Epstein-Barr Virus Induction of the Hedgehog Signalling Pathway Imposes a Stem Cell-Like Phenotype on Human Epithelial Cells – Implications for the Pathogenesis of Nasopharyngeal Carcinoma

by

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is endemic in Southern China and South East Asia, causally linked to Epstein-Barr virus (EBV) infection, and frequently shows dysregulation in a number of stem cell maintenance signalling pathways. This thesis has endeavoured to investigate the status of one of these pathways; the Hedgehog (HH) signalling pathway, in NPC tumours, and reveals the novel finding that EBV is able to active the HH signalling pathway through autocrine induction of the SHH ligand in the C666.1 authentic EBV-positive NPC-derived cell line and latently infected epithelial carcinoma cell lines. This study demonstrates that constitutive engagement of the HH pathway in EBV-infected epithelial cells *in vitro* induces the expression of a number of stemness-associated genes and imposes stem-like characteristics. Using epithelial cells expressing individual EBV latent genes, this study also investigates the viral protein responsible for HH dysregulation demonstrating that EBNA1, LMP1 and LMP2A are all capable of inducing SHH ligand and activating the HH pathway, but only LMP1 and LMP2A are able to induce expression of stemness-associated marker genes. These findings not only identify a role for dysregulated HH signalling in NPC oncogenesis but also provide a novel rationale for therapeutic intervention.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AITL	Andioimmunoblastic I-cell lymphoma
ALL	Acute lymphoblastic leukaemia
ATL	Adult T-cell lymphoma
BARF1	BamH1-A reading frame-1
BART	BamA rightward transcript
BCC	Basal cell carcinoma
BCL2	B-cell lymphoma 2
BCR	B-cell receptor
BHRF1	BamH reading frame 1
BKV	BK virus
BL	Burkitt's lymphoma
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenic protein
BRDU	Bromodeoxyuridine
BRLF1	BamHI R fragment leftward open reading frame 1
BZLF1	BamHI Z fragment leftward open reading frame 1
CCND	Cyclin D family of proteins
CD21	a.k.a. EBV receptor CR2
CD44v6	CD44 variant 6
CDK1	Cyclin-dependent kinase 1
CFLAR	CASP8 and FADD-like apoptosis regulator
CMV	Cyclomegavirus
CR2	Complement receptor 2
CSC	Cancer stem cell
DHH	Desert hedgehog ligand
DNA	Deoxyribonucleic acid
ds-	double stranded
EBER	EBV encoded RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EBV-GC	EBV positive Gastric Carcinoma
EBV-HL	EBV positive Hodgkin's Lymphoma
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
FGF	Foetal growth factor
GANT	GLI-antagonist
GAS1	Growth arrest specific protein 1
GBM	Glioblastoma multiforme
GC	Gastric carcinoma
GCB	Gemcitabine
GCOS	GeneChip operating software
GCV	Valganciclovir

GLIA	GLI transcription factor activator form
GLIR	GLI transcription factor repressor form
GSK3β	Glycogen synthase kinase 3β
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HERV	Human endogenous retrovirus
hESC	Human embryonic stem cell
HH	Hedgehog
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HMTV	Human mammary tumour virus
HNSCC	Head and neck squamous cell carcinoma
HOK	Human oral keratinocytes
HPV	Human papilloma virus
HSC	Hematopoietic stem cell
HSV	Human herpes simplex virus
HTLV-1	Human T-cell lymphoma virus, type 1
IE	Immediate-early
IHC	Immunohistochemistry/Immunohistochemical
IHH	Indian hedgehog ligand
IM	Infectious mononucleosis
ISH	*in situ* hybridisation
IVL	Involucrin
JAK	Janus kinases
JCV	JC virus
JNK	c-Jun N-terminal kinase
kb	kilo base
KRT	Keratin
KS	Kaposi-Sarcoma
KSHV	Kaposi-Sarcoma associated virus
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LMPC	Laser microdissected purified cells
LOR	Loricrin
LRP	Low density lipoprotein receptor-related protein
MAPK	Mitogen activated protein kinase
MCV	Merkel cell virus
MEK	MAPK kinase 1
MHC	Major histocompatibility complex
mi-	micro
MYC	Myelocytomatosis viral oncogene
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLK	Nemo-like kinase
non-SCID	Non obese diabetic/severe combined immunodeficient
NPC	Nasopharyngeal carcinoma
NSC	Neural stem cell
OCT4	Octamer binding protein 4

OHL	Oral hairy leukoplakia
P75NTR	P75 neurotrophin receptor
PCR	Polymerase chain reaction
PI3K	Phosphoinositol-3 kinase
PKC	Protein kinase C
PLC	Phospholipase C
PML	Promyelocytic leukaemia
PTCH	Patched receptor
PTEN	Phosphatase and tensin homolog
PTLD	Post-transplant lymphoproliferative disease
QPCR	Semi-quantitative PCR
rEBV	Recombinant wild type EBV
RMA	Robust multi-array average
RNA	Ribonucleic acid
RP	Rank product
RSV	Rous sarcoma virus
RT-PCR	Reverse transcription-PCR
NSCLC	Non-small cell lung carcinoma
SAPK	SAP kinase
SHH	Sonic hedgehog ligand
siRNA	small interfering RNA
SMO	Smoothened receptor
SP	Side population
ss-	single stranded
STAT	Signal transducer and activator of transcription
SUFU	Suppressor of fused
SV40	Simian virus 40
T/NK	T/natural killer
TA	Transit amplifying
TGFβ	Tumour growth factor beta
TPA	12-O-tetradecanoylphorbol-13-acetate
TTV	Torque teno virus
UCNT	Undifferentiated carcinoma of the nasopharyngeal type
VPA	Valproic acid
WHO	World health organisation
XMRV	Xenotropic murine leukaemia virus-related virus

CHAPTER ONE: INTRODUCTION

"As ideas are preserved and communicated by means of words, it necessarily follows that we cannot improve the language of any science, without at the same time improving the science itself; neither can we, on the other hand, improve a science without improving the language or nomenclature which belongs to it."

— Antoine-Laurent Lavoisier

Elements of Chemistry (1790), trans. R. Kerr, Preface, xiv-v.

1.1 Introduction to cancer

The earliest evidence of human cancer is found in 1.5 million year old hominid remains (Capasso, 2005). Whilst examples of neoplasms are rare in ancient populations, early Egyptian mummification has preserved cases of what has been interpreted as nasopharyngeal carcinoma (NPC), a disease which is the subject of this thesis (Strouhal, 1978). It is estimated that approximately 15% of all malignancies endemic to Dynastic Egypt were NPC, a cancer linked to Epstein-Barr virus (EBV) infection and exposure to chemical carcinogens, factors that are strongly associated with the development of this disease today (Capasso and Mariani-Costantini, 1994, Capasso, 2005). There are written accounts of possible cancerous tumours and growths from many ancient cultures worldwide, dating from a few centuries BC back to the Babylonian *Code of Hammurabi* (1750 BC) (Javier and Butel, 2008, David and Zimmerman, 2010). However, our scientific understanding of cancer as a disease only began during the early 19th century. The discovery that infectious agents, in particular viruses, were linked to the development of certain types of cancer led ultimately to the discovery of the first human DNA tumour virus, Epstein-Barr virus (EBV), in the 1960s (Epstein et al., 1965).

1.2 The biology of cancer

Cancer is a disease of deregulated cell growth caused by the accumulation of mutations in the cellular genome. These events are generated through heritable changes in DNA sequence or epigenetic modification, a process that involves post-translational modification of histone proteins and gene silencing through DNA methylation. Alterations in expression of non-coding RNAs, or cellular microRNAs, also regulate gene expression (Sandoval and Esteller, 2012). Collectively, these

changes alter the expression of genes regulating cell growth and differentiation, the consequence of which is aberrant or uncontrolled cell proliferation. There are over 200 types of cancer and tumour subtypes identified in humans alone, with tumours falling into five categories: carcinoma, sarcoma, leukaemia, lymphoma and myeloma. The discovery of oncogenes and tumour suppressor genes, which, when mutated or inactivated, lead to a dominant gain or a suppressive loss of function respectively, has increased our understanding of the mechanisms involved in deregulated cell proliferation (Vogelstein and Kinzler, 2004). It is now well established that multiple genetic alterations are responsible for the acquisition of a fully malignant phenotype. This multi-step process is supported by the observation that cancer incidence does not have a linear relationship with age. Many cancers display an age dependent increase in incidence that would suggest the presence of around five rate-limiting steps which must be overcome before malignant transformation is achieved (Miller, 1980, Stephens et al., 2012). In their 2000 review, Hanahan and Weinberg identify six classic cellular processes whose dysregulation is required for cell transformation and ultimately malignant progression. Cells must achieve self-sufficiency in growth signals and insensitivity to anti-growth signals; they must be able to evade apoptosis; acquire limitless reproductive potential; invade tissue and become metastatic, and hijack adipogenesis to provide sufficient nutrients for continued cell growth (Hanahan and Weinberg, 2000). Additional hallmarks are now known to include deregulation of cellular genes and the ability to avoid immune destruction, whilst genome instability and mutation, and tumour-promoting inflammation and microenvironment can also promote oncogenic progression (Hanahan and Weinberg, 2011). The acquisition of such mutations and characteristics often occurs spontaneously and sequentially, allowing cells with a malignant phenotype to develop over time.

1.3 The role of infectious agents in cancer

The link between infectious agents and cancer has been appreciated for over a 100 years. Chronic infection with certain strains of mycoplasma, bacteria and viruses has been associated with increased cancer risk (Huang et al., 2001, Mager, 2006, Talbot and Crawford, 2004). Persistent infection with low virulence mycoplasmas, such as *Mycoplasma penetrans* and *Mycoplasma fermentans*, is associated with significant alterations in host cell gene expression and malignant transformation (Tsai et al., 1995). Similarly, infection with certain strains of bacteria is linked to increased cancer risk, presumably as a result of chronic inflammation and the production of bacterial-derived metabolites that act as carcinogens. Chronic inflammation and its association with a predisposition to cancer development was first observed by Virchow (1858b), who suggested chronic irritation could give rise to cellular proliferation and the development of cancer (Virchow, 1863). Chronic inflammation gives rise to an environment that inhibits apoptosis, induces angiogenesis and compromises cell mediated immunity, which is believed to be a pre-requisite for malignant transformation (Macarthur et al., 2004). However, the role of inflammation in the development of cancer is not clear cut, and acute inflammation has been seen to cause tumour regression. Observations by Bruns, in 1866, of an erysipelas bacterial infection that cured multiple skin sarcomas led to experiments to show artificial erysipelas bacterial infection of sarcoma and carcinoma could cause tumour regression (Bruns, 1988, Fehleisen, 1882). Similarly Coley et al., (1991) found that repeated infection of malignant tumours with erysipelas bacteria lead to tumour regression. Despite these findings it is still recognised that inflammation can be tumour promoting, and the recruitment of inflammatory cells can induce cancer cell proliferation, invasion and angiogenesis (Grivennikov et al., 2010).

1.4 Viruses and cancer

The link between viruses and cancer was first described by Ellermann and Bang (1908) following the discovery that filtered extracts from chicken leukaemia cells could pass the disease to healthy non-infected chickens. Similarly, work by Peyton Rous in 1909 showed that cell free filtrates purified from chicken fibrosarcomas, were able to produce sarcomas when injected into healthy chickens (Rous, 1911). It was later discovered that the filterable agent responsible was an RNA retrovirus, subsequently named Rous sarcoma virus (RSV). The viral transforming gene responsible for promoting sarcoma development (Martin et al., 1979) was subsequently identified as v-Src*,* a constitutively active form of the human c-Src proto-oncogene (Martin, 2004). This discovery led to the identification of cellular oncogenes, as it was later discovered that many acutely transforming retroviruses had acquired cellular proto-oncogenes involved in growth regulation.

The discovery of RSV led to the identification of the first mammalian tumour viruses in the 1950s. For a timeline of oncogenic virus discovery please see Figure 1. 1. However, it was not until 1960s that the first human tumour virus was discovered by Tony Epstein and Yvonne Barr following the visualisation of herpesvirus-like particles in a cell line derived from a Burkitt's Lymphoma (BL), reviewed by (Javier and Butel, 2008). Human tumour viruses are now known to include the double stranded DNA (dsDNA) viruses Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Kaposi-sarcoma associated virus (KSHV), high risk Human papillomaviruses (HPV), and the RNA retrovirus human T-cell lymphoma virus type I (HTLV-I). Viruses that are associated with human cancer are summarised in Table 1. 2 (adapted from McLaughlin-Drubin and Munger, 2008). Collectively, these viruses are responsible for an estimated 10-

20% of cancer incidence worldwide (zur Hausen, 1991, Eckhart, 1998, Kalland et al., 2009). Table 1. 1 shows the infection-attributed cancer incidence of 2008. For a recent review on human oncogenic viruses see (Moore and Chang, 2010).

Table 1. 1 Infection-attributed cancer incidence (2008)

(modified from de Martel et al., 2012)

Agent	**Developing countries (%)**	**Developed countries (%)**	**World (%)**
Hepatitis B virus (HBV)/ Hepatitis C virus (HCV)	32.0	19.4	29.5
Human papillomavirus (HPV)	30.2	29.2	30.0
H. pylori	28.9	46.2	32.5
Epstein-Barr virus (EBV)	5.9	3.9	5.4
Kaposi sarcoma herpesvirus (KSHV)	2.4	1.0	2.1
Human T cell lymphotropic virus 1 (HTVL-1)	0.0	0.4	0.1
Liver flukes	0.1	0.0	0.1
Schistosomes (bladder)	0.4	0.0	0.3
Total (n)	1.6M	0.41M	2M
Total for all new cases (%)	**22.9**	**7.4**	**16.1**

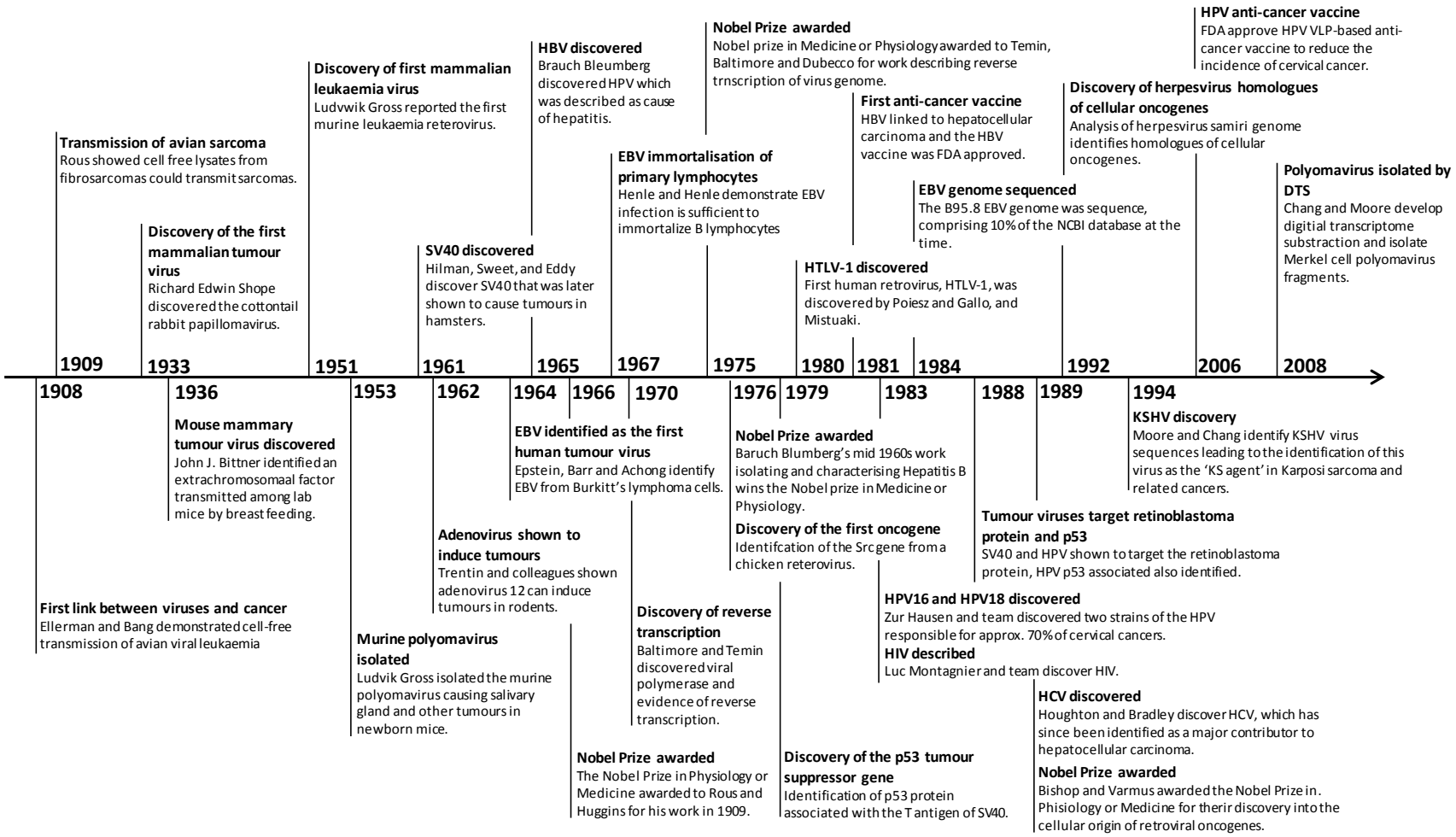


Figure 1. 1 Timeline of oncogenic virus discovery showing key events in human oncogenic virus research

Key date in the discovery of oncogenic viruses relating to human cancer. Abbreviations used SV40; Simian Virus 40, EBV; Epstein-Barr Virus, HBV; Herpes B Virus, HTLV-1; Human T-cell Lymphotropic Virus, HPV; Human Papilloma Virus, HCV; Herpes C Virus, KSHV; Kaposi Sarcoma Herpesvirus.

