin column membrane and incubated for 1 minute followed by centrifugation at 6,000g for 1 minute to elute the DNA. The DNA concentration was measured using a Nanodrop 2000 spectrophotometer (ThermoFisher, USA) and the DNA sample was stored in -20°C.

### 3.7.2 Total RNA extraction

Cells were trypsinised and pelleted when they reached 60% confluency. Total RNA was extracted using a RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, 350µl RLT buffer containing 3.5µl β-mercaptoethanol (Bio-basic, Canada) was added to the cell pellet to lyse the cells. The lysate was transferred to a QIAshredder column and centrifuged at 15,871g for 2 minutes to homogenize the lysed cells and to reduce viscosity of the lysate. The flow through was mixed with 350µl of 70% ethanol and the mixture was loaded to a RNeasy Mini spin column followed by centrifugation at 8,000g for 15 second. The flow through was discarded and 350µl RW1 buffer was added to the spin column and centrifuged at 8,000g for 15 seconds to wash the spin column. After discarding the flow through, DNase1 solution was loaded directly onto the spin column membrane and incubated at room temperature for 15 minutes to digest DNA. Before centrifuging at 8,000g for 15 seconds, 350µl RW1 buffer was added to the spin column. The flow through was discarded and the column was further washed twice with 500µl RPE buffer and then transferred to a new 1.5ml eppendorf tube. RNA was eluted from the spin column membrane by adding 40µl elution buffer and centrifuge at 8,000g for 15 second. The RNA samples were stored in -80°C until use.

### 3.7.3 cDNA synthesis

The purified total RNA was used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied biosystems, USA) according to the manufacturer's instructions. RNase free water was added to 1µg RNA to obtain a final volume of 10µl. To prepare 10µl of reverse transcription master mix, 2µl of 10x RT buffer, 2µl of 10x RT random primers, 0.8µl of 25x dNTP mix, 1µl MultipScribe$^{TM}$

Reverse Transcriptase and 4.2µl RNAse-free water were mixed in a 1.5ml eppendorf tube. The reverse transcription master mix was added to the 10µl of the RNA samples and mixed well. The tubes were briefly centrifuged and cDNA synthesis was carried out in a thermal cycler (Applied biosystems, USA). The thermal cycler condition was programmed as follows: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes and 4°C for 10 minutes. The synthesized cDNA was diluted using nuclease-free water (Gibco Life Technologies, USA) at a 1:20 ratio and stored in -20°C.

### 3.7.4 Polymerase chain reaction (PCR)

PCR amplification was performed by using either 1µl of the diluted cDNA or 100ng of extracted DNA as the template. To prepare a PCR master mix, 12.5µl Hot-Start DNA polymerase (Promega, USA), 1.25µl of 100µM forward primer, 1.25µl of 100µM reverse primer and 9µl sterile water was mixed in an eppendorf tube. The master mix was added to the diluted cDNA to give a final volume of 25µl and mixed well. PCR was carried out in a thermal cycler (Applied biosystems, USA) using the programs as follows: 94°C for 2 minutes, 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 1 minutes, 72°C for 10 minutes and 4°C for 10 minutes. The primer sequences of GAPDH and TGFβR2 are listed in Table 3.3.

Table 3.3. PCR primer sequences

| **Gene** | **Primer sequences** | **PCR product size (bp)** |
|---|---|---|
| GAPDH | Forward: 5'CCACCCATGGCAAATTCCATGGC 3'<br>Reverse: 5'TCTAGACGGCAGGTCAGGTCCAC 3' | 600 |
| *TGFBR2* | Forward: 5'ACTTGGAGCGAGGAACTCCT 3'<br>Reverse: 5'CACAACGCCTTTCCTGCTCG 3' | 900 |

### 3.7.5 Agarose gel electrophoresis

PCR products were analyzed by electrophoresis using 2% agarose gels. To prepare 20ml of agarose gel solution, 0.4g agarose powder (1st Base, Singapore) was weighed and dissolved in 20ml of 1x Tris/Borate/EDTA buffer (TBE; 1st Base, Singapore) by boiling in a conical flask. The agarose solution was allowed to cool down before addition of 0.5µg/ml ethidium bromide (EtBR; Sigma-Aldrich, USA). The solution was poured into a casting tray with a gel comb inserted and left for 20 minutes for the gel to harden. The gel was then placed into an electrophoresis tank (Bio-rad, USA) containing 1x TBE buffer and the comb was removed. After mixing 5µl of PCR products with 1µl of 6x DNA dye (Promega, USA), the sample was loaded into the well along with either 100bp or 1kb ladder (Promega, USA). Gel electrophoresis was performed at 100V for 40 minutes and the PCR products were visualized using a UVP Gel-doc imaging system (UVP, US).

### 3.7.6 Real-time quantitative (qPCR)

#### 3.7.6.1 Probe-based qPCR for gene expression

Commercially available Taqman® gene expression assays (Applied biosystems, USA) were used to determine the expression of two genes: GAPDH (4326317E) and *TGFBR2* (Hs00947893_m1). To determine the expression of EBV genes, customized primers and probes according to the published sequences (Bell et al., 2006) were used (Table 3.4). Firstly, 5µl of the diluted cDNA was loaded into a MicroAmp® Fast Optical 96-well plate (Applied biosystems, USA). Master mixes for Taqman® gene expression assays and assays using customized primers and probes were prepared as shown in Table 3.5 and Table 3.6, respectively. The master mix was added to the cDNA

and the plate was sealed with a MicroAmp® optical adhesive film (Applied biosystems, USA). The plate was briefly centrifuged at 112g for 5 minutes and loaded to an ABI 7500 fast real-time PCR system (Applied biosystems, USA). GAPDH was served as the endogenous control to normalize the expression of target genes and each reaction was done in triplicate. Analysis was performed using the delta delta Ct (ΔΔCt) method using the 7500 software v2.0 (Applied biosystems, USA). A cycle threshold (Ct) value is defined as the number of cycles required for the fluorescent signal to exceed background level. Ct value of each gene was then normalized against the Ct value of GAPDH to obtain ΔCt values. The expression of target genes relative to references samples was calculated using the following formula: $2^{-\Delta\Delta Ct}$.

Table 3.4. Primer and probe sequences for EBV genes

| **Gene** | **Sequences** |
|---|---|
| EBNA1 | Primer Forward : 5'GTGCGCTACCGGATGGC 3'<br>Primer Reverse : 5'CATGATTCACACTTAAAGGAGACGG 3'<br>Probe : TCCTCTGGAGCCTGACCTGTGATCG |
| LMP1 | Primer Forward : 5'AATTTGCACGGACAGGCATT 3'<br>Primer Reverse : 5'AAGGCCAAAAGCTGCCAGAT 3'<br>Probe : TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT |
| LMP2A | Primer Forward : 5'CGGGATGACTCATCTCAACACATA 3'<br>Primer Reverse : 5'GGCGGTCACAACGGTACTAACT 3'<br>Probe : CAGTATGCCTGCCTGTAATTGTTGCGC |

Table 3.5. Master mix for commercially available TaqMan® gene expression assays

| **Reagents** | **Volume (µl)** |
|---|---|
| 2x FastStart Universal Probe master mix (Roche, Germany) | 10 |
| TaqMan® gene expression assays consist of a pair of unlabeled primer and Taqman® probe labeled with FAM at 5'end | 1 |
| Human GAPDH TaqMan® gene expression assay consist of a pair of unlabeled primer and Taqman® probe labeled with VIC at 5'end | 1 |
| Sterile distilled water | 3 |
| Total | 15 |

Table 3.6. Master mix for assays using customized primers and probes

| **Reagents** | **Volume (µl)** |
|---|---|
| 2x FastStart Universal Probe master mix (Roche, Germany) | 12.5 |
| 3µM Forward primer | 2.5 |
| 3µM Reverse primer | 2.5 |
| 5µM probe labeled with FAM at 5'end | 1 |
| Human GAPDH TaqMan® gene expression assay consist of a pair of unlabeled primer and Taqman® probe labeled with VIC at 5'end | 1 |
| Sterile distilled water | 0.5 |
| Total | 20 |

### 3.7.6.2 Dye-based qPCR for gene expression

The expression of EBV lytic genes, *BZLF1*, was determined using a QuantiNova® SYBR® green PCR kit (Qiagen, Germany) according to the manufacturer's protocol. The primer sequences of *BZLF1* are shown in Table 3.7. Briefly, 5µl of the diluted cDNA was loaded into a 96-well plate and a master mix containing 10µl of 2x QuantiNova SYBR Green PCR Master Mix, 0.1µl QN ROX reference dye, 1.4µl of 10µM forward primer, 1.4µl of 10µM reverse primer and 2.1µl sterile distilled water was added to the diluted cDNA. The plate was sealed with an adhesive film and briefly centrifuged at 112g for 5 minutes before loading to an ABI 7500 fast real-time PCR system. GAPDH was served as the endogenous control to normalize the expression of target genes. Experiments were performed in triplicate for each reaction. Analysis was performed using the delta delta Ct (ΔΔCt) method using the 7500 software v2.0. A cycle threshold (Ct) value is defined as the number of cycles required for the fluorescent signal to exceed background level. Ct value of each gene was then normalized against the Ct value of GAPDH to obtain ΔCt values. The expression of target genes relative to references samples was calculated using the following formula: $2^{-\Delta\Delta Ct}$.

Table 3.7. Primer sequences for *BZLF1*

| Gene | Sequences |
|---|---|
| *BZLF1* | Primer Forward : 5'CCGGCTTGGTTAGTCTGTTG3'<br>Primer Reverse : 5'AGCTTATGCAGCACCTCAGC3' |

### 3.7.7 Plasmid preparation

#### 3.7.7.1 Bacterial transformation

A 25µl aliquot of DH5α competent *E. coli* cells was thawed on ice prior to the addition of 100ng plasmid DNA. The plasmid-DH5α mixture was incubated on ice for 30 minutes before placing in a Thermomixer®R (Eppendorf, USA) for heat shock at 42°C for 45 seconds. The mixture was then immediately returned to ice for 2 minutes followed by the addition of 200µl Terrific-broth (TB). The cells were incubated at 37°C for 1 hour in an orbital shaker. 50µl of the transformed cells were plated onto TB-agar plates containing carbenicillin (100ug/ml; Fisher Scientific, USA) and incubated at 37°C for 16 hours.

#### 3.7.7.2 Plasmid DNA extraction

Following bacterial transformation, single bacterial colonies on the TB-agar plate were picked with sterile pipette tips and grown in 5ml TB containing 100µg/ml carbenicillin overnight at 37°C in an orbital shaker. The overnight bacterial culture was used for plasmid DNA extraction.

Plasmid DNA was extracted from 1.5ml of the overnight bacteria culture using a NucleoSpin® Plasmid DNA purification kit (Macherey-Nagel, Germany) according to the manufacturer's instruction. Briefly, the bacteria cells were pelleted by centrifugation

at 11,000g for 30 seconds. The supernatant was discarded and the cells were re-suspended in 250µl A1 buffer. The cells were lysed in 250µl A2 buffer at room temperature for 5 minutes. To neutralize the lysis buffer, 350µl A3 buffer was added and the mixture was centrifuged at 11,000g for 5 minutes. The supernatant was transferred to a NucleoSpin® Plasmid QuickPure column and centrifuged at 11,000g for 1 minute. The flow through was discarded and 450µl AQ buffer was added into the column followed by centrifugation at 11,000g for 3 minutes to wash the column. The flow through was discarded and the column was centrifuge at 11,000g for 3 minutes to remove any remaining solution. 40µl AE buffer was added to the column and the plasmid DNA was eluted by centrifugation at 11,000g for 1 minute. The concentration of the plasmid DNA was measured using a Nanodrop 2000 spectrophotometer.

To obtain higher concentration of plasmid DNA, 3ml of the overnight bacteria culture were transferred to 250ml TB containing 100µg/ml carbenicillin and incubated overnight at 37°C in an orbital shaker. Plasmid DNA from 250ml overnight bacterial culture was extracted using a Qiagen® Plasmid Purification Maxi kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, bacteria cells were pelleted by centrifugation at 3381g for 15 minutes. The supernatant was decanted and the cells were re-suspended in 10ml P1 buffer containing RNaseA. The cells were then lysed in 10ml P2 buffer at room temperature for 5 minutes. To neutralize the lysis buffer, 10ml pre-chilled P3 buffer was added to the cells and incubated on ice for 15 minutes. The mixture was centrifuged at 19,000g for 30 minutes at 4°C. Meanwhile, a Qiagen-tip 500 column (DNA binding column) was equilibrated by addition of 10ml QBT buffer and allowed to drain by gravity flow. The supernatant of the lysed cells was transferred to the Qiagen-tip 500 column and allowed to drain by gravity flow. The column was washed twice with 30ml QC buffer. 15ml of QF buffer was added to the column and the elute was collected in a 15ml tube. Subsequently, 10.5ml isopropanol

was added to the elute and the mixture was centrifuged at 18,000g for 30 minutes at 4°C. The supernatant was decanted carefully and the precipitated plasmid DNA was washed with 5ml of 70% ethanol. The plasmid DNA was pelleted by centrifugation at 18,000g for 10 minutes at 4°C. The supernatant was removed carefully and the DNA pellet was air-dried for 10 minutes. The plasmid DNA was re-suspended in 450µl TE (Tris-EDTA) buffer and the concentration of the plasmid DNA was determined using a Nanodrop 2000 spectrophotometer. The plasmid DNA was stored in -20°C.

#### 3.7.7.3 Preservation and retrieval of bacterial cultures

A bacterial glycerol stock was prepared by mixing 800µl of overnight bacteria culture with 200µl of 75% sterile glycerol and stored in -80°C. To recover the bacteria cells, a small amount of frozen bacteria was scraped into 5ml TB containing 100µg/ml of carbenicillin and incubated overnight at 37°C in an orbital shaker.

## 3.8 Western blotting

### 3.8.1 Protein isolation

Cells were grown in 100mm$^2$ cell culture dishes and harvested when they reached 70% confluency. Cells were washed with DPBS, scraped and pelleted by centrifugation at 16,363g for 2 minutes. The cells were lysed in 40µl of NP40 lysis buffer (150mM sodium chloride, 1% NP-40, 50mM Tris, pH 8.0) or 1x RIPA buffer (Biotech, Canada) containing Protease Inhibitor Cocktail Set III (Calbiochem, Germany) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, USA). The lysate was sonicated for 2 minutes and incubated on ice for 30 minutes prior to

centrifugation at 16,363g for 30 minutes at 4°C. The supernatant (crude protein) was transferred to a pre-chilled 1.5ml eppendorf tube and stored at 80°C.

### 3.8.2 Determination of protein concentration

Protein concentrations were determined using a Bradford Protein Assay kit (Bio-rad, USA) according to the manufacturer's instructions. Briefly, protein samples were prepared in eppendorf tubes by mixing 1µl of the protein lysate and 9µl of DPBS. 250µl of 1x Bradford dye reagent was added into a 96-well plate. 5µl of the protein samples, DPBS and BSA (0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.5µg/µl) was added to the 1x Bradford dye reagent. The samples were incubated at room temperature for 5 minutes and the absorbance was measured at 595nm using an Infinite 200 Pro NanoQuat microplate reader. Protein concentrations were determined from the standard curve generated using the BSA standards.

### 3.8.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein lysates (30µg) were mixed with 2x Laemmli sample buffer (Bio-rad, USA) containing 5% β-mercaptoethanol (Bio-rad, USA) and boiled at 95°C for 5 minutes. Bio-rad apparatus was used for SDS-PAGE. To prepare one gel, 7ml of 10% pre-mixed EZ-run$^{TM}$ protein gel solution (Fisher Scientific, USA), 4.2µl tetramethylethylenediamine (TEMED; Merck, Germany) and 10% ammonium persulfate (APS; Merck, Germany) were mixed in a 15ml-falcon tube. The gel solution was poured into a gel cassette. A gel comb was inserted and removed after the gel had solidified. The gel was transferred into electrophoresis tank containing 1x EZ-run$^{TM}$ running buffer (Fisher Scientific, US). The denatured protein samples and 3.5µl

Precision Plus Protein™ All Blue Standards (Bio-rad, USA) were loaded into the wells. The proteins were separated at 100V for 2 hours.

### 3.8.4 Protein transfer and detection

Following SDS-PAGE, the separated proteins were electro-transferred onto a polyvnylidene difluoride (PVDF) membrane (Millipore, MA) using a Mini Trans-Blot® Cell (Bio-rad, USA) according to the manufacturer's instruction. A transfer sandwich (fiber pad-filter papers-gel-membrane-filter papers-fiber pad) was prepared and placed into a buffer tank containing transfer buffer (25mM tris, 190mM glycine, 20% methanol). Protein transfer was carried out at 100V for 2 hours. The membrane was then blocked with blocking buffer containing 5% non-fat milk in Tris-Buffered Saline (TBS) with 0.1% Tween 20 (TBST) for 1 hour at room temperature followed by incubation with primary antibodies diluted in blocking buffer (Table 3.8) for overnight at 4°C. The next day, the membrane was washed three times in TBST for 5 minutes each time. The membrane was subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blocking buffer at room temperature for 1 hour. After three washes in TBST for 15 minutes each and a final wash in TBS for 5 minutes, the membrane was incubated with WesternBright Sirius chemiluminescence (ECL) substrate (Advansta, USA) for 5 minutes. The target proteins were visualized using an Odyssey fc imaging system (Li-COR Biosciences, USA) and β-actin was used as a loading control.

Table 3.8. List of primary antibodies used in Western blotting

| Antibody | Species | Dilution | Manufacturer |
|---|---|---|---|
| Anti-TGFR-2 | Mouse monoclonal | 1:500 | Abcam, US |
| Anti-Total Smad2 | Rabbit polyclonal | 1:1000 | Cell Signalling Technology, USA |
| Anti-Total Smad3 | Rabbit polyclonal | 1:1000 | Cell Signalling Technology, USA |
| Anti-Total Smad4 | Rabbit polyclonal | 1:1000 | Cell Signalling Technology, USA |
| Anti-Phospho-Smad2 (Ser465/467) | Rabbit polyclonal | 1:1000 | Cell Signalling Technology, USA |
| Anti-Phospho-Smad3 (Ser463/464) | Rabbit monoclonal | 1:1000 | Cell Signalling Technology, USA |
| Anti-SIRT1 | Rabbit polyclonal | 1:10000 | Santa-cruz, USA |
| Anti-β-Actin | Mouse monoclonal | 1:3000 | Sigma-Aldrich, USA |
| Anti-Involucrin | Mouse monoclonal | 1:1000 | ThermoFisher, USA |

## 3.9 Fluorescent staining techniques

### 3.9.1 Immunofluorescence staining (IF)

Cells were seeded in 24-well plates at 2.5 x $10^3$ cells per well and allowed to grow for 14 days after EBV infection. The cells were washed twice with DPBS before fixing with 4% paraformaldehyde (PFA) at room temperature for 30 minutes. The cells were then permeabilised in methanol for 10 minutes, followed by washing twice with DPBS and blocking with 5% BSA in DPBS for 30 minutes. After washing with DPBS, the cells were incubated overnight with primary antibodies diluted in 1% BSA-DPBS (Table 3.9) at 4°C. The following day, the cells were washed three times in DPBS containing 0.1% Tween 20 (DPBST) for 5 minutes each time followed by incubation with secondary antibodies in 1% BSA-DPBS for 1 hour at room temperature in the dark. After three washes in DPBST with 15 minutes each time, the cells were mounted with fluoroshield containing DAPI (Abcam, USA) and viewed under a fluorescence microscope (Leica MICROSYSTEMS, Germany). Photographs at 20x magnification

were taken and the staining intensity was analyzed using image J software (National Institute of Health, USA).

Table 3.9. List of primary antibodies used in IF

| Antibody | Species | Dilution | Manufacturer |
|---|---|---|---|
| Anti-$p16^{INK4a}$ | Rabbit monoclonal | 1:100 | Abcam, US |
| Anti-$p21^{WAF1}$ | Mouse monoclonal | 1:100 | Santa Cruz, USA |
| Anti-Involucrin | Mouse monoclonal | 1:100 | ThermoFisher, USA |

### 3.9.2 Fluorescence in situ hybridization (FISH)

#### 3.9.2.1 Sample preparation

FISH was performed in NP550hTert cells at day 14 of post-EBV infection to detected EBV DNA. The EBV-positive cells were seeded in a 6-well plate at 2.5 x $10^4$ cells per well and pelleted by centrifugation at 112g for 8 minutes. The cells were re-suspended in 2ml of 0.8% sodium citrate (Sigma-Aldrich, USA) and incubated at 37°C for 15 minutes. 5ml washing solution (1:3 ratio of acetic acid to methanol) was added to the cells and incubated at 37°C for 5 minutes followed by centrifugation at 135g for 4 minutes. The cells were re-suspended in 5ml washing solution and pelleted by centrifugation at 13,523g for 4 minutes. The washing steps were repeated three times. After the final centrifugation, the cells were re-suspended in 50µl washing solution. 15µl of the cell suspension was spread onto a microscope slide (ThermoFisher, USA) in a circular direction and the slide was stored at room temperature for one week until staining.

#### 3.9.2.2 Staining

One week after the cells were fixed on the microscope slide, the slide was submerged in 0.1mg/ml RNase (Sigma-Aldrich, USA) solution in 2x saline sodium citrate (SCC; Merck, Germany) for 1.5 hours at 37°C. The slide was then rinsed with water and submerged in 2x SSC at room temperature for 10 minutes followed by submerging in 0.025mg/ml proteinase K solution (Sigma-Aldrich, USA) for 15 minutes at 37°C. After rinsing the slide with water followed by incubation in 2x SCC at room temperature for 10 minutes, the slide was submerged in cold 3% PFA for 10 minutes. The slide was then submerged in 2x SCC for 10 minutes, dehydrated by sequential incubation in graded alcohol (70%, 85% and 95% ethanol for 2 minutes each time) and air-dried at room temperature for 30 minutes. The samples was then denatured by incubating in 70% formamide in 2x SCC at 80°C for 4 minutes. After dehydrating again in graded alcohol (70%, 85% and 95% ethanol for 2 minutes each time), the slide was dried using a stream of nitrogen gas. Meanwhile, 1µl biotin-labeled EBV probe was mixed with 2.5µl hybridization buffer (Cytocell, UK) in an eppendorf tube. The probe was denatured in 70% formamide/2x SCC at -80°C for 5 minutes followed by 37°C for 20 minutes. 3.5µl of the denatured EBV probe was then applied to the cells. A cover slip was placed on top of the cells and the edge of the slide was sealed with rubber cement to avoid evaporation during overnight incubation at 37°C. The following day, the rubber cement and cover slip were removed and the slide was washed twice in 50% formamide/2x SCC at 45°C for 6 minutes each time. 45µl of detection solution containing Cy3-conjugated streptavidin (Cytocell, UK) was applied to the cells and the slide was incubated in the dark at 37°C for 30 minutes. The slide was washed twice in 2x SCC at 45°C for 6 minutes each time and dehydrated using graded alcohol (70%, 85% and 95% ethanol for 2 minutes each time). After drying using stream of nitrogen gas, the slide was mounted in mounting solution containing DAPI (Applied spectra

imaging, Italy). A cover slip was placed on top of the cells and photographs at 100x magnification were taken.

### 3.10 Statistical analysis

One way analysis of variance (ANOVA) was used for statistical analysis in cell proliferation assays where more than one variable is tested. Meanwhile Student T-test were used in luciferase reporter assays, SA-β-Gal assays, differentiation assay, IF and qPCR. The statistical analyses were performed using GraphPad PRISM 5.0 software (GraphPad, La Jolla, USA). For FISH analyses, online software https://www.medcalc.org/calc/comparison_of_proportions.php was used to calculate the significant differences between the test and control groups. P values $<0.05$ were considered as statistically significant and all the graphs were plotted using GraphPad PRISM 5.0 software.

# CHAPTER 4: EFFECT OF DISRUPTING THE TGF-BETA SIGNALLING PATHWAY ON THE OUTCOME OF EBV INFECTION IN NPE CELLS

## 4.1 Introduction

Undifferentiated NPC is strongly associated with EBV infection. Deletion of *p16* or overexpression of *CCND1* are commonly found in primary NPC tissues and these genetic alterations have been shown to contribute to the susceptibility of immortalised NPE cells to stable EBV latent infection (Tsang et al., 2012; Tsang et al., 2010). Once infected, EBV latent genes drive the malignant transformation of NPE cells by inducing additional genetic alterations (Tsao et al., 2015). A microarray study has shown that the *TGFBR2* gene was down-regulated in primary NPC tissues compared to non-cancerous NP tissues (Fang et al., 2008). Subsequently, these results were supported by other research groups who showed that *TGFBR2* mRNA and protein expression were reduced in NPC tissues and NPC cell lines compared to non-cancerous NP tissues and NP69 cells, respectively (Lyu et al., 2014; Zhang et al., 2012), implying that the TGF-β signalling pathway is de-regulated in NPC. Furthermore, a negative correlation was identified between the expression of TGFR-1 and TGFR-2 and that of EBER1/2 genes, suggesting a role for TGF-β signalling in the pathogenesis of EBV-associated NPC (Zhang et al., 2012). However, the role of TGF-β signalling in regulating stable EBV infection in NPE cells has not been studied.

TGF-β signalling is mediated through its serine/threonine kinase receptors, TGFR-1 and TGFR-2. Binding of activated TGF-β ligands (TGF-β1, TGF-β2 and TGF-β3) to TGFR-2 leads to the recruitment and phosphorylation of TGFR-1 (Derynck et al., 2001). This, in turn, activates Smad2 or Smad3, which form complexes with Smad4, leading to transcription of various target genes (Feng et al., 2000). In normal epithelial cells, the addition of exogenous TGF-β1 results in a G1 cell cycle arrest and this effect

is reversible, where the cells continue to proliferate in the absence of exogenous TGF-β1 (Shipley et al., 1986). A number of studies have shown that, genetic abnormalities of various components within the TGF-β signalling pathway cause defects in TGF-β1-mediated growth arrest (Kretschmer et al., 2003; Papageorgis, 2015; Piek et al., 2001; Wang et al., 1997). For example, resistance to TGF-β1-mediated growth inhibition was shown in a EBV-positive NPC cell line, C666.1 (Dr. CW. Dawson, University of Birmingham, unpublished data), which is consistent with the loss of TGFR-2 expression reported in these cells (Wood et al., 2007).

It has been reported previously that EBV infection in HONE1, an undifferentiated NPC-derived cell line is much higher compared to the well-differentiated NPC-derived cell line, HK1 (Tsang et al., 2010). Furthermore, our preliminary data demonstrated that HONE1 that were not growth inhibited by TGF-β1 were susceptible to EBV infection, whereas differentiation-competent squamous epithelial cells (e.g. HaCaT), which were unable to sustain EBV infection were responsive to the cytostatic effects of TGF-β1 (Dr. CW. Dawson, University of Birmingham, unpublished data). Therefore, the hypothesis of the present study is that disruption of the TGF-β signalling pathway contributes to stable EBV maintenance in epithelial cells. This chapter describes the development of *in vitro* models to study the impact of TGF-β signalling pathway disruption on the outcome of EBV infection in NPE cells.