Adapted from Moore and Chang (2010)

Table 1. 2 Human tumour viruses

(adapted from McLaughlin-Drubin and Munger, 2008)

Virus	Family	Genome	Human Tumours
HUMAN ONCOGENIC VIRUSES			
Hepatitis C virus (HCV)	*Flaviviridae*	dsRNA	Hepatocellular carcinoma
Hepatitis B virus (HBV)	*Hepadnaviridae*	Partially dsDNA	Hepatocellular carcinoma
Epstein-Barr virus (EBV)	*Herpesviridae*	dsDNA	Nasopharyngeal carcinoma (NPC), gastric carcinoma (EBV-aGC), Burkitt's, Immunoblastic Hodgkin's, post-transplant and NK/T-cell lymphomas
Kaposi's Sarcoma herpesvirus (KSHV)	*Herpesviridae*	dsDNA	Kaposi's sarcoma (KS), primary effusion lymphoma, Castleman's disease
Human papillomavirus (HPV)	*Papillomaviridae*	dsDNA	Cervical, oropharynx and other anogenital cancers
Merkel cell virus (MCV)	*Papillomaviridae*	dsDNA	Skin cancer
Human T cell lymphotropic virus 1 (HTLV-1)	*Retroviridae*	ssRNA	Adult T-cell leukaemia (ATL)
Human immunodeficiency virus (HIV-1)	*Retroviridae*	ssRNA	Indirectly associated with: B-cell lymphoma, Kaposi's sarcoma

1.4.1 Viruses implicated in cancer

Whilst oncogenic viruses which express viral oncogenes can contribute directly to carcinogenesis, there are a number of viruses that may contribute indirectly to oncogenic transformation. For instance, cutaneous HPV types have been implicated in the aetiology of skin cancer but there is insufficient mechanistic evidence suggesting that they are directly carcinogenic (Bouvard et al., 2009). Human immunodeficiency virus (HIV) causes CD4+ T cell depletion which contributes to increased cancer risk due to decreased immune surveillance (Tuttleton et al., 2011).

In the polyomaviridae family, the ubiquitous and wide spread JC virus (JCV) and BK virus (BKV) have been implicated in the aetiology of human cancer; however, their role in carcinogenesis is still debated (Sarid and Gao, 2011). Similarly a potential

indirect role for retroviruses in human cancer has been proposed. Human endogenous retroviruses (HERVs) have been reported to contribute to a range of cancers, including breast, ovarian, prostate and melanoma, whilst the human mammary tumour virus (HMTV) has been implicated in the development of breast cancer (McLaughlin-Drubin and Munger, 2008). While current knowledge suggests the contribution of these viruses to human cancer is indirect, the future discovery of viral oncogenes within cancer cells may point toward a direct role for these viruses, and others, in cancer.

1.4.2 DNA and RNA viruses

DNA and RNA viruses are classified into seven categories in the Baltimore classification system (Baltimore, 1971). The Baltimore classification and examples of human viruses in each group are shown in Table 1. 3. There are 21 different families of virus that have vertebrate hosts. This variety and the sheer number of different viruses that populate the viral family and sub-family classes demonstrate the success of these infectious agents.

Table 1. 3. The Baltimore classification system including examples of human viruses

Group	Type	Example: *Family* e.g. Genus – species
I	dsDNA viruses	***Herpesviridae*** e.g. Lymphocryptovirus - human herpesvirus 4 (a.k.a. Epstein-Barr virus; EBV)
II	ssDNA viruses	***Parvoviridae*** e.g. Erythrovirus - parvovirus B19
III	dsRNA viruses	***Reoviridae*** e.g. Orthoreovirus – mammalian reovirus
IV	Positive sense ssRNA viruses	***Flaviviridae*** e.g. Hepacivirus - hepatitis C virus
V	Negative sense ssRNA viruses	***Paramyxoviridae*** e.g. Morbillivirus – measles virus
VI	Reverse Transcribing RNA viruses	***Retroviridae*** e.g. Lentivirus - human immunodeficiency virus 1
VII	Reverse Transcribing DNA viruses	***Hepadnaviridae*** e.g. Orthohepadnavirus - hepatitis B virus

1.4.3 RNA tumour viruses

RNA viruses, such as HCV, RSV and murine leukaemia virus, mostly contain a single stranded (ss) RNA sequence; however, many possess double stranded genomes. ssRNA viruses can be classified as positive sense, negative sense or ambisense

whereas dsRNA possess both sense and antisense strands (Nguyen and Haenni, 2003). Positive sense viruses have RNA that can be immediately translated into protein whereas negative sense viral RNA has to first be transcribed to positive sense (often by a viral encoded RNA polymerase), ambisense viral RNA has regions of both positive and negative sense. Retroviruses also possess a ssRNA genome, however, these viruses are not classed as ssRNA viruses by the Baltimore classification system as they replicate following integration of viral DNA, produced by a viral reverse transcriptase, into the host genome (Coffin, 1992).

RNA viruses are associated with a number of human tumours. HCV is a positive ssRNA flavivirus that has been associated with some lymphomas and hepatocellular carcinomas (Choo et al., 1989), whilst HTLV-I, a positive ssRNA retrovirus, is associated with adult T-cell leukaemia (ATL) (Poiesz et al., 1980).

1.4.4 DNA tumour viruses

The DNA tumour viruses contain viral genomes in the form of DNA. As with the RNA viruses there are dsDNA, ssDNA and reverse transcribing DNA viruses (King et al., 2011). DNA viruses are most often double stranded, replicating using a DNA-dependant DNA polymerase. Like reverse transcribing RNA viruses, which replicate through a DNA intermediate, there exist DNA viruses that replicate through an RNA intermediate. These viruses, including HBV, are classified in a separate group, VII, by the Baltimore classification system (Baltimore, 1971).

DNA viruses that have been associated with malignant transformation include high-risk HPV, EBV, KSHV, and HBV (Inman et al., 2001).

1.4.5 Herpesviruses family

The *Herpesviridae* is a large family of DNA viruses that infect vertebrate hosts, and are responsible for both human and animal disease. Within this family there are three subfamilies: alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae.

The alphaherpersvirinae includes the Simplexvirus and Varicellovirus genera. Examples of viruses in this subfamily are the human herpes simplex virus (HSV)-1, HSV-2 and varicella zoster virus. These viruses are neurotropic, replicate in the musocal epithelium, and establish latency in the sensory ganglia of the nervous system (reviewed by Smith, 2012). The betaherpesvirinae genera are the cytomegalovirus, roseolovirus, and muromegalovirus. Viruses in this subfamily include human herpesvirus (HHV)-6 and HHV-7, and murine cytomegavirus (CMV) and establish latency in the secretory glands (Chen and Hudnall, 2006). The gammaherpesvirinae genera are lymphocryptovirus and rhadinovirus. Viruses in this last subfamily include EBV, Herpesvirus saimari (HVS), and KSHV (Damania, 2004). Oncogenic herpesviruses, and the human disease with which they are associated, can be found in Table 1. 2.

1.5 Epstein-Barr virus (EBV)

EBV was identified in 1964 (Epstein et al., 1964), following the visualisation of a novel herpesvirus-like particle in an electron micrograph of lymphoid cells grown from

a Burkitt's Lymphoma (BL) (Epstein et al., 1965, Henle and Henle, 1966). EBV was later associated with other malignancies such as nasopharyngeal carcinoma (NPC) where, like endemic BL, NPC patients showed elevated antibodies to immediate early and late EBV antigens (Henle et al., 1970). The consistent presence of EBV DNA in the neoplastic cells of both BL and NPC implicates a causative role for EBV in the pathogenesis of both tumours (zur Hausen et al., 1970, Klein et al., 1974).

Establishment of EBV as a human tumour inducing virus involved experiments on marmoset and owl monkeys in which inoculation with EBV was sufficient to induce lymphoma in some animals (Epstein et al., 1973, Shope et al., 1973). Additionally, EBV is associated with a number of other human malignancies including Hodgkin's lymphoma (HL), lymphoproliferative disorders in immunocompromised individuals, T-cell lymphomas and EBV-associated gastric carcinoma (EBV-aGC) (Okano, 1998).

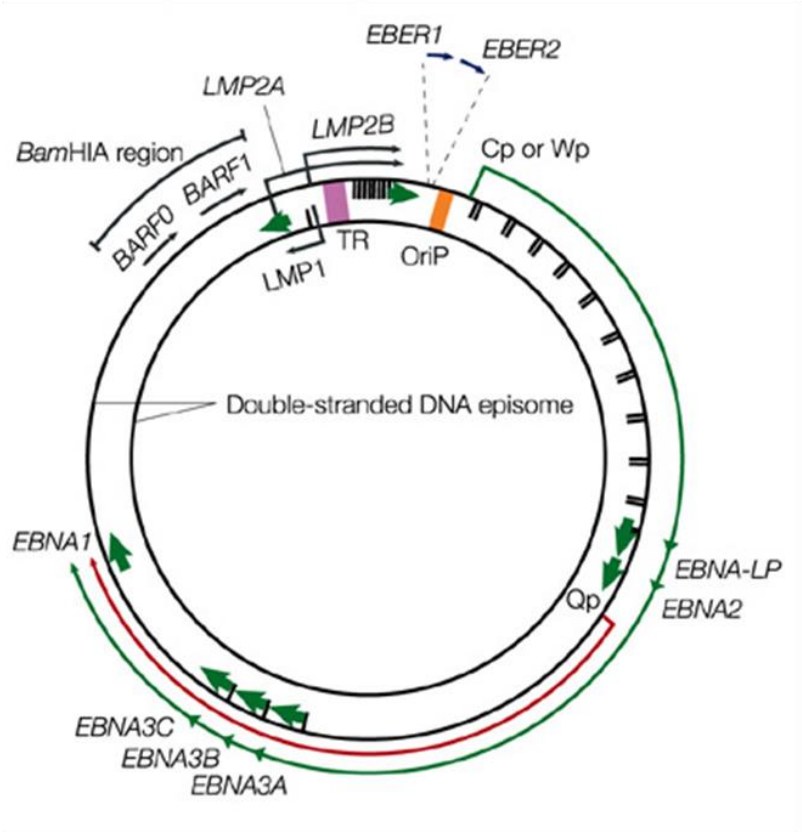
1.5.1 The EBV genome

The EBV genome is a linear, double-stranded 172kb DNA which encodes nearly 100 viral genes (Kieff and Rickinson, 2007). Upon release from the EBV capsid in the host nucleus (Pellet and Roizman, 2007) a covalently closed episome is generated through fusion of terminal repeat (TR) sequences (Adams and Lindahl, 1975, Kintner and Sugden, 1979, Raab-Traub and Flynn, 1986). The linear form of EBV DNA has variable numbers of these direct tandem 0.5kb repeats at each terminus, as well as 3kb internal repeat sequences which in combination serve to effectively divide the genome into unique long and short regions (Hayward et al., 1980). There are two major EBV types, Type-1 (prevalent worldwide) and Type-2 (common in parts of

Africa), which are distinguished by their EBV nuclear antigen 2 (EBNA2) sequences (Tzellos and Farrell, 2012).

The prototype EBV strain B95-8, which has a 11.8kb deletions compared to other EBV stains (Parker et al., 1990), was the first human herpesvirus genome to be fully cloned and sequenced (Baer et al., 1984), and regions of the EBV genome were designated by their position on a *Bam*HI restriction endonuclease map (Murray and Young, 2001) (Figure 1. 2). The expression pattern of EBV genes in NPC, and the role of expressed latent proteins in the manipulation and dysregulation of cellular signalling are covered later in this chapter.

A.



B.

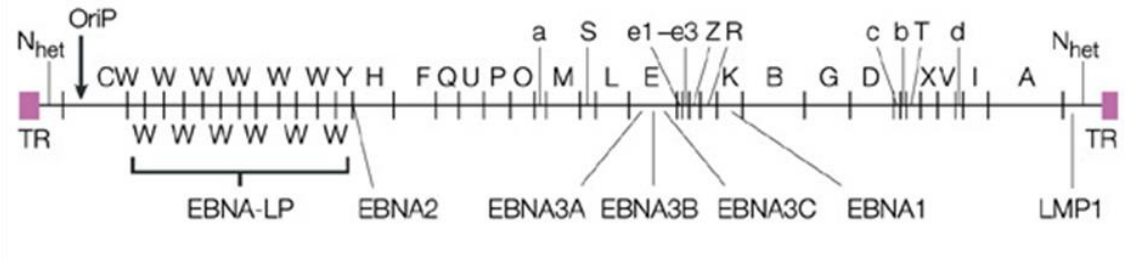


Figure 1. 2. The EBV Genome
(A) Showing the EBV dsDNA episome. Exons encoding EBV latent proteins are marked, under respective promoters, with direction of transcription. The terminal repeat region (pink) is required from LMP2 gene transcription and is generated through re-circularisation of the EBV genome upon terminal repeat fusion. *OriP* (orange) is the origin of EBV replication. (B) B95-8 EBV prototype *Bam*HI restriction endonuclease map showing the location of the EBV latent proteins open reading frames. Fragments labelled A-Z in descending size order.

Figure from: Young, L.S. & Rickinson, A.B. (2004)

1.5.2 EBV biology

EBV is a ubiquitous virus infecting the vast majority (greater than 90%) of the world's population asymptomatically (Babcock et al., 1998, Cohen, 2000). Infection of naïve individuals occurs early in childhood and is usually asymptomatic; however, infection in developed countries often occurs following early adolescence and is associated with symptomatic disease presenting with clinical features of infectious mononucleosis (IM) (Vetsika and Callan, 2004).

As with other herpesviruses, EBV demonstrates a biphasic life cycle, adopting both lytic and latent forms of infection. Latent infection in B-cells is complex and linked to the state of B-cell differentiation. To date, at least four latency states have been identified (Young and Rickinson, 2004). In latently infected memory B-cells, the site of long-term persistence, expression of viral transcripts is severely restricted to the non-coding RNAs; the so-called Latency 0 program. The lack of expression of EBV protein products allows the virus to evade the host immune system (Rickinson and Kieff, 2007). Viral latency states are discussed in section 1.7. Replication of the virus requires lytic induction with the expression of over 80 viral protein products that leads to the production and release of new, infectious viral particles. This lytic induction appears in both the B-cells and epithelial cells (Kieff and Rickinson, 2007); indeed, there is evidence that the epithelial tissue is EBV's natural site of replication, occurring even in healthy individuals, and is believed to have a role in virus amplification prior to shedding (Stetler-Stevenson et al., 1997). Peripheral infected B lymphocytes will undergo periodic reactivation when circulating through the oropharynx producing new virions, by amplifying the genome more than 100-fold (Contreras-Brodin et al., 1991). These virions are shed into the saliva, allowing for

the spreading of infection through person-to-person contact (Kieff and Rickinson, 2007). The transmission of EBV through the saliva, as the main vehicle for transferral, is followed by a symptomatic disease in naïve individuals which is affectionately known as “The Kissing Disease”. EBV can also be transmitted via organ and bone marrow transplantation (Cen et al., 1991, Haque et al., 1996, Teira et al., 2006).

Lytic replication is induced by the simultaneous expression of BamHI Z fragment leftward open reading frame 1 (BZLF1) and BamHI R fragment leftward open reading frame 1 (BRLF1), two immediate-early (IE) genes that encode transcription factors Zta and Rta respectively (Baer et al., 1984). These two transactivators are both required for lytic DNA replication; however, they can both induce lytic reactivation (Feederle et al., 2000). Lytic induction triggers the temporal, and ordered cascade of viral gene expression; early (E) genes, required for viral DNA replication and nucleotide metabolism, and subsequent late (L) viral genes, which support viral DNA amplification and produce the structural components required for viron packaging (Pellet and Roizman, 2007, Kieff and Rickinson, 2007).

The induction of lytic replication appears to be differentiation dependent, induced *in vivo* in B lymphocytes by the differentiation of memory cells into plasma cells (Laichalk and Thorley-Lawson, 2005) and in squamous epithelial cells, where virus replication is linked to the keratinocyte differentiation programme. Indeed, *in situ* hybridisation (ISH) for EBV DNA in oral hairy leukoplakia (OHL), a benign hyperproliferative disease caused by opportunistic EBV infection in immunosuppressed acquired immunodeficiency syndrome (AIDS) and post-

transplant patients, is found only in the upper, terminally differentiated layers of the tongue (Niedobitek et al., 1991). Furthermore, OHL demonstrated differentiation associated BZLF1 expression (Young et al., 1991). *In vitro* studies using the differentiation-competent SCC12F cell line showed that the BZLF1 promoter was found to contain an differentiation-specific response element that was activated in response to terminal differentiation (Karimi et al., 1995).

1.6 EBV infection of B-cells and epithelial cells

1.6.1 EBV infection of B-cells *in vitro*

Infection of B-cells and epithelial cells *in vitro* occurs through distinct mechanisms. EBV infection of B-cells requires binding of the major capsid glycoprotein gp350, to the known EBV receptor CR2 (CD21) on the B-cell surface. Membrane fusion is then initiated through the binding of gp42 to major histocompatibility complex (MHC) class II molecules (Iizasa et al., 2012).