## 4.2 Response of NPE and NPC cells to TGF-β1-mediated growth inhibition

To select cells that were responsive to TGF-β1-mediated growth inhibition, MTT assays were performed following the treatment of cells with TGF-β1 for 24, 48 and 72 hours. Six cell lines were used, namely four immortalised NPE cell lines (NP69, NP361hTert, NP460hTert, NP550hTert), HK1 and HONE1. Among these, the growth of four cell lines, NP361hTert, NP460hTert, NP550hTert and HK1, was significantly inhibited 40-60% following TGF-β1 treatment (Figure 4.1A-D, $p<0.05$). NP69 and HONE1 were not responsive to TGF-β1-mediated growth inhibition (Figure 4.1E&F). Given that NP361hTert, NP460hTert and NP550hTert are non-malignant NPE cells that represent more suitable models than NPC cells in studying the mechanism of EBV infection, these three cell lines were selected for subsequent experiments.

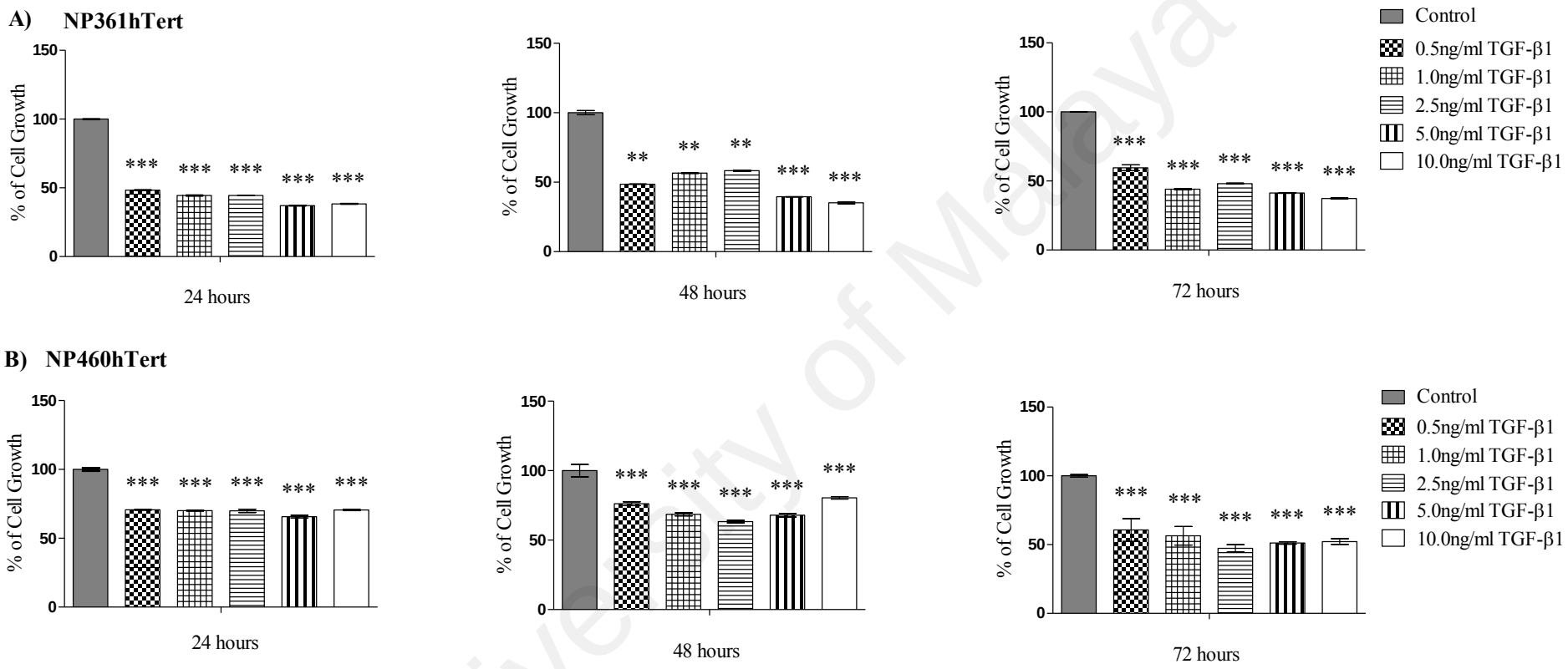
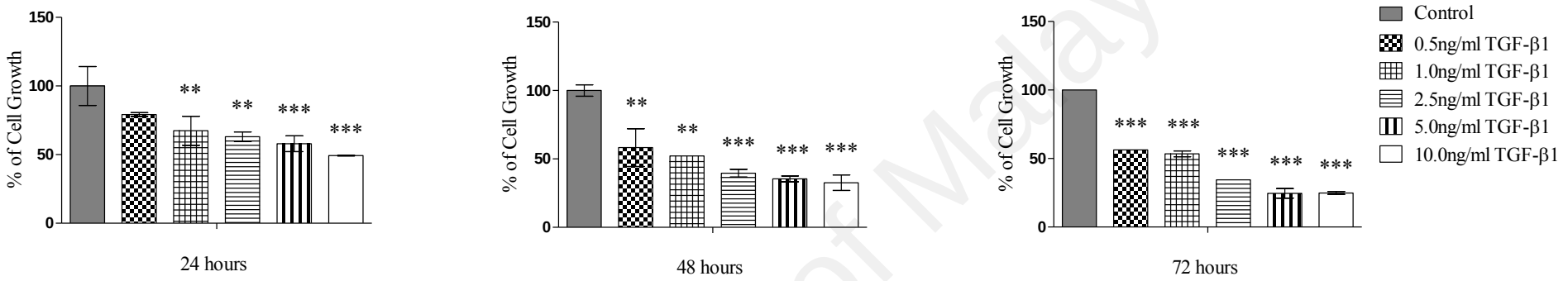



**Figure 4.1. Responsiveness of immortalised NPE and NPC cell lines to TGF-β1-mediated growth inhibition.** MTT assays were used to examine the growth of epithelial cells following treatment with a range of TGF-β1 concentrations. Compared to controls, the growth of A) NP361hTert, B) NP460hTert, C) NP550hTert and D) HK1 were significantly inhibited by TGF-β1. No growth inhibition was observed in E) NP69 and F) HONE1 following TGF-β1 treatment. The data presented are representative of three independent experiments. *** denotes p-value <0.001 and ** denotes p-value <0.01.

**C) NP550hTert**




**D) HK1**

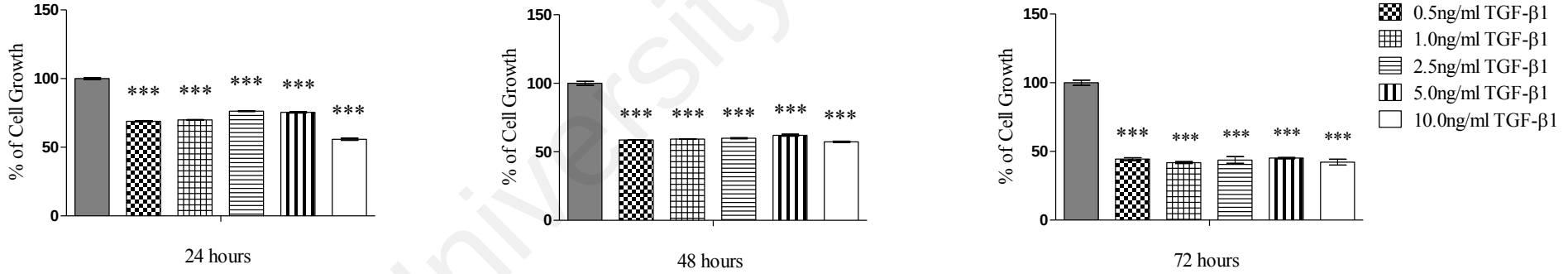
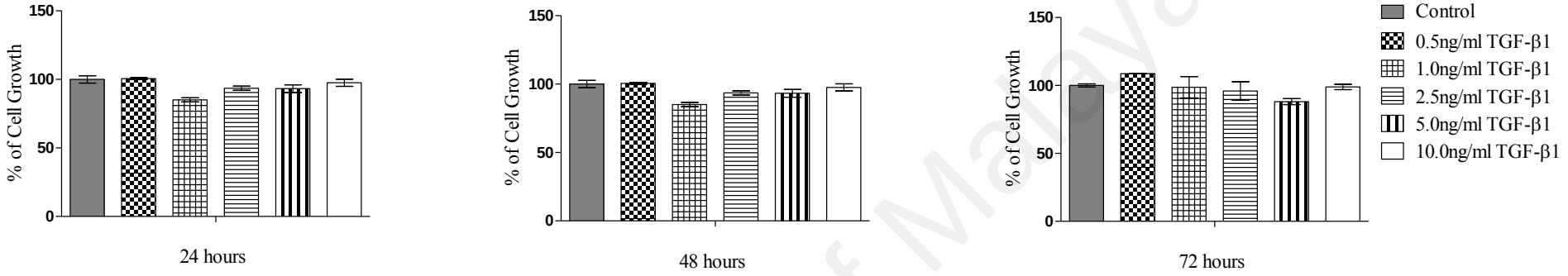



**Figure 4.1, continued**

**E) NP69**




**F) HONE1**

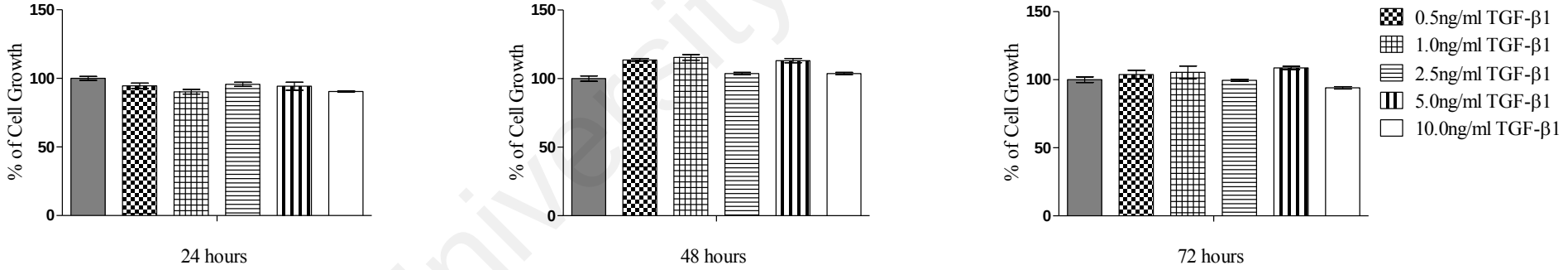



**Figure 4.1, continued**

## 4.3 Generation and validation of cells with disrupted TGF-β signalling

To inhibit TGF-β signalling in NP361hTert, NP460hTert and NP550hTert, two different approaches were used, by targeting different genes within the TGF-β signalling pathway (*TGFBR1* and *TGFBR2*).

### 4.3.1 Inhibition of TGFR-1 kinase with SB431542

The first approach was to target the TGFR-1 kinase by treating cells with a chemical inhibitor, SB431542 (Callahan et al., 2002; Inman et al., 2002; Matsuyama et al., 2003). Data from our laboratory have shown that SB431542 treatment of mink lung epithelial cells (MLEC) transfected with a constitutively active *TGFBR1* significantly decreased Smad3-dependent transcriptional activity (Appendix A), indicating that SB431542 is a selective inhibitor of TGFR-1, thereby inactivating the TGF-β/Smad signalling pathway. Following treatment of cells with SB431542 for 72 hours, the levels of phosphorylated Smad2 (pSmad2) were decreased in all three NPE cell lines, NP361hTert, NP460hTert and NP550hTert. A maximum reduction in the levels of pSmad2 was observed at 5μM of SB431542 in NP361hTert and NP460hTert (Figure 4.2A&B). In NP550hTert the maximum reduction was observed at 10μM of SB431542 (Figure 4.2C). To further confirm the disruption of TGF-β signalling, cell proliferation assays were performed to determine the effect of SB431542 on the responsiveness of these cells to TGF-β1-mediated growth inhibition. Following treatment with SB431542, NP361hTert, NP460hTert and NP550hTert cells grew faster than cells treated with diluent (ethanol) (Figure 4.3; $p<0.05$), demonstrating that the cells treated with SB431542 are less responsive to growth inhibition mediated by TGF-β1.

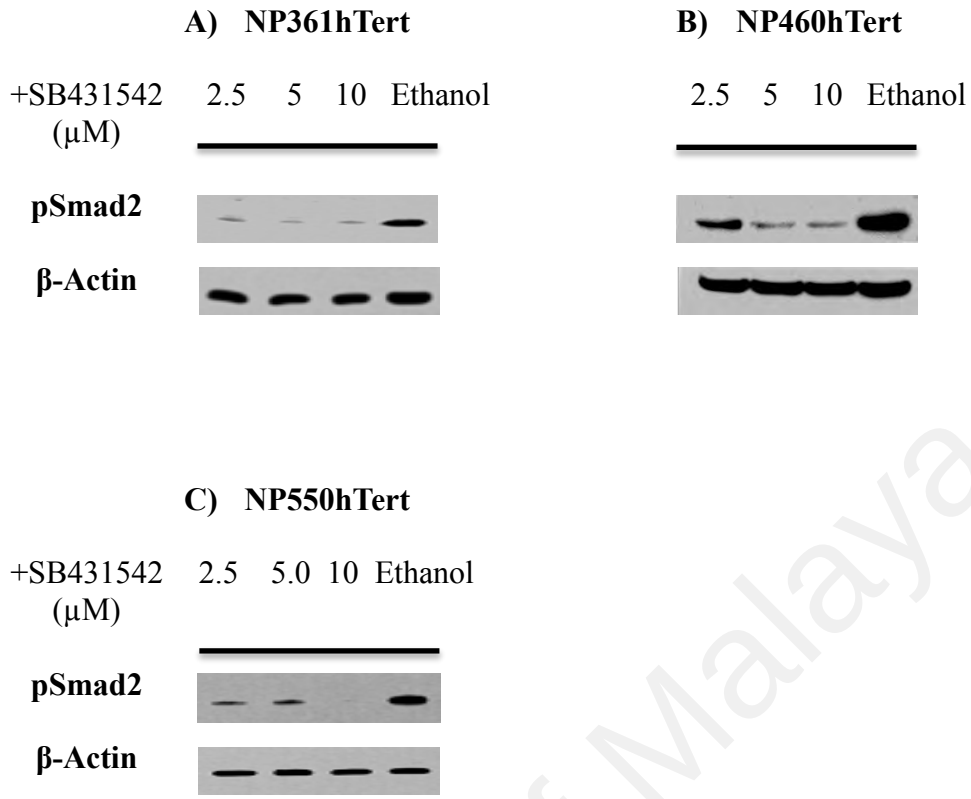



**Figure 4.2. Inhibition of TGFR-1 kinase reduced expression of pSmad2 in NPE cells.** The levels of pSmad2 in A) NP361hTert, B) NP460hTert and C) NP550hTert were decreased following treatment of cells with SB431542 (2.5, 5 and 10μM) for 72 hours prior to the addition of 1ng/ml TGF-β1 for 1 hour, as determined by Western blotting. Diluent (ethanol)-treated cells served as controls. Representative images from two independent experiments are presented.

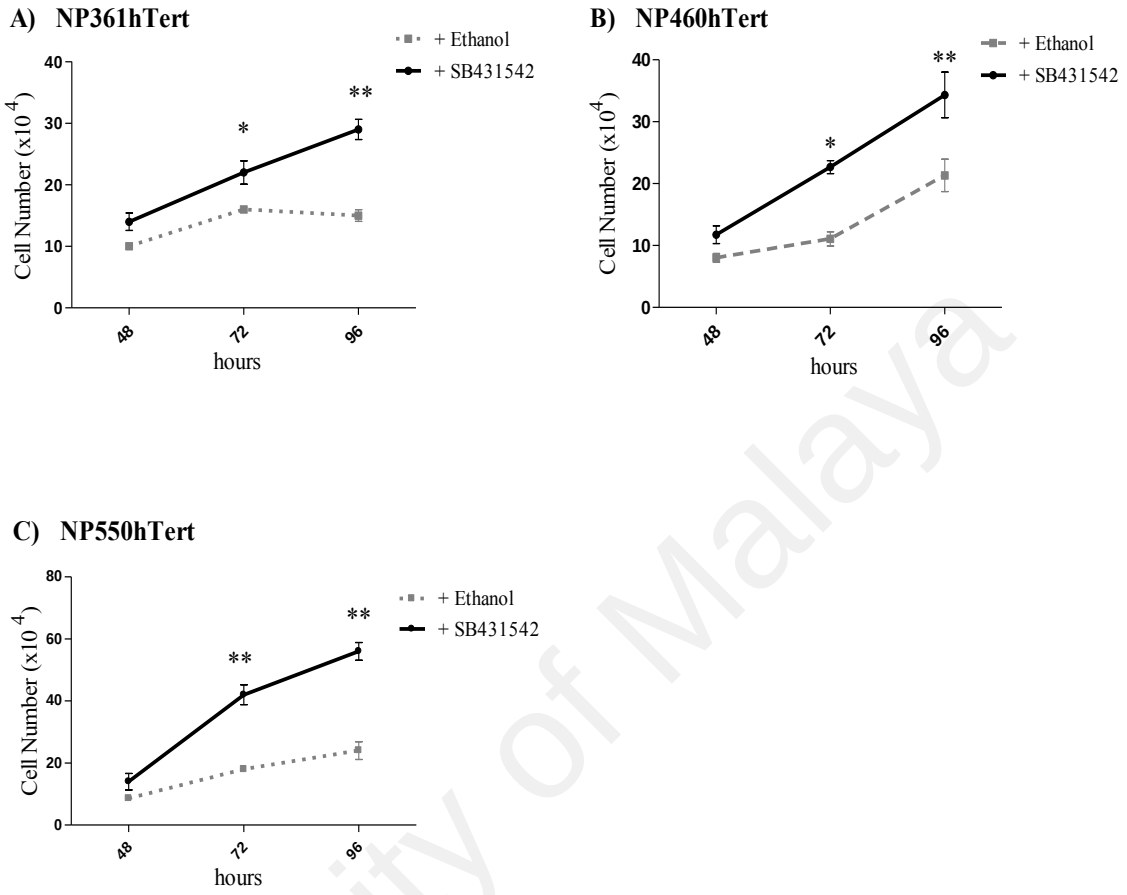



**Figure 4.3. Inhibition of TGFR-1 kinase abrogated TGF-β1-mediated growth inhibition in NPE cells**. Reduced growth inhibitory responses in A) NP361hTert, B) NP460hTert and C) NP550hTert treated with 10μM SB431542 for 30 minutes followed by addition of 1ng/ml of TGF-β1 for 48, 72 and 96 hours compared to their respective controls, with diluent (ethanol)-treated cells. The data presented are representative of two independent experiments. ** denotes p-value <0.01 and * denotes p-value <0.05.

To examine whether TGF-β/Smad-dependent transcription is lost following SB431542 treatment, NP361hTert, NP460hTert and NP550hTert were transiently transfected with *SMAD2* ($ARE_3$-Luc) or *SMAD3* (pCAGA12-luc) luciferase reporter plasmids. Upon addition of TGF-β1, an increase in Smad2-dependent transcriptional activity with fold changes of 11.5-fold, 5.3-fold and 5.9-fold were shown in NP361hTert, NP460hTert and NP550hTert, respectively (Figure 4.4; $p<0.05$). Treatment with SB431542 prior to TGF-β1 stimulation resulted in a small increase in the Smad2-dependent transcriptional activity in NP361hTert (1.3-fold) and NP460hTert (2.5-fold; $p<0.05$) while no change was evident in NP550hTert (Figure 4.4). Similarly, addition of TGF-β1 resulted in an increase in the Smad3-dependent transcriptional activity with fold changes of 8.5-fold, 12.1-fold and 30.2-fold in NP361hTert, NP460hTert and NP550hTert, respectively (Figure 4.5; $p<0.05$). Treating these cells with SB431542 prior to TGF-β1 stimulation abolished their Smad3-dependent transcriptional activities (Figure 4.5). Taken together, these results confirmed that SB431542 treatment was able to functionally disrupt TGF-β/Smad signalling in these cell lines.

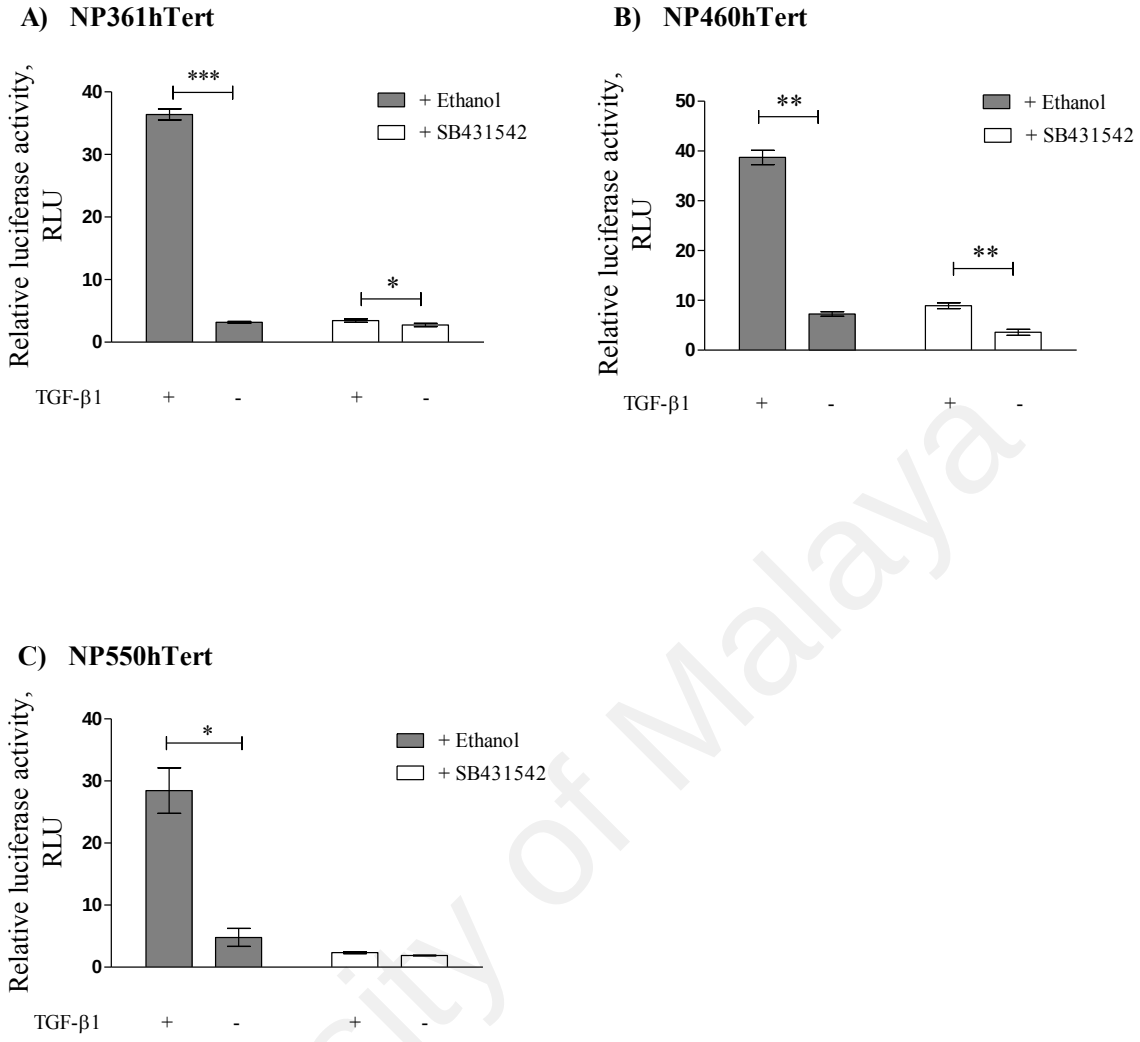



**Figure 4.4. Inhibition of TGFR-1 kinase reduced Smad2-dependent transcriptional activity in NPE cells.** ARE3-Luc reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in Smad2-dependent transcriptional activity in A) NP361hTert (11.5-fold), B) NP460hTert (5.3-fold) and C) NP550hTert (5.9-fold). Treatment with 10μM SB431542 prior to TGF-β1 stimulation resulted in a small increase of Smad2-dependent transcriptional activity in NP361hTert (1.3-fold) and NP460hTert (2.5-fold) while no change was observed in NP550hTert. The data presented are representative of two independent experiments. ***denotes p-value <0.001, ** denotes p-value <0.01 and * denotes p-value <0.05.

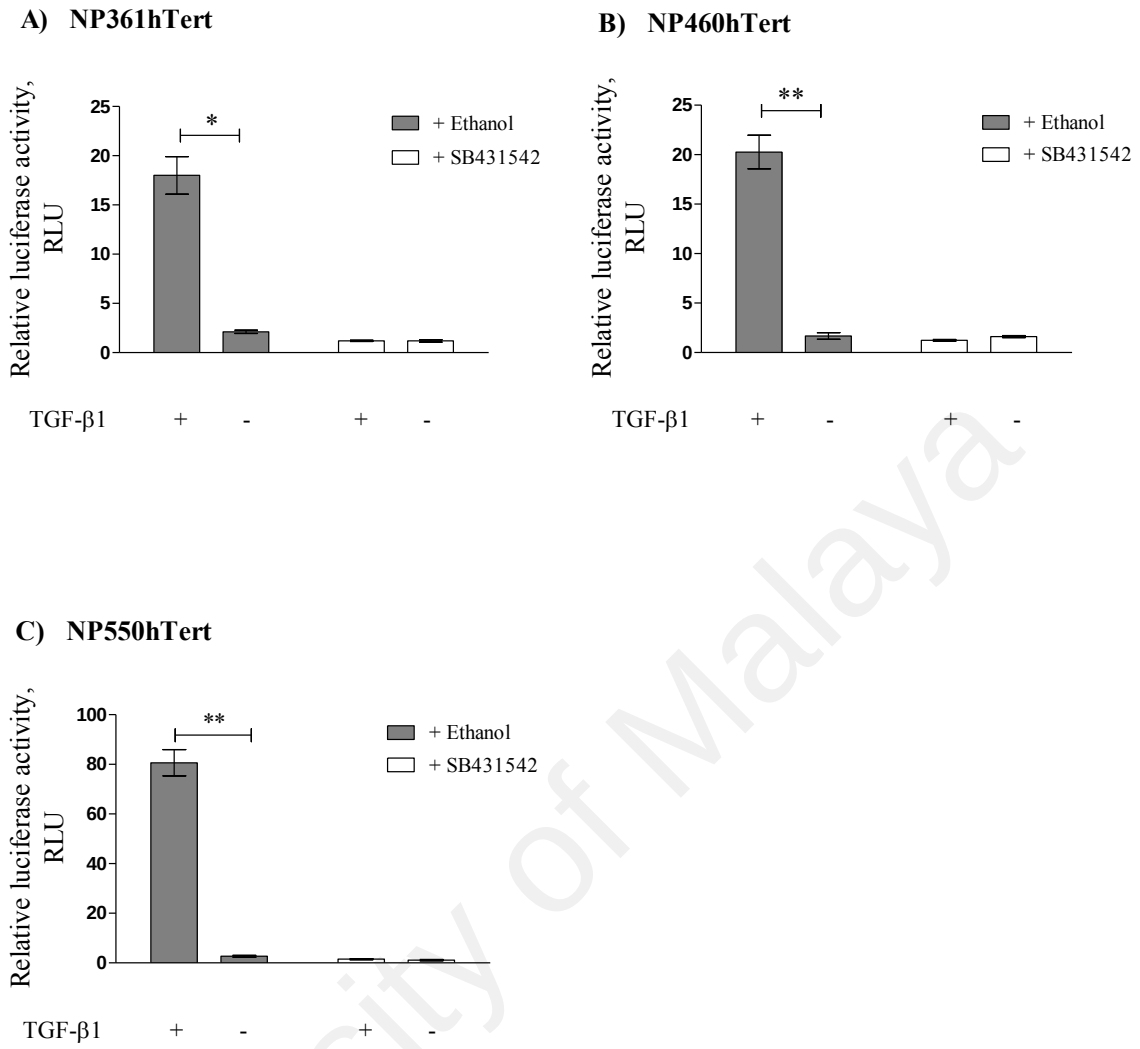



**Figure 4.5. Inhibition of TGFR-1 kinase reduced Smad3-dependent transcriptional activity in NPE cells.** pCAGA12-luc reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in Smad3-dependent transcriptional activity in A) NP361hTert (8.5-fold), B) NP460hTert (12.1-fold) and C) NP550hTert (30.2-fold). No increase in Smad3-dependent transcriptional activities in 10μM SB431542-treated cells prior to TGF-β1 treatment. The data presented are representative of two independent experiments. ** denotes p-value <0.01 and * denotes p-value <0.05.

### 4.3.2 Overexpression of a *DnTGFBR2*

The second approach to inhibit TGF-β signalling was to target the *TGFBR2* gene by overexpressing a *DnTGFBR2* cDNA in NP361hTert, NP460hTert and NP550hTert. *DnTGFBR2* encodes a truncated TGFR-2 protein with no cytoplasmic domain and overexpression of a *DnTGFBR2* in human oral carcinoma cell lines has been demonstrated to result in a functional loss of TGF-β signalling (Paterson et al., 2001). The NPE cells were transduced with lentiviruses carrying a *DnTGFBR2* cDNA, puromycin-resistant cells were pooled and designated as *DnTGFBR2*/NP361hTert, *DnTGFBR2*/NP460hTert and *DnTGFBR2*/NP550hTert. QPCR analyses using a probe that detects wild type and *DnTGFBR2* showed that the expression of *TGFBR2* mRNA was 12.2-fold, 5.9-fold and 17.2-fold higher in the *DnTGFBR2*/NP361hTert, *DnTGFBR2*/NP460hTert and *DnTGFBR2*/NP550hTert, respectively, compared to their corresponding vector controls (pLVX-Puro) (Figure 4.6, $p<0.001$). In addition, overexpression of DnTGFR-2 protein was demonstrated in the *DnTGFBR2*-expressing cells compared to their respective vector controls (Figure 4.7). To confirm the TGF-β signalling pathway was disrupted in these cells, the expression of pSmad2 following TGF-β1 treatment was determined by Western blotting. Compared to their respective vector controls all the three *DnTGFBR2*-expressing cell lines showed a decrease in the levels of pSmad2, with the maximum reduction at 15 minutes of post-TGF-β1 treatment (Figure 4.8). In addition, a significant reduction in the growth inhibitory responses to TGF-β1 was noted in all the three *DnTGFBR2*-expressing cell lines compared to their corresponding vector controls (Figure 4.9; $p<0.05$).

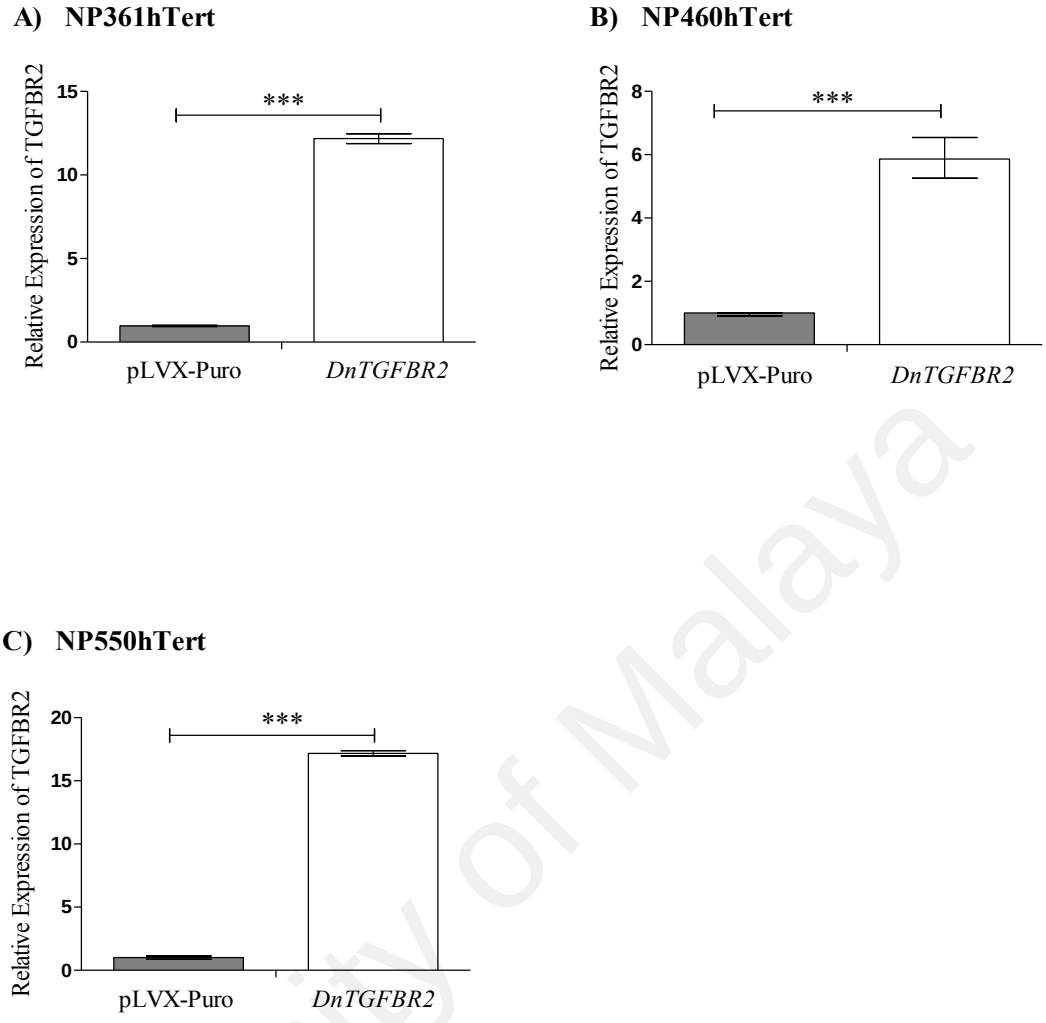



**Figure 4.6. Expression of *TGFBR2* in *DnTGFBR2*-expressing cells.** QPCR analyses showed that the levels of *TGFBR2* mRNA were significantly higher in A) *DnTGFBR2*/N361hTert, B) *DnTGFBR2*/NP460hTert and C) *DnTGFBR2*/NP550hTert compared to their respective vector controls (pLVX-Puro). The data presented are representative of two independent experiments. *** denotes p-value <0.001.

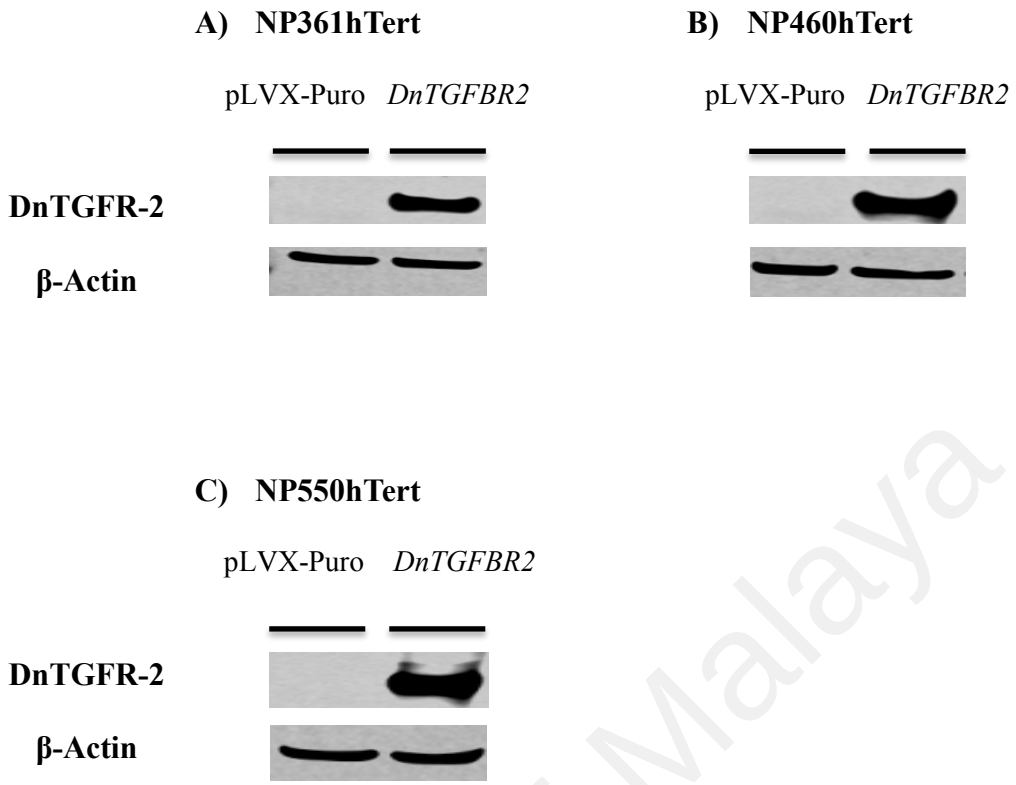



**Figure 4.7. Expression of DnTGFR-2 in *DnTGFBR2*-expressing cells.** Western blotting analyses showed that the DnTGFR-2 was overexpressed in A) *DnTGFBR2*/N361hTert, B) *DnTGFBR2*/NP460hTert and C) *DnTGFBR2*/NP550hTert compared to their respective vector controls (pLVX-Puro).

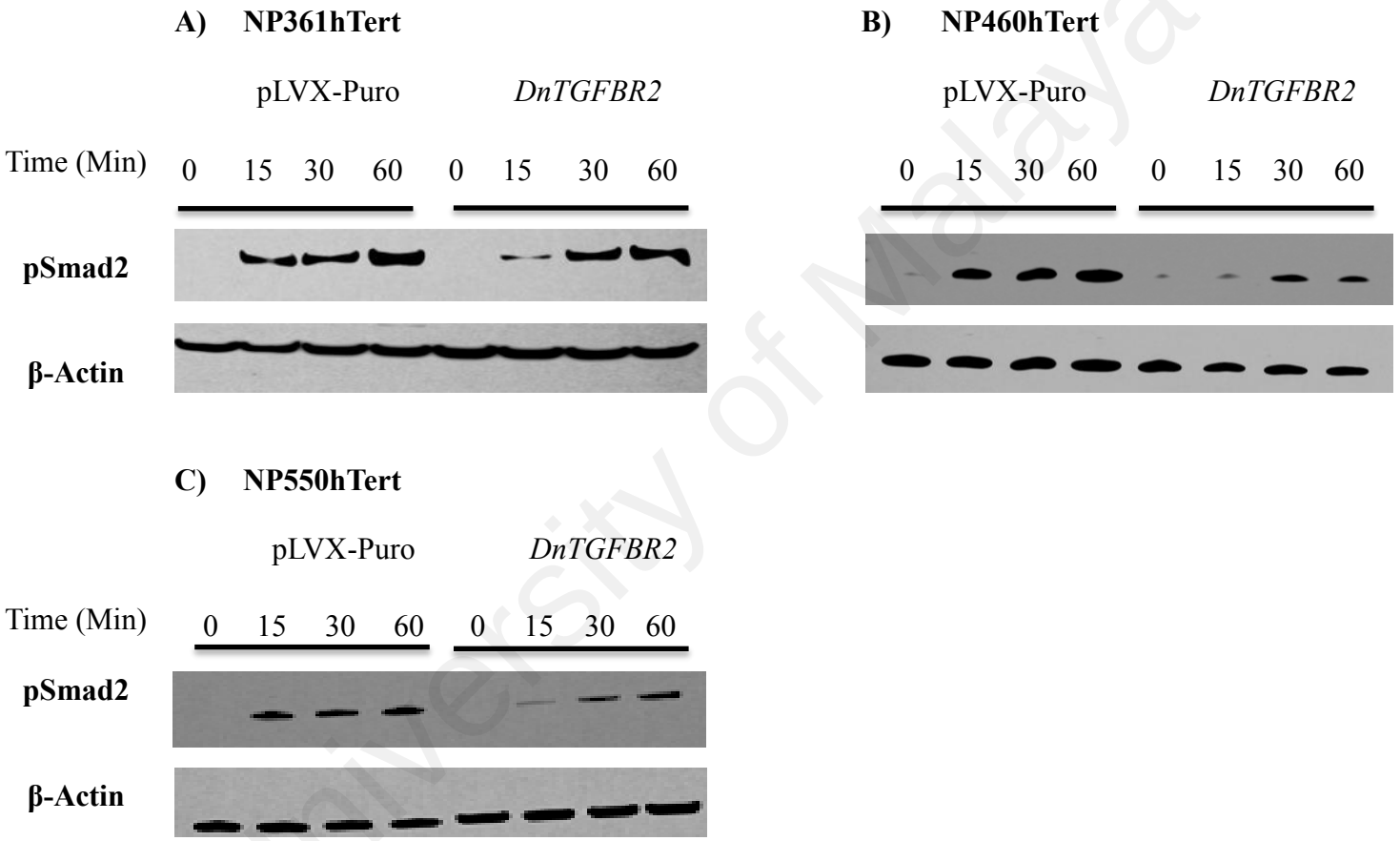



**Figure 4.8. Expression of pSmad2 in *DnTGFBR2*-expressing cells.** Western blotting analyses showed that the levels of pSmad2 in A) *DnTGFBR2*/NP361hTert, B) *DnTGFBR2*/NP460hTert and C) *DnTGFBR2*/NP550hTert were decreased following 1ng/ml TGF-β1 treatment compared to their respective vector controls (pLVX-Puro). Representative images from three independent experiments are presented.

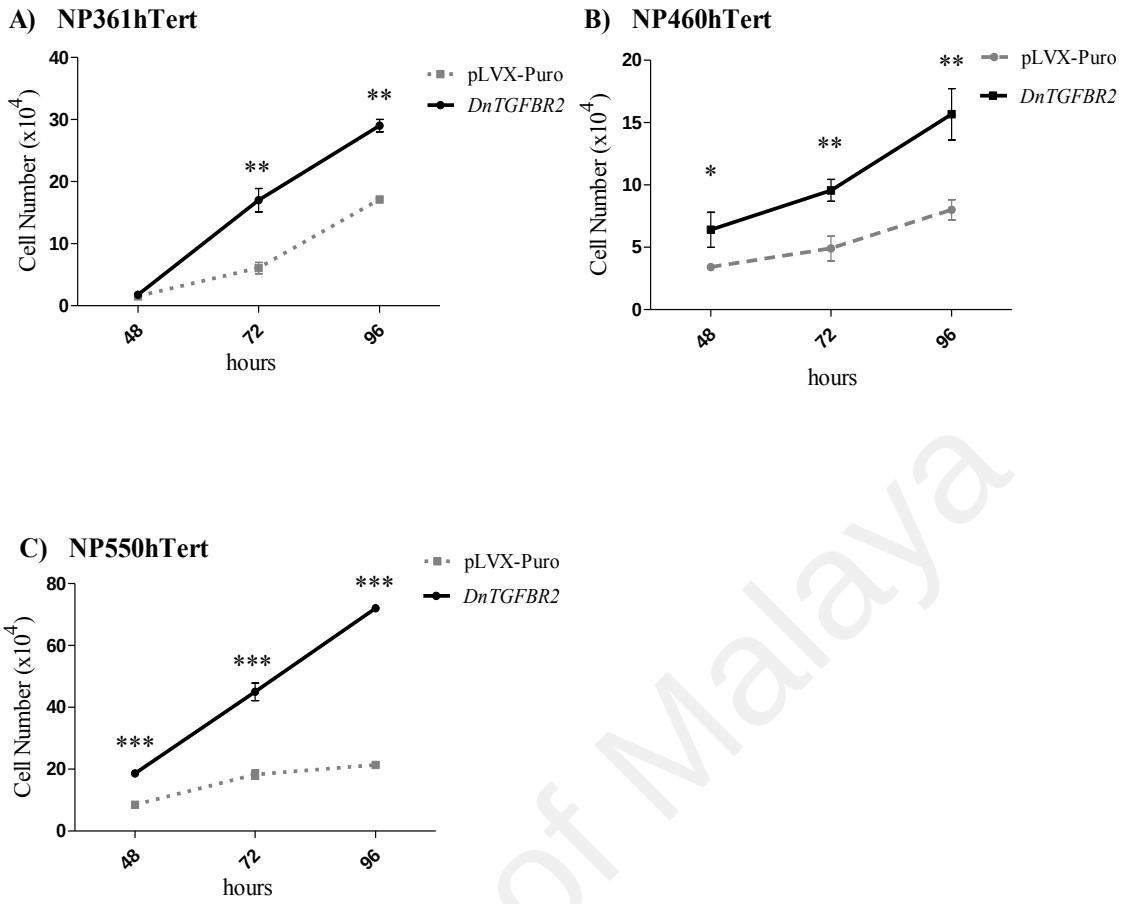



**Figure 4.9. TGF-β1-mediated growth suppression in *DnTGFBR2*-expressing cells.** Reduced growth inhibitory responses towards TGF-β1 (1ng/ml) in A) *DnTGFBR2*/NP361hTert, B) *DnTGFBR2*/NP460hTert and C) *DnTGFBR2*/NP550hTert compared to their respective vector controls (pLVX-Puro). The data presented are representative of two independent experiments. *** denotes p-value <0.001, ** denotes p-value <0.01 and * denotes p-value <0.05.

To determine the extent to which TGF-β/Smad-dependent transcription was abrogated in the *DnTGFBR2*-expressing cells, NP361hTert, NP460hTert and NP550hTert were transiently transfected with *SMAD2* or *SMAD3* luciferase reporter plasmids. Upon addition of TGF-β1, an increase in Smad2-dependent transcriptional activity was observed, with fold changes of 8.8-fold, 3.5-fold and 5-fold in the vector controls of NP361hTert, NP460hTert and NP550hTert, respectively (Figure 4.10; $p<0.05$). In the *DnTGFBR2*-expressing cells, TGF-β1 stimulation resulted in a small increase of Smad2-dependent transcriptional activity in NP460hTert (1.5-fold) and NP550hTert (3-fold) while no change was evident in NP361hTert (Figure 4.10). Similarly, the addition of TGF-β1 increased Smad3-dependent transcriptional activity with fold changes of 28.6-fold, 17-fold and 17.4-fold in NP361hTert, NP460hTert and NP550hTert, respectively (Figure 4.11; $p<0.05$). In the *DnTGFBR2*-expressing cells, TGF-β1-induced Smad3-dependent transcriptional activity was markedly reduced in NP361hTert (7.1-fold) and NP550hTert (2.0-fold), while no change was observed in NP460hTert cells after TGF-β1 stimulation (Figure 4.11). Taken together, these results confirmed the functional disruption of TGF-β/Smad signalling in cells expressing *DnTGFBR2*.

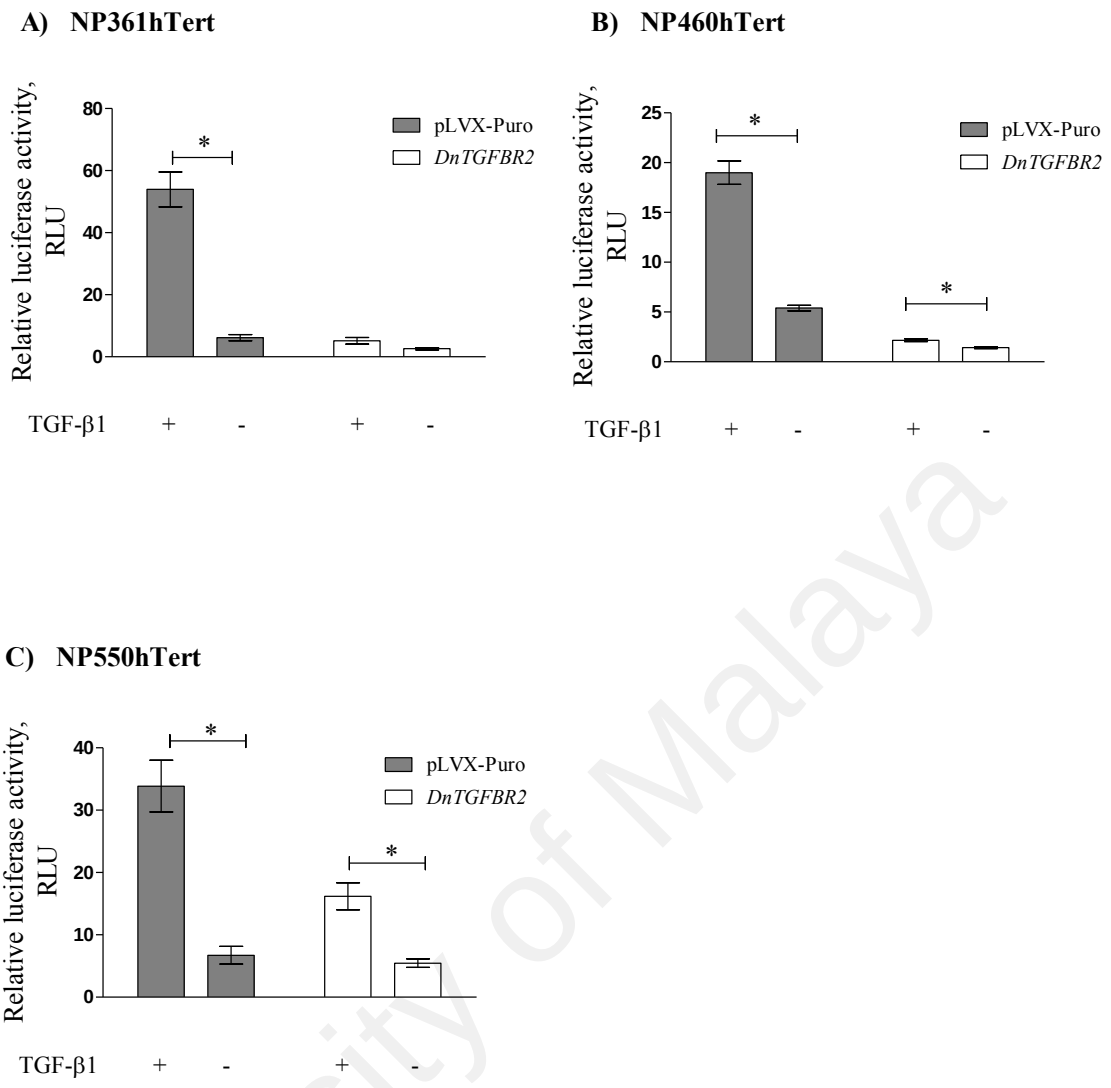



**Figure 4.10. Reduction of Smad2-dependent transcriptional activity in *DnTGFBR2*-expressing cells**. Luciferase reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in Smad2-dependent transcriptional activity in the vector controls (pLVX-Puro) A) NP361hTert (8.8-fold), B) NP460hTert (3.5-fold) and C) NP550hTert (5-fold). In *DnTGFBR2*-expressing cells, only small increased of Smad2-dependent transcriptional activity in NP460hTert (1.5-fold) and NP550hTert (3-fold) while no change was observed in NP361hTert after TGF-β1 treatment. The data presented are representative of two independent experiments. * denotes p-value <0.05.