Much of our understanding of the biology of EBV relates to its interaction with B-lymphocytes. Indeed, the B-lymphotropic nature of EBV is borne out by findings that EBV can readily infect and transform normal resting B-lymphocytes *in vitro* into lymphoblastoid cell lines (LCLs), which are capable of indefinite proliferative growth (Henderson et al., 1977, Miller, 1984).

1.6.2 EBV infection of epithelial cells *in vitro*

Whilst the generation of EBV infected B-lymphocytes is a relatively easy process, infection of epithelial cells in culture is problematic, attributed in part to the lack of the

known EBV receptor, CR2 (CD21) (Nemerow et al., 1985). In contrast to B-cells, epithelial cell infection appears to be gp350/220 independent and therefore does not require CR2. Studies with cell free infectious virus show infection requires the binding of BMRF2 to cell surface integrins; binding of gH/gL to αvβ5 initiates fusion with the plasma membrane. Interestingly, gp42 actually impedes epithelial cell infection (reviewed in Odumade et al., 2011). Whether the same molecules play a role in B-cell to epithelial cell "transfer" infection is currently unknown.

However, epithelial cells stably expressing CR2, although amenable to infection, quickly lose the virus, perhaps reflecting a propensity of this cell type to adopt a lytic rather than a latent form of infection *in vitro* (Knox et al., 1996, Li et al., 1992). In recent years it has become possible to stably infect a range of epithelial cell lines with recombinant forms of EBV (rEBV) through co-cultivation with Akata BL cells replicating EBV or primary B-cells coated with EBV particles (Imai et al., 1998, Shannon-Lowe et al., 2006). However, the fact that only a small proportion of cells are amenable to infection, whilst most lose the EBV genome upon cell line generation, suggests that only a sub-population of cells can support a stable latent infection (Li et al., 1992). These findings support the existence of distinct populations of cells within these cell lines, which differ in their biological properties relating to their ability to support stable latent infection (Li et al., 1992). Furthermore isolated EBV infected clones have been shown to be slowly cycling and fail to respond to inducers of terminal differentiation (Tsao et al., 2012).

Additional methods of infection are likely to exist *in vivo*; studies involving EBV infection of gastric carcinoma cell lines, which are negative for the CR2 receptor,

showed these cells are amenable to infection through a mechanism independent of CR2 (Yoshiyama et al., 1997). It was thus discovered that cell-cell contact has an important role in the infection and spread of the virus in epithelial cells. Studies by the Takada lab, in 1998, showed that co-cultivation of epithelial cells with recombinant EBV producing B-cells, produced low level infection at rates roughly 800 times that of non-co-cultivated cells. However, few of these acutely infected cells could give rise to EBV positive cell lines and of the cell lines produced only one showed NPC-like latency II viral protein expression whilst the other showed LCL-like latency expression program III (Imai et al., 1998).

It has been postulated that EBV may only be able to establish a stable latent infection in an undifferentiated or more primitive epithelial cell environment. This is supported by studies showing that only undifferentiated NPC or adenocarcinoma cell lines give rise to stable EBV infection highlighting the need for an undifferentiated cellular environment for the establishment of persistent latent infection (Knox et al., 1996, Tsao et al., 2012). NPC tumour cells are found to express B-cell lymphoma 2 (BCL2), ΔNp63 and B lymphoma Mo-MLV insertion region 1 homolog (BMI1) (Lu et al., 1993, Song et al., 2006, Fotheringham et al., 2010), a phenotype reminiscent of the cells residing in the basal layer of stratified squamous epithelium. It has thus been hypothesised that the population of cells originally establishing a latent infection in undifferentiated NPC, and gastric carcinoma, are either the adult stem cell pool or cells that harbour a stem cell/basal cell like phenotype. The possibility that stem cells could undergo malignant transformation into tumour initiators in cancer goes partway to explaining the activity of diverse developmental signalling pathways, increased DNA repair and ABC transporter-mediated drug efflux. It would seem possible that

instead of acquiring these attributes over time, slowly converting a differentiated cell towards a phenotype more commonly associated with stem cells, the generation of oncogenic potential may happen in a cell which already has these properties.

1.6.2.1 EBV infection and persistence *in vivo*

To establish long-term latency in the infected host, EBV has evolved a strategy to exploit the B-cell differentiation programme. EBV adopts a number of latency programmes which facilitate entry of EBV-infected B-cells into the long-lived memory B-cell pool; the site of long-term persistence (Babcock and Thorley-Lawson, 2000).

During initial infection, virus enters lymphoid tissue within the Waldeyers ring (pharyngeal lymphoid tissue), where it crosses the surface epithelium to infect naïve B-cells, activating them into proliferating blasts through the expression of the so-called “growth program”. This latency program (Latency III), is associated with the expression of 10 viral genes, which include two non-translated RNAs; EBV encoded RNA 1 and 2 (EBER1/2), six nuclear proteins; EBNA1, 2, 3A, 3B, 3C, LP, three membrane proteins; latent membrane protein 1, 2A and 2B (LMP1, LMP2A/2B), BamH1-A reading frame 1 (BARF1) early lytic gene and a family of microRNAs encoded within the BamH reading frame 1 (BHRF1) and BamA rightward transcripts (BARTs) (Riley et al., 2012). Transit of these blasts into the germinal centre allows cells to undergo a germinal centre reaction and the antigen-driven somatic hypermutation of their immunoglobulin genes. In the germinal centre, latently infected cells switch to the so-called “default program” where viral gene expression is restricted to EBNA1, LMP1, LMP2A/2B and expression of the non-coding EBER1/2

and BHRF1 and BART miRNAs. Upon exiting the germinal centre as memory B-cells, latently infected cells either express EBNA1 and the non-coding EBER1/2, and BHRF1 and BART miRNAs (the EBNA1 only programme) or the non-coding EBER1/2, and BHRF1 and BART miRNAs in the absence of any viral proteins (Latency 0). The latter is considered to be the site of long-term persistence as these latently infected cells are quiescent and invisible to the immune system. A proportion of latently infected B-cells are induced into lytic cycle in response to terminal differentiation signals, with cells differentiating into plasma cells. At this stage, transient infection of squamous pharyngeal epithelium is thought to occur, amplifying the release of infectious virus. Whether, latent infection of epithelium plays a role in EBV persistence is still unknown (reviewed by Thorley-Lawson and Gross, 2004). The EBV latency programs and the gene products expressed in these states are shown in Table 1. 2 (Young and Rickinson, 2004). Also included are the known EBV-associated malignancies and the latency states adopted in these cancers.

Table 1. 4. Types of EBV latency and associated malignancies

Latency Type	Latent Products Expressed	Associated Malignancies
I	EBNA1 expression mediated through the Qp promoter, EBERs, and BART RNAs	Burkitt's lymphoma
II Default	EBNA1 (Qp), LMP1, LMP2A, LMP2B , EBERs, and BART RNAs	Nasopharyngeal Carcinoma, Hodgkin's and EBV-positive T-cell lymphomas
III Growth	EBNA1-6 mediated through Wp/Cp, LMP1, LMP2A, LMP2B, and EBERs.	Post-transplant lymphoproliferative disorders
0	EBERs and BART RNAs. Perhaps additional expression of LMP2 and EBNA1	Proposed to occur in peripheral resting memory B-cells

1.7 Latency programs in EBV-associated malignancies

The latency program adopted in EBV-associated malignancies is most likely dictated by the state of cell differentiation and controlled by tissue and/or cell-type-specific transcription factors. With the exception of post-transplant lymphoproliferative disorder (PTLD), all known EBV-associated malignancies display a restricted form of EBV latency, with the majority displaying a Latency II or default latency program. The latency I program is characteristic of endemic BL, where viral gene expression is limited to Qp promoter-driven expression of EBNA1, and expression of EBER1/2 and BHRF1 and BART-encoded miRNAs. Latency II, also referred to as the default programme, is characteristic of NPC, EBV associated gastric carcinoma (EBV-aGC), Hodgkin's disease and certain EBV-positive T-cell lymphomas. Here viral gene expression is restricted to Qp-driven EBNA1 and variable expression of LMP1 and/or LMP2A/2B and expression of EBER1/2 and BHRF1 and BART-encoded miRNAs. The latency III, or growth programme, as exemplified *in vitro* by LCLs, is also observed in PTLD. Here, the full complement of latent genes are expressed, with Cp/Wp-driven EBNA1, 2, 3A, 3B, 3C and LP, high levels of LMP1 and LMP2A/2B, and expression of EBER1/2, and BHRF1 and BART-encoded miRNAs. Whilst these classifications work as a general rule of thumb there are exceptions; an additional "Wp-restricted" latency has been reported in a subset of BL tumours, which harbour an EBNA2 deletion (Kelly et al., 2002).

The function of products seen in a type II latency program, the expression profile which is associated with NPC, will be discussed later in this chapter.

1.7.1 EBV associated lymphoma malignancies

EBV infection is implicated in the aetiology of B-cell lymphomas; BL, Hodgkin's lymphoma (HL), PTLD and immunoblastic lymphoma, and an T/NK-cell malignancy that is caused by the expansion of transformed EBV-positive T-cells (Epstein et al., 1964, Ziegler et al., 1982, Weiss et al., 1989, Shapiro et al., 1988, Jones et al., 1988).

1.7.1.1 Burkitt's lymphoma (BL)

EBV was first discovered in a case of BL, a lymphatic cancer (Epstein et al., 1964). BL is a non-Hodgkin's lymphoma which is classified into three forms: endemic, sporadic and AIDS-associated. The endemic form is seen in equatorial Africa and displays a strong association with EBV infection and the geographical distribution of malaria. Endemic BL shows typical symptoms of rapid aggressive swelling of the jaw and facial bone. The sporadic form is less associated with EBV, between 10-20% are EBV positive, and more commonly involves distal ileum, cecum, ovaries, kidney or the breast (Epstein and Achong, 1979). BL is also associated with HIV infection, the AIDS-associated form of the disease that occurs in the early stages of HIV immunosuppression (Beral et al., 1991), as well as acute lymphoblastic leukaemia, and is seen in post-transplant patients owing to an impaired immune system. All forms of BL contain a chromosomal translocation that results in oncogenic cMYC deregulation (Rickinson and Kieff, 2007, Gutierrez et al., 1992). Around 70-80% of patients demonstrate the hallmark t(8;14)(q24;q32) translocation causing the juxtaposition of the *MYC* gene to enhancer element of the heavy chain immunoglobulin gene. Other translocations seen in this disease, t(2;8)(p:12;q24) and

t(8;22)(q24;q11), place *MYC* under the kappa and lambda light chain enhancer element control respectively (reviewed in Molyneux et al., 2012).

1.7.1.2 Hodgkin's lymphoma (HL)

HL can be classified as one of four subtypes of which one, the mixed-cellularity subtype – a common form of this disease – is strongly associated with EBV infection. In 1966, MacMohon first proposed an infective aetiology for this disease (MacMahon, 1966). Whilst the mechanism behind how EBV gives rise to oncogenic transformation in this disease is not fully understood, it is now known that EBV positive HL accounts for between 20-50% of cases in Europe and Northern America, and a substantially higher percentage of cases occurring in underdeveloped countries. Indeed, up to 100% EBV positivity has been reported in tumours from populations within Kenya and Peru. Hodgkin's lymphoma and its association with EBV are reviewed by (Flavell and Murray, 2000).

1.7.1.3 Post-transplantation lymphoproliferative disorders (PTLDs)

EBV is also associated with lymphomas which arise in immunosuppressed individuals as a result of impaired EBV-specific cellular immune responses. Most PTLDs are B-cell lymphoproliferative disorders associated with EBV infection; however rare T-cell lymphoproliferations, that are less commonly associated with EBV, are occasionally reported after renal transplantation (Frias et al., 2000). Approximately 90% of all PLTDs are reported to be EBV positive (EBV-PTLD), which demonstrate a type III latency program, expressing all EBV latent gene products, that is also seen in lymphoblastoid B-cell lines, and lymphomas occurring as a result of

congenital immunodeficiency or acquired immunodeficiency syndrome (AIDS) (Gottschalk et al., 2005).

1.7.1.4 T and natural killer cell lymphomas

EBV is primarily a B lymphotropic virus; however, it is also capable of infecting a number of other cell types including T-cells and epithelial cells. Whilst EBV is associated with a number of T-cell lymphomas, it is thought to be play an important role in the aetiology of peripheral angioimmunoblastic T-cell lymphoma (AITL) and extra nodal nasal type T/NK-cell lymphoma (Carbone et al., 2008). AITL is the most common peripheral T-cell lymphoma, accounting for around 1-2% of all non-Hodgkin's lymphomas (Jarrett et al., 2006), displaying EBV positive B-lymphocytes and B immunoblasts, and rarely non-neoplastic T-cells. EBV infection is seen only in a sub-population of cells in this lymphoma, and as such is believed to occur subsequent to oncogenic transformation, or is not required by and lost from malignant cells (Carbone et al., 2008). Nasal type T/NK-cell lymphoma are found in the nasal and upper aerodigestive passage and are strongly associated with EBV infection, demonstrating an NPC-like latency gene expression pattern and increased expression of EBV-induced genes (Chiang et al., 1996a, Huang et al., 2010b).

1.7.2 EBV associated epithelial malignancies

Whilst the association of EBV infection with tumours of lymphoid tissue is well-established, the virus is also implicated in the aetiology of certain epithelial tumours; namely, undifferentiated NPC, a proportion of diffuse gastric adenocarcinoma (EBV-aGC) and a subset of rare lymphoepithelia-like carcinomas (LELC) of the

salivary gland, lung, thymus, gastric and intra-hepatic biliary epithelium (Hippocrate et al., 2011). However, unlike the situation in B lymphocytes, comparatively little known about the interaction between EBV and normal epithelial cells or the role that EBV plays in the transformation of these epithelial cell types.

It is postulated that the establishment of latent EBV infection in epithelial tissue may promote malignant transformation (Herbst and Niedobitek, 2006). Whilst there is an obvious association of EBV with NPC, the links between EBV and gastric cancer, salivary gland and Lymphoepithelial-like carcinomas (LELC) are much weaker. Whilst both NPC and EBV-aGC carry monoclonal viral genomes (Raab-Traub and Flynn, 1986, Imai et al., 1994), suggesting that EBV infection must have occurred prior to the expansion of the final malignant clone, the stage at which EBV participates in the carcinogenic process is currently unknown but is discussed in the context of NPC in the following section.

1.7.2.1 Nasopharyngeal Carcinoma (NPC)

NPC is a tumour of the mucosal epithelium of the nasopharynx (Regaud, 1921, Schmincke, 1921) that is associated with a number of genetic and dietary factors (e.g. preserved foods that are high in nitrosamines) (Yu and Yuan, 2002). While endemic to parts of South-East Asia and China, NPC displays medium incidence rates in parts of North Africa, the Mediterranean basin and Greenland, and overall has low worldwide incidence (Yu and Yuan, 2002) as seen in Figure 1. 3. It is estimated that 84,400 new cases of NPC occurred in 2008 with 51,600 deaths

(mortality to incidence ratio of 0.61) representing 0.7% of the global cancer burden and making NPC the 24th most common cancer worldwide (Jemal et al., 2011).

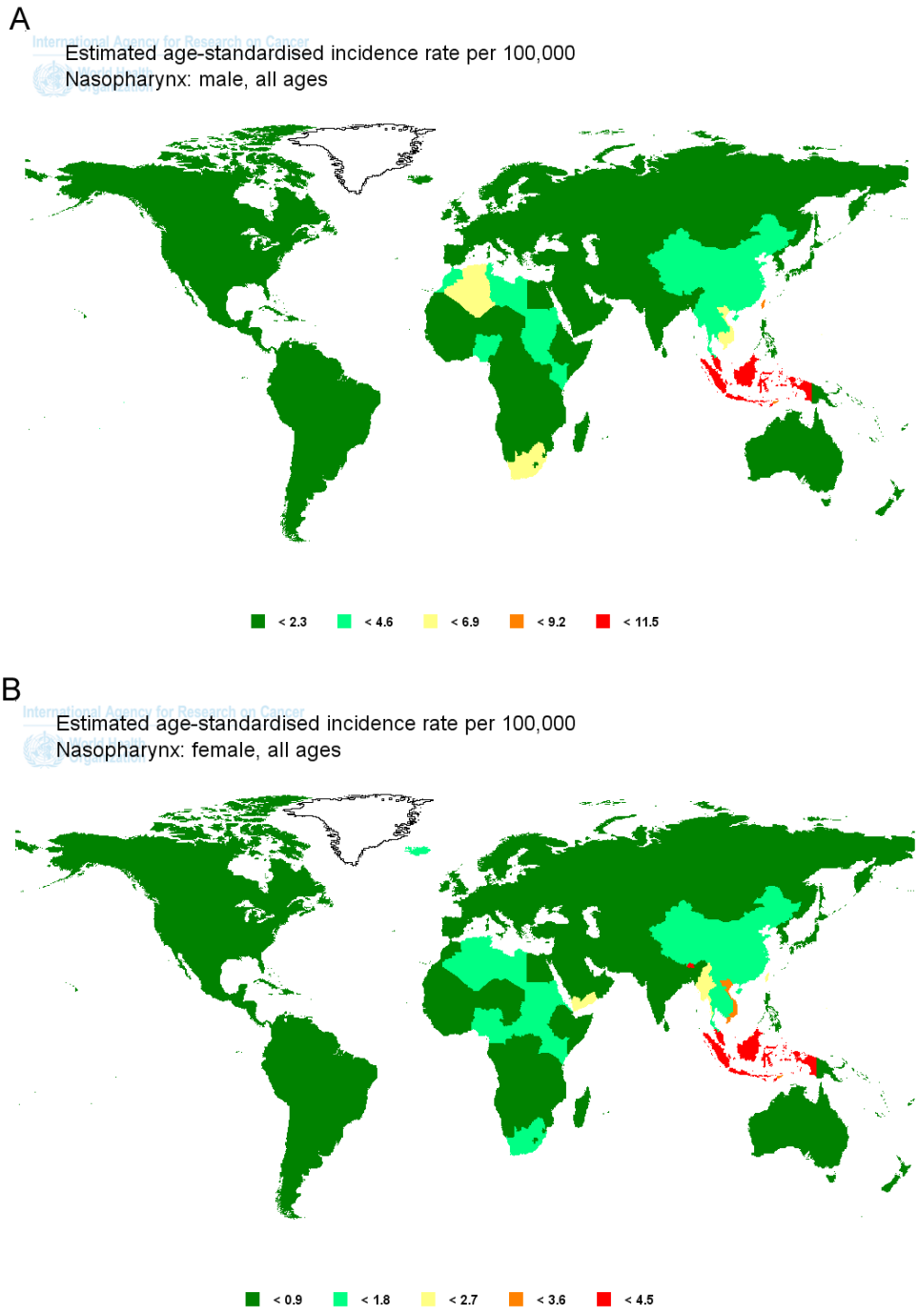


Figure 1. 3. Global NPC incidence
The GLOBOCAN2008 maps for NPC incidence in (A) male and (B) female.
From Sun et al., (2011)

There are two main types of NPC as defined by the World Health Organisation (WHO); keratinising squamous cell carcinoma (Type I) and non–keratinising squamous cell carcinoma (differentiated; Type II, and undifferentiated; Type III) (Wei et al., 2011). It is non-keratinising Type II and III that show a strong association with EBV latent infection; EBV is not generally associated with Type I NPC (Neel et al., 1983, Pathmanathan et al., 1995).