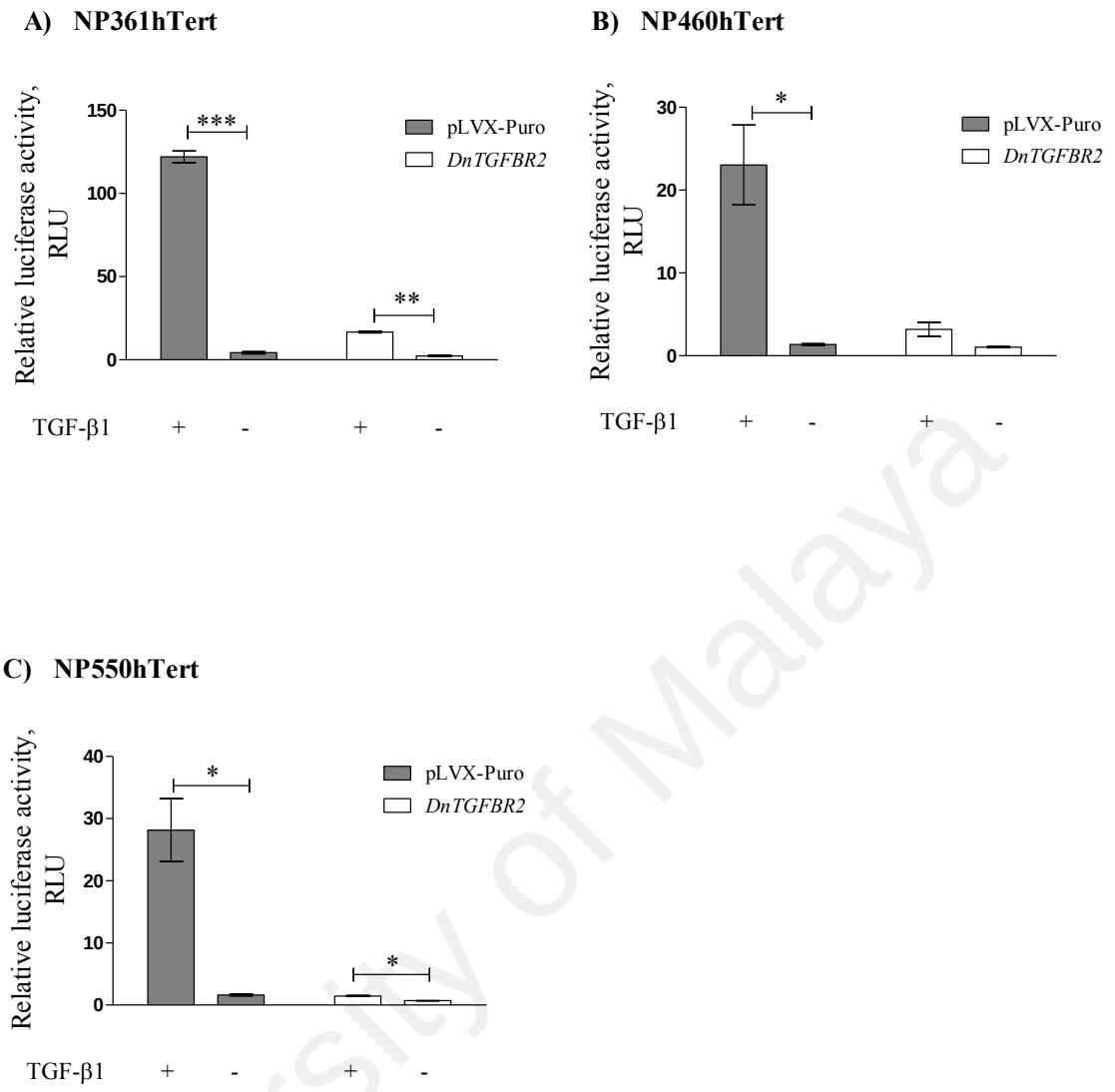



**Figure 4.11. Reduction of Smad3-dependent transcriptional activity in *DnTGFBR2*-expressing cells**. Luciferase reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in Smad3-dependent transcriptional activity in the vector controls (pLVX-Puro) A) NP361hTert (28.6-fold), B) NP460hTert (17-fold) and C) NP550hTert (17.4-fold). In *DnTGFBR2*-expressing cells, increased of Smad3-dependent transcriptional activity in NP361hTert (7.1-fold) and NP550hTert (2.0-fold) while no change was observed in NP460hTert after TGF-β1 stimulation. The data presented are representative of two independent experiments. ***denotes p-value <0.001, ** denotes p-value <0.01 and * denotes p-value <0.05.

## 4.4 Effect of disrupting the TGF-β signalling pathway on the outcome of EBV infection

### 4.4.1 Inhibition of TGFR-1 kinase with SB431542 resulted in an increased numbers of EBV-positive cells

To examine how the inhibition of TGFR-1 kinase with SB431542 affects the outcome of EBV infection, NP361hTert, NP460hTert and NP550hTert cells were infected with a GFP-tagged recombinant EBV (Akata strain). The EBV-infected cells were isolated using FACS, placed back into culture and treated with either SB431542 or with diluent (ethanol) every 48 hours for 7, 14 or 21 days. At the desired time point, the cells were harvested and subjected to FACS analysis. In NP361hTert and NP460hTert, SB431542 treatment led to a consistent increase in the numbers of EBV-positive cells from day 7 to day 21 (Figure 4.12A&B; $p<0.05$). Similarly, treatment of NP550hTert with SB431542 significantly increased the percentage of EBV-positive cells at day 14 and 21 post-EBV infection (Figure 4.12C; $p<0.05$).

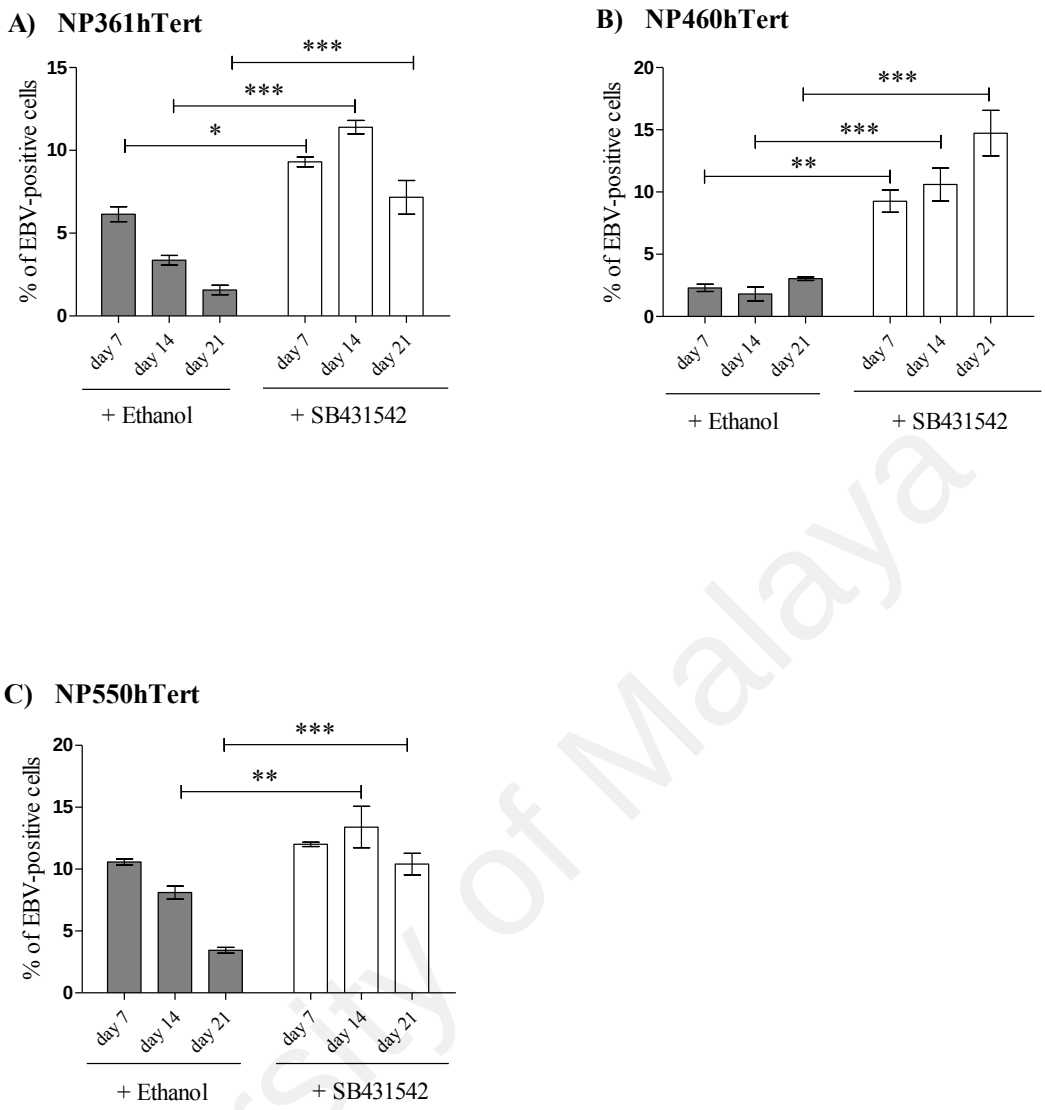



**Figure 4.12. Inhibition of TGFR-1 kinase increased numbers of EBV-positive cells in NPE cells.** Following 10µM SB431542 treatment, FACS analyses showed higher percentages of EBV-positive cells in A) NP361hTert, B) NP460hTert and C) NP550hTert, compared to their respective controls, with diluent (ethanol)-treated cells. The data presented are representative of three independent experiments. ***denotes p-value <0.001, ** denotes p-value <0.01 and * denotes p-value <0.05.

To further support these observations, the isolated EBV-positive NP550hTert cells were analysed by FISH, using a probe that detects BamH1 region of EBV DNA, 14 days post infection. The results demonstrated that the percentage of EBV-positive cells, as well as the copy number of EBV episomes per EBV-positive cell, was higher in NP550hTert following SB431542 treatment compared to the controls, with diluent (ethanol)-treated cells (Figure 4.13, $p<0.05$).

### 4.4.2 Expression of *DnTGFBR2* resulted in an increased numbers of EBV-positive cells

To examine how the disruption of *TGFBR2* affects the outcome of EBV infection, the three *DnTGFBR2*-expressing cell lines and their respective vector controls (pLVX-Puro) were infected with a GFP-tagged recombinant EBV (Akata strain). The EBV-positive cells were isolated using FACS and grown for 7, 14 or 21 days. The percentage of EBV-positive cells was significantly higher in *DnTGFBR2*/NP361hTert cells than that of the vector controls at day 21 post-EBV infection (Figure 4.14A; $p<0.05$). Similarly, a significant increase in the percentage of EBV-positive cells was evident in both the *DnTGFBR2*/NP460hTert (Figure 4.14B) and *DnTGFBR2*/NP550hTert (Figure 4.15C) cells at day 14 and 21 post-EBV infection ($p<0.05$).

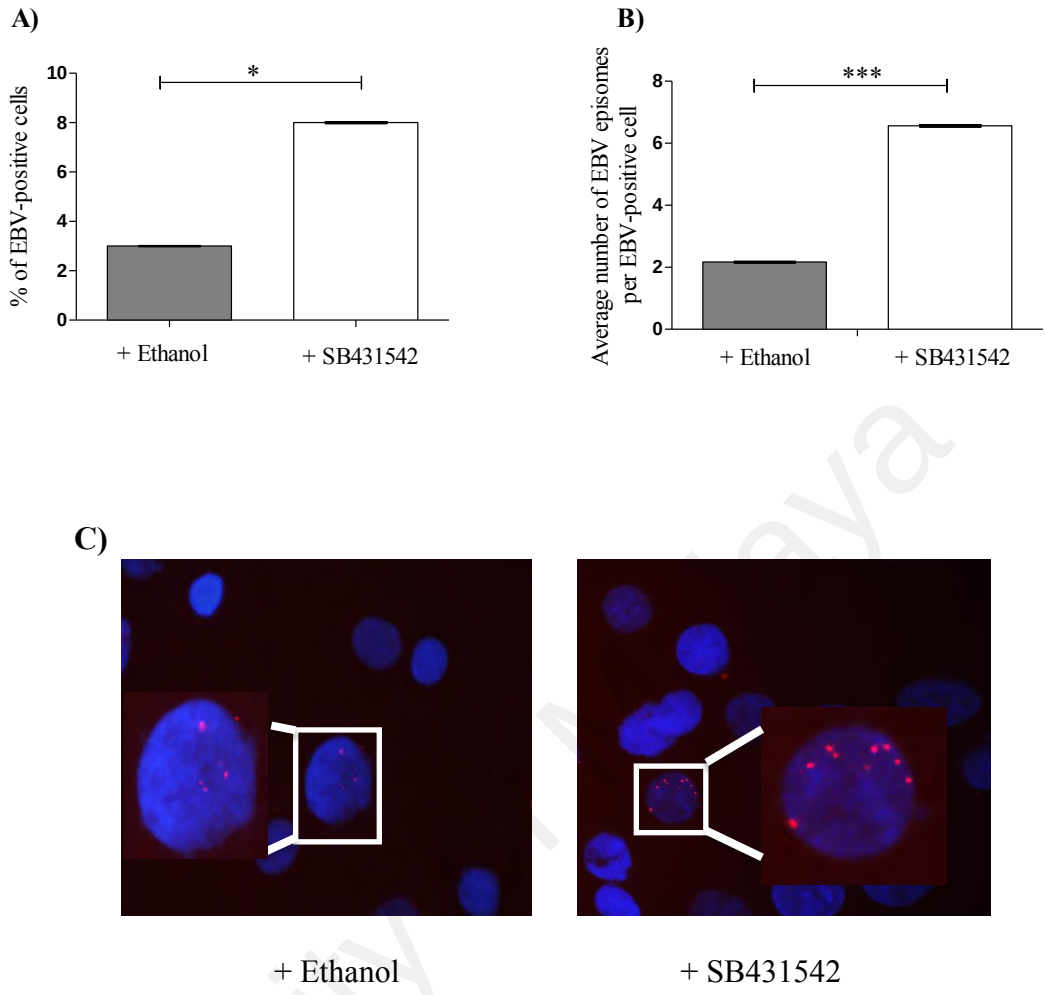



**Figure 4.13. Inhibition of TGFR-1 kinase increased numbers of EBV-positive in NP550hTert.** At day 14 post-EBV infection, the number of EBV-positive cells in NP550hTert following 10µM SB431542 treatment was determined by FISH analyses. A) A higher percentage of EBV-positive cells was observed in cells treated with SB431542 than that of with diluent (ethanol)-treated cells; B) Among the EBV-positive cells, a higher copy number of EBV episome per cell was shown in cells treated with SB431542 than those treated with diluent (ethanol); C) Representative images at 100x magnification, showing EBV episomes (red dots) in EBV-infected cells. ***denotes p-value <0.001 and * denotes p-value <0.05.

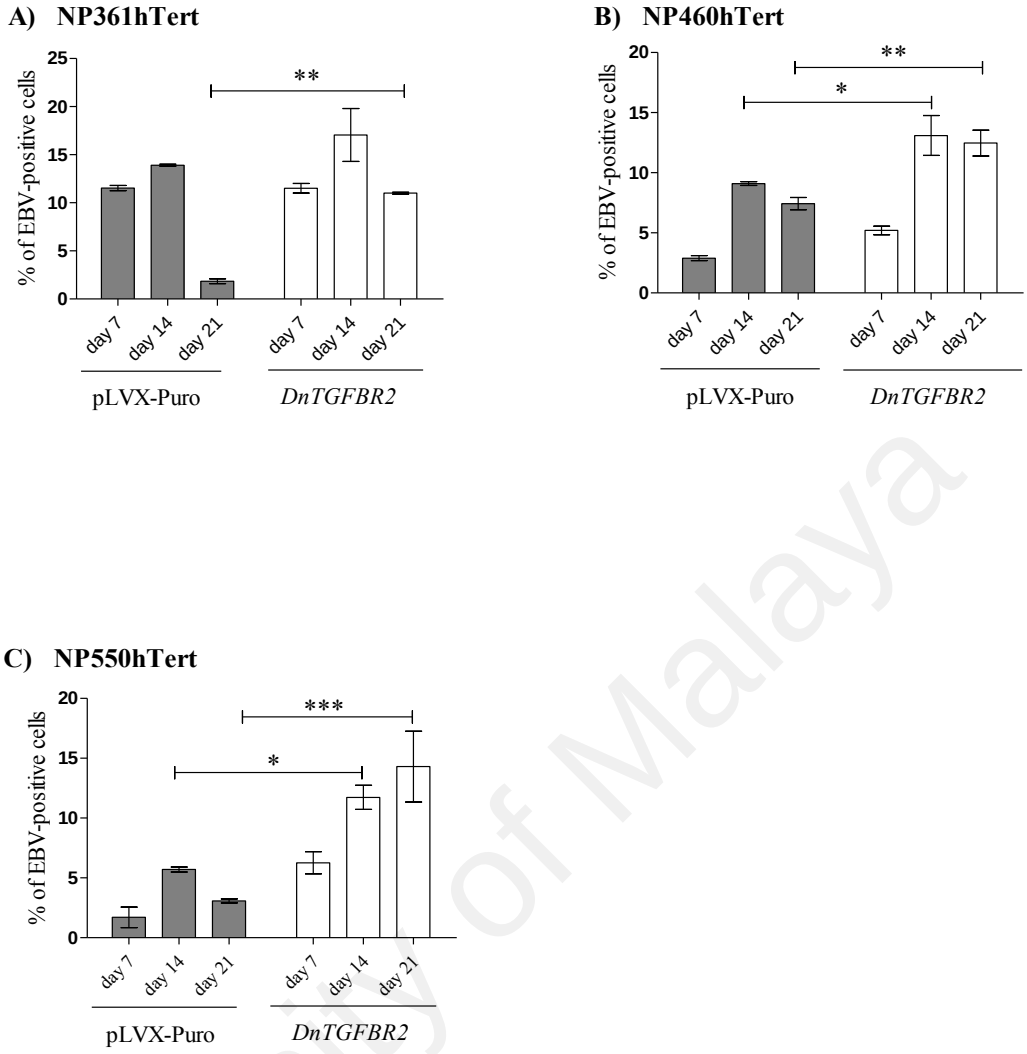



**Figure 4.14. Increased numbers of EBV-positive cells in *DnTGFBR2*-expressing cells.** FACS analyses showed that of the numbers of EBV-positive cells were higher in A) *DnTGFBR2*/NP361hTert, B) *DnTGFBR2*/NP460hTert and C) *DnTGFBR2*/NP550hTert, compared to their respective vector controls (pLVX-Puro). The data presented are representative of three independent experiments. *** denotes p-value <0.001, ** denotes p-value <0.01 and * denotes p-value <0.05.

The isolated NP550 (*DnTGFBR2* and pLVX-Puro) EBV-positive cells were subjected to FISH analysis 14 days after EBV infection. The results demonstrated that the percentage of EBV-positive cells, as well as the copy number of EBV episomes per EBV-positive cell, was higher in the *DnTGFBR2*-expressing cells compared to the vector controls (Figure 4.15, $p<0.05$).

Taken together, the results suggested that inhibition of TGFR-1 kinase with SB431542 and overexpression of a *DnTGFBR2* may facilitate the maintenance of EBV genomes in NPE cells.

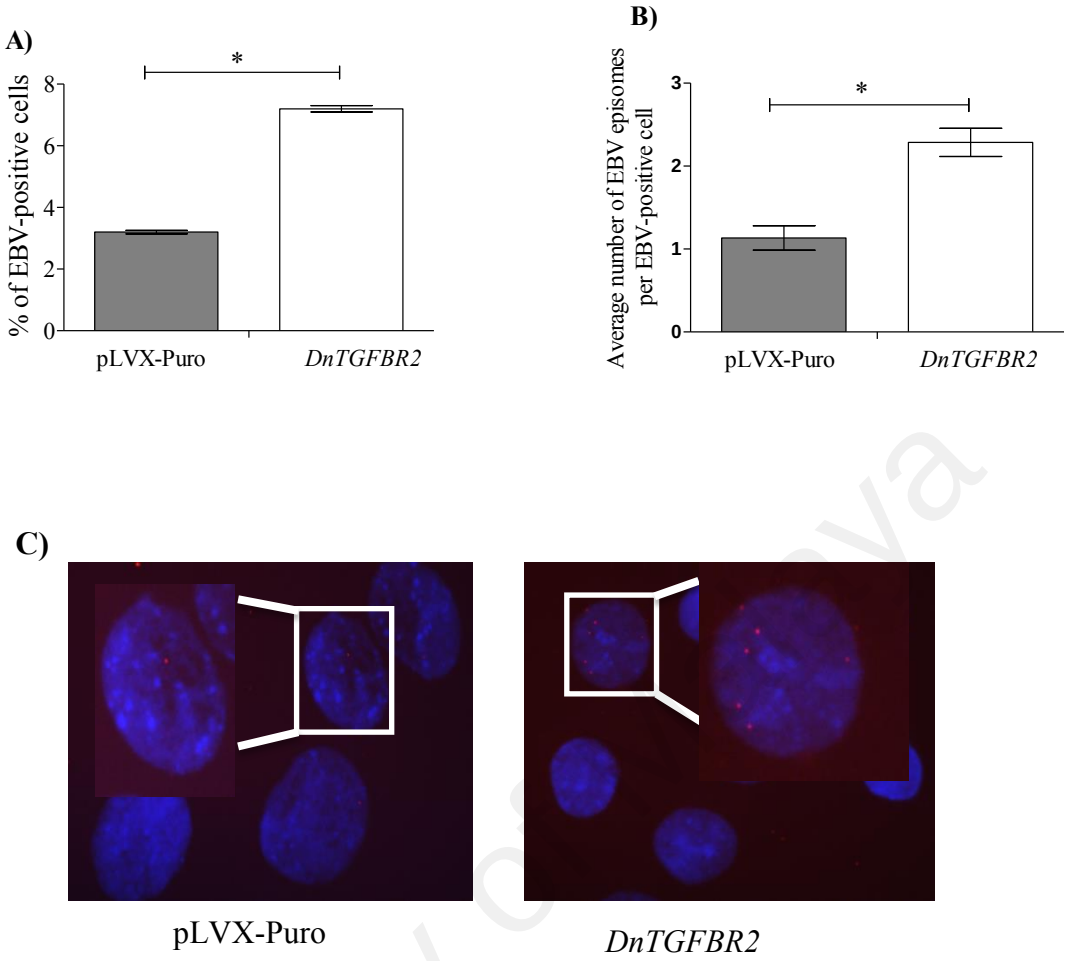



**Figure 4.15. Increased number of EBV-positive cells in *DnTGFBR2*/NP550hTert.** At day 14 post-EBV infection, the number of EBV-positive cells in *DnTGFBR2*/NP550hTert was determined by FISH analyses. A) A higher percentage of EBV-positive cells was demonstrated in *DnTGFBR2*-expressing cells compared to the vector controls (pLVX-Puro); B) Among the EBV-positive cells, a higher copy number of EBV episome per cell was shown in *DnTGFBR2*-expressing cells compared to the vector controls (pLVX-Puro); C) Representative images at 100x magnification, showing EBV episomes (red dots) in EBV-infected cells. The data presented are representative of two independent experiments. * denotes p-value <0.05.

## 4.5 Summary

This study examined the effects of disruption TGF-β signalling on the outcome of EBV infection in NPE cells. The responsiveness of six cell lines, NP69, NP361hTert, NP460hTert, NP550hTert, HK1 and HONE1 to TGF-β1-mediated growth inhibition was first determined using MTT assays. The growth of NP361hTert, NP460hTert, NP550hTert and HK1 was significantly inhibited by 40-60% following TGF-β1 treatment. In contrast, NP69 and HONE1 were not responsive to TGF-β1-mediated growth inhibition. Consistent with our preliminary results, the undifferentiated NPC cell line, HONE1 was not responsive to TGF-β1-mediated growth inhibition, while TGF-β1 inhibited the growth of the well-differentiated cell line, HK1. Non-malignant immortalised NPE cells are likely to represent more suitable model systems than NPC cell lines to study the mechanism of EBV infection. Therefore, NP361hTert, NP460hTert and NP550hTert were chosen for subsequent experiments.

Two different approaches were used to disrupt TGF-β signalling in these, namely, inhibition of TGFR-1 kinase with chemical inhibitor SB431542 and overexpression of a dominant negative *TGFBR2* (*DnTGFBR2*) gene. The disruption of TGF-β/Smad signalling pathway was confirmed in SB431542-treated cells and *DnTGFBR2*-expressing cells by the demonstration of a reduced growth inhibitory response to TGF-β1, inhibition of TGF-β1-induced phosphorylation of Smad2 and loss of Smad2/3-dependent transcription activity. Next, the effects of disrupting the TGF-β signalling pathway on the outcome of EBV infection were examined. Compared to the controls, inhibition of TGFR-1 kinase with SB431542 and overexpression of *DnTGFBR2* resulted in higher percentages of EBV-positive cells in all the three cell lines. In agreement with these observations, an increase in copy number of EBV

episomes was demonstrated in SB431542-treated and *DnTGFBR2*-expressing NP550hTert cells.

Collectively, the results of this study suggest that disruption of the TGF-β signalling pathway may facilitate the maintenance of the EBV genomes in NPE cells.

# CHAPTER 5: THE EFFECT OF DISRUPTING THE TGF-BETA SIGNALLING ON CELLULAR DIFFERENTIATION, EBV-INDUCED SENESCENCE AND EBV LYTIC CYCLE INDUCTION

## 5.1 Introduction

Non-keratinizing NPC was initially known as lymphoepithelioma of the nasopharynx due to the prominent reactive lymphocytes in the tumour microenvironment (Hang et al., 1991; Herait et al., 1987). Lymphoepithelioma-like carcinoma (LELC) is defined as an undifferentiated carcinoma with intense lymphoplasmacytic infiltrates (Gulley et al., 1995). Apart from NPC, EBV infection has also been detected in LELC of other organs such as stomach, esophagus, hepatobiliary tract, lung, thymus, and the salivary gland (Burke et al., 1990; Castro et al., 2001; Mori et al., 1994; Tsai et al., 1996; Vortmeyer et al., 1998), suggesting a close relationship between differentiation status of the host cells and EBV infection. There are also some evidence to support a link between differentiation status and stable maintenance of EBV in epithelial cells. It was shown that inhibition of differentiation prevent the EBV-infected cells from entering lytic cycle, whilst support stable EBV maintenance in these cells (Knox et al., 1996; Tsang et al., 2012). TGF-β signalling is known to induce differentiation in epithelial cells (Derynck & Akhurst, 2007; Kowanetz et al., 2004; Wells et al., 2009). Disruption of TGF-β signalling might suppress cellular differentiation, which in turn might facilitate stable EBV infection. To test this hypothesis, the differentiation status of NPE cell lines following the disruption of TGF-β signalling was examined.

EBV infection has been shown to induce senescence and growth inhibition in NPE cells, which in turn prohibited the stable maintenance of EBV (Tsang et al., 2012). Recently, a study has shown that EBV-infected primary human B cells initially undergo

a period of hyperproliferation leading to senescence-like growth arrest at the G1/S phase of the cell cycle (McFadden et al., 2016), further strengthening the link between EBV infection and senescence. Given that TGF-β signalling has been shown to induce senescence in primary mouse embryo fibroblasts (Zerlanko et al., 2012), the effect of TGF-β signalling disruption on EBV-induced senescence was investigated.

The regulation of the latent and lytic switch plays a crucial role in the persistence of EBV genomes in infected cells (Kenney & Mertz, 2014; Tsang et al., 2014). It has been shown that the establishment of stable EBV infection in B-cells and epithelial cells requires the suppression of *BZLF1*, an EBV gene that controls the switch from latent to lytic cycle (Murata et al., 2012; Tsang et al., 2012). In EBV positive B-cells and gastric cancer cell lines, exogenous addition of TGF-β1 could activated the expression of *BZLF1* that led to the reactivation of EBV lytic cycle (Fahmi et al., 2000; Fukuda et al., 2001). To investigate whether disruption of TGF-β signalling inhibit the induction of EBV lytic cycle in NP550hTert, the expression of *BZLF1* post-EBV infection was determined. NP460hTert cells were not used in these experiments because they habour a *p16* deletion and a *p16*-resistant form of CDK4 has been shown to counteract the EBV-induced growth arrest and senescence in NPE cells, enabling persistent infection of EBV in these cells. (Tsang et al., 2012). Therefore, NP361hTert and NP550hTert cells were used to determine the mechanisms associated with TGF-β signalling disruption in facilitating the maintenance of EBV genomes in NPE cells.

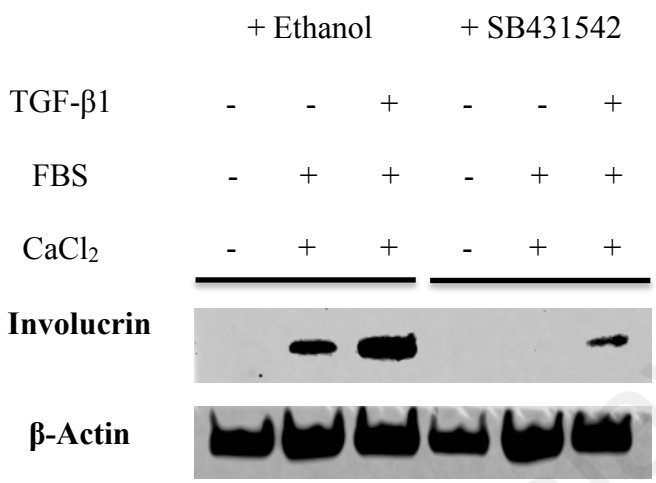
## 5.2 Disruption of TGF-β signalling suppressed cellular differentiation of NPE cells

Given that $CaCl_2$ and FBS are commonly used to induce differentiation of epithelial cells in culture (Borowiec et al., 2013; Tsang et al., 2012), they were used to induce differentiation in NP361hTert and NP550hTert following inhibition of TGFR-1 kinase with SB431542 or overexpression of *DnTGFBR2*. Involucrin was used as an indicator of cellular differentiation (Chou et al., 2004).

### 5.2.1 Inhibition of TGFR-1 kinase with SB431542 suppressed cellular differentiation of NPE cells

Prior to the addition of $CaCl_2$, FBS and/or TGF-β1, NP361hTert and NP550hTert cells were treated with 10μM SB431542 for 30 minutes to disrupt TGF-β signalling. The media were replaced every alternate day for 14 days before the cells were collected for Western blot analysis. The expression of involucrin was increased in NP361hTert and NP550hTert following treatment with $CaCl_2$ and FBS, and the expression was further enhanced by the addition of TGF-β1 (Figure 5.1). In contrast, treatment with SB431542 suppressed the differentiation of both cell lines, as shown by the lower expression of involucrin compared to diluent (ethanol)-treated cells (Figure 5.1). In line with the Western blotting results, immunofluorescence staining demonstrated that the expression of involucrin in NP550hTert was detectable following treatment with $CaCl_2$ and FBS, and the expression was further enhanced by TGF-β1 (Figure 5.2). Inhibition of TGFR-1 with SB431542 reduced the expression of involucrin in these cells (Figure 5.2).

**A) NP361hTert**




**B) NP550hTert**

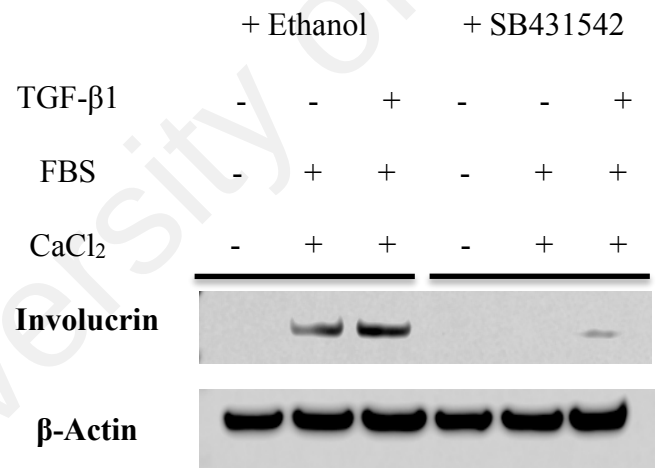



**Figure 5.1. Inhibition of TGFR-1 kinase suppressed the expression of involucrin in NPE cells.** Western blotting analyses showed that compared to the diluent (ethanol)-treated cells, the levels of involucrin in A) NP361hTert and B) NP550hTert were lower in cells pre-treated with 10μM SB431542 for 30 minutes prior to the addition of 2.8mM $CaCl_2$ and 10% FBS in the presence or absence of 1ng/ml TGF-β1 for 14 days. Representative images from two independent experiments are presented.

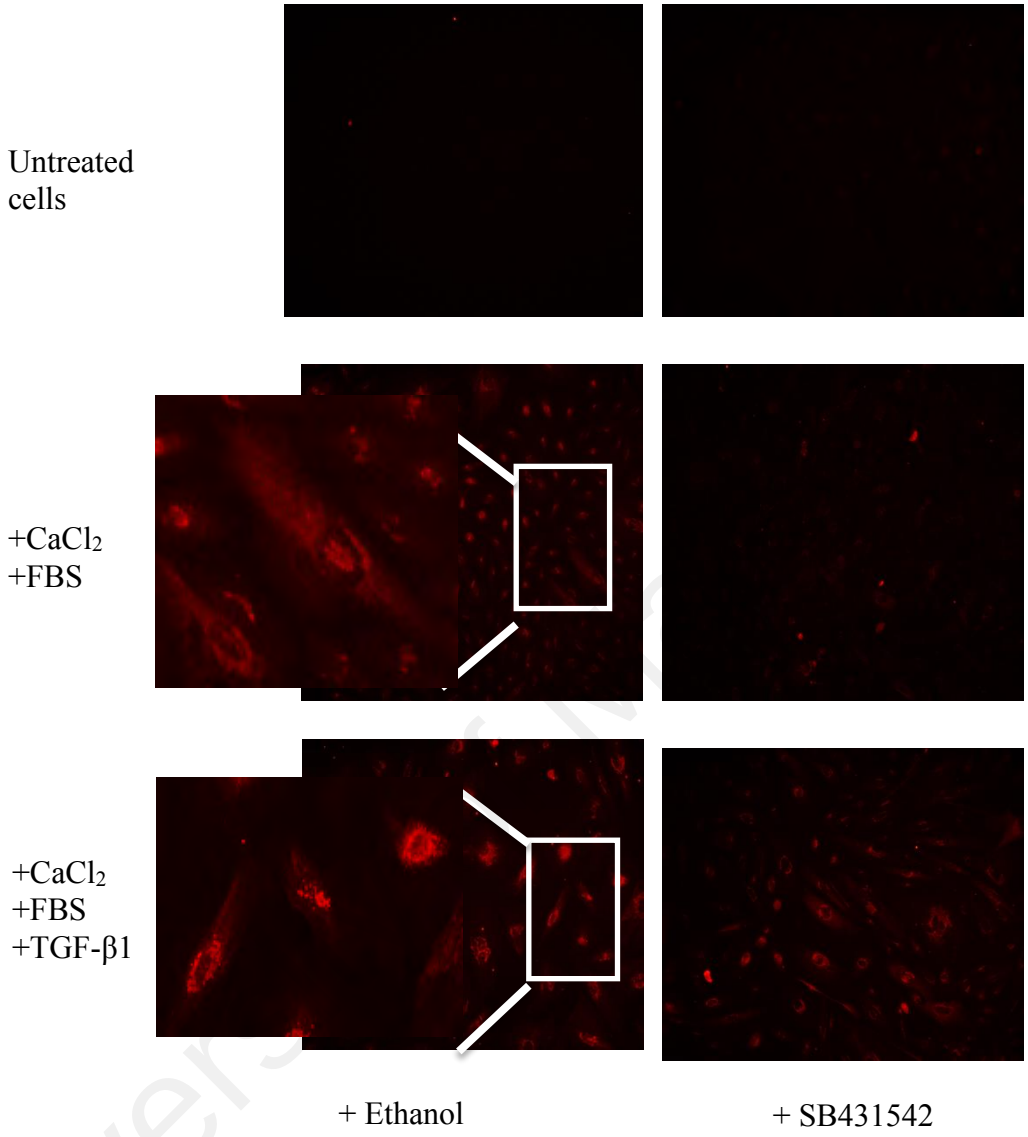


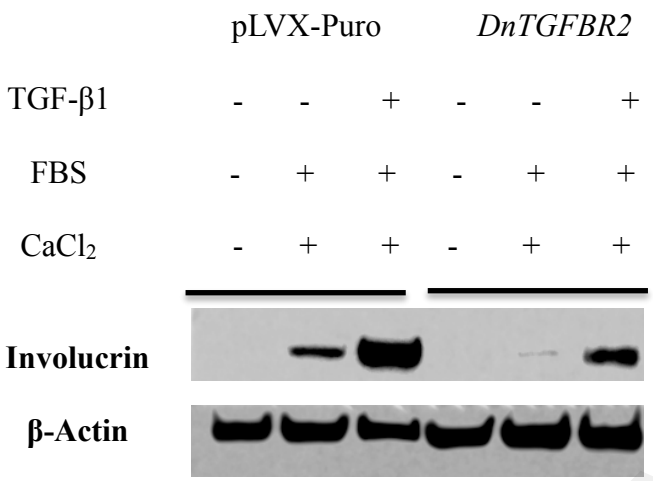

**Figure 5.2. Inhibition of TGFR-1 kinase suppressed the expression of involucrin in NP550hTert.** Immunofluorescence analyses showed that compared to the diluent (ethanol)-treated cells, the expression of involucrin in NP550hTert were lower in cells treated with 10μM SB431542 for 30 minutes prior to the addition of 2.8mM $CaCl_2$ and 10% FBS in the presence or absence of 1ng/ml TGF-β1 for 14 days. Involucrin was not detected in cells that were not treated with any of the reagents (Untreated cells). Representative images at 20x magnification from two independent experiments are presented.

### 5.2.2 Expression of *DnTGFBR2* suppressed cellular differentiation of NPE cells

*DnTGFBR2*-expressing cells and their respective vector controls were treated with $CaCl_2$ and FBS in the presence or absence of TGF-β1. The media were replaced every alternate day for 14 days before the cells were collected for Western blotting analysis. In vector controls, the expression of involucrin was increased in NP361hTert and NP550hTert following treatment with $CaCl_2$ and FBS, and the expression was further enhanced by the addition of TGF-β1 (Figure 5.3). In contrast, differentiation was suppressed in cells expressing *DnTGFBR2*, as shown by the reduced levels of involucrin (Figure 5.3). In accordance with these results, immunofluorescence staining of NP550hTert demonstrated that the expression of involucrin in the vector controls was induced following treatment with $CaCl_2$ and FBS, and the expression was further enhanced with the addition of TGF-β1 (Figure 5.4). Compared to the vector controls, the *DnTGFBR2*-expressing cells expressed lower levels of involucrin following these treatments (Figure 5.4).

Taken together, these results demonstrated that disruption of TGF-β signalling suppressed cellular differentiation in NPE cells.

**A) NP361hTert**




**B) NP550hTert**

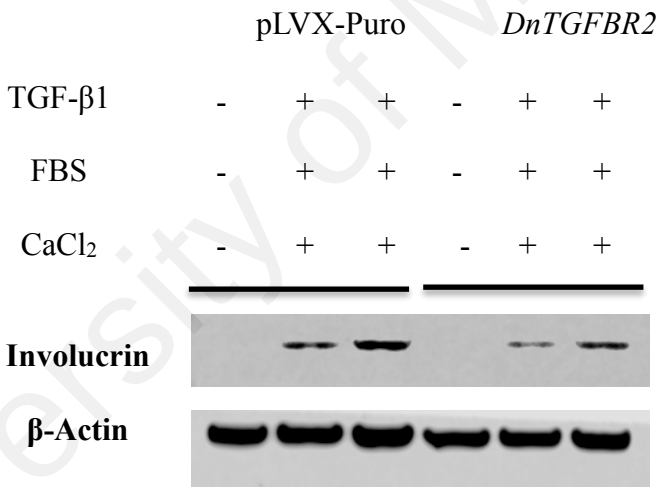



**Figure 5.3. Decreased expression of involucrin in *DnTGFBR2*-expressing cells.** Western blotting analyses showed that the levels of involucrin in A) *DnTGFBR2*/NP361hTert and B) *DnTGFBR2*/NP550hTert were lower following treatment with 2.8mM $CaCl_2$ and 10% FBS in the presence or absence of 1ng/ml TGF-β1 for 14 days compared to their respective vector controls (pLVX-Puro). Representative images from two independent experiments are presented.

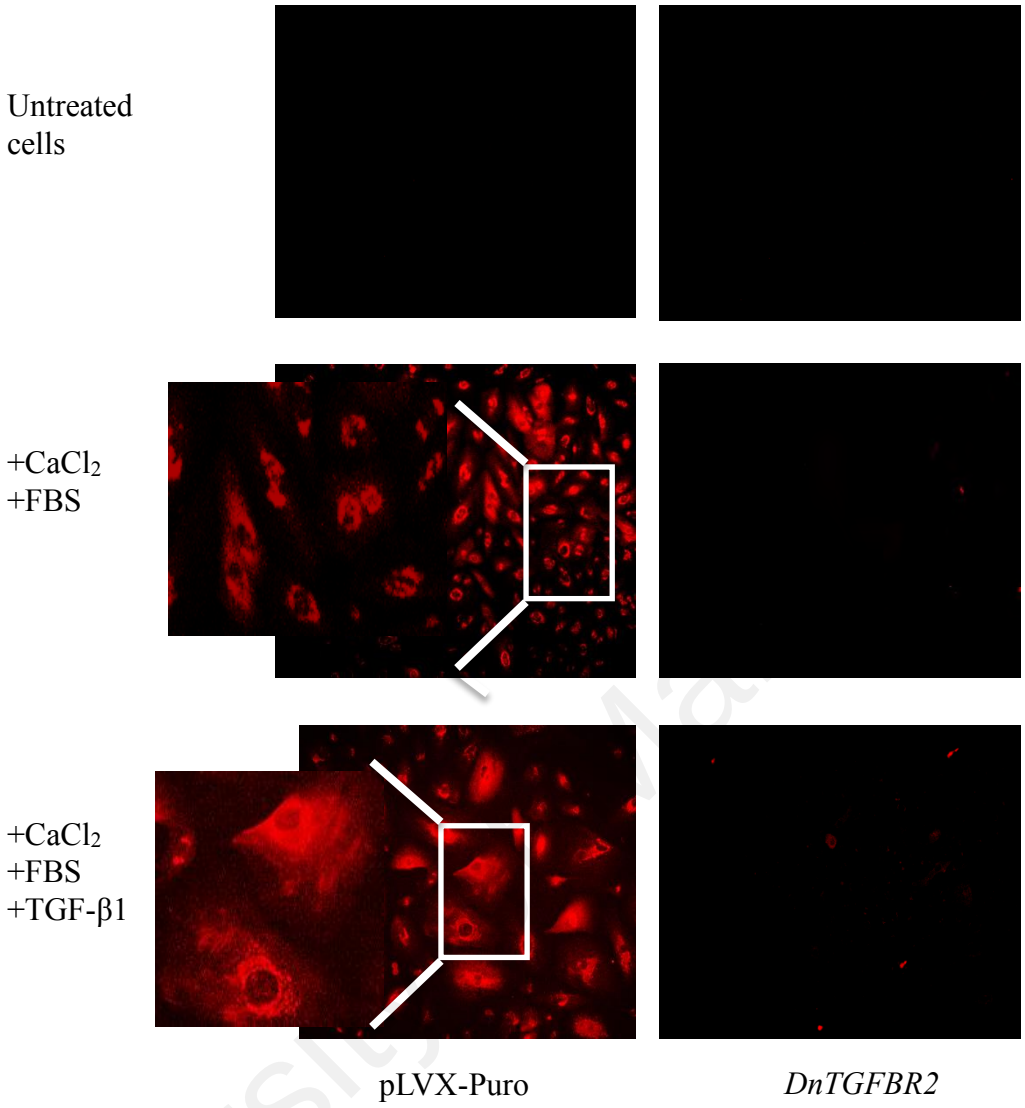



**Figure 5.4. Decreased expression of involucrin in *DnTGFBR2*/NP550hTert.** Immunofluorescence analyses showed that the levels of involucrin in *DnTGFBR2*/NP550hTert were lower following treatment with 2.8mM $CaCl_2$ and 10% FBS in the presence or absence of 1ng/ml TGF-β1 for 14 days compared to the vector controls (pLVX-Puro). Involucrin was not detected in cells that were not treated with any of the reagents (Untreated cells). Representative images at 20x magnification from two independent experiments are presented.

## 5.3 Disruption of TGF-β signalling suppressed EBV-induced senescence in NPE cells

Initially, to examine whether disruption of TGF-β signalling could override senescence in NPE cells, SA-β-Gal assays were performed (Dimri et al., 1995). Consistenly lower percentages of senescent cells were observed in the SB431542-treated cells and *DnTGFBR2*-expressing cells in both cell lines compared to their corresponding controls (Figure 5.5A&B; $p<0.05$) after seven days of culturing, suggesting that distruption of TGF-β signalling might suppress cellular senescence in NPE cells. However, unlike a NPC cell line (HK1), an oral cancer cell line (H400) and a culture of normal human oral fibroflasts (NHOF1), the number of SA-β-Gal-positive cells was considerably high (approximately 40-50%) in the untreated parental cells of NP361hTert and NP550hTert (Figure 5.5C). These observations were puzzling because parental cells of NP361htert and NP550hTert grew in a normal culture condition and did not show any sign of senescence.

Therefore, instead of using SA-β-Gal assays, the expression of three other markers of senescence (SIRT1, p16 and p21) were examined in the subsequent experiments. SIRT1 is a NAD-dependent protein deacetylase that modulates various cellular functions such as cell cycle and loss of SIRT1 expression was positively correlated with senescence (Huang et al., 2008; Sasaki et al., 2006). The expression of SIRT1 was determined by Western blotting analysis and the levels of p16 and p21 were determined by immunofluorescence analyses.

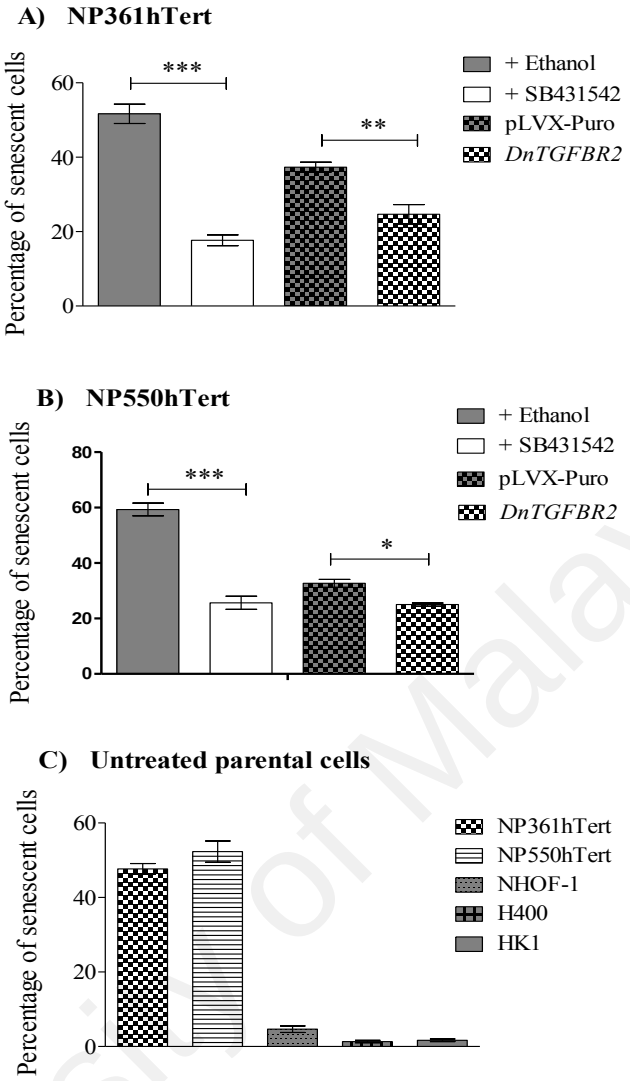
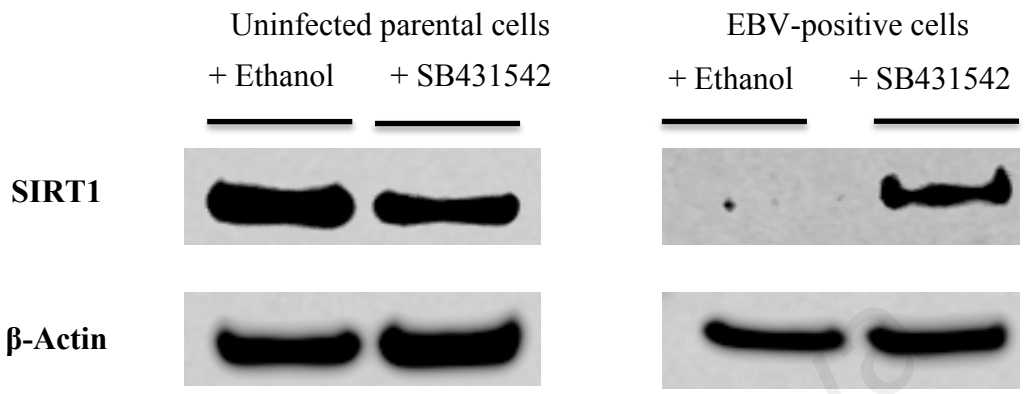



**Figure 5.5. Disruption of TGF-β signalling decreased the numbers of senescent cells.** Compared to the diluent (ethanol)-treated cells or vector controls (pLVX-Puro), treatment with 10μM SB431542 or overexpression of *DnTGFBR2* decreased the percentages of senescent cells in (A) NP361hTert and (B) NP550hTert; however, higher percentages of senescent cells in untreated parental cells of NP361hTert and NP550hTert were demonstrated compared to NHOF-1, H400 and HK1 (C). The data presented are representative of two independent experiments. *** denotes p-value <0.001, ** denotes p-value <0.01 and * denotes p-value <0.05.

### 5.3.1 Inhibition of TGFR-1 kinase with SB431542 suppressed EBV-induced senescence in NPE cells

To examine whether disruption of TGF-β signalling could override EBV-induced senescence, NP361hTert and NP550hTert were infected with a GFP-tagged recombinant EBV and the EBV-positive cells were isolated and cultured for 14 days in the presence of SB431542 or diluent (ethanol). Western blotting analysis showed that the expression of SIRT1 protein was detected in both uninfected parental cell lines (Figure 5.6), indicating that senescence did not occur in these cells. In the EBV-positive cells, the expression of SIRT1 protein was lost following treatment with the diluent (ethanol), indicating the induction of senescence. Notably, treatment of both cell lines with SB431542 protected the cells from EBV-induced cellular senescence, as shown by the restoration of SIRT1 protein expression (Figure 5.6).

**A) NP361hTert**




**B) NP550hTert**

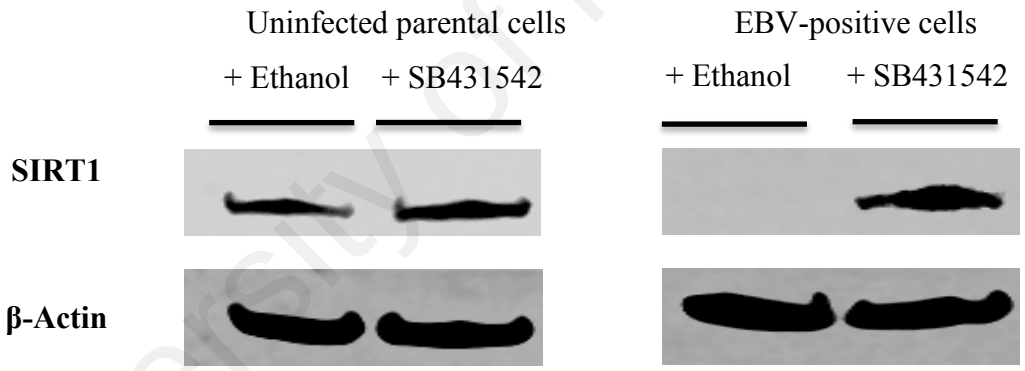



**Figure 5.6. Inhibition of TGFR-1 kinase resulted in the restoration of SIRT1 protein following EBV infection** . The expression of SIRT1 protein was detected in uninfected parental cells of A) NP361hTert and B) NP550hTert. EBV infection led to loss of SIRT1 expression in the diluent (ethanol)-treated cells. Addition of 10μM SB431542 restored the expression of SIRT1 in the EBV-positive cells. Representative images from two independent experiments are presented.

To further confirm the disruption of TGF-β signalling suppressed EBV-induced senescence, the expression of p16 and p21 was determined in NP550hTert by immunofluorescence analysis. While no p16 expression was observed in the uninfected parental cells, p16 was readily detected in the EBV-positive NP550hTert cells treated with the diluent (ethanol), indicating that senescence was induced following EBV infection (Figure 5.7). The expression of p16 was noticeably reduced in the EBV-positive cells treated with SB431542 (Figure 5.7; $p<0.05$). Similar results were obtained with the expression of p21 (Figure 5.8; $p<0.05$).