NPC was first associated with EBV following the finding that the high antibody titres to the EBV-associated immediate early/late structural antigens are seen in serological studies from BL patients were also present in individuals with NPC (Old et al., 1966, Henle et al., 1970, Henle and Henle, 1976). This association was later confirmed with the discovery of clonal EBV genomic DNA in the neoplastic cells of NPC (Wolf et al., 1975, Andersson-Anvret et al., 1977). Furthermore, latent EBV infection, as detected by *in situ* hybridisation to the viral EBER RNAs, has been demonstrated in almost 100% of type II and type III tumours (Raab-Traub et al., 1987). Latent infection with EBV is postulated to play a critical function in the aetiology of non-keratinising NPC, with tumour cells showing the presence of monoclonal EBV episomes indicating that EBV infection must have occurred prior to expansion of the final malignant clone (Raab-Traub and Flynn, 1986, Raab-Traub, 2002). EBV is not normally found in histologically normal nasopharyngeal epithelium (Sam et al., 1993) and is also absent from the abundant lymphomal infiltrate seen in Type III undifferentiated NPC.

Undifferentiated carcinomas that show similar characteristics to Type III NPC exist as carcinomas of the tonsils, lungs, thymus, stomach, skin and cervix. These carcinomas are referred to as undifferentiated carcinomas of nasopharyngeal type (UCNT). EBV association with other UCNTs has been investigated and, whilst undifferentiated stomas carcinomas show a high degree of EBV infection (Shibata et al., 1991), other UCNTs are not seen to be consistently EBV positive (Fujii et al., 1993, Raab-Traub et al., 1991, Weinberg et al., 1993, Dadmanesh et al., 2001).

The role of EBV in the pathogenesis of NPC is still being elucidated; disease progression involves multiple mutational and epigenetic events, seen to occur frequently at progressive stages of the disease (reviewed by Lo, et al., 2013). These events can be seen in Figure 1. 4 (Young and Rickinson, 2004) where it is proposed that EBV latent infection occurs in a low-grade pre-invasive lesion that will support EBV latent infection, and leads to further cell deregulation and ultimately NPC.

Genome wide alleleotyping has identified allelic deletions of numerous chromosomes implicated in NPC pathogenesis; of these, 3p, 9p and 14p are detected in 85% of primary tumours (reviewed by Lo et al., 2012). Tree models investigating the order of transformational events in NPC have identified the 3p deletion and chromosome 12 gain as early event in NPC pathogenesis whilst also finding evidence that two pathways to cellular dysregulation may exist; the first involving loss of 9p and 13q with the gain of 1q, and the second loss of 14q, 16q, 9q and 1p (Shih-Hsin Wu, 2006). Allelic imbalances allow the inactivation of tumour suppressor genes and overexpression of oncogenes; for instance commonly deleted regions 9p21 and 3p21.3 contain tumour suppressors p15, p16 INK4/ARF and DAPK1, and RASSF1A

and RARβ2 genes respectively, whilst overexpression of cyclinD1, which is present in over 90% of NPC primary tumours, can be achieved through amplification of 11q13.3 (Lo et al., 2012).

Epigenetic inactivation of the RASSF1A and p16 tumour suppressors is also achieved through hypermethylation allowing progression towards dysplastic nasopharyngeal epithelium. Indeed, inactivation of p16 or overexpression of cyclinD1 is believed to be required for cell proliferation and establishment of a stable latent EBV infection (Tsang et al., 2012). Hypermethylation of other genes including CHRF, RIZ1, WIFI, TIG1, THY1 and many more have been reported in NPC, of which the former three, in conjunction with p16 and RASSF1A hypermethylation, have been proposed as a means of early NPC detection (Hutajulu et al., 2011).

Other early modifications that contribute to the initial establishment of a low-grade pre-invasive phenotype include BCL2 overexpression, telomerase dysfunction and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) activation. Whilst this cell signalling activation occurs via a 12p13.3 amplicon, further cell signalling dysregulation occurs following EBV infection with LMP1 and LMP2A driven proliferation and transformation (Lo et al., 2012). Figure 1. 4 shows the current theory behind NPC pathogenesis detailing the key events in oncogenic transformation.

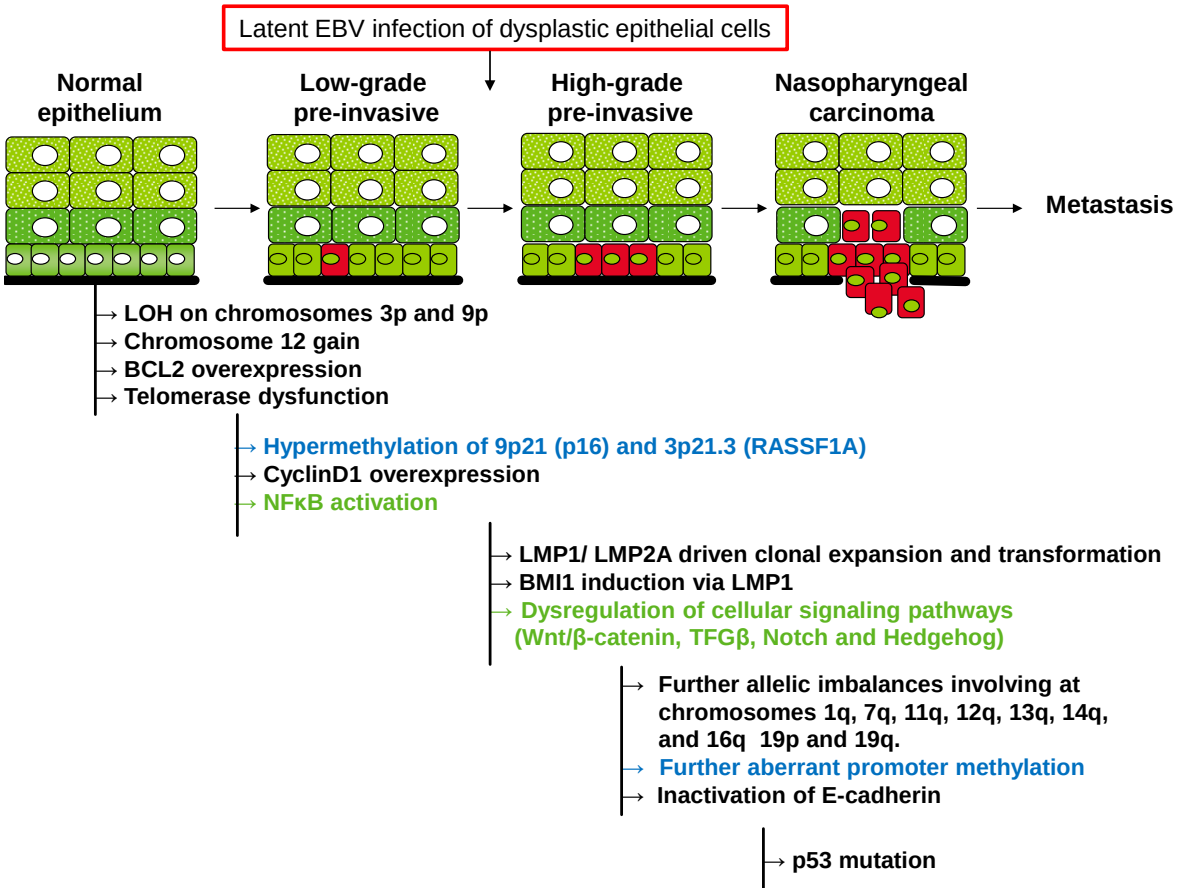


Figure 1. 4. Schematic showing the current theory behind NPC pathogenesis
Multiple mutational and epigenetic modifications accompany EBV infection and cell signalling dysregulation in the onset of NPC. Mutational modifications include chromosomal imbalancing, the mutation of cell cycle modulators, cyclinD1 and p53, and other cell maintenance proteins, BCL2 and E-cadherin, and loss of telomerase function. Epigenetics modifications (presented in blue), involving the hypermethylation of gene promoter regions, and the dysregulation in multiple cell signalling pathways (green) is also observed.

Adapted from Young and Rickinson (2004) and Lo, Chung and To (2012)

1.7.2.2 EBV-associated Gastric Carcinoma (EBV-aGC)

Like NPC, gastric carcinoma (GC) is an epithelial malignancy that is frequently associated with EBV. It was estimated that 989,600 new cases of stomach cancer occurred in 2008, representing 8% of global cancer incidence and 10% of total deaths (mortality to incidence ratio 0.75) (Jemal et al., 2011). Whilst EBV positive gastric carcinoma (EBV-aGC) has been reported to account for around 10% of worldwide GC incidence (Strong et al., 2013), the number of cases of GC per year means there is a greater incidence of EBV-aGC than NPC.

EBV-aGC is a non-endemic disease, with incidence throughout the world, but like EBV positive NPC EBV-aGC shows substantial geographic variation thought to be caused by ethnic and genetic differences (Lee et al., 2009, Murphy et al., 2009). The highest proportion of EBV-aGC to non EBV-aGC occurs in Germany and the USA, with 18% and 16% respectively (Takada, 2000). EBV-aGC was originally proposed to display the same latency II program as NPC; however, early studies were unable to demonstrate LMP1 expression, as such, viral gene expression was proposed to more closely represent a latency I program, as observed in Burkitt's lymphoma, with occasional, additional expression of LMP2A at low levels (Imai et al., 1994, Sugiura et al., 1996). A more recent study of EBV expression in EBV-aGC was able to detect very low expression of LMP1, in addition to LMP2 and other EBV latent genes, and identified EBV positivity in 17% of 71 GC cases (Strong et al., 2013). Additionally this study identified two distinct forms of EBV infection; less than 200 EBV reads per sample and more than 30,000 EBV reads per sample, which the authors suggest should be considered functionally different in relation to therapeutic intervention and clinical responsiveness (Strong et al., 2013). Further regard for the EBV status of GC

is required to direct therapeutic intervention for EBV-aGC as a separate disease to EBV negative GC. EBV-aGC are more commonly hypermethylated, and demethylating agent inducing EBV lytic replication may result in the lysis of stable infected GC cells (for a review see Iizasa et al., 2012).

1.7.2.3 Lymphoepithelioma-like carcinomas (LELCs)

In addition to NPC and gastric cancer, EBV is also linked to the development of LELCs of the salivary gland, lung, thymus, and intra-hepatic biliary epithelium (Hippocrate et al., 2011). LELCs have histopathological features similar to undifferentiated NPC, but the low incidence of LELC at sites other than the nasopharynx and gastric epithelium means the EBV status and viral contribution to these cancers is still being investigated. Interestingly the EBV positivity of LELCs around the body appears to differ greatly; in a study of 32 cases of lung LELC in southern China 94% showed EBER positivity, compared to 0% of non-LELC lung cancers, suggesting a causal link between EBV and the disease (Han et al., 2000), whereas EBV *in situ* hybridisation was negative in all 35 cases of urinary tract LELCs (Mori et al., 2013). In breast cancer EBV positivity has been reported in between 10-51% of tumour samples but with less than one in a thousand cells harbouring EBV the contribution of the virus to disease progression, if any, is likely to be very different from that seen in NPC or EBV-aGC (Hippocrate et al., 2011).

1.8 EBV gene expression and the pathogenesis of NPC and EBV-aGC

Unlike B-cells, EBV infection of epithelial cells is not associated with growth transformation. It is now generally accepted that epithelial cell infection with EBV is

associated with lytic replication and the loss of the viral genome. This is exemplified in studies on OHL *in vivo* and EBV: epithelial-cell infection systems *in vitro* (Greenspan et al., 1985, Tsao et al., 2012). It has been postulated that NPC or EBV-aGC development occurs as a result of EBV infection of initiated or pre-neoplastic epithelia cells (Greenspan et al., 1985), which are impaired in their ability to differentiate. While evidence for this is still controversial, *in vitro* studies show that stable, latently EBV-infected clones of squamous differentiation-competent SV40-immortalised keratinocytes have lost their ability to differentiate (Li et al., 1992, Knox et al., 1996).

Like other EBV-associated malignancies, with the exception of PTLD, viral gene expression is restricted in both lymphoid (BL, HL, T/NK) and epithelial malignancies (NPC, EBV-aGC), with tumours displaying a latency II or default programme. In this form of latency, viral gene expression is restricted to EBNA1, LMP1, LMP2A/2B, BARF1 and the non-coding EBER1/2 and BART-derived miRNAs. Numerous *in vivo* and *in vitro* studies have shown that all of these gene products can impact on epithelial cell biology (Frappier, 2012, Dawson et al., 2012, Takada, 2012, Lung et al., 2013). The biological properties of these proteins and RNAs are discussed in the following section.

1.9 EBV latent proteins

1.9.1 Latent membrane protein 1 (LMP1)

LMP1 is an approximately 62kDa, six membrane spanning, integral membrane protein that localises to lipid raft domains within the plasma membrane and to intracellular endosomes. LMP1 possesses a short cytoplasmic N-terminal and longer, 200 amino acid, cytoplasmic C-terminal containing three functional signalling domains (CTAR1, CTAR2, and CTAR3) (the structure of LMP1 is reviewed in Meckes Jr and Raab-Traub, 2011). The structure and signalling domains of LMP1, and signalling proteins that engage with the dimerised form of this EBV latent protein are depicted in Figure 1. 5 (reproduced from Dawson et al., 2012).

LMP1 was the first EBV latent protein found to possess oncogenic properties, transforming established rodent fibroblast cell lines *in vitro* and rendering them tumourigenic *in vivo* (Wang et al., 1985). LMP1 plays a critical role in B-cell transformation, as naive B-cells infected with a recombinant rEBV deleted for LMP1 are not transformed *in vitro* (Kaye et al., 1993). And, with the exception of endemic BL, most EBV positive B-cell cancers express LMP1 (Young et al., 1989). This contrasts with NPC where expression appears to be both variable and heterogeneous (Tsao et al., 2002). Whilst early RT-PCR-based studies identified expression of LMP1 at the mRNA level in between 80-92% of NPC samples (Brooks et al., 1992; Chen et al., 1995), immunohistochemical (IHC) staining studies did not corroborate these findings, identifying expression of LMP1 at the protein level in anywhere between 20–100% of cases. Here, LMP1 expression can be uniform or patchy, and display low, intermediate or high levels of expression (Khabir et al.,

2005, Chen et al., 2010). More recent studies support these early observations and show that LMP1 expression is extremely variable, in the range of 20-65% of samples (Lo et al., 2013, Chen et al., 2012). These findings have raised questions regarding the role that LMP1 plays in the pathogenesis of NPC

LMP1 has pleiotropic effects when expressed in epithelial cells, inducing morphological change, a blockade in epithelial cell differentiation, blocking apoptosis, promoting migration and angiogenesis, reducing growth factor requirement, and altering cell cycle regulation, all of which can contribute to malignant transformation (Dawson et al., 1990, Lo et al., 2003, Everly et al., 2004, Dawson et al., 2008). LMP1 has also been shown to induce cancer stem cell (CSC)-like characteristics and an epithelial-mesenchymal transition (EMT) in NPC cell lines (Kondo et al., 2011, Horikawa et al., 2011), and contributes to genomic instability by inhibiting DNA repair (Gruhne et al., 2009).