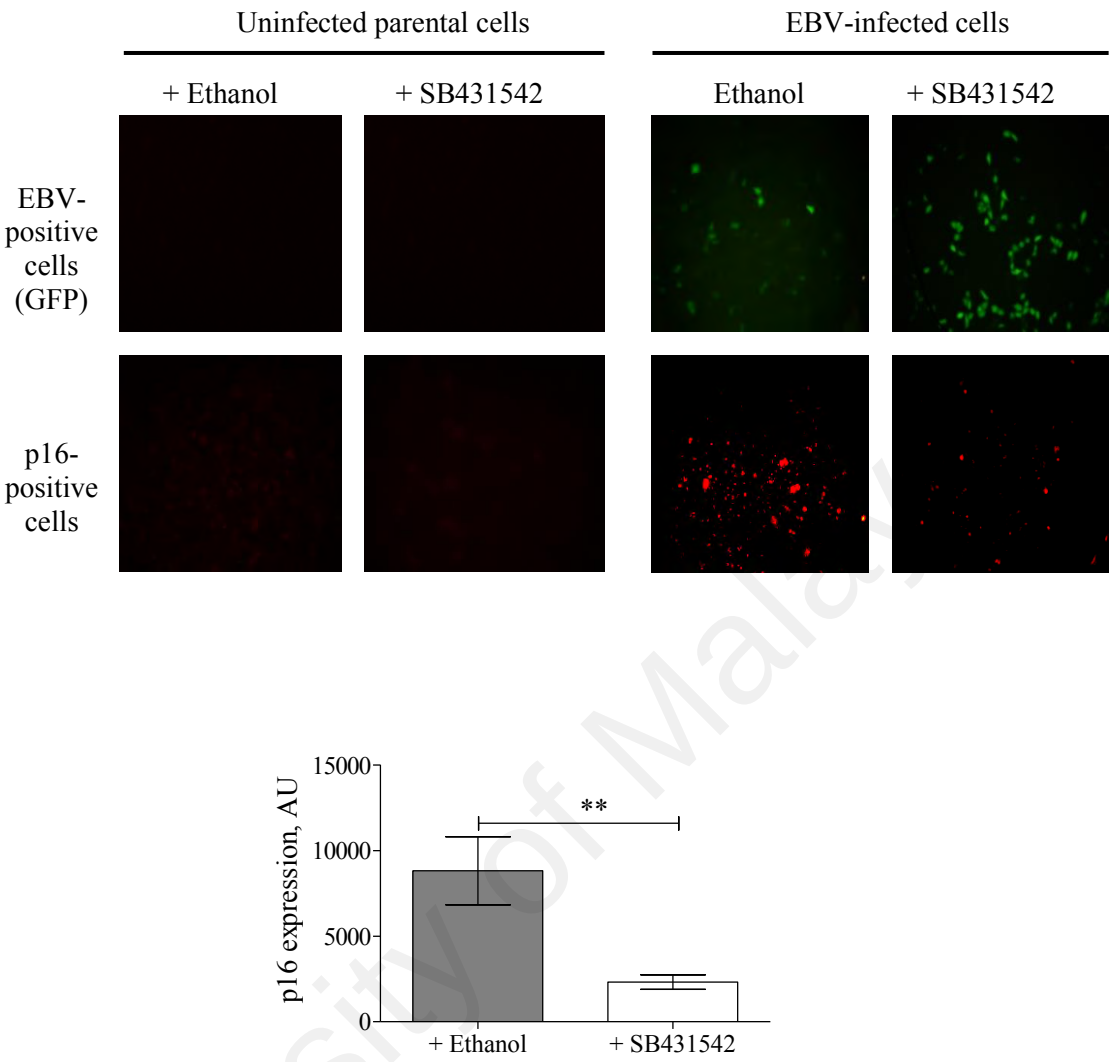



**Figure 5.7. Inhibition of TGFR-1 kinase decreased p16 expression in EBV-positive NP550hTert.** No p16 expression was detected in the uninfected parental cells of NP550hTert. In EBV-positive cell populations, p16 expression was reduced following treatment with 10μM SB431542 compared to the diluent (ethanol) treatment. The signal intensity was measured as integrated intensity (AU) per cell using image J software. Representative images at 20x magnification from two independent experiments are presented. ** denotes p-value<0.01.

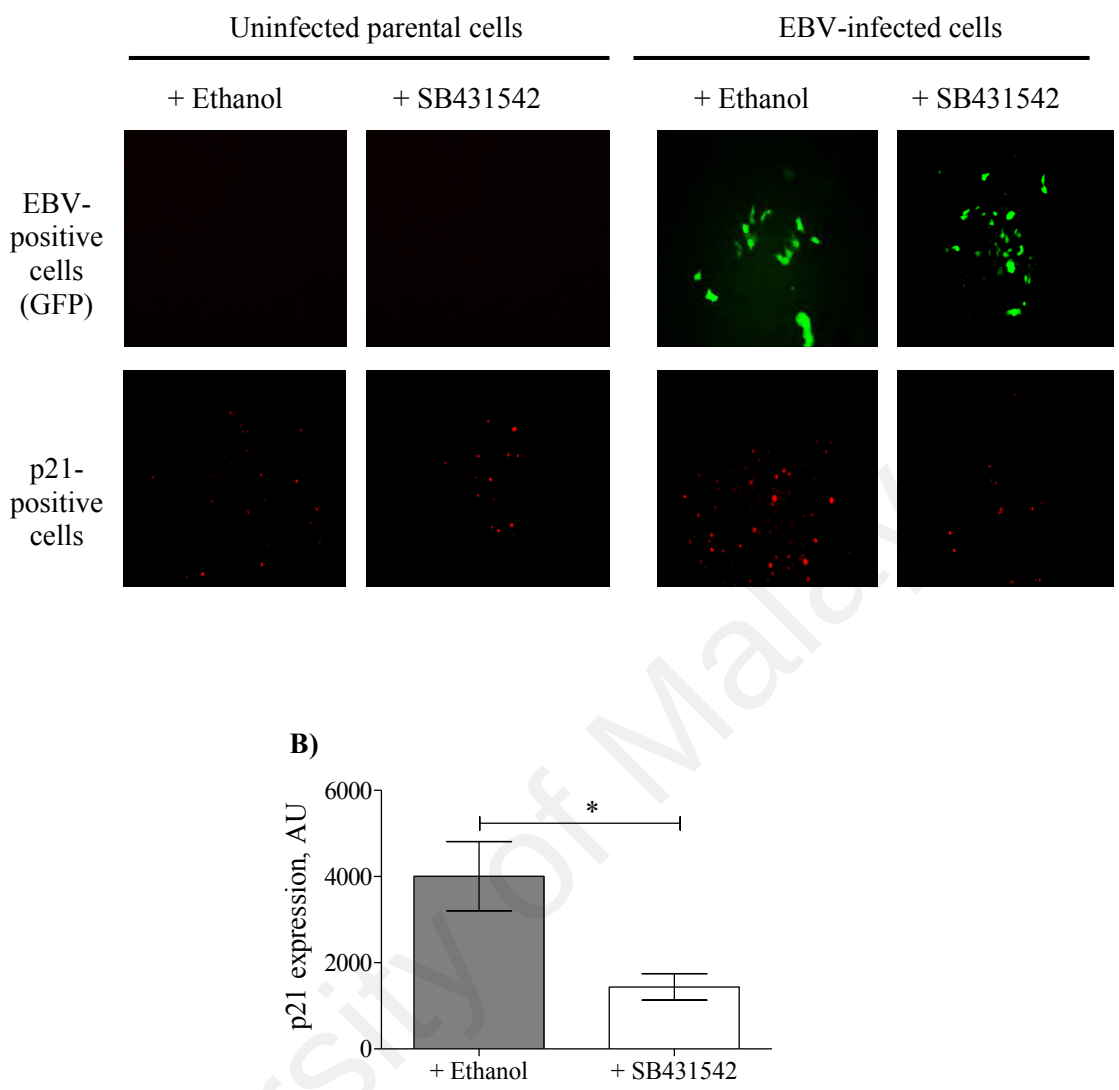


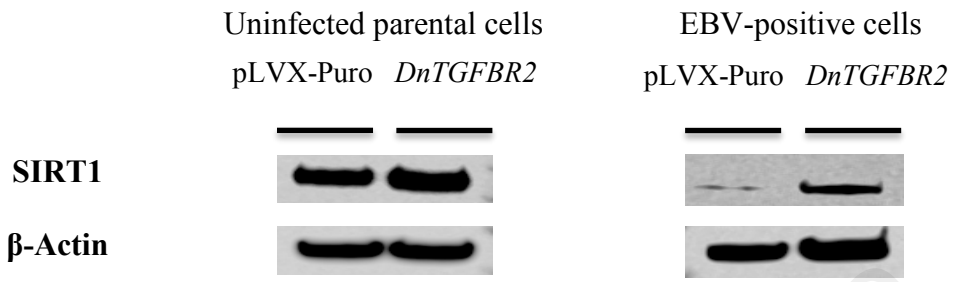

**Figure 5.8. Inhibition of TGFR-1 kinase decreased p21 expression in EBV-positive NP550hTert.** Compared to the EBV-infected cells, weak p21 expression was detected in the uninfected parental cells of NP550hTert. In EBV-positive cell populations, the p21 expression was reduced following treatment with 10μM SB431542 compared to the diluent (ethanol) treatment. The signal intensity was measured as integrated intensity (AU) per cell using image J software. Representative images at 20x magnification from two independent experiments are presented.* denotes p-value<0.05.

### 5.3.2 Suppression of EBV-induced senescence in *DnTGFBR2*-expressing cells

NP361hTert and NP550hTert were infected with a GFP-tagged recombinant EBV and the EBV-positive cells were isolated and cultured for 14 days. Western blot analysis showed that the expression of SIRT1 protein was detected in both uninfected parental cell lines (Figure 5.9), indicating there is no sign of senescence. The expression of SIRT1 was lost in the vector controls following EBV infection, indicating the induction of senescence. Notably, overexpression of a *DnTGFBR2* in both cell lines protected the cells from EBV-induced cellular senescence, as shown by the restoration of SIRT1 protein expression (Figure 5.9). To further confirm the disruption of TGF-β signalling suppressed EBV-induced senescence, the expression of p16 and p21 was determined in NP550hTert by immunofluorescence analysis. While no p16 expression was observed in the uninfected parental cells, p16 was readily expressed in the EBV-positive vector controls, indicating that senescence was induced following EBV infection (Figure 5.10). The expression of p16 was noticeably reduced in the EBV-positive cells expressing *DnTGFBR2* (Figure 5.10; $p<0.01$). Similar results were obtained with the expression of p21 (Figure 5.11; $p<0.01$).

Taken together, these results showed that disruption of TGF-β signalling protected NPE cells from EBV-induced senescence, representing a possible mechanism to support stable EBV infection in these cells.

**A) NP361hTert**




**B) NP550hTert**

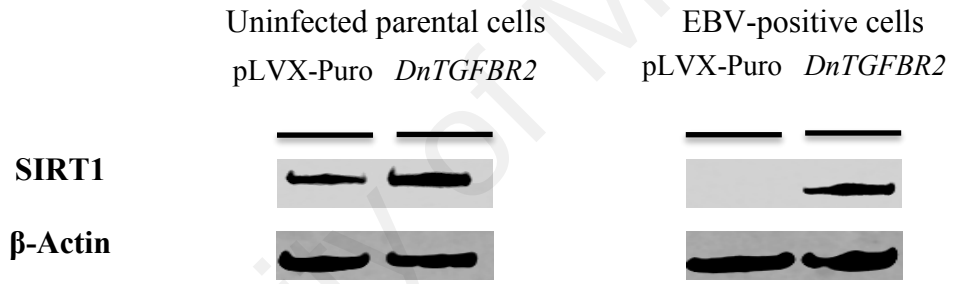



**Figure 5.9. Expression of *DnTGFBR2* resulted in the restoration of SIRT1 protein following EBV infection.** The expression of SIRT1 protein was detected in uninfected parental cells of A) NP361hTert and B) NP550hTert. Following EBV infection, the expression of SIRT1 protein was lost in the vector controls (pLVX-Puro) while detected in the *DnTGFBR2*-expressing cells. Representative images from two independent experiments are presented.

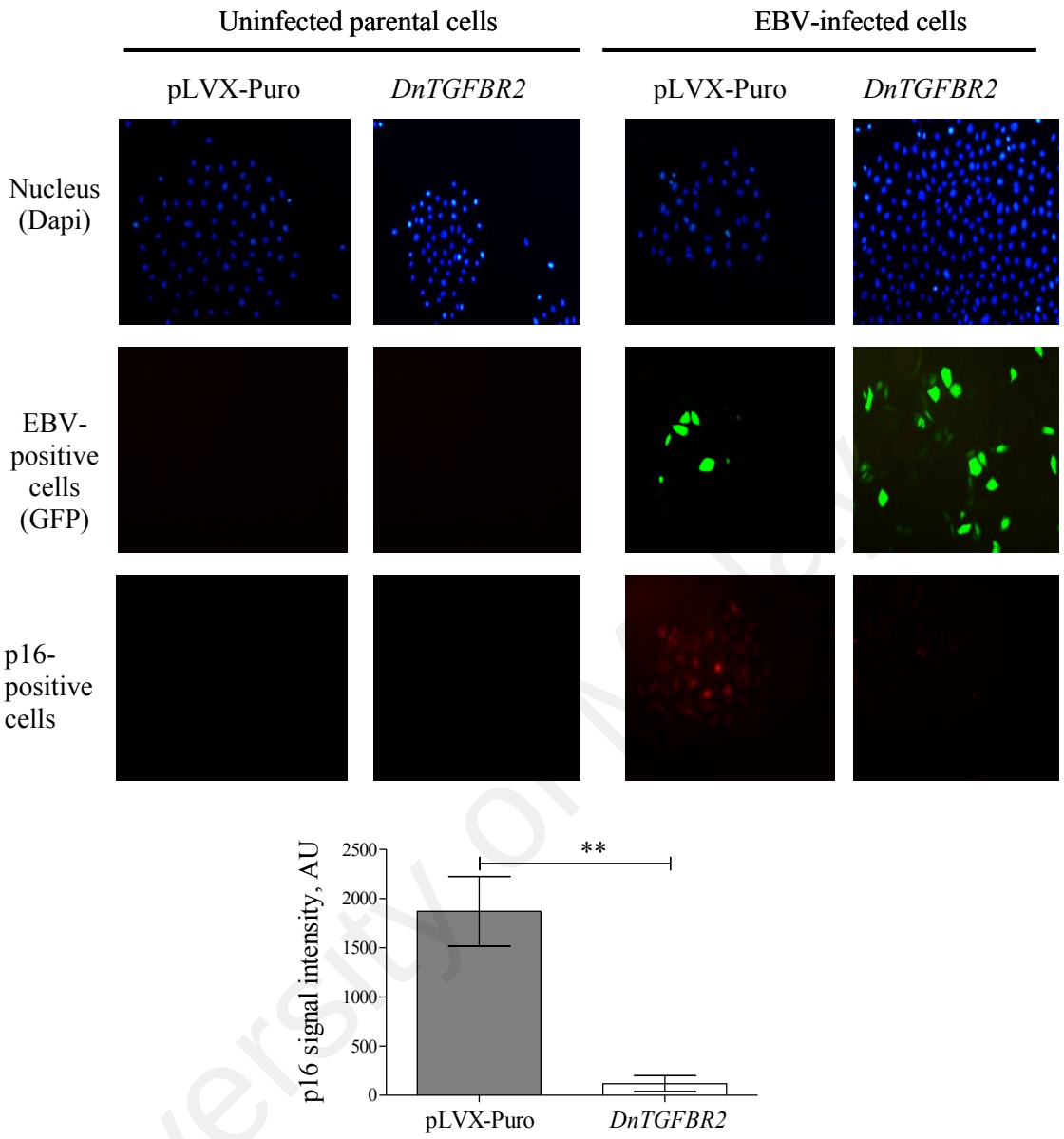



**Figure 5.10. Decreased p16 expression in EBV-positive *DnTGFBR2*/NP550hTert.** No p16 expression was detected in the uninfected parental cells of NP550hTert. Following EBV infection, the expression of p16 was reduced in *DnTGFBR2*-expressing NP550hTert compared to the vector controls (pLVX-Puro). The signal intensity was measured as integrated intensity (AU) per cell using image J software. Representative images at 20x magnification from two independent experiments are presented. ** denotes p-value<0.01.

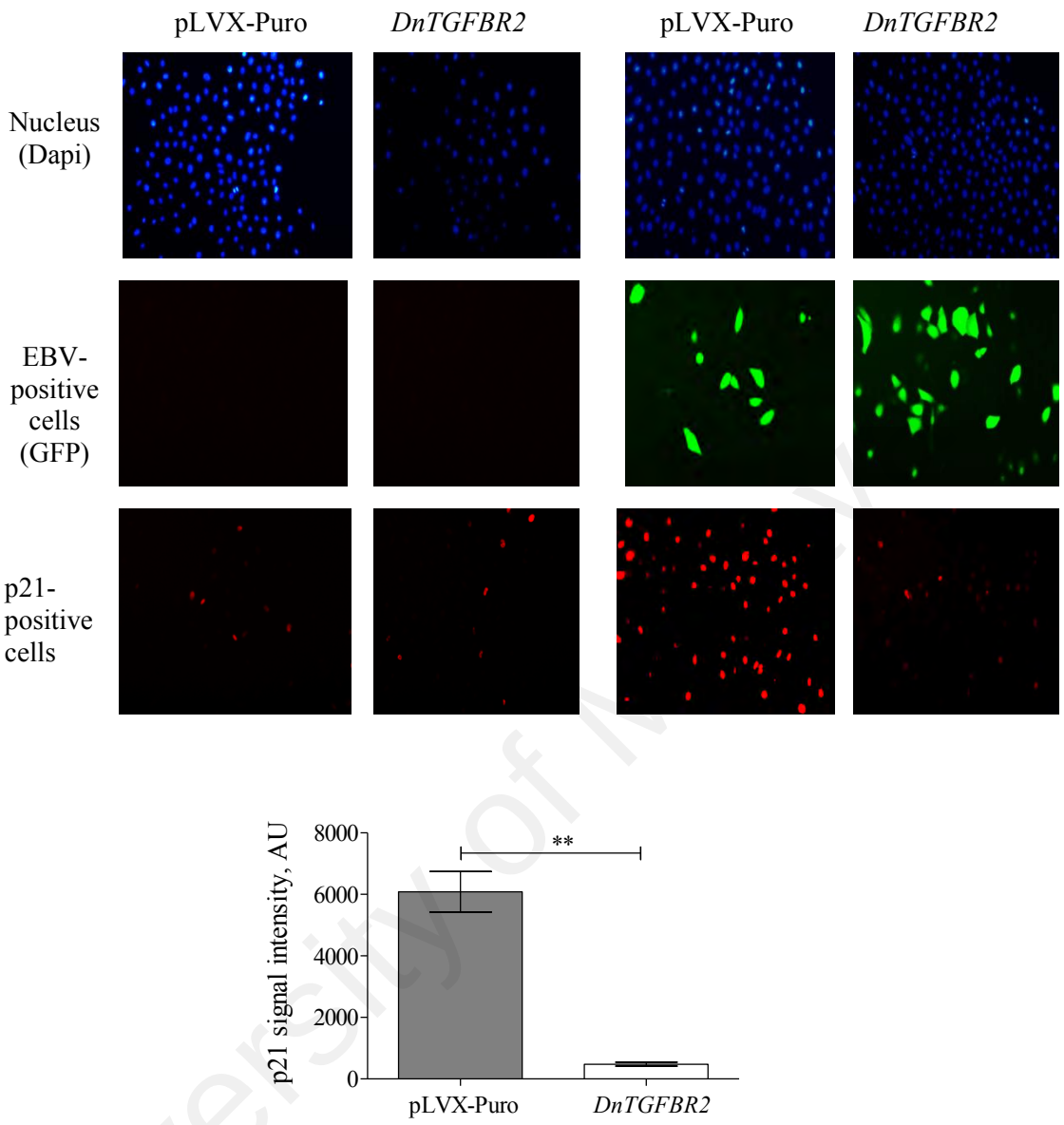



**Figure 5.11. Decreased p21 expression in EBV-positive *DnTGFBR2*/NP550hTert.** Compared to the EBV-infected cells, week p21 expression was detected in the uninfected parental cells of NP550hTert. Following EBV infection, the expression of p21 was reduced in *DnTGFBR2*-expressing NP550hTert compared to the vector controls (pLVX-Puro). The signal intensity was measured as integrated intensity (AU) per cell using image J software. Representative images at 20x magnification from two independent experiments are presented. ** denotes p-value<0.01.

## 5.4 Disruption of TGF-β signalling inhibited the induction of EBV lytic cycle in NPE cells

### 5.4.1 Inhibition of TGFR-1 kinase with SB431542 decreased expression of the EBV-encoded *BZLF1* gene in NP550hTert

To determine if disruption of TGF-β signalling suppressed the induction of EBV lytic cycle, the expression of *BZLF1* mRNA was measured following treatment with SB431542. NP550hTert cells were infected with a GFP-tagged recombinant EBV and the EBV-positive cells were isolated and cultured in growth media containing either 10μM SB431542 or diluent (ethanol) for 14 days. QPCR analysis showed that compared to the diluent (ethanol)-treated cells, the expression of *BZLF1* was significantly down-regulated in cells treated with SB431542 following EBV infection (Figure 5.12; $p<0.05$).

### 5.4.2 Decreased expression of the EBV-encoded *BZLF1* gene in *DnTGFBR2*/NP550hTert

The NP550hTert cells expressing *DnTGFBR2* or vector controls were infected with a GFP-tagged recombinant EBV and the EBV-positive cells were isolated and cultured for 14 days. Compared to the vector controls, the expression of *BZLF1* was significantly down-regulated in the *DnTGFBR2*-expressing cells following EBV infection (Figure 5.13; $p<0.05$). Collectively, these results suggested that disruption of TGF-β signalling could suppress the induction of EBV lytic cycle, a mechanism that might support stable EBV latent infection in NPE cells.

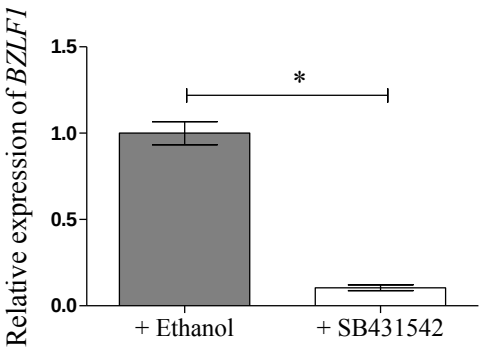



**Figure 5.12. Inhibition of TGFR-1 kinase decreased *BZLF1* expression in EBV-positive NP550hTert.** QPCR analysis showed that compared to the diluent (ethanol)-treated cells, the expression of *BZLF1* mRNA was significantly down-regulated in NP550hTert treated with 10μM SB431542 following EBV infection. The data presented are representative of two independent experiments. * denotes p-value <0.05.

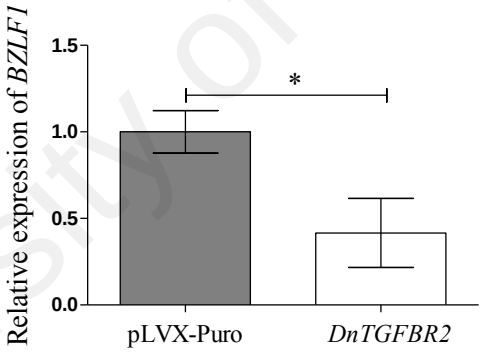



**Figure 5.13. Decreased *BZLF1* expression in EBV-positive *DnTGFBR2*/NP550hTert.** QPCR analysis showed that compared to the vector controls (pLVX-Puro), the expression of *BZLF1* mRNA was significantly down-regulated in *DnTGFBR2*-expressing cells following EBV infection. The data presented are representative of two independent experiments. * denotes p-value <0.05.

## 5.5 Summary

The results of the previous chapter (Chapter 4) showed that the disruption of TGF-β signalling facilitated the maintenance of EBV in three NPE cell lines (NP361hTert, NP460hTert and NP550hTert). In this chapter, possible mechanisms that are responsible for these effects were investigated in two NPE cell lines, NP361hTert and NP550hTert.

Most, if not all, undifferentiated NPCs are associated with EBV infection. To examine whether the disruption of TGF-β signalling could influence the differentiation of NPE cells that lead to EBV maintenance, NP361hTert and NP550hTert were treated with $CaCl_2$ and FBS in the presence or absence of TGF-β1 and the levels of involucrin protein was measured. Inhibition of TGFR-1 kinase with SB431542 and overexpression of *DnTGFBR2* reduced the expression of involucrin in both cell lines. These data suggested that disruption of TGF-β signalling suppressed cellular differentiation in NPE cells, which might support EBV maintenance in these cells.

EBV infection has been shown to induce senescence and growth inhibition in NPE cells (Tsang et al., 2012), implying that repression of senescence might be essential for EBV maintenance. To examine whether disruption of TGF-β signalling could override EBV-induced senescence, the expression of three senescence markers (SIRT1, p16 and p21) was determined in EBV-positive NP361hTert and NP550hTert cells following inhibition of TGFR-1 kinase with SB431542 and overexpression of *DnTGFBR2*. Western blot analysis showed that the expression of SIRT1 protein was detected in both uninfected parental cell lines. Following EBV infection, the expression of SIRT1 protein was lost in the control cells, indicating the induction of senescence.

Notably, treatment of both cell lines with SB431542 and overexpression of a *DnTGFBR2* protected the cells from EBV-induced senescence, as shown by the restoration of SIRT1 protein expression. In agreement with these data, immunofluorescence analysis showed that SB431542 treatment and *DnTGFBR2* expression resulted in a noticeable reduction of p16 and p21 expression following EBV infection in these cells. These results demonstrated that disruption of TGF-β signalling protected NPE cells from EBV-induced senescence that in turn facilitated persistence of EBV infection in these cells.

Suppression of EBV-encoded *BZLF1*, the gene responsible for the latent to lytic switch, is required to establish stable EBV maintenance in B-cells and epithelial cells (Murata et al., 2012; Tsang et al., 2012). To examine whether disruption of TGF-β signalling could suppress EBV lytic cycle induction, the expression of *BZLF1* was determined in NP550hTert following EBV infection. Compared to the diluent (ethanol)-treated cells and vector controls, the expression of *BZLF1* mRNA was significantly down-regulated in SB431542-treated and *DnTGFBR2*-expressing NP550hTert cells following EBV infection, suggesting that disruption of TGF-β signalling may support latent EBV infection in these cells by suppressing the induction of EBV lytic cycle.

Taken together, these data suggest that disruption of TGF-β signalling facilitates stable EBV infection in NPE cells through a variety of mechanisms. These include the suppression of cellular differentiation, protection against EBV-induced senescence and inhibition of EBV lytic cycle reactivation

# CHAPTER 6: EFFECT OF CRISPR/CAS9-MEDIATED *TGFBR2* KNOCKOUT ON THE OUTCOME OF EBV INFECTION IN NP460HTERT

## 6.1 Introduction

It is now recognised that epithelial cells carrying pre-malignant genetic alterations (for example, *p16* deletion or *CCND1* overexpression) are susceptible to stable EBV infection (Tsao et al., 2017). Among the immortalised NPE cell lines, only NP460hTert which harbors a *p16* deletion can maintain EBV genomes in prolonged culture (Tsang et al., 2010). The results of Chapter 4 showed that disruption of TGF-β signalling by inhibition of TGFR-1 kinase with SB431542 or overexpression of *DnTGFBR2* facilitated stable EBV infection in three immortalised NPE cell lines; namely, NP361hTert, NP460hTert and NP550hTert. To confirm these data and to conclusively demonstrate a role for TGF-β in regulating stable EBV infection in NPE cells, the present study used the CRISPR/Cas9 system to knockout *TGFBR2* in NP460hTert. This is a powerful approach to study gene function and edits cell genomes by co-expressing the Cas9 endonuclease with a single guide RNA (sgRNA) specific to the target gene (Cong et al., 2013). Cas9 endonuclease is guided by the sgRNA to introduce double-stranded breaks in the target DNA sequences, resulting in the loss-of-function of the target gene. The results presented in this chapter describe the outcome of EBV infection in NP460hTert following *TGFBR2* knockout.