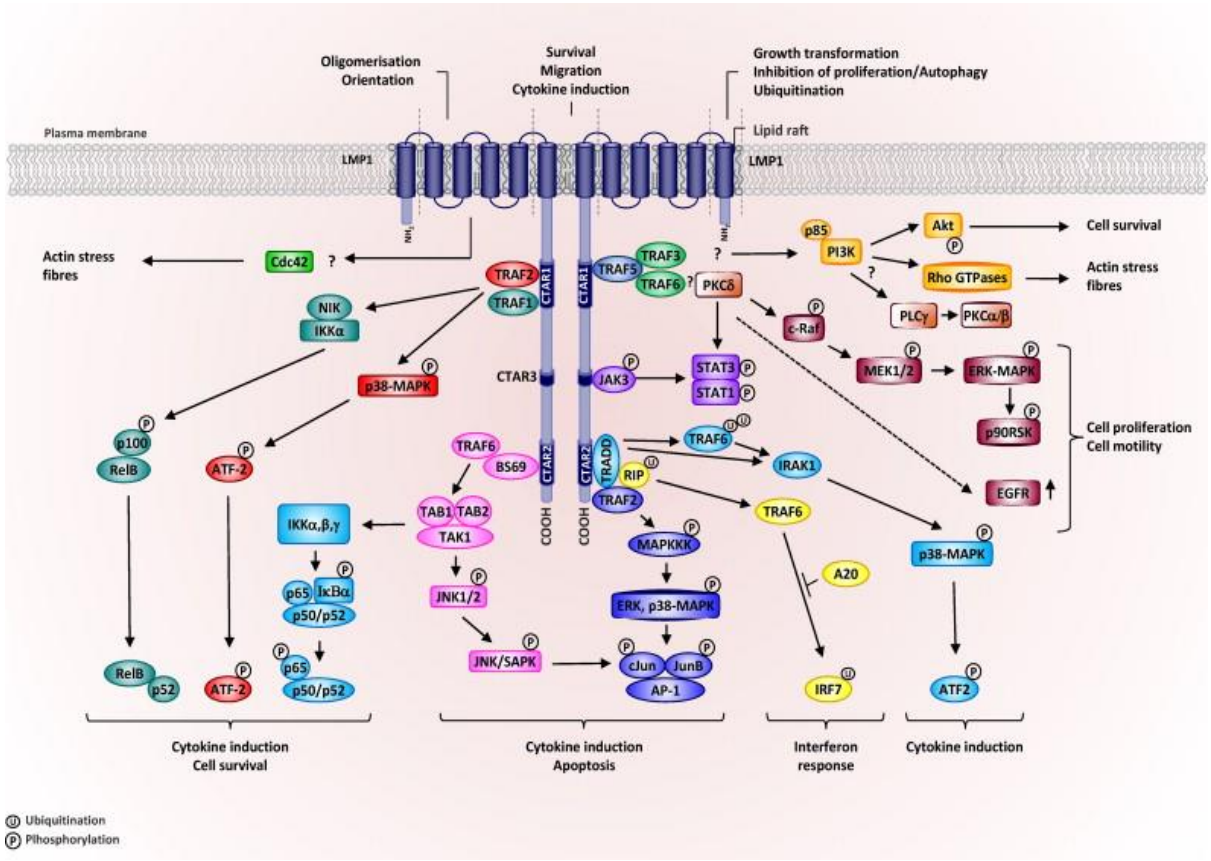


Figure 1. 5. Activation of cell signalling pathways by LMP1
Signalling is achieved through three carboxyl terminal signalling domains, CTAR1, CTAR2 and CTAR3. Recruitment of signalling adapter proteins (TRAF, TRADD, RIP), and BS69 and Janus kinase (JAK)-3 proteins allows activation of the NFκB, JNK/SAPK, PI3K/Akt, ERK-MAPK, PLC/PKC and JAK/STAT signalling pathways. Signalling activation impacts on cell phenotype by influencing cell proliferation, survival, motility and invasion.

Figure reproduced from Dawson et al., (2012)

These effects are achieved through activation of a multitude of cell signalling pathway that include NFκB, c-Jun N-terminal kinase/SAP kinase (JNK/SAPK), phosphatidyl inositol-3 kinase (PI3K)/Akt, extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK), phospholipase C/protein kinase C (PLC/PKC) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) (see Figure 1. 5). Collectively, activation of these pathways is required for various facets of LMP1-induced effects. These signalling pathways are also seen to be deregulated in other cancers of epithelial origin as discussed later in this chapter.

1.9.2 Latent membrane protein 2 (LMP2)

The LMP2 gene codes two mRNA products, LMP2A and LMP2B, initiated from two different promoters (Sample et al., 1989). These two proteins are highly similar in their structure containing 12 hydrophobic transmembrane domains, and a cytoplasmic C-terminal domain. Additionally, LMP2A possesses an N-terminal cytoplasmic domain, which is not present in LMP2B, containing motifs that bind tyrosine kinases Lyn, Syk, and the ubiquitin ligases Nedd4/Itchy (Dawson et al., 2012). Original studies demonstrated that LMP2A acts to maintain viral latency by modulating signalling from the B-cell receptor (BCR) (Miller et al., 1994, Miller et al., 1995). While LMP2B may also possess intrinsic signalling capabilities, it is currently thought to act as a competitive regulator of LMP2A (Rovedo and Longnecker, 2007).

Like LMP1, LMP2A has been shown to act on multiple signalling pathways (Figure 1. 6 reproduced from Dawson et al., 2012), and in carcinoma cells can activate

PI3K/Akt pathway and modulate STAT and NFκB signalling pathways (Scholle et al., 2000, Stewart et al., 2004). The consequences of LMP2 expression in epithelial tissues range from inhibition of cell differentiation (Fotheringham et al., 2010) and increased migration (Allen et al., 2005, Fotheringham et al., 2012) to the induction of an epithelial to mesenchymal transition (EMT) in NPC cells (Kong et al., 2010). The role of LMP1 and LMP2 in the pathogenesis of NPC is reviewed in (Dawson et al., 2012).

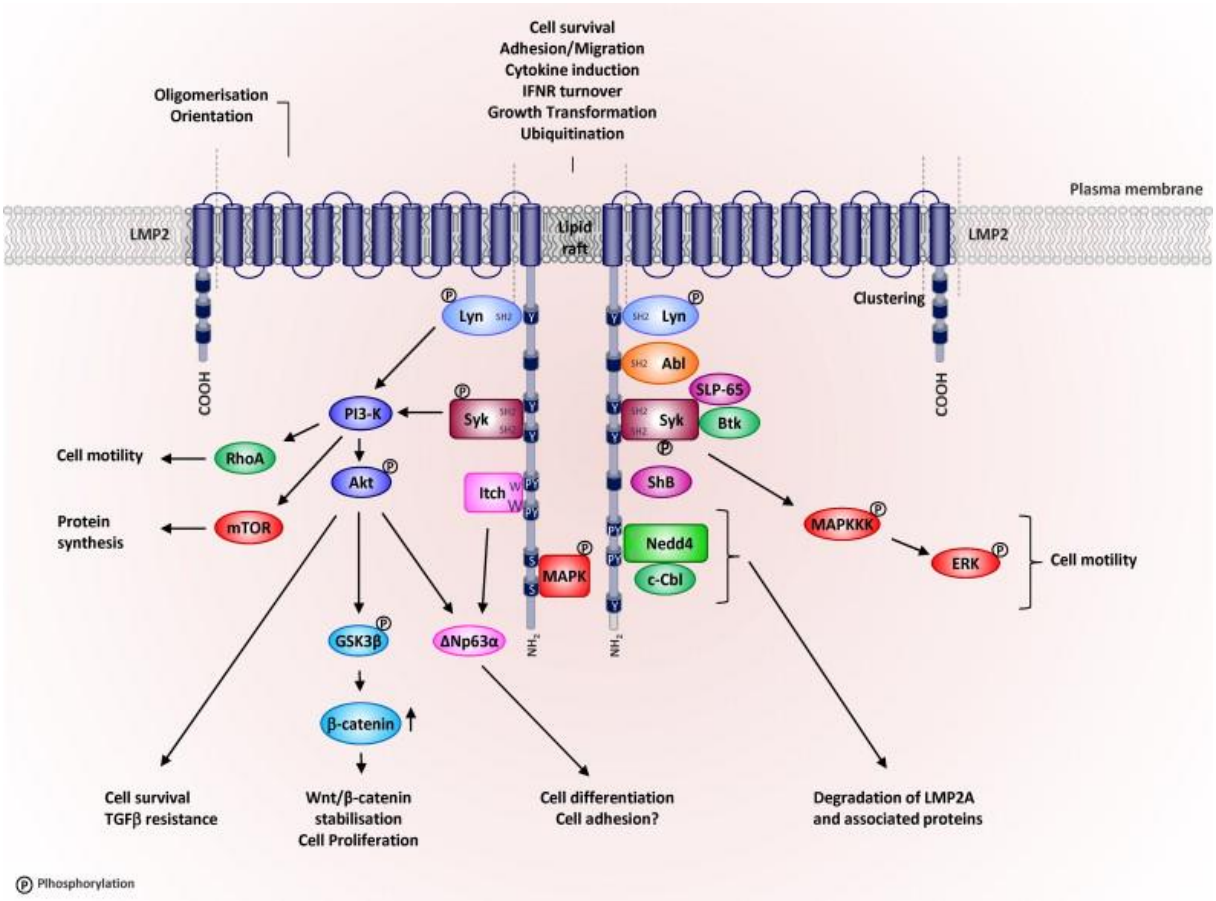


Figure 1. 6. Activation of cell signalling pathways by LMP2
Signalling occurs through motifs in the amino terminus of LMP2A contains which bind tyrosine kinases Lyn, Syk, and the ubiquitin ligases Nedd4/Itchy. Activation of PI3K/Akt, JNK/SAPK, ERK-MAPK and WNT/β-catenin signalling pathways impacts on cell phenotype; influencing cell growth, inhibiting apoptosis and differentiation, and contributing transformation.

Figure reproduced from Dawson et al., (2012)

1.9.3 Epstein-Barr virus nuclear antigen 1 (EBNA1)

EBNA1 is a 641 amino acid nuclear phosphoprotein containing a glycine-alanine repeat region (Fischer et al., 1997), nuclear localisation sequence and DNA binding/dimerisation domain (Ambinder et al., 1991). EBNA1 transcription is driven from three viral promoters; Cp/Wp, Qp and Fp. The Wp promoter drives EBNA1 expression upon initial infection B-cell infection, and is also active in a latency III transcription programme. In latency I and II programmes EBNA1 transcripts are initiated from the Qp promoter (Bakos et al., 2007) as the Cp promoter is silenced (Chen et al., 1999). The Fp promoter has been reported to drive EBNA1 expression upon entry into lytic cycle (Zetterberg et al., 1999).

Like LMP1 and LMP2, EBNA1 is also seen to influence cell signalling pathways; modulating transforming growth factor β (TGFβ) and STAT1 signalling (Flavell et al., 2008, Wood et al., 2007), inhibiting NFκB (Valentine et al., 2010), and destabilising p53 leading to cell immortalisation (Holowaty et al., 2003, Sheng et al., 2006). The immortalisation of EBV positive lymphomas is also promoted by EBNA1, via oxidative stress induced telomere dysfunction (Kamranvar and Masucci, 2011).

EBNA1 influences cell biology through a number of mechanisms; by promoting tumourigenicity and chemotherapy resistance (Cheng et al., 2010), inducing metastasis (Kaul et al., 2007), disrupting PML bodies (Sivachandran et al., 2008, Sivachandran et al., 2010) and, in B-cells, conferring resistance to apoptosis (Lu et al., 2011) and enhancing migration (Murakami et al., 2005). EBNA1 also promotes genomic instability and, like LMP1, has been demonstrated to induce reactive oxygen

species in BL (Gruhne et al., 2009). EBNA1 additionally regulates viral Cp and LMP1 promoters (Guha et al., 1990), and is required for the maintenance and replication of the EBV genome (Gregory et al., 1990).

1.9.4 EBV-encoded RNAs (EBERs)

EBERs are small non-coding non-polyadenylated RNAs that are expressed in all forms of EBV latency (Takada and Asuka, 2001). Although not essential for B cell transformation and viral replication in vitro (Takada, 2012), the EBER RNAs are thought to modulate innate immune responses (Takada, 2012). Due to their abundant expression, in-situ hybridisation (ISH) of the EBERs is commonly used as an indicator of stable latent infection with EBV (Takada, 2012). High resolution ISH has suggested a close association with the Golgi apparatus and rough endoplasmic reticulum (Takada, 2012).

There are two EBER RNAs; EBER1 (167bp) and EBER2 (172bp), which have similar tertiary structures comprising multiple short stem-loops (Takada, 2012). Both EBER1 and EBER2 are thought to contribute to the malignant phenotype of EBV lymphoid and epithelial cancers through modulation of innate immunity and induction of pro-inflammatory cytokines (Iwakiri and Takada, 2010; Mrinal and Takada, 2010; reviewed by Takada, 2012). Indeed EBER is seen to induce type-1 interferons and activate NF-κB signalling. EBER also has a role conferring resistance to IFN and FAS mediated apoptosis and inhibiting PKR phosphorylation of eLF-2α so blocking its antiviral effects (Takada, 2012).

1.9.5 The EBV-encoded micro-RNAs (miRNAs)

EBV has been found to encode 25 EBV miRNA precursors and 48 mature miRNAs from two transcripts; BHRF1 and the BamHI-A rightward transcripts (BARTs). Whilst the BHRF1-encoded miRNAs are restricted to B-lymphocytes (Pfeffer et al., 2004), BART miRNAs are detected in all types of latent infection and are highly expressed in NPC and EBV-aGC (Qiu et al., 2011). The BHRF1 transcript encodes the three miRNA precursors; ebv-miR-BHRF1-1, -2 and -3 that produce 4 mature miRNAs, and the BART region encodes the remaining 22 miRNA precursors producing 44 mature miRNAs. These mature BART miRNAs are produce from two clusters; cluster 1 located between BART exon I and IB encodes ebv-miR-BART1, -BART3 to 6, and –BART15 to 17, and cluster 2 located between exon IV and V encodes ebv-miR-BART2-3p and 2-5p (Zhu et al., 2009, Lung et al., 2009, Cai et al., 2006, Grundhoff et al., 2006, Pfeffer et al., 2004).

Recently it has become apparent that BHRF1 and BART miRNAs have a role in modulating cellular phenotype by targeting both cellular and viral genes. For instance, ebv-miR-BART22, which is highly expressed in NPC, targets LMP2A and has a role in immune evasion (Lung et al., 2009), whilst ebv-miR-BART5 modulates the expression of p53 up-regulated modulator of apoptosis (PUMA) promoting apoptosis resistance (Choy et al., 2008). Viral and cellular targets of the BART miRNAs, and associated cellular consequences, can be found in Table 1. 5.

Table 1. 5. BART miRNA viral and cellular targets and cellular consequence

Modified from Lung et al., 2013.

Target	miRNA	Cellular consequence
BALF5 DNA polymerase	BART2-5p v-snoRNA^{22pp}	Maintain latency
BHRF1 DNA polymerase	BART10-3p	Unknown
LMP1 Signalling protein	BART1-5p, 16, 17-5p BART19-5p, 5 BART9	Anti-apoptotic Immune evasion Promotes NK/T cell growth
LMP2A Signalling protein	BART22	Immune evasion
APC WNT inhibitor	BART19-3p, 7, 17-5p	Pro-oncogenic
Bim Proapoptotic protein	BART cluster 1	Anti-apoptotic
BRUCE Anti-apoptotic protein	BART15	Pro-apoptotic
CASP3 Proapoptotic protein	BART1-3p, 16	Anti-apoptotic
CAPRIN2 WNT-signalling enhancer	BART13-3p	Anti-apoptotic
DICE1 Tumour suppressor	BART3	Pro-oncogenic
DICER miRNA synthesis	BART6-5p	Unknown
IPO7 Nuclear importer	BART3, BART16	Immune evasion
MICB NK cell ligand	BART2-5p	Immune evasion
NLK WNT inhibitor	WNT inhibitor	Pro-oncogenic
NLPR3 Inflammation regulator	BART15	Immune evasion
PUMA Proapoptotic protein	BART5-5p	Anti-apoptotic
T-bet T-box transcription factor	BART20-5p	Anti-apoptotic
TOMM22 Mitochondrial Bax proapoptic receptor	BART16	Anti-apoptotic
WIF1 WNT inhibitor	BART19-3p	Pro-oncogenic

1.9.6 BamH1-A reading frame-1 (BARF1)

BARF1 located in BamHI-A fragment of the EBV genome and was originally identified as an early lytic protein being restricted to the lytic replication in B-cells (Lu et al., 2006). BARF1 is highly expressed in NPC and EBV-aGC tumours in the absence of spontaneous lytic induction (Takada, 2012). Although identified as a viral homologue of CSF1, BARF1 has been shown to possess oncogenic and immunomodulatory properties in epithelial cells and B-cells, and can immortalise fibroblasts and activate anti-apoptotic BCL2 production (Hoebe et al., 2012).

1.10 The cancer stem cell hypothesis

1.10.1 Stem cells

Stem cells are undifferentiated progenitor cells with an infinite life span, high self-renewal capacity, and the ability to generate differentiated cell progenitors of multiple cell lineages (Mimeault and Batra, 2006). In 1963, McCulloch and Till first described the regenerative capacity of transplanted mouse bone marrow cells identifying cells that had limitless self-renewal capabilities (Becker et al., 1963) having previously published the discovery of radiation sensitive cells of the marrow (McCulloch and Till, 1960, Till and McCulloch, 1961). It is now known that many distinct stem cell types exist. ESCs, found in the foetal tissues and umbilical cord blood, are the only true totipotent cells capable of differentiating into multiple cell types. Multipotent adult stem cells share properties with totipotent ESCs; such as limitless self-renewal capacity, high proliferative capacity, high telomerase activity and slow rates of division (Sales et al., 2007, Harrison et al., 1978, Hoshino et al., 1967). These specialised cells exist in many adult tissues and organs such as the brain, skin,

heart, bone marrow, intestines, lungs kidneys, pancreas, liver and eyes. However, unlike the totipotent ESCs from which they are derived, adult stem cells have a restricted differentiation potential; as such they can only generate a limited number of lineage-restricted progenitor cells (Mimeault and Batra, 2006). Adult stem cells reside, quiescently, in specialized niches within organs and are responsible for normal tissue maintenance and repair, they are also essential in regeneration and repair of tissue after damage.

Self-renewal of somatic stem cells produces daughter cells with the same self-renewal capacity, as well as a more differentiated transit amplifying (TA) cells, which have a lower self-renewal capacity but higher cycling speed (Clarke and Fuller, 2006, Barker et al., 2007, Pastrana et al., 2011). These progeny TA cells are responsible for the generation of the bulk of the tissue by undergoing terminal differentiation. The current understanding of the epidermal stem cell niche is that a single stem cell and ~10 TA cells that undergo several rounds of amplification in the basal layer prior exiting the niche and terminally differentiating, switching between different keratin expression profiles as they differentiate up through the skin layers (reviewed in Fuchs, 2009a). This is also true of the intestinal epithelium, where stem cells reside at the base of the crypts and produce the TA cells which undergo several rounds of amplification prior to differentiation as they progress up to the lumen (reviewed in Simons and Clevers, 2011).

The self-renewal capacity of stem cells is demonstrated by the self-renewal and differentiation of isolated cells in primary transplants such that they are able to

reconstitute the whole spectrum of differentiated cells from the tissue from which they are derived (Uchida et al., 2000). This process is then repeated in subsequent serial transplantations in which the same phenotype differentiation and self-renewal of the population is required. This has so far been demonstrated for adult stem cells residing in the hematopoietic system, bone marrow, and breast and prostate epithelium (Matsuzaki et al., 2004, Stingl et al., 2006, Krause et al., 2001, Shackleton et al., 2006, Leong et al., 2008).

To investigate the properties of stem cells, ESC lines derived from the inner cell mass of the blastocyst in early embryo development, have been established (Thomson et al., 1998). Human ESCs express markers common to undifferentiated cells such as octamer-binding protein (OCT4), NANOG, SOX2, CD9 and CD24 (Hoffman and Carpenter, 2005). When grown in simplified culture or transplanted *in vivo* ESC differentiate into all foetal and adult stem cells and progenitor cells found in the adult body (Evans and Kaufman, 1981). Hence human ESCs are potential tools for a range of applications, from regenerative medicine to drug discovery and toxicology, studies of human development and as unique model for cancer progression. For these applications it is necessary that we understand the molecular mechanisms of human ESC self-renewal, maintenance and differentiation. Our understanding of the signalling pathways involved in human ESC self-renewal is incomplete. Several signalling pathways are thought to be important in ESC maintenance and function including epidermal growth factor (EGF), fibroblast growth factor (FGF), TGFβ, hedgehog (HH), WNT/β-catenin, PI3K and NOTCH signalling pathways, which will feature in the later part of this introduction.

1.10.2 Cancer stem cells (CSCs)

The role of CSCs in the initiation and maintenance of cancers is not a new concept. It has long been postulated that cancers arise from normal long-lived stem cell populations which acquire numerous genetic modifications. A cancer derived from a mutated stem cell would already possess limitless self-renewal capacities, subjugating the need for this property of cancerous cells. This idea is countered by a theory that supposes non stem cells can acquire stem cell “like” characteristics during genetic modification that precedes cancer cell formation (Pardal et al., 2003).

Recamier was the first to propose that dormant embryonic cells residing in adult tissues could be the origin of cancer over 100 years ago in his treatise “Recherches sur le traitement du cancer” dated 1829. In this work he coined the term “métastase” as meaning the spread of cancer (Carr and Carr, 2005). This “embryonic rest hypothesis of cancer origin” was also proposed by Remak in 1854 (Remak, 1855) and Virchow in 1858 (Virchow, 1858a), and was later refined by Durante and Cohnhiem who described these embryonic cells in adult tissues as dormant until activated at the onset of cancer (Sell, 2004). The displacement and activation of germ cells as the origin of tumours was postulated by John Beard in the early 20th century, a theory that continues to receive support today (Ratajczak et al., 2010).

The existence of CSCs had been proposed as early as 1829 (Carr and Carr, 2005), however it was not until 1994 that the first evidence for their existence came to light following the finding that a small sub-population of acute myeloid leukaemia cells could initiate leukaemia in immunodeficient mice (Lapidot et al., 1994). Isolation of CSCs from solid tumours has proved more technically difficult. However, cells with

the ability to initiate new tumours in non-obese diabetic/severe combined immunodeficient (NON/SCID) mice were first identified as a minor CD44 positive, but CD24 and lineage dependent marker negative ($CD44^{+}/CD24^{-/LOW}/Lineage^{-}$) population of breast cancer cells in 2002 (Al-Hajj et al., 2003). Since this time CSCs, defined as a subpopulation of tumour cells capable of repopulating the phenotype of the tumour over serial passages and expressing a mixture of tissue dependent "CSC-markers", have been identified in numerous solid tumours (Visvader and Lindeman, 2008).

The CSC hypothesis states that only this subpopulation of cells is capable of regenerating the tumour, the bulk of the tumour does not have this capability. Furthermore, it is believed that the tumour mass comes from the differentiation of a CSC such that on differentiation the vast majority of the tumour cells lose their stem like properties. It is the similarity of the self-renewal and differentiation mechanisms between normal and CSCs that make this such an attractive theory (Reya et al., 2001, Pardal et al., 2003).

Whilst the origin of cancer is still debated, many tumours, including most NPC and EBV-aGC, display an undifferentiated phenotype (Niedobitek et al., 1992). This would occur if either the cancer develops from differentiated cells that acquires transforming mutations and dedifferentiates prior to expansion, or if an undifferentiated stem cell acquires multiple mutations allowing dysregulation of the cell towards a cancer like phenotype. The possibility that stem cells undergo malignant transformation into tumour initiators in cancers not only explains the acquisition of limitless self-renewal but goes partway to explaining activity of diverse

developmental signalling pathways, increased DNA repair and high rates of ABC transporter-mediated drug efflux, which would normally be associated with stem cells. It would seem possible that instead of acquiring these attributes over time, slowly converting a differentiated cell towards a phenotype more commonly associated with stem cells, the mutation may occur in a cell which already has these properties.

1.10.3 Isolation of CSCs

In recent years considerable progress has been made in identifying populations of cells within the proliferative compartments of both lymphoid and epithelial tissues that display characteristics of multipotent stem cells. The gold-standard for characterising stem cells is demonstrated by serial transplantation experiments involving the isolation of precursor cells, frequently sorted from the bulk population on the expression of putative stem cell markers, and transplantation into nude mice to form new tumours at which point the tumour is dissociated, cell are resorted and transplanted again (Uchida et al., 2000). In these studies putative stem cells will continue to regenerate tumours characteristic of the original tumour from which the cell was derived through these serial transplantations. However, other techniques for establishing the presence of a stem cell population have also been reported. Stem cells possess the ability to form tumourspheres in highly defined serum-free medium in the absence of an adhesive substrate (technique reviewed by Pastrana et al., 2011), they are often slow cycling and so can be identified as the label retaining population in bromodeoxyuridine (BrdU) label retaining studies (reviewed by Fuchs, 2009b), and various cancer stem cell populations have been shown to differentially

express markers on the cell surface although these markers are highly lineage dependent and as such many markers appear in the literature.

Integrin profiling, particularly the expression of β1 integrin, has been used to characterise numerous putative stem cell populations from normal epithelial tissues (Jones and Watt, 1993, Schlotzer-Schrehardt and Kruse, 2005, Lathia et al., 2010); studies have demonstrated some stem cell populations express cell surface β1 integrin at levels 2-3 fold higher than their transit amplifying progeny (Hotchin et al., 1995). In epidermal keratinocytes overexpression of β1 integrins or constitutive activation of the MAPK kinase 1 (MEK) (an important effector protein downstream of integrins) leads to expansion of the stem cell pool (Zhu et al., 1999, Haase et al., 2001) underscoring the importance of integrin signalling in the maintenance of the stem cell phenotype. Similarly depletion of this integrin in intestinal stem cells leads to an increase in differentiation due to an impaired HH signalling pathway (Jones et al., 2006), and β1 integrin has also been reported to modulate NOTCH signalling in the maintenance of an stem cell phenotype in breast and neural stem cells (Campos et al., 2006, Brisken and Duss, 2007).

Isolation of putative CSC populations in neuroblastoma, glioblastoma, breast and lung cancer cell lines has been achieved by utilising the increased expression of the ABC transporter proteins, believed to confer cells with increased resistance to chemotherapy reagents (Hirschmann-Jax et al., 2004). Characterisation of putative stem cells, and the identification of extracellular markers, has allowed the isolation of subpopulations of cells by fluorescence activated cell sorting – a technique that has

proved invaluable in the characterisation of populations of cells that make up the bulk of the tumour.

As previously mentioned, stem cell markers are highly tissue dependent however there are number of markers that consistently appear in the literature in relation to epithelial tumours. CD133 has been identified as a CSC in multiple tissues (Clevers, 2011); in prostate cancer CD133, Trop-2 and α2β1 integrin are reported to mark CSCs (Trerotola et al., 2010), whilst in head and neck squamous cell carcinoma (HNSCC) and NPC markers such as CD133 and CD44 are reported to identify the CSC population (Sayed et al., 2011, Su et al., 2011, Zhuang et al., 2013).

Stem cells can also be identified by expression of stem cell maintenance proteins such as the polycomb protein BMI1, homeobox protein NANOG, and transcription factors such as OCT4 and SOX2 (Chambers et al., 2003, Claudinot et al., 2005, Lee et al., 2006, Boyer et al., 2006). These markers of "stemness" are not only expressed or over-expressed in a range of undifferentiated carcinomas *in vivo*, but *in vitro* studies support a role for these proteins in maintaining an "undifferentiated" or stem cell-like phenotype (Park et al., 2003, Kashyap et al., 2009), indeed as few as four stem cell factors (OCT4, SOX2, NANOG and LIN28) are sufficient to reprogram more committed cells to adopt an undifferentiated state (Yu et al., 2007, Pardo et al., 2010). Other novel proteins, such as the transcriptional repressor Zfp145 and MTS24, have also been reported as markers of stem cells (Claudinot et al., 2005).

Stemness related transcription factors expressed in the putative stem cell population of HNSCC cell lines have recently been investigated. Spheroid derived HNSCC cells, a subpopulation of cells capable of forming spheres in serum free medium, showed marked increase in the expression of SOX2, NANOG and OCT3/4 whilst also demonstrating increased expression of EMT related genes (Chen et al., 2011). This study also demonstrated an increased expression of aldehyde dehydrogenase (ADHD) in the sphere forming cells, which has been shown to identify CSCs in HNSCC and other epithelial cancers (Chen et al., 2009, Clay et al., 2010, Chen et al., 2011).

1.10.4 Evidence for CSCs in NPC

As with other carcinomas of the head and neck, considerable interest has focused on the possibility that NPC may originate from a CSC population. The presence of CSC in NPC is supported by studies on biopsies of NPC tumours which demonstrate expression of ESC proteins such as BMI1, SOX2 and OCT4, and the isolation of tumour initiating cells from an undifferentiated NPC cell line, CNE2 (Song et al., 2006, Wang et al., 2007, Zhang et al., 2010, Luo et al., 2013). A “side population” of cells enriched for putative CSCs has been isolated from the CNE2 cell line on differential efflux of Hoechst dye, a DNA binding drug exported by the ABC transporter (Wang et al., 2007). These cells were able to form tumour in non-obese diabetic/severe combined immunodeficient mice with 20 times fewer cells than the unsorted population.

In the NPC biopsies a small population of cells were found to express embryonic stem cell markers, OCT4 and SOX2, these cells also showed deregulated NOTCH1 signalling which was proposed to regulate the NPC CSC population (Zhang et al., 2010). Further interrogation of the role of NOTCH in the CSC-like population of NPC has demonstrated this signalling pathway is required for the self-renewal of the side population cells in CNE1 and CNE2 cell lines. Furthermore inhibition of NOTCH signalling reduced the ability for unsorted cells to form tumours (Yu et al., 2012).

1.11 Deregulation of signalling pathways associated with "stemness" and cancer

Various signalling pathways are associated with development and regulating normal cell growth. A number of these: HH, WNT/β-catenin, NOTCH, TGFβ, FGF, play essential roles in maintaining populations of stem cells within a defined stem cell "niche" (Katoh, 2007a). Aberrant regulation of these pathways is known to give rise to tumours, underscoring the importance of these pathways in oncogenesis. This section will briefly introduce some of the pathways known to influence stem cell self-renewal, which are dysregulated in tumours with a particular emphasis on the HH signalling pathway.

The HH signalling pathway is intrinsically entwined with the WNT, TGFβ, NOTCH and FGF transduction pathways, which together constitute a signalling network responsible for the maintenance of CSCs (Katoh and Katoh, 2006). Whilst there are many review papers on stem cell maintenance signalling pathways and their interactions, in relation to the HH signalling pathway and cancer, reviews by

Merchant and Takebe provide an overview of the stem cell signalling network relevant to this thesis (Merchant and Matsui, 2010, Takebe et al., 2011).

In the maintenance of the embryonic stem cells, the TGFβ family of proteins act as growth and differentiation factors and direct embryonic stem cell fate commitment (Kitisin et al., 2007); the WNT pathway regulates stem-cell self-renewal, embryonic patterning and cell commitment (Alonso and Fuchs, 2003, Huelsken and Birchmeier, 2001); MAPK signalling activates proliferation and induces differentiation (Orford and Scadden, 2008), and the NOTCH pathway directs differentiation to maintain cell fate commitment and the expansion of the stem cell pool (Stier et al., 2002, Chiba, 2006).

Transcriptional profiling comparing the expression of genes in embryonic stem cells ESCs, neural stem cells (NSCs), and hematopoietic stem cells (HSC) has identified the upregulation of several members of the TGFβ family of proteins, as well as members of the JAK/STAT pathway, in all three types of stem cell, indicating the importance of activated TGFβ signalling in these populations (Ramalho-Santos et al., 2002). Other pathways involved in ESC maintenance also influence adult stem cell systems; NOTCH signalling regulates the size of the stem cell compartment in multiple adult progenitor cells (Androutsellis-Theotokis et al., 2006) and regulates cellular-fate in mammary progenitor cells (Dontu et al., 2004), WNT signalling is seen to maintain self-renewal in stem cell populations not limited to breast, brain and the hematopoietic system (Reya and Clevers, 2005), while bone morphogenic protein (BMP) signalling suppresses WNT in intestinal precursor cells to inhibit this process (He et al., 2004). There are many examples of cross-talk between different stem cell signalling pathways; in the intestine an interplay between the WNT and NOTCH

pathways is involved in epithelial cell renewal and differentiation (Nakamura et al., 2007b), and many signalling pathways have downstream targets that feature in other signalling pathways. These interactions of stem cell signalling pathways are poorly understood but it is likely that stem cell growth, differentiation and fate determination, and the maintenance of the stem cell pool, is highly dependent on the interactions of multiple signalling pathways.

WNT5A regulates both the canonical and non-canonical WNT signalling cascades in a context dependant manner (Katoh, 2007b). Canonical signalling occurs through the frizzled receptor and LRP5/LRP6 co-receptor to activate the β-catenin, TCF/LEF, BCL9/BXL9L and PYGO1/PYGO2 nuclear complex and induce downstream targets such as MYC (He et al., 1998) and JAG1 (Katoh, 2006). Transcription of *Dkk1* is also activated and implicated in negative feedback control on this pathway (Chamorro et al., 2005, Hirata et al., 2011). In contrast, the non-canonical WNT signalling is activated at the tumour stromal interface and is transduced through the Frizzled receptor and ROR2/RYK co-receptor inducing RhoA, JNK, PKC, NFAT and NLK signalling cascades (reviewed by Katoh, 2007a). The non-canonical WNT signalling pathway has roles in cellular polarity, adhesion and movement and is activated in tumour invasion and metastasis (Ling et al., 2009).

1.11.1 The Hedgehog (HH) Signalling Pathway

The HH signalling pathway is critical for stem cell maintenance; it plays an essential role in development, morphogenesis, and patterning, by dictating tissue patterning in multiple organs (reviewed by McMahon et al., 2003). The *hh* gene was discovered in

Drosophila as a secreted signalling protein expressed in stripes within the *Drosophila* embryo with a role in dictating embryonic segment polarity (Tashiro et al., 1993). There are three mammalian *hh* homologs desert hedgehog (DHH), Indian hedgehog (IHH) and sonic hedgehog (SHH). The indispensability of HH signalling is demonstrated by the severe congenital mutations and neonatal lethality seen in IHH and SHH targeted gene disruption mouse models (Chiang et al., 1996b, St-Jacques et al., 1999, Hayhurst and McConnell, 2003), and holoprosencephaly in humans, a developmental defect of the forebrain and midface caused by SHH mutation (Roessler et al., 1996). Additionally, in epidermal stem cell populations, SHH is required for normal proliferation as treatment with cyclopamine, a SHH inhibitor, decreased stem cell proliferation (Zhou et al., 2006). SHH has been shown to directly regulate the cell-cycle as human keratinocytes overexpressing SHH from a transfected vector no longer exit S and G2/M in response to calcium, an inducer of terminal differentiation (Fan and Khavari, 1999, Roy and Ingham, 2002).

Activated HH ligand is produced by post translational modification of the HH precursor ligands; autocatalytic cleavage from 45kD to 19kD and a palmitoyl acid and cholesterol moiety addition at the N-terminus and C-terminal respectively (Pepinsky et al., 1998, Porter et al., 1996).