## 6.2 The effect of *TGFBR2* knockout on the outcome of EBV infection in NP460hTert

### 6.2.1 Generation and validation of CRISPR/Cas9-mediated knockout of *TGFBR2* in NP460hTert

To knockout *TGFBR2* in NP460hTert using CRISPR/Cas9, cells were co-transfected with two plasmids separately encoding Cas9 endonuclease and sgRNAs. Two sgRNAs (sgRNA1 and sgRNA2) were designed to target exon 1 of the *TGFBR2* gene. A cell which carries both the sgRNAs is expected to have a deletion of 600bp and yield a PCR product of 300bp that represents intronic region flanking exon 1 (with exon 1 being deleted). The transfected cells were grown in puromycin and single colonies selected to isolate monoclonal cell populations. Out of 12 clones, five (C1-C5) were able to grow continuously. PCR analysis showed that two clones (C3 and C4) displayed the expected size of the amplicon (300bp), indicating a successful deletion of *TGFBR2* gene in these cells (Figure 6.1). Given that C3 showed a stronger band than C4, it was selected for subsequent experiments and designated as CRISPR9/NP460hTert.

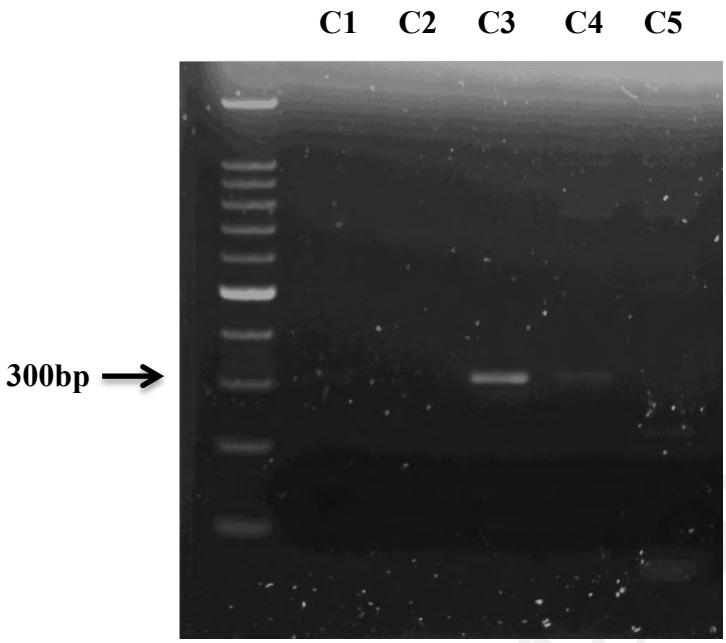



**Figure 6.1. CRISPR/Cas9-mediated knockout of *TGFBR2* in NP460hTert.** PCR analysis showed that among the five clones, only C3 and C4 displayed the expected size of the amplicon (300bp), indicating a successful deletion in the exon 1 of *TGFBR2*. The band from C4 was very faint and difficult to be visualized on copied images.

To further validate the knockout of *TGFBR2* in CRISPR9/NP460hTert cells, Western blot analysis showed that the CRISPR9/NP460hTert cell line did not express TGFR-2 (Figure 6.2). Similarly, pSmad2 was not detected in the CRISPR9/NP460hTert cells following treatment with TGF-β1 (Figure 6.3). To examine whether TGF-β/Smad-dependent transcription was lost in the CRISPR9/NP460hTert, the cells were transiently transfected with *SMAD2*- or *SMAD3*-dependent luciferase reporter plasmids. Upon addition of TGF-β1, an increase in Smad2-dependent transcriptional activity with a fold change of 3.4-fold was shown in NP460hTert (Figure 6.4; $p<0.01$). In contrast, no change in Smad2-dependent transcriptional activity was shown in CRISPR9/NP460hTert following TGF-β1 treatment (Figure 6.4). Similarly, addition of TGF-β1 resulted in an increase in Smad3-dependent transcriptional activity with a fold change of 9.7-fold in NP460hTert (Figure 6.5; $p<0.01$), whilst no change was evident in CRISPR9/NP460hTert cells (Figure 6.5). Taken together, these results confirmed the functional loss of TGF-β/Smad signalling in the CRISPR9/NP460hTert cells.

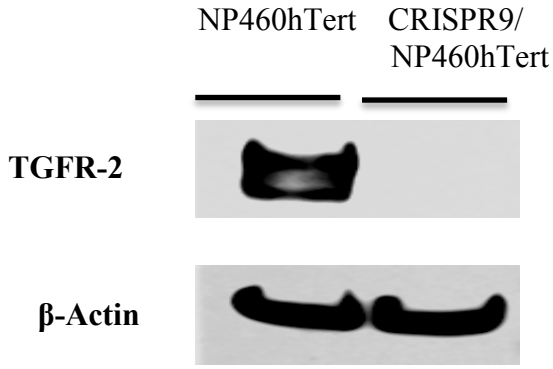



**Figure 6.2. Loss of TGFR-2 expression in CRISPR9/NP460hTert cells.** The expression of TGFR-2 was readily detected in the parental controls (NP460hTert), but not in CRISPR9/NP460hTert. Representative images from two independent experiments are presented.

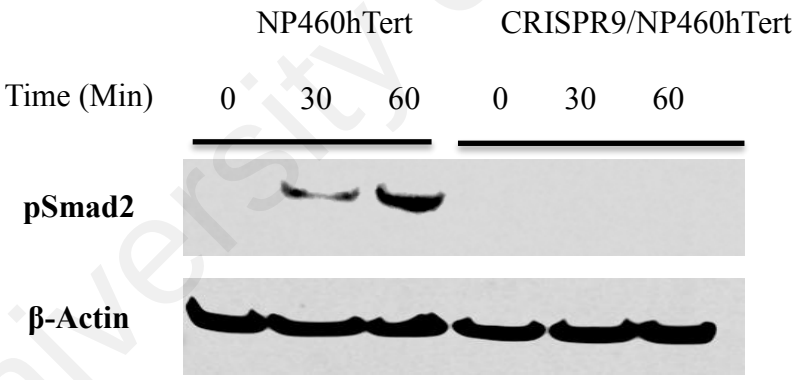



**Figure 6.3. Expression of pSmad2 in CRISPR9/NP460hTert cells.** No expression of pSmad2 was detected in CRISPR9/NP460hTert following treatment with 1ng/ml TGF-β1 for 30 and 60 minutes. Representative images from two independent experiments are presented.

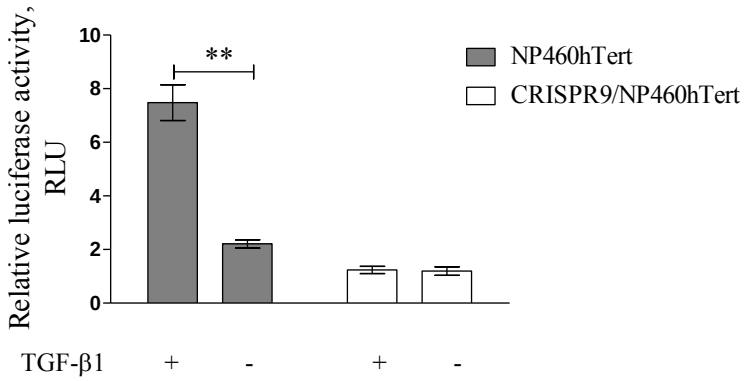



**Figure 6.4. Reduction of Smad2-dependent transcriptional activity in CRISPR9/NP460hTert cells.** Luciferase reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in Smad2-dependent transcriptional activity in parental controls (NP460hTert). However, no change in Smad-dependent transcriptional activity was observed in CRISPR9/NP460hTert following TGF-β1 stimulation. The data presented are representative of two independent experiments. ** denotes p-value<0.01.

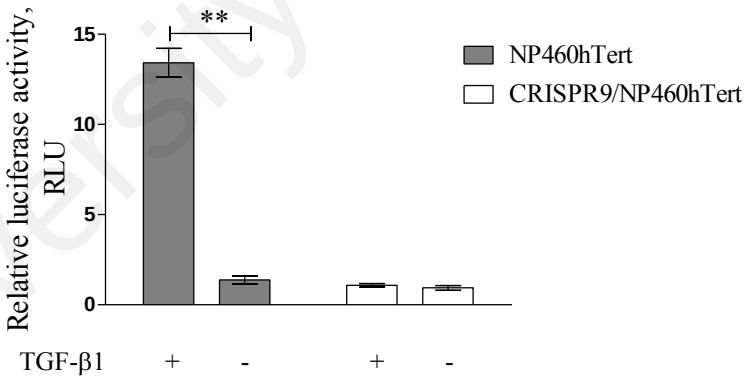



**Figure 6.5. Reduction of Smad3-dependent transcriptional activity in CRISPR9/NP460hTert cells.** Luciferase reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in Smad3-dependent transcriptional activity in parental controls (NP460hTert). However, no change in Smad3-dependent transcriptional activity was evident in CRISPR9/NP460hTert following TGF-β1 stimulation. The data presented are representative of two independent experiments. ** denotes p-value<0.01.

### 6.2.2 Increased numbers of EBV-positive cells in CRISPR9/NP460hTert

Data in Chapter four showed that expression of a *DnTGFBR2* cDNA resulted in a more persistent EBV infection of NP460hTert cells. To examine whether knockout of *TGFBR2* would also lead to the same outcome, CRISPR9/NP460hTert cells were infected with a GFP-tagged recombinant EBV (Akata strain) and the EBV-positive cells were isolated and grown for 7, 14 and 21 days. Compared to the parental controls (NP460hTert), the numbers of EBV-positive cells in CRISPR9/NP460hTert cells were significantly higher at each time point (Figure 6.6; $p<0.001$), confirming the previous results showing that disruption of TGF-β signalling facilitated the maintenance of EBV genomes in NP460hTert.

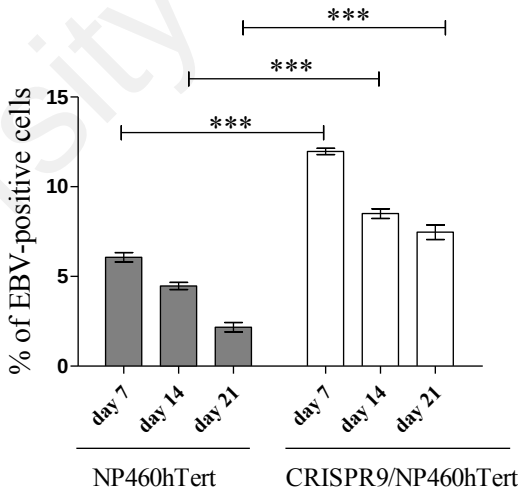



**Figure 6.6. Increased numbers of EBV-positive cells in EBV infected CRISPR9/NP460hTert cells.** FACS analyses showed that the numbers of EBV-positive cells were higher in CRISPR9/NP460hTert compared to the parental controls (NP460hTert). The data presented are representative of two independent experiments. *** denotes p-value <0.001.

## 6.3 Re-expression of *TGFBR2* in CRISPR9/NP460hTert impaired the maintenance of EBV infection

To rescue the loss of *TGFBR2*, CRISPR9/NP460hTert cells were transduced with lentiviruses carrying either a wild-type *TGFBR2*, or an empty vector. The expression of TGFR-2 in the CRISPR9/NP460hTert-expressing *TGFBR2* was confirmed by Western blotting (Figure 6.7). To validate the restoration of functional TGF-β signalling in these cells, Western blotting of pSmad2 was performed following the addition of TGF-β1 for 30 and 60 minutes. Whilst no expression of pSmad2 was observed in the vector controls, pSmad2 was readily detected in the *TGFBR2*-expressing CRISPR9/NP460hTert following TGF-β1 stimulation (Figure 6.8). To examine if the re-expression of *TGFBR2* influenced the maintenance of EBV genomes, *TGFBR2*-expressing CRISPR9/NP460hTert cells were infected with a GFP-tagged recombinant EBV (Akata strain) and the EBV-positive cells were isolated and grown for 7, 14 and 21 days. Compared to the vector controls, the numbers of EBV-positive cells in the *TGFBR2*-expressing CRISPR9/NP460hTert were significantly decreased at each time point (Figure 6.9; $p<0.05$), further confirming that a functional TGF-β signalling pathway plays an important role in facilitating stable EBV infection in NPE cells.

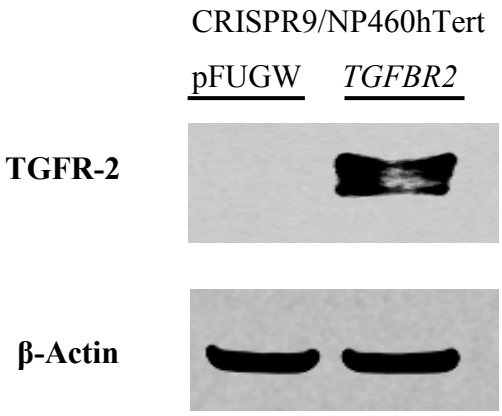



**Figure 6.7. Expression of TGFR-2 in *TGFBR2*-expressing CRISPR9/NP460hTert.** Expression of TGFR-2 was shown in CRISPR9/NP460hTert transduced with a wild type *TGFBR2* compared to the vector controls (pFUGW). Representative images from two independent experiments are presented.

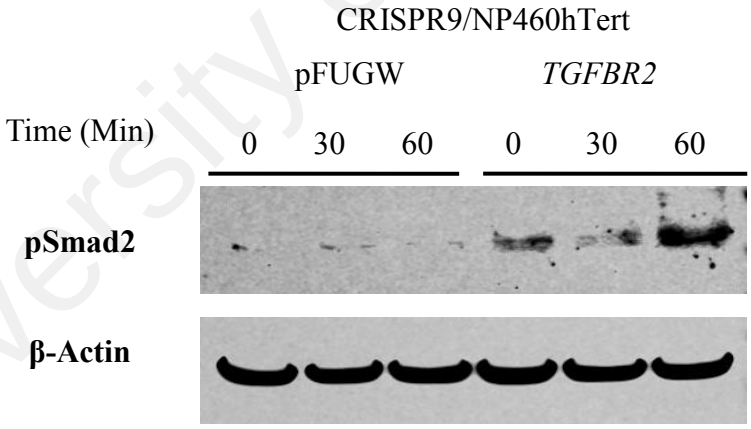



**Figure 6.8. Expression of pSmad2 in *TGFBR2*-expressing CRISPR9/NP460hTert.** Expression of pSmad2 was shown in CRISPR9/NP460hTert transduced with a wild type *TGFBR2* following treatment with 1ng/ml TGF-β1 for 30 and 60 minutes. Representative images from two independent experiments are presented.

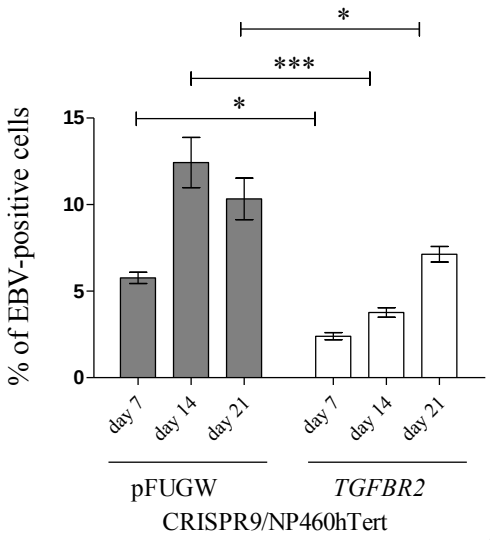



**Figure 6.9. Restoration of *TGFBR2* decreased the numbers of EBV-positive cells in CRISPR9/NP460hTert.** FACS analyses showed that the numbers of EBV-positive cells were decreased in *TGFBR2*-expressing CRISPR9/NP460hTert compare to the vector controls (pFUGW). The data presented are representative of two independent experiments. * denotes p-value <0.05 and *** denotes p-value <0.001.

## 6.4 Summary

Previous chapters have described that disruption of TGF-β signalling facilitated the maintenance of EBV genomes in NPE cell lines, most likely through the suppression of cellular differentiation, EBV-induced senescence, and EBV lytic cycle reactivation. In this chapter, the effects of TGF-β signalling disruption on EBV persistence was further confirmed in NP460hTert following CRISPR/Cas9-mediated knockout of the *TGFBR2*. The *TGFBR2* knockout cells (CRISPR9/NP460hTert) were generated by transfecting NP460hTert with plasmid DNA encoding Cas9 endonuclease and sgRNAs targeting exon 1 of the *TGFBR2*.

Having confirmed the loss of TGFR-2 in the CRISPR9/NP460hTert, the disruption of TGF-β/Smad signalling pathway was verified by the inhibition of TGF-β1-induced phosphorylation of Smad2 and loss of Smad2/3-dependent transcriptional activity. Next, these cells were infected with a recombinant EBV and, compared to the parental controls (NP460hTert), the *TGFBR2* knockout cells sustained higher numbers of EBV-positive cells. To rescue the loss of *TGFBR2*, CRISPR9/NP460hTert cells were transduced with lentiviruses carrying a wild type *TGFBR2*. The expression of TGFR-2 and TGF-β1-induced phosphorylation of Smad2 was restored in the *TGFBR2*-expressing CRISPR9/NP460hTert. EBV infection of *TGFBR2*-expressing CRISPR9/NP460hTert showed that restoration of *TGFBR2* impaired the maintenance of EBV genomes. Collectively, these results demonstrate that loss of TGF-β signalling is a critical event in supporting stable EBV infection in NPE cells.

# CHAPTER 7: DISCUSSION

## 7.1 Introduction

The contribution of EBV infection to the development of NPC is thought to be a consequence of the establishment of virus latency in epithelial cells displaying pre-malignant genetic changes. Indeed, NPC cells carry monoclonal EBV genomes, indicating that EBV infection takes place before the expansion of the malignant cells. Although EBV replication has been found within oral epithelial cells (Niedobitek et al., 1991b), it has been a challenge to establish persistent EBV latent infection in epithelial cells *in vitro*. To date, only one study has investigated a mechanistic link between pre-malignant genetic changes and EBV infection, which demonstrated that inactivation of *p16* or overexpression of *CCND1*, two characteristic features of NPC tumours, contributed to stable EBV infection in immortalised NPE cells (Tsang et al., 2012). Interestingly, we noted that less-differentiated NPC cell lines (e.g. HONE1) did not respond to TGF-β1-mediated growth inhibition but were susceptible to EBV infection. In contrast, differentiation-competent squamous epithelial cell lines (e.g. immortalised oral keratinocytes, OKF6), which could not sustain EBV infection were responsive to the cytostatic effects of TGF-β1. These observations suggested that disruption of TGF-β signalling might be a crucial event in supporting stable EBV latent infection in epithelial cells. There is evidence to suggest that TGF-β signalling is de-regulated in NPC. In particular, reduced expression of both TGFR-1 and TGFR-2 has been reported in primary NPC tissues and cell lines (Fang et al., 2008; Lyu et al., 2014; Sriuranpong et al., 2004; Zhang et al., 2012). Although de-regulation of TGF-β signalling has been shown to promote NPC tumorigenesis, the functional significance of this pathway in regulating EBV latency has not been investigated.

The present study aimed to investigate the influence of TGF-β signalling disruption on the outcome of EBV maintenance in NPE cells. Three immortalised NPE cell lines (NP361hTert, NP460hTert and NP550hTert) with disrupted TGF-β signalling were generated by inhibiting the TGFR-1 kinase using a chemical inhibitor (SB431542) or by the overexpression of a dominant negative *TGFBR2* (*DnTGFBR2*) gene. Following EBV infection, the number of cells carrying EBV genomes was significantly higher in these cells compared to their respective controls. Having shown that disruption of TGF-β signalling contributed to the maintenance of EBV genomes in NPE cells, the possible mechanisms that were responsible for these effects were investigated. The results showed that disruption of TGF-β signalling significantly suppressed cellular differentiation in response to serum/calcium and TGF-β1, protected cells from EBV-induced senescence and inhibited the induction of the EBV lytic cycle. To conclusively demonstrate the role of TGF-β signalling in regulating persistent EBV infection in NPE cells, the *TGFBR2* gene was knocked out in NP460hTert cells using the CRISPR/Cas9 system. Similarly, increased numbers of EBV-positive cells were observed in the *TGFBR2* knockout cells and these effects were reversed following re-expression of a wild-type *TGFBR2* gene.

## 7.2 The effect of TGF-β signalling disruption on the outcome of EBV infection in NPE cell lines

### 7.2.1 TGF-β1-mediated growth inhibition

The responsiveness of six epithelial cell lines, namely four immortalised NPE cells (NP69, NP361hTert, NP460hTert, NP550hTert), a well-differentiated NPC-derived cell line (HK1) and an undifferentiated NPC-derived cell line (HONE1),

towards TGF-β1-mediated growth inhibition was first determined. As expected, HONE1 cells that were able to sustain EBV infection did not respond to the cytostatic effects of TGF-β1. In line with our unpublished observations that HK1 could not sustain EBV genomes without numerous cycles of selection, its growth was suppressed in response to TGF-β1 treatment. This might be due to the fact that HK1 was derived from a well-differentiated NPC and its cellular characteristics do not favor the maintenance of EBV genomes. It would be informative to examine whether these cells harbor any defects in the components of the TGF-β signalling pathway that contribute to the differences in their responsiveness to TGF-β1. Interestingly, the SV40T-immortalised NP69 cells did not respond to TGF-β1-mediated growth inhibition. These observations can be partly explained by the data showing that overexpression of the SV40-T antigen can override the suppressive effects of TGF-β1 on c-MYC expression, thereby allowing c-MYC to inhibit the expression of p15, p21 and p27 induced by TGF-β1 (Laiho et al., 1990; Laiho et al., 1991; Pietenpol et al., 1990). Further, SV40-T antigen can directly bind Rb protein and trigger the release of E2F proteins, resulting in cell cycle progression (Hume & Kalejta, 2009). Among the three telomerase-immortalised NPE cell lines, only NP460hTert, which harbor a *p16* deletion can maintain EBV genomes after multiple cycles of selection, while NP361hTert and NP550hTert are only able to sustain EBV infection after overexpressing *CCND1* (Tsang et al., 2012; Tsang et al., 2010). However, their responsiveness towards cytostatic effects of TGF-β1 was similar, suggesting that the degree of inhibition did not directly reflect the susceptibility to EBV infection.

### 7.2.2 Disruption of TGF-β signalling and EBV infection

The TGF-β receptors; *TGFBR1* and *TGFBR2* are essential molecules within the TGF-β signalling pathway. Given that reduced expression of these receptors is a frequent feature of NPC (Fang et al., 2008; Lyu et al., 2014; Sriuranpong et al., 2004; Zhang et al., 2012), they were selected as targets to disrupt the TGF-β signalling pathway. The kinase activity of TGFR-1 was inhibited using a chemical inhibitor, SB431542 (Inman et al., 2002; Matsuyama et al., 2003) and data from our laboratory have shown that SB431542 is a selective inhibitor of TGFR-1 kinase and the TGF-β signalling pathway (Appendix A). The *DnTGFBR2* construct has been previously used and its expression was sufficient to inhibit the function of *TGFBR2* (Paterson et al., 2001). To infect the NPE cells with a recombinant EBV (Akata strain), the established co-culture method was used given its ability to infect epithelial cells more efficiently than cell-free infection using the viral supernatant harvested from EBV-producing cell lines (Imai et al., 1998). Following EBV infection, GFP-positive cells were isolated and cultured for 7, 14 or 21 days. Compared to the respective controls, treatment with SB431542 or overexpression of *DnTGFBR2* resulted in higher percentages of EBV-positive cells in all three cell lines tested. These results were not affected by the EBV infection rates as an infection rate of approximately 10% was observed in all the cells examined. Interestingly, TGF-β1 was previously shown to enhance the infection rate of EBV in both the primary and immortalised NPE cells, possibly by up-regulating the integrin receptors (Tsang et al., 2010). Taken together, it can be speculated that TGF-β1 treatment can enhance the EBV infection efficiency of epithelial cells, but once infected, the cells need to develop resistance to TGF-β1-induced growth inhibition in order to maintain the EBV genomes.

The present study was designed to determine the percentage of EBV-positive cells sustained post infection over a 21-day period. Attempts were made to passage the cells after 21 days, however, the loss of EBV genomes was rapid, and re-growth of EBV-positive cells after passaging was not successful. It has previously been shown that EBV-positive cells grew significantly slower compared to their EBV-negative counterparts (Tsang et al., 2010). As such it was possible that in a culture of mixed cell populations, the EBV-negative cells outgrew the EBV-positive cells, and eventually leading to the loss of cells carrying EBV genomes. To overcome this hurdle, multiple cycles of selection would be required to enrich the EBV infected cells and to achieve persistent EBV infection in prolonged culture (personal communication with Prof. Dr. George Tsao from the University of Hong Kong). It is anticipated that cells with the disrupted TGF-β signalling would achieve a stable EBV infection quicker than their respective controls.

During the course of this study, one of our collaborators, Prof. Dr. Kwok-Wai Lo from the Chinese University of Hong Kong, showed that loss-of-function mutations or deletions in key molecules within the TGF-β signalling pathway occur in a significant proportion of primary NPC tissues using whole genome sequencing (unpublished data). To conclusively demonstrate a role for TGF-β signalling in regulating persistent EBV infection in epithelial cells, CRISPR/Cas9-mediated knockout of the *TGFBR2* gene was performed. Similar to the results obtained from the inhibition of TGFR-1 kinase with inhibitor SB431542 and overexpression of *DnTGFBR2*, numbers of EBV-positive cells were higher following the knockout of *TGFBR2*. More importantly, re-expression of a wild-type *TGFBR2* impaired the ability of cells to maintain the EBV genomes, providing convincing evidence that disruption of TGF-β signalling supports stable EBV infection in NPE cells.

## 7.3 Cellular responses to TGF-β that regulate the maintenance of EBV genomes in NPE cells

### 7.3.1 Effect on cellular differentiation and EBV lytic cycle

Undifferentiated NPC is invariably associated with EBV infection and also known as lymphoepithelioma of the nasopharynx. Lymphoepithelioma-like carcinoma (LELC) is defined as an undifferentiated carcinoma with an intense lymphoplasmacytic infiltrates (Gulley et al., 1995). Apart from NPC, EBV infection has also been detected in LELC of other organs such as stomach, esophagus, hepatobiliary tract, lung, thymus and salivary gland (Burke et al., 1990; Castro et al., 2001; Mori et al., 1994; Tsai et al., 1996; Vortmeyer et al., 1998), suggesting a close relationship between the loss of differentiation of the host cells and EBV infection. The regulation of the latent and lytic switch is crucial in the maintenance of EBV genomes in the infected cells (Kenney & Mertz, 2014; Tsang et al., 2014). Suppression of the EBV-encoded immediate early gene, *BZLF1*, which controls the switch from latent to lytic cycle, is required for the establishment of stable EBV infection in epithelial and B cells (Murata et al., 2012; Tsang et al., 2012). It was shown that differentiation of epithelial and B cells induced by the cellular differentiation regulator B-lymphocyte-induce maturation protein 1 (BLIMP1) triggered EBV lytic reactivation in latently infected B cells (Reusch et al., 2015). These observations suggest that latently infected cells need to suppress cellular differentiation and override viral lytic induction in order to establish persistent latent infection. In line with this concept, an early study using SVK epithelial cells showed that compared to acutely infected cells, clones that stably carried EBV genomes were impaired in their ability to differentiate and enter the virus lytic cycle (Knox et al., 1996). Using an organotypic raft culture of telomerase-immortalised normal oral

keratinocytes, lytic replication of EBV was confined to the more differentiated cell layers and this process was synergistically mediated by Kruppel-like factor 4 (KLF4) and BLIMP1 (Nawandar et al., 2015). In *CCND1*-overexpressing NPE cells, the maintenance of EBV genomes was accompanied by the suppression of serum-induced differentiation and low expression of *BZLF1* (Tsang et al., 2012). These data suggest that stable maintenance of EBV in epithelial cells require an undifferentiated cellular environment that links to the suppression of viral lytic cycle.