Drosophila HH ligands function by binding to transmembrane receptors, Hip1, Patched 1 (PTCH1), and Patched 2 (PTCH2), relieving the tonic inhibition of receptor bound Smoothened (SMO). Once depressed, Smo activates HH signalling through the activation and nuclear transport of Ci, a *Drosophila* transcription factor, which regulates gene transcription. Whilst mammalian HH signalling functions in much the

same way, three vertebrate homologues of Ci exist, GLI1, GLI2 and GLI3, which may have independent and distinct repressor and activator activities that are cell context dependent (Ruiz i Altaba et al., 2002 and references there in).

GLI1 and GLI2 act primarily as transcriptional activators, whereas GLI3 exists as both a transcriptional activator and repressor. In the absence of HH signalling, GLI3 is sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase-3β and casein kinase 1 (CK1) prior to polyubiquitination via ubiquitin ligase β-TrCP and processing by the proteasome to produce an N-terminal fragment that acts as a transcriptional repressor. GLI2 also has an amino-terminal repressor domain and in the absence of SHH is phosphorylated and processed through interaction with β-TrCP, however processing to the repressor form is inefficient and where it does happen largely leads to the degradation by the proteasome (reviewed in Simpson et al., 2009).

In the presence of active HH signalling, SHH acts to inhibit the cleavage of GLI2 and GLI3 allowing the full length proteins to act as transcriptional activators. GLI1 cannot be processed and acts only as a strong transcriptional activator although does not appear to be essential as $GLI1^{-/-}$ mice are viable and exhibit a normal phenotype (Park et al., 2000). GLI1 and GLI2 have been shown to have overlapping functions and whilst some studies suggest low level GLI1 expression can rescue GLI2 mutants *in vivo* (Bai and Joyner, 2001), other studies have found a distinct, and essential, role for GLI2 in lung and hair follicle development, initial SHH responsiveness and ectopic activation which cannot be rescued by GLI1 (Bai et al., 2002, Mill et al., 2003).

Activated transcription factors are translocated to the nucleus where they activate downstream target transcription. The primary cilica and intraflagellar transport (IFT) proteins facilitate HH signalling; allowing the import and export of PTCH, SMO, and the GLI transcription factors via the Kif3 motor complex and retrograde dynein motors, and promoting GLI activation. Indeed mutations that disrupt the IFT proteins and Kif3a cause phenotypes associated with HH signalling defects (reviewed in Pan et al., 2013). A schematic of the HH signalling pathway can be seen in Figure 1. 7.

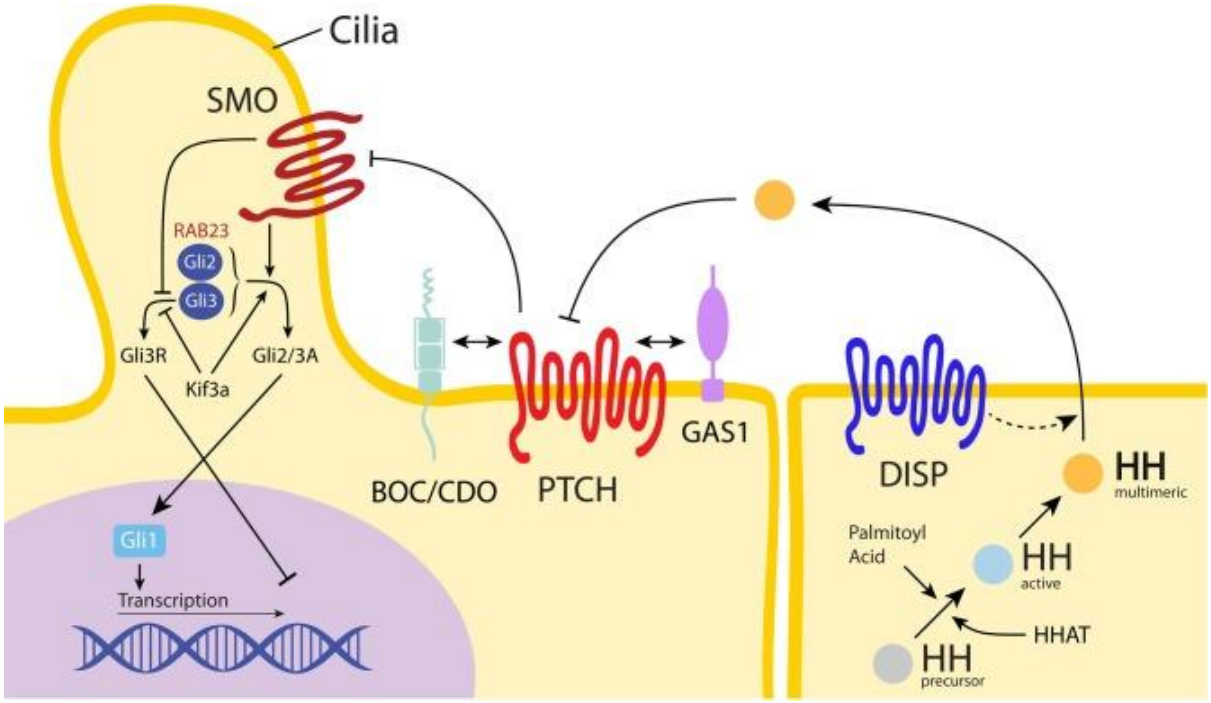


Figure 1. 7. Schematic of the HH Signalling Pathway
Modified activated oligomeric HH ligand (yellow) is released from Dispatched (DISP) and secreted into the intracellular space where it is able to act in a paracrine or autocrine manner. HH ligand binds to Patched (PTCH) cell membrane receptor with assistance from GAS1, BCO and CDO which relieves inhibition of Smoothened (SMO). Activated SMO moves to the cilia where it promotes the activation of GLI2/3 (GLI2/3A) and inhibits repressor of transcription GLI3R. Activated GLI transcription factors are translocated to the nucleus to activate downstream HH target expression. Arrow represents activation, flat headed arrow represents inhibition.

From Pan et al., (2013)

1.11.2 Regulators of the HH Signalling Pathway

Positive regulators of the HH pathway include Growth arrest-specific protein (GAS1), Hedgehog acyltransferase (HHAT), Caveolin1 (CAV1), CDO, BOC, Fused (FU) and ZIC2. Loss of HHAT leads to decreased HH secretion demonstrating this protein is needed for HH ligand modification (Dennis et al., 2012), CAV1 is also proposed to facilitate SHH ligand by assisting vesicular packaging for intracellular transport (Mao et al., 2009), whilst other studies have demonstrated a negative regulatory function for CAV1 (Capozza et al., 2012). GAS1 and BOC/CDO are theorised to be SHH co-receptors for PTCH; however, an interaction between these proteins has remained elusive. GAS1 is negatively transcribed in relation to HH pathway activity, and both $GAS1^{-/-}$ and $CDO^{-/-}$ mutants show similar HH target expression profiles to those of $SHH^{-/-}$ mice and display severe defects in regard to notochord and craniofacial defects that partially recapitulate those seen in $SHH^{-/-}$ mice (Allen et al., 2007). CDO/GAS1 and CDO/BOC double mutants also exhibit severe defects, although the viability of $BOC^{-/-}$ mice suggests this protein has a similar function to CDO which is compensating for its loss (reviewed in Pan et al., 2013).

FU, also known as STK36, is a positive regulator of the GLI transcription factors and its expression, like GLI1, has been demonstrated to rescue lymphoma cells grown in the presence of cyclopamine (Dierks et al., 2007). ZIC2 is also reported to positively regulate GLI transcription by increasing nuclear retention of GLI1 (Chan 2011).

Multiple negative regulators of the HH signalling pathway exist and inhibit the pathway at different points. Suppressor of fused (SUFU) negatively regulates the

modification of GLI3 into its repressor or activator forms (Humke et al., 2010) and RAB23 has been identified through epigenetic studies, with RAB23/GLI2 double knockout mice, to inhibit with the processing of GLI2 into a transcriptional activator (Eggenschwiler et al., 2006). DAZ interacting protein 1 (DZIP1) promotes GLI turnover, a process that is regulated by the phosphorylation of DZIP1 by casein kinase 2 and inhibited by B56-containing protein phosphatases (Jin et al., 2011). Additional regulation of the HH pathway involves the expression of GLI1 and PTCH1, HH pathway components, as downstream targets (Lee et al., 1997, Goodrich et al., 1996).

1.11.3 Targets of activated GLI transcription factors

The three vertebrate GLI proteins bind conserved target sequences to activate downstream target transcription (Villavicencio et al., 2000), of note is the common GLI1 and GLI2 DNA binding motif, GACCACCCA, which allows for some functional redundancy between these two proteins (Yan et al., 2011). Classical transcriptional targets of an activated HH pathway include FOXM1 (Teh et al., 2002), BCL2 (Regl et al., 2004, Bigelow et al., 2004) and Cyclin D proteins, CyclinD1 and CyclinD2 (Mill et al., 2003), additionally activated GLI proteins target HH pathway components, PTCH1 (Ågren et al., 2004) and GLI1 (Dai et al., 1999), creating a positive feedback loop (Katoh and Katoh, 2006). In addition to the BCL2 and Cyclin D proteins, which have a role in cell proliferation and survival, other direct targets of GLI transcription influence cellular phenotype by regulating cell cycle (Cyclin E and cMYC), adipogenesis (vascular endothelial growth factor VEGF, and angiopotetins-1 and 2), epithelial to mesenchymal transition (SNAIL and MUC5A) and self-renewal (BMI1,

SOX2, PAX3) (Perrot et al., 2012). Recently gene expression profiling and chromatin immunoprecipitation have allowed the identification of many putative GLI targets, however these have yet to be confirmed as global transcriptional targets in all human cell types (Vokes et al., 2007, Lee et al., 2010, Vokes et al., 2008).

1.11.4 Interaction of HH signalling pathway with other stem cell maintenance pathways

The HH pathway feeds into other stem cell maintenance pathways via downstream targets that feature in the WNT family, such as WNT5A (Mullor et al., 2001, Cohen, 2003, Cohen, 2010), and TGFβ family, and BMPs, such as BMP4 (Astorga and Carlsson, 2007). These signalling pathways may function to regulate one another; indeed, co-expression of HH and BMP -2,-4 and -6 suggests interaction between these two pathways (Bitgood and McMahon, 1995) and TGFβ has been shown to induce SHH ligand expression (Chung and Fu, 2013). Additionally, TGFβ can induce the expression of GLI1 in a GLI2 dependent manner, and this induction is also SMAD3 dependent (Javelaud et al., 2012).

Ligand independent activation of the HH pathway is also achieved through the TNF-α (Wang et al., 2012c) and WNT pathways (Borycki et al., 2000), and via mutant KRAS (Collins et al., 2012), whilst the NFκB signalling pathway may contribute to SHH ligand induction (Nakashima et al., 2006, Kasperczyk et al., 2009). The PI3K/Akt pathway regulates (Ramaswamy et al., 2012) also positively regulate HH, whereas NOTCH, GsPCR and PTEN signalling downregulate GLI1 transcription (Katoh and Katoh, 2009b, Schreck et al., 2010, Xu et al., 2008) and Akt-GSKβ3 signalling,

initiated by osteopontin, influences GLI1 distribution and activation (Das et al., 2013). Finally crosstalk and co-operation between the HH and EGF (Palma and Ruiz i Altaba, 2004, Bigelow et al., 2005), and WNT signalling pathways has also been described (Yang et al., 2008a). The consequences of an activated HH signalling pathway in relation to tumourgenesis are outlined in Figure 1. 8 (reviewed by Kar et al., 2012).

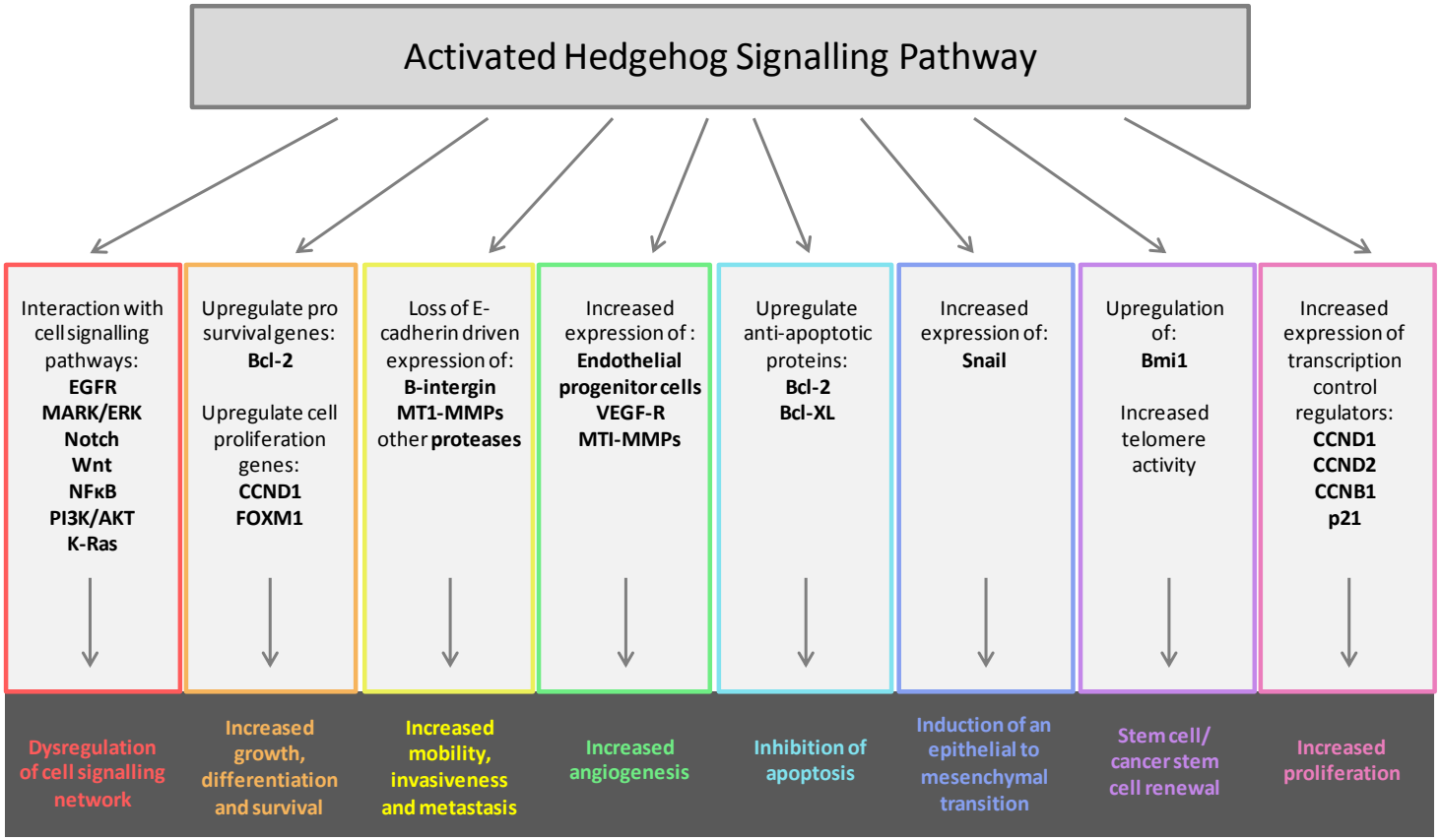


Figure 1. 8. Consequences of an activated HH signalling pathway
Schematic showing cellular consequences of activated HH signalling pathway downstream targets and other cell maintenance pathways with which the HH signalling pathway interacts.

Adapted from Kar S, et al., (2012)

1.12 The HH signalling pathway in cancer

HH signalling is seen in both the ESC and adult stem cell compartments (Lai et al., 2003, Palma and Ruiz i Altaba, 2004) as well as being implicated in numerous cancers (Xie and Epstein, 2011) such as basal cell carcinoma (BCC), a tumour arising in the undifferentiated epidermal stem cell population (Grachtchouk et al., 2011), and glioblastoma multiforme (GBM) (Dahmane et al., 2001, Bar et al., 2007). Indeed, GLI1 was first identified from its overexpression in malignant glioma (Kinzler et al., 1987) and is identified as an oncogene due to its ability to transform primary cells in concert with adenovirus E1A (Ruppert et al., 1991). Overexpression of GLI1 has been identified in many cancers including BCC (Dahmane et al., 1997, Ghali et al., 1999), B-cell lymphoma (Rao et al., 1998), meduloblastoma, glioma and other brain tumours (Kinzler et al., 1987, Salgaller et al., 1991, Reifenberger et al., 1996, Pasca di Magliano and Hebrok, 2003) and childhood sarcomas (Roberts et al., 1989, Khatib et al., 1993) in which the scale of upregulation is correlated with tumour grade (Stein et al., 1999). GLI1 overexpression is also associated with breast cancer and can predict early relapse (ten Haaf et al., 2009, Li et al., 2012), whilst the expression of GLI2 is correlated with poor prognosis in hepatocellular carcinoma (Zhang et al., 2013). Furthermore, the chromosomal location of the GLI gene is frequently rearranged or amplified in human cancers (Stein et al., 1999).

1.12.1 Mechanisms of HH signalling pathway activation

HH activation in BCC is largely the consequence of frequent UV-induced mutations in PTCH and SMO leading to constitutive HH signalling (Villavicencio et al., 2000, Daya-Grosjean and Couve-Privat, 2005, Daya-Grosjean and Sarasin, 2005). Indeed

more than 90% of sporadic BCC has a mutation in a single PTCH allele and 10% display activating mutations in SMO (Roewert-Huber et al., 2007). As such HH pathway activation in the initiation and maintenance of BCC is ligand independent; mutations occur downstream of the HH ligand, this is also true of meduloblastoma which most commonly involves mutations in PTCH or SMO receptors (Thompson et al., 2006).