A role for TGF-β signalling in the regulation of epithelial cell differentiation has been demonstrated. A number of studies have shown that TGF-β signalling can induce differentiation of the immortalised skin keratinocyte cell line, HaCaT (Buschke et al., 2011; Cho et al., 2004; Descargues et al., 2008; Matsumoto et al., 1990). In agreement with these previously published findings, results obtained from this study demonstrate that exogenous TGF-β1 enhances expression of involucrin in NPE cells treated with serum/calcium, while such effects were suppressed following disruption of TGF-β signalling. These data support the notion that loss of differentiation is a critical determinant in enabling epithelial cells to sustain EBV genomes.

The contribution of TGF-β signalling to induction of the EBV lytic cycle has also been documented. EBV-positive BL and gastric epithelial cells were found to produce TGF-β1, which could stimulate EBV lytic reactivation, as, evidenced by the induced expression of *BZLF1* (Fahmi et al., 2000; Fukuda et al., 2001). Loss of functional *TGFBR2* in EBV-infected BL cells resulted in insensitivity to TGF-β1-induced lytic cycle reactivation (Fukuda et al., 2006). In support of these data, the results of the present study showed that inhibition of TGFR-1 kinase and overexpression of *DnTGFBR2* in NPE cells led to a decrease in the *BZLF1* mRNA expression following

EBV infection. It has been proposed that an abortive lytic cycle is the initial mode of primary EBV infection of epithelial and B cells (Ma et al., 2012). Therefore, it is possible that EBV infection of NPE cells induces the production of TGF-β1 and the defects in TGF-β signalling confer resistance to TGF-β1-mediated EBV lytic induction. Recently, the differentiation of epithelial cells was shown to stimulate the expression of LMP1, which was required for an efficient EBV lytic reactivation (Nawandar et al., 2017). Although it is recognised that *BZLF1* alone is sufficient to be an indicator for lytic cycle (Kolman et al., 1996; Le Roux et al., 1996; Ragoczy et al., 1998; Schelcher et al., 2005), it would be informative to demonstrate that the expression of LMP1 and other EBV lytic genes was also suppressed in cells with disrupted TGF-β signalling.

### 7.3.2 Effect on EBV-induced senescence

Senescence is a process by which cells irreversibly stop dividing and enter a state of permanent growth arrest without undergoing cell death (Munoz-Espin & Serrano, 2014). Senescence can be induced by diverse stimuli, including shortening of telomeres (replicative senescence), increasing levels of ROS and DNA damage (stress-induced senescence), activation of oncogenes (oncogene-induced senescence) and increasing levels of pro-inflammatory cytokines (senescence-associated secretory phenotype) (Campisi & d'Adda di Fagagna, 2007; Munoz-Espin & Serrano, 2014; Salama et al., 2014). EBV is a human oncovirus and its ability to induce senescence in normal cells has been reported. EBV infection of normal B cells has been shown to result in hyperproliferation of infected cells with accumulating DNA damage, leading to a G1/S phase growth arrest or senescence (Alfieri et al., 1991; Nikitin et al., 2010). To overcome this obstacle, EBV infected B cells express EBNA-2 and EBNA-LP to induce expression of cyclin D2, which leads to cell cycle progression and immortalisation

(Sinclair et al., 1994). Similarly, EBV infection of normal epithelial cells has been shown to induce growth arrest and senescence (Dittmer et al., 2008; Lu et al., 2010; Tsang et al., 2012; Tsang et al., 2010). The expression of LMP1 in fibroblast cells was shown to inhibit senescence by down-regulating expression of p16 (Ohtani et al., 2003; Yang et al., 2000). In NPE cells, overexpression of *CCND1* was sufficient to override senescence induced by EBV infection in a p16- and p21-dependent manner (Tsang et al., 2012).

To examine whether disruption of TGF-β signalling could override EBV-induced senescence in NPE cells, the expression of SIRT1, p16 and p21 was measured in the isolated EBV-positive cells at day 14 post-infection. SA-β-Gal is frequently used to measure senescence, but this assay could not be used in the present study because of high levels of false positive staining in NPE cells. However, SIRT1, which is a NAD-dependent protein deacetylase that modulates various cellular functions such as cell cycle progression, is down-regulated in senescent cells and can be used as a marker of late senescence (Huang et al., 2008; Sasaki et al., 2006). Following EBV infection of NPE cells, inhibition of TGFR-1 kinase with SB431542 and overexpression of *DnTGFBR2* restored the expression of SIRT1, which was accompanied by the reduction of p16 and p21 levels. These findings indicate that disruption of TGF-β signalling protected cells from EBV-induced senescence that in turn, might facilitate stable, persistent infection with EBV. These findings are consistent with the role of TGF-β signalling in inducing senescence of epithelial cells. Exogenous TGF-β1 was shown to induce ROS-dependent senescence in hepatocellular carcinoma cells in a p21- and p15-dependent manner (Senturk et al., 2010). Further, TGF-β signalling has been shown to promote oncogenic Ras-induced senescence in human mammary epithelial cells (HMEC) by inducing the expression of p21 (Cipriano et al., 2011; Lin et al., 2012).

### 7.3.3 TGF-β signalling and EBV infection

It is well-recognised that once EBV latency is established in pre-malignant epithelial cells, EBV-encoded genes regulate multiple signalling pathways to drive the development of NPC. During the course of this study, a number of signalling pathways were identified to be de-regulated by EBV infection in NPC, including lysophosphatidic acid (LPA) signalling (Appendix B). Interestingly, a previous study by Dr. CW. Dawson (University of Birmingham, UK) has shown that EBV-encoded EBNA1 repressed TGF-β1-induced transcription by increasing Smad2 turnover (Wood et al., 2007). Taken together, a possible loop between EBV infection and TGF-β signalling can be proposed in which cells with defects in TGF-β signalling are susceptible to EBV infection, once infected, the EBV-positive cells express EBNA1 to impair TGF-β signalling which in turns supports the maintenance of latency in these cells.

## 7.4 Limitations of the study

Although the study was carefully designed to address the specific research questions, there were a number of limitations, which could potentially influence the broader conclusions drawn from the data.

The GFP (EBV)-positive cells were isolated 48 hours post-EBV infection and cultured for over 21 days. A rapid loss of EBV genomes was observed after passaging the cells and therefore stable latent infection was not established. Multiple cycles of sorting to select the EBV-positive cells are required to prove that cells with disrupted TGF-β signalling could proliferate and maintain EBV genomes in prolonged culture.

Disruption of TGF-β signalling was shown to suppress cellular differentiation and to inhibit EBV lytic cycle induction in the NPE cells. Due to some technical issues, a direct link showing the maintenance of EBV genomes in cells treated with differentiation inducing reagents could not be established. It would be more informative to demonstrate that disrupting TGF-β signalling in cells with impaired ability to differentiation could sustain EBV genomes.

The mRNA expression of *BZLF1* was reduced in cells with disrupted TGF-β signalling following EBV infection. It will be more persuasive to show the reduction of *BZLF1* at the protein levels. Further, the suppressive effect of defective TGF-β signalling on lytic cycle induction would be more compelling if decreased expression of other immediate early lytic genes, such as *BRLF1*, were shown.

## 7.5 Future work

The results of the present study showed that disruption of TGF-β signalling facilitated EBV infection in the NPE cells. There are several areas of research arising from this work that are likely to form a fundamental basis for a better understanding of the contribution of EBV infection to the development of NPC.

Given that high levels of TGF-β1 were found in sera of NPC patients (Sun et al., 2007; Xu et al., 1999) and TGF-β1 represents the prototype of the three TGF isoforms, the present study used only TGF-β1 in the experiments. It will be informative to investigate whether TGF-β2 and TGF-β3 are also highly expressed in NPC and exhibit similar effects as TGF-β1.

Inhibition of TGFR-1 kinase with SB431542 or overexpression of *DnTGFBR2* was demonstrated to suppress cellular differentiation in response to calcium/serum as well as TGF-β1. Several cellular transcription factors, such as BLIMP1, are key mediators of differentiation in epithelial cells (Nawandar et al., 2015; Reusch et al., 2015) understanding the mechanisms engaged by TGF-β signalling in the differentiation process would strengthen our understanding of the events conferring a suitable cellular environment for EBV infection of epithelial cells.

NPC is the consequence of aberrant establishment of EBV latent infection in NPE cells. Developing cell lines with defective TGF-β signalling that stably maintain EBV genomes would provide important insights into the underlying mechanisms contributing to the maintenance of EBV infection in epithelial cells. Further, TGF-β1 was previously shown to enhance the infection rate of EBV in NPE cells (Tsang et al., 2010) and the present study demonstrated that disruption of TGF-β signalling facilitated persistent EBV infection. It would be compelling to elucidate the mechanisms employed by TGF-β signalling in regulating these events.

Overexpression of *CCND1* or a *p16*-resistant form of *CDK4* was shown to counteract the EBV-induced growth arrest and senescence in NPE cells, thus enabling persistent infection of EBV (Tsang et al., 2012). Similar results were obtained with the disruption of TGF-β signalling in the NPE cells. Taken together, these observations suggest that defects in cell cycle control may support stable EBV latent infection in NPE cells. Studies to investigate whether these events are mutually exclusive or synergistic are greatly warranted.

# CHAPTER 8: CONCLUDING REMARKS

EBV infection is believed to be an initiating event in the development of NPC. Evidence suggests that epithelial cells harboring specific genetic changes are susceptible to persistent EBV infection, and that key genetic alterations or pathway disruption is required for the establishment of stable latent infection. This is the first study to show that disruption of TGF-β signalling supports stable EBV infection in NPE cells. These results provide crucial information which leads to a better understanding of the genetic events associated with the progression of NPC.

There is some evidence to show that TGF-β signalling is de-regulated in NPC. The present study showed that disrupting TGF-β signalling in NPE cells by inhibiting TGFR-1 kinase using a chemical inhibitor (SB431542) or overexpression of a *DnTGFBR2* gene facilitated the maintenance of EBV genomes in three NPE cell lines (NP361hTert, NP460hTert and NP550hTert). These results were further confirmed by knocking out the *TGFBR2* gene in NP460hTert cells using the CRISPR/Cas9 system. Significantly, restoration of wild-type *TGFBR2* in these cells reversed the effects caused by *TGFBR2* knockout. To determine the possible mechanisms responsible for these effects, the present study showed that disruption of TGF-β signalling in NPE cells resulted in the suppression of cellular differentiation, EBV-induced senescence and EBV lytic cycle induction. Taken together, these results imply that an intact TGF-β signalling pathway is required for epithelial cells to initiate a protective cellular response that prevents stable EBV infection. Loss of such protective mechanisms resulting from de-regulated TGF-β signalling during the early stages of NPC pathogenesis facilitates EBV infection and subsequent tumour progression.

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## LIST OF PUBLICATIONS

1. LF Yap, S Velapasamy, HM Lee, S Thavaraj, R Pathmanathan, W Wei, K Vrzalikova, MH Ibrahim, A Khoo, SW Tsao, IC Paterson, GS Taylor, CW Dawson and PG Murray. Down-regulation of LPA receptor 5 to aberrant LPA signalling in EBV-associated nasopharyngeal carcinoma. Journal of pathology. 2016;235(3):456-465
2. Sharmila Velapasamy, Christopher W. Dawson, Lawrence S. Young, Ian C. Paterson and Lee Fah Yap. The dynamic roles of TGF-β signalling in EBV-associated cancers. Cancers. 2018; 10, 247.
3. S Velapasamy, KW Lo, G Chung, CM Tsang, LS Young, CW Dawson, SW Tsao, IC Paterson and LF Yap. Disruption of transforming growth factor-beta signalling supports stable EBV infection in nasopharyngeal epithelial cells. (Manuscript under preparation, to be submitted to PNAS).

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ORIGINAL PAPER

# Down-regulation of LPA receptor 5 contributes to aberrant LPA signalling in EBV-associated nasopharyngeal carcinoma

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## Abstract

**Undifferentiated nasopharyngeal carcinoma (NPC) is a highly metastatic disease that is consistently associated with Epstein–Barr virus (EBV) infection. In this study, we have investigated the contribution of lysophosphatidic acid (LPA) signalling to the pathogenesis of NPC. Here we demonstrate two distinct functional roles for LPA in NPC. First, we show that LPA enhances the migration of NPC cells and second, that it can inhibit the activity of EBV-specific cytotoxic T cells. Focusing on the first of these phenotypes, we show that one of the LPA receptors, LPA receptor 5 (LPAR5), is down-regulated in primary NPC tissues and that this down-regulation promotes the LPA-induced migration of NPC cell lines. Furthermore, we found that EBV infection or ectopic expression of the EBV-encoded LMP2A was sufficient to down-regulate LPAR5 in NPC cell lines. Our data point to a central role for EBV in mediating the oncogenic effects of LPA in NPC and identify LPA signalling as a potential therapeutic target in this disease.**


Keywords: nasopharyngeal carcinoma; Epstein–Barr virus; lysophosphatidic acid; LPA receptor

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No conflicts of interest were declared.

## Introduction

Nasopharyngeal carcinoma (NPC) is a cancer with high metastatic potential that is particularly prevalent in South East Asia and southern China [1]. Radiotherapy is effective against early-stage NPC; however, over 75% of cases present with late-stage disease and there are significant rates (~30%) of distant metastases subsequent to treatment in these cases [2]. Furthermore, most survivors of NPC have an impaired quality of life due to the location of the tumour at the base of the skull and in close proximity to many vital structures. Unfortunately, our current understanding of the molecular basis of NPC is still inadequate to inform any personalized treatment strategies.

Unlike other head and neck cancers, NPC is consistently associated with Epstein–Barr virus (EBV) infection [3]. EBV latent protein expression in NPC is restricted to Epstein–Barr nuclear antigen (EBNA) 1, latent membrane proteins (LMP1 and LMP2), and BARF1. Although the exact contribution of EBV to the pathogenesis of NPC is still to be elucidated, it is well recognized that EBV alters many functional properties that are involved in tumour progression. NPC characteristically presents with a prominent lymphocyte infiltration, indicating that the tumour microenvironment can influence some of the malignant features of NPC tumour cells. The consistent expression of EBV proteins in NPC cells has led to several clinical trials of adoptive T-cell therapy or vaccination to boost the immune response to these antigens [4]. While there was evidence of a clinical response in some patients, it is not known why other patients did not respond, which may be due to varying degrees of immunosuppression in the tumour microenvironment [5].

Given that distant metastasis remains a major cause of death for NPC patients, we focused on the possibility that lysophosphatidic acid (LPA), a bioactive lipid that

cancers

MDPI

Review

# The Dynamic Roles of TGF-β Signalling in EBV-Associated Cancers

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**Abstract:** The transforming growth factor-β (TGF-β) signalling pathway plays a critical role in carcinogenesis. It has a biphasic action by initially suppressing tumorigenesis but promoting tumour progression in the later stages of disease. Consequently, the functional outcome of TGF-β signalling is strongly context-dependent and is influenced by various factors including cell, tissue and cancer type. Disruption of this pathway can be caused by various means, including genetic and environmental factors. A number of human viruses have been shown to modulate TGF-β signalling during tumorigenesis. In this review, we describe how this pathway is perturbed in Epstein-Barr virus (EBV)-associated cancers and how EBV interferes with TGF-β signal transduction. The role of TGF-β in regulating the EBV life cycle in tumour cells is also discussed.

**Keywords:** TGF-β signalling; Epstein-Barr virus; nasopharyngeal carcinoma; gastric cancer; B-cell lymphoma

## 1. Introduction

The transforming growth factor-beta (TGF-β) superfamily is a group of multifunctional proteins comprising more than 40 members that are clustered in several subfamilies, which include TGF-β, activins/inhibins, bone morphogenetic proteins (BMPs), nodal and growth differentiation factors (GDFs) [1,2]. The prototypic member, TGF-β1, is produced by a diverse range of cell types and modulates cell proliferation, migration, adhesion, differentiation and survival [2,3]. Consequently, a malfunctioning TGF-β pathway is central to many diseases including cancer. TGF-β functions as a tumour suppressor by inhibiting the growth of untransformed epithelial, endothelial and lymphoid cells [4–6] and resistance to TGF-β is regarded as one of the crucial steps in malignant progression [2,7]. In the early stages of cancer development, TGF-β signalling functions as a tumour suppressor by inhibiting cell cycle progression from G1 to S phase and inducing apoptosis, senescence and differentiation [2,5,8,9]. Conversely, in late stage disease, it acts as a tumour promoter by inducing epithelial-to-mesenchymal transition (EMT), migration, invasion, metastasis, angiogenesis and immune suppression [2,8–12]. Frequently, cancer cells become resistant to the tumour suppressive effects of TGF-β, however functional TGF-β signalling often persists in these cells enabling TGF-β-induced tumour promoting phenotypes [13–16]. Accumulating evidence has revealed that the TGF-β signalling pathway is targeted by many oncogenic viruses, including Epstein-Barr virus (EBV), during the course of tumorigenesis [17].

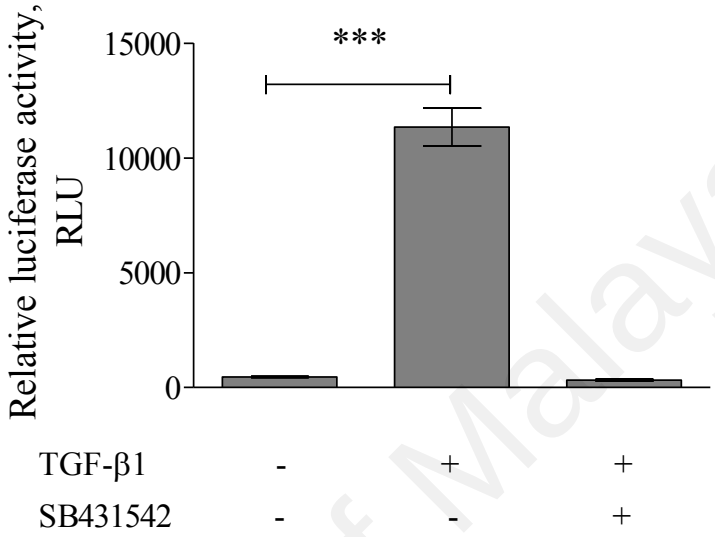
## LIST OF PRESENTATION

1. V Sharmila. Transforming Growth Factor β (TGF-β) signalling in the regulation of EBV infection and differentiation of squamous epithelium. Presented: PhD Proposal Seminar, University Malaya, Kuala Lumpur. (2013).
2. V Sharmila, CW Dawson, IC Paterson, LF Yap. Transforming Growth Factor β (TGF-β) signalling in the regulation of differentiation and EBV infection in NP69 and OKF6. Presented: 2nd NPC research day, University Malaya, Kuala Lumpur. (2013).
3. V Sharmila, CW Dawson, IC Paterson, LF Yap. Transforming Growth Factor β (TGF-β) signalling in the regulation of EBV infection of nasopharyngeal epithelium. Presented: 3rd NPC Day, Institute of Medical Research, Malaysia. (2014).
4. V Sharmila, CW Dawson, IC Paterson, LF Yap. Transforming Growth Factor β (TGF-β) signalling regulates stable EBV infection in nasopharyngeal epithelial cells. Attended: 6th Regional Conference On Molecular Medicine (RCMM) In Conjunction With 2nd National Conference For Cancer Research, Sunway Putra Hotel, Kuala Lumpur. (2015).
5. V Sharmila. Transforming Growth Factor β (TGF-β) signalling in the regulation of EBV infection and the role of BILF1 in NPC. Presented: PhD Candidature Defense, University Malaya, Kuala Lumpur. (2016).

6. V Sharmila, CW Dawson, CM Tsang, SW Tsao, IC Paterson, LF Yap. Transforming Growth Factor β (TGF-β) Signalling Regulates EBV Persistence in Nasopharyngeal Epithelial Cells. Presented: 5th NPC Research Day, Institute of Medical Research, Kuala Lumpur. (2016). **Awarded the best oral presentation.**
7. V Sharmila. Transforming Growth Factor-β (TGF-β) Signalling in the Regulation of EBV Infection. Presented: Three Minutes Thesis (UM3MT) 2016 competition. Faculty of Dentistry, University of Malaya, Kuala Lumpur. (2016). **Awarded the best oral presenter at faculty level.**
8. V Sharmila. Transforming Growth Factor-β (TGF-β) Signalling in the Regulation of EBV Infection. Presented: Three Minutes Thesis (UM3MT) 2016 competition. University of Malaya, Kuala Lumpur. (2016).
9. V Sharmila, CW Dawson, CM Tsang, SW Tsao, IC Paterson, LF Yap. Transforming Growth Factor β Signalling Regulates EBV Persistence in Nasopharyngeal Epithelial Cells. Presented: Global Lessons on Cancer Pathogenesis from Insights into Geographically Restricted Tumor of the Nasopharynx, Gordon Research Conference. The Hong Kong University of Science and Technology, Hong Kong (2016). **Awarded the best poster presentation.**
10. V Sharmila, SW Tsao, CM Tsang, CW Dawson, IC Paterson, LF Yap. The Role of Transforming Growth Factor-β Signalling in Persistent EBV Infection of Nasopharyngeal Epithelial Cells. Presented: 4th International Anatomical Sciences & Cell Biology Conference, University of Hong Kong, Hong Kong. (2016).

## APPENDIX A

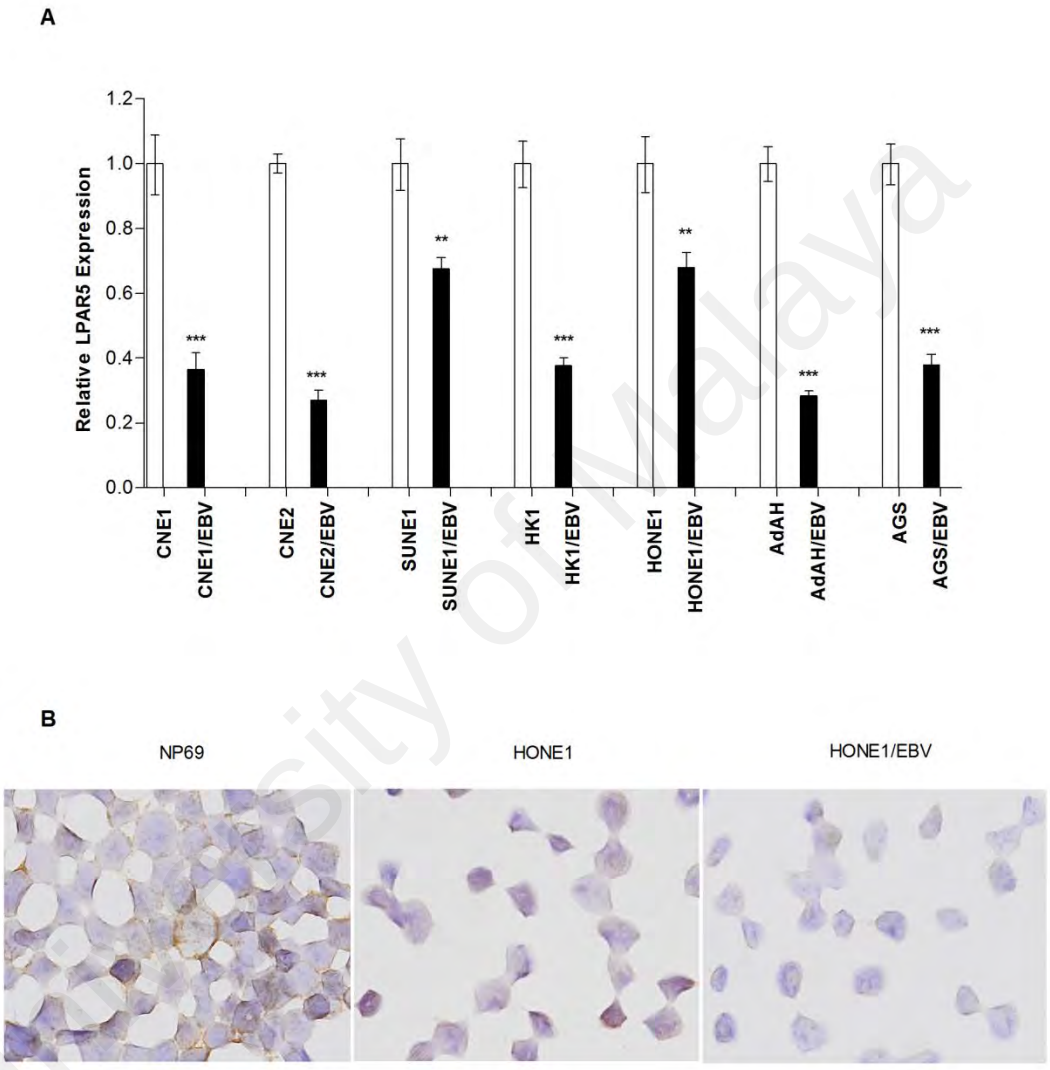
**Chemical inhibitor, SB431542 specifically inhibit TGFR-1 kinase activation**




pCAGA12-luc reporter analyses showed that TGF-β1 stimulation (1ng/ml) led to a significant increase in SMAD3-dependent transcriptional activity in MLEC transfected with constitutively active *TGFBR1*. No changes in SMAD3-dependent transcriptional activities in 10μM SB431542-treated cells following TGF-β1 treatment. *** denotes p-value <0.001

## APPENDIX B

**De-regulation of LPA signalling in NPC following EBV infection**




A) qPCR analysis showed that the expression of LPAR5 levels was significantly reduced in five NPC cell lines (CNE1, CNE2, SUNE1, HK1, and HONE1) and two epithelial cell lines (AdAH and AGS) following EBV infection. B) Immunocytochemical analysis showed that expression of LPAR5 was reduced HONE1 cells following EBV infection. NP69 cells were used as a positive control.