The HH signalling pathway can also become aberrantly activated by overexpression of SHH, which in transgenic mice and human fibroblasts is sufficient to induce the onset of BCC (Grachtchouk et al., 2000, Oro et al., 1997, Fan et al., 1997). Similarly GLI1 RNA injected into the epidermis is also capable of inducing BCC formation (Dahmane et al., 1997), however expression of an activated mutant form of SMO was insufficient for the induction of BCC (Grachtchouk et al., 2003), and studies using mutated SMO in pancreatic cancer have since identified a paracrine nature of HH signalling such that oncogenic SMO induces HH activation in the stromal cells (Tian et al., 2009). Tumour cells in this paracrine HH ligand-dependent model are responsible for HH signalling activation in the stromal cells that feeds back inducing alternative signalling pathway to maintain the tumour (Tian et al., 2009). Whilst this study does not rule out the existence of ligand independent mechanisms for pancreatic cancer initiation and maintenance, indeed constitutively activating mutations are associated with this cancer (Kar et al., 2012), it highlights the complex interaction between the stroma and tumour cells that is responsible for HH stimulated tumourgenesis in at least a subset of this disease.

A paracrine HH ligand dependent nature has been demonstrated in a number of other human cancers including those of the breast, colon, prostate and ovaries which demonstrate HH activity in the adjacent stroma in response to tumour produced ligand (O'Toole et al., 2011, Yauch et al., 2008, Teglund and Toftgard, 2010). Additional autocrine signalling mechanisms have been suggested for prostate, lung, ovarian, colon and upper gastrointestinal cancers (Teglund and Toftgard, 2010, Kar et al., 2012). It would appear that in a number of cancers HH signalling may act through both autocrine and paracrine mechanisms, for instance prostate cancer where the former is implicated in tumour initiation and the latter in establishing communication with the stroma and cancer progression (Kar et al., 2012).

While it would be unwise to dismiss autocrine HH signalling entirely, further support of a paracrine mechanisms for HH signalling in pancreatic and colon cancer follows the findings that proposed autocrine HH signalling in these tumours may be inadequate when considering the off target effects of cyclopamine at high doses and lack of cyclopamine induced growth inhibition at SMO specific levels (Varnat et al., 2009, Pasca di Magliano et al., 2006, Kolterud et al., 2009), and the low levels of HH activity in tumour cells (Yauch et al., 2008, Berman et al., 2003).

Interestingly whilst it would appear that in certain cancers HH activity in the stroma may be more important than in the tumour cells themselves. Yauch et al. (2008) noted that this does not rule out a role for HH activity in the stem cell population. Future studies may tease out further intricacies in the HH signalling pathway that might well support multiple methods of HH activation within the tumour cell and

stroma at different stages of tumourgenesis and subsequent maintenance that are likely to be highly tumour dependent.

1.12.2 Targeting the HH signalling pathway in tumour therapy

The involvement of HH in the initiation and maintenance of numerous cancers has encouraged research into the therapeutic application of HH pathway inhibitors. Cyclopamine is a plant alkaloid that directly inhibits the HH signalling pathway through its interaction with SMO. Multiple SMO inhibitors have been produced that act by a variety of mechanisms (interacting with SMO directly to prevent processing into its active form or inhibiting SMO transport to the cilia) and have progressed into clinical trials (reviewed in Onishi and Katano, 2011). Vismodegib is a cyclopamine-competitive SMO inhibitor that has recently been approved by the US Food and Drug Administration's (US FDA). Whilst many of these inhibitors have been demonstrated to disrupt HH signalling, continued use of Vismodegib and other SMO inhibitors is associated with mutations in the SMO receptor leading to drug resistance (Sandhiya et al., 2013).

In tumours dependent on SHH ligand overexpression, aberrant HH signalling can be disrupted through with use of SHH blocking antibody 5E1 or the small molecular SHH inhibitor, robotnikinin (Merchant and Matsui, 2010). However, therapies relying on ligand targeting to inhibit the pathway may induce PTCH or SMO mutations, leading to resistance. In order to decrease the development of drug resistance and to target tumours resulting in downstream HH pathway mutations, such as meduloblastomas that arise in $SUFU^{-/-}$ mice (Lee et al., 2007a), the HH pathway must be inhibited

downstream of SMO. Multiple downstream pathway inhibitors of HH exist (e.g. GLI-antagonists GANT58 and GANT61, zerumbone, arcyriaflavin C and physalin F) which inhibit GLI transcription, whilst small molecular HH protein inhibitors (HPIs) act to block components of the HH signalling cascade downstream of SMO (Hyman et al., 2009). With the identification of HH signalling dysregulation in an increasing number of cancers, the future of targeted cancer therapies will likely involve HH inhibitors.

This thesis investigates the contribution of a dysregulated HH signalling pathway in NPC, interrogates the relationship between EBV and HH dysregulation, and attempts to establish the cellular consequences of EBV induced HH activation.

CHAPTER TWO: MATERIALS AND METHODS

There is one thing even more vital to science than intelligent methods; and that is, the sincere desire to find out the truth, whatever it may be.

— Charles Sanders Peirce

Pragmatism as a Principle and Method of Right Thinking (1997), 266.

2.1 Cell culture

2.1.1 Basic media

Tissue culture media purchased is outlined below. Supplements added through 0.2 µm syringe filters (PALL Life Sciences, US). All media were stored at 4 ^{o}C. Antibiotics added to all media unless stated in method.

RPMI 1640: Liquid in sterile 500ml bottles (Sigma Aldrich, US). Supplemented with 2 mM L-glutamine, 25 mM Hepes and adjusted to pH 7.0.

Dulbecco's Modified Eagle's Medium (DMEM): Liquid in sterile 500ml bottles (Sigma Aldrich, US). Supplemented with 2 mM L-glutamine, 25 mM Hepes and adjusted to pH 7.0.

Keratinocyte serum-free medium: Liquid in sterile 500 ml bottles from (Gibco®, Invitrogen, US). Supplemented with 25 mg bovine pituitary extract.

2.1.2 Supplements and sterile solutions

*Foetal Calf Serum (FCS)***:** Liquid in sterile 500ml bottles pre-screened for viral and mycoplasma contamination (Gibco®, Invitrogen, US). Stored as 50 ml aliquots at -20 ^{o}C.

Penicillin/Streptomycin: Liquid in sterile 100 ml bottles containing 10,000 units/ml penicillin and 10 mg/ml streptomycin (Sigma Aldrich, US). Used at 2.5 ml/500 ml media. Stored at 4 ^{o}C.

Ciprofloxacin: Liquid in sterile 250 ml bottles of concentration 200 mg/ml (Bayer, Germany). Used at 2.5 ml/500 ml media. Stored at room temperature.

Fibronectin: Solution of 0.1% from human plasma. Supplied at 1 mg/ml in 1-5 ml bottles (Sigma Aldrich, US). Stored at 4 ^{o}C.

TrypLE™ Express Stable Trypsin Replacement: 1x solution in 100 ml sterile bottles containing EDTA buffer and phenol red (Gibco®, Invitrogen, US), stored at 4 ^{o}C.

Geneticin disulphate salt (G418): Powder (Sigma Aldrich, US). 5 g powder dissolved in 100 ml SDW producing a stock solution of 50 mg/ml. Stored as filter sterilised 5ml aliquots at -20 ^{o}C.

Phosphate Buffered Saline (PBS): 100 tablets of 0.8 g NaCl, 0.02 g KCL, 0.115 g Na_2HPO_4, 0.02 g KH_2PO_4 (Oxid LTD). 100 tablets dissolved in 10 litres distilled water. Stored as 50 ml aliquots following 20 min autoclave sterilisation at 15 psi and 120 ^{o}C.

*Ethylenediaminetetra acetic acid (EDTA)***:** Powder (Difco™, BD Biosciences, US) 0.02% autoclaved solution made up by dissolving 0.5 g in 2.5 litres PBS and adjusting to pH 7.0, stored at 4 ^{o}C.

Dimethyl Sulphoxide (DMSO): Liquid in 500 ml sterile bottle (Fisher Scientific, US), stored at room temperature.

2.2 Cell lines

All cell lines were maintained at 37 ^{o}C and 5% CO_2 in complete growth medium (5% FCS, antibiotic supplemented RPMI unless otherwise stated).

C666-1: subclone of stable EBV positive cell line derived from a human undifferentiated nasopharyngeal carcinoma (NPC) xenograft of Southern Chinese origin (Cheung et al., 1999). Maintained in 10% FCS supplemented RPM1. Plastic tissue culture dishes were treated with 10% fibronectin in PBS for an hr at 37 ^{o}C prior to use.

Primary Tonsil Keratinocytes: human, normal primary oral keratinocytes, maintained in keratinocyte media.

OKF6: human, retroviral hTert-immortalised, normal oral epithelial cell line (Opitz et al., 2001), maintained in keratinocyte media.

NP460: human, Chinese derived, hTert-immortalised normal nasopharyngeal cell line (Li et al., 2006), maintained in keratinocyte media.

YCCEL1: human, Korean derived, naturally EBV positive gastric carcinoma cell line (Seo et al., 2013).

CNE2: human EBV negative cell line isolated from undifferentiated NPC biopsy. Original EBV status unknown (Sizhong et al., 1983).

CNE1: undifferentiated, human, EBV negative NPC derived cell line (Sizhong et al., 1983).

SUNE1: poorly differentiated, human, EBV negative NPC derived cell line (Chen et al., 1998).

H103: EBV negative, human squamous cell carcinoma derived cell line (Prime et al., 1990). Maintained in 10% FCS supplemented RPMI.

HONE1: EBV negative cell line derived from an EBV-positive undifferentiated Chinese NPC biopsy (Glaser et al., 1989).

ADAH: EBV negative, human, immortalised cell line derived from an adenocarcinoma of the nasopharynx (Takimoto, 1979).

AGS: human, tumourigenic immortalised cell line derived from an undifferentiated gastric carcinoma (Barranco et al., 1983).

CNE2, SUNE1, CNE1, HONE1, AGS and ADAH rEBV: cell line stably infected with recombinant Akata strain of EBV containing neomycin resistance cassette. Maintained under neomycin drug selection.

HONE1 NEO, EBNA1, LMP1, LMP2A and LMP2B: cell lines generated from individual clones of parental HONE1 transfected with EBV gene or neomycin control plasmid, maintained under neomycin drug selection.

2.2.1 Maintenance of cell lines

Adherent epithelial cells were grown on plastic tissue culture flasks in humid incubators at 37 ^{o}C with 5% CO_2. Adherent cells were grown to 80% confluence, pre-confluence log phase growth prior to passage. Subculture involved gently washing cells once in 1x PBS and incubating for a maximum of 10-15 min in a covering of trypsin replacement solution at 37 ^{o}C. Once detached cells were recovered in an excess of FCS containing complete growth medium, and pelleted by centrifugation at 1,000rpm for 5 min at room temperature. Supernatant was discarded and cells resuspended in 5 ml of supplemented growth medium ready for re-plating at required density in fresh growth media as established by haemocytometer counts.

2.2.2 Cryopreservation of cell lines

Pre-confluent cells, in log phase of growth, were trypsinised and pelleted as above prior to resuspension in 1ml of freezing medium. Freezing medium contained 50% supplemented growth medium, 40% FCS and 10% DMSO cyroprotectant. Aliquots of 1 x10^6 cells were dispensed into freezing ampoules (Nunc, Denmark) and placed into a cryopreservation container (Mr Frosty, Nalgene, US). The container was then placed into 80 ^{o}C freezer for at least 4 hr before the ampules were transferred to liquid nitrogen for long term storage.

2.2.2.1 Recovery of cryopreserved cells

Frozen ampoules were rapidly thawed in a 37 ^{o}C water bath. Cells were transferred to sterile universal (Sterilin, Thermo Scientific, US) and warm supplemented growth medium was added drop wise. Cells were left for 5-10 min prior to centrifugation at 1,500rpm for 5 min at room temperature. The supernatant containing the DMSO was removed and cells resuspended in supplemented growth medium before plating out.

2.3 Generation of EBV positive cell lines

Generation of SUNE1, CNE1 and H103 cell lines was necessary for this thesis. All other cell lines had previously been generated. A cross linked Akata Burkett's lymphoma (Akata BL) cell line was utilised to infect epithelial cells with neomycin cassette containing wild type EBV (Stewart et al., 2004). Akata cells were pelleted and resuspended at 2 x10^{6} cell/well in fresh 10% BL media, to which 1 µl/ml anti Human IgG cross linker was added. Cells were incubated for 2-3 hr in a 9 cm dish prior to recovery of cell suspension and resuspension at 2 x10^{6} cell/ml in fresh media. Cells were left for 72-96 hr prior to plating at 3 x10^{5} B-cells with 1x10^{5} epithelial cells/well (3:1 ratio), in a 12 well plate. Epithelial cells attached over the following 48-72 hr at which point the Akata cells were washed off and EBV infected, polyclonal population of epithelial cells was selected for by G418 treatment.

2.4 Bacterial cell culture for the production of plasmid DNA

2.4.1 Preparation of competent cells

E.coli strain XL1-Blue cell aliquots were stored at -80 ^{o}C in 20% (v/v) glycerol. Aliquots were recovered onto LB media plates containing 15 g bacto-agar per litre of

LB media, and incubated overnight at 37 ^{o}C. Isolated colonies were removed into 2ml SOB and placed back at 37 ^{o}C until the cell density rose to 5 x10^{7} cells/ml (OD_{600} = 0.5). Cells pelleted by centrifugation at 2,500 x g at 4 ^{o}C for 10 min (Sorvall SS-34 centrifuge, Du Pont Instruments, US) prior to resuspension in 50 ml pre-chilled RF1 (100 mM RbCl, 50 mM $MnCl_2$, 10 mM potassium acetate pH 5.8, 10 mM $CaCl_2$, 15% (w/v) glycerol)

2.5 Generation of SHH plasmid

Plasmids were supplied (Addgene, US) as bacterial colonies in agar, and bulked up overnight in 400 ml broth (shaking at 37 ^{o}C) prior to plasmid extraction using purelink plasmid miniprep system (Invitrogen, US) according to manufacturer's instructions. DNA resuspended in DEPC water at 1 µg/µl. FastDigest® (Thermo Scientific, US), Fast simultaneous plasmid vector linearization and dephosphorylation reaction mixtures are presented in Table 2.1 and 2.2 respectively. Reactions were incubated at 37 ^{o}C for 5 min.

Table 2. 1 Reaction mixture for plasmid digestion

Reagent	Volume (µl)
13996 pBS hShh CT#401 (Addgene, US) (1µl/µl)	2
10x FastDigest® buffer (Thermo Scientific, US)	4
FastDigest® enzyme	2
Nuclease free water	32

Table 2. 2 Reaction mixture for plasmid linearization and dephosphorylation.

Reagent	Volume (µl)
Plasmid DNA	1
10x FastDigest® buffer	2
FastDigest® enzyme	1
FAST AP™ Thermosensitive Alkaline Phosphate	1
Nuclease free water	15

Backbone pBABE-puro plasmid (1764, Addgene, US) was digested with EcoRI and alkaline phosphatase. pBS hShh CT#40 (113996, Addgene, US) plasmid was digested with EcoRI prior to loading into an agarose gel. Plasmid maps of pBS hShh and pBABE puro are shown in Figure 2.1 A and B respectively. The SHH insert gene was excised from the agarose gel under UV and purified with a GeneJet Gel Extraction Kit (Thermo Scientific, US). Linear DNA sample concentrations were analysed on a Nanodrop (Fisher Scientific, UK) prior to ligation. Rapid DNA ligation (Fermentas, US) protocol was used to ligate 10-100 ng of linearised vector DNA with SHH insert DNA at a 3:1 molar excess using 4µl of 5x rapid ligation buffer (Fermentas, US) and 1 µl of 5 µg/µl T4 DNA ligase made up to 20 µl with H_2O. Cells were heat shocked prior to plating on ampicillin plates (20 ml agar/LB broth containing 100 µg/ml ampicillin). Cells were plated at 2 densities using 5 and 10 µl of reaction mixture on two plates alongside a control pBABE and control complement cell plate, and left at 37 ^{o}C overnight. Colonies were picked and put in 3 ml LB broth containing 100 µg/ml ampicillin and left shaking at 37 ^{o}C overnight. 1.5 ml LB culture used for miniprep (QIAprep Spin Miniprep Kit, Qiagen, US) according to manufacturer's protocol, prior to diagnostic digest with bglII and HindIII restriction enzymes. Remaining LB culture containing correct plasmid construct was bulked up.

2.5.1 Visualisation of digest products

Digest products of pBS hShh were visualised in a gel from which the SHH gene, 1567bp, was excised (Figure 2. 2). Following ligation of pBABE-puro and SHH insert were bulked up and purified. Insert presence and orientation was established by analytical digestion using BglII and HindIII enzymes sites for which both appear once in the pBABE SHH plasmid. Digestion of pBABE SHH in the correct orientation would give bands at 5kb and 1.5kb, where as a SHH insert in the wrong direction would give rise to a 6Kb and 560bp bands. Digestion was seen to produce linearised products of the pBABE puro backbone without insert in 4 of the 5 ligations tested, and produced two ligation products in the remaining digest. Lane 3 of Figure 2. 2 shows the two digestion products present at 5kb and 1.5kb in addition to undigested plasmid present at 6.5Kb.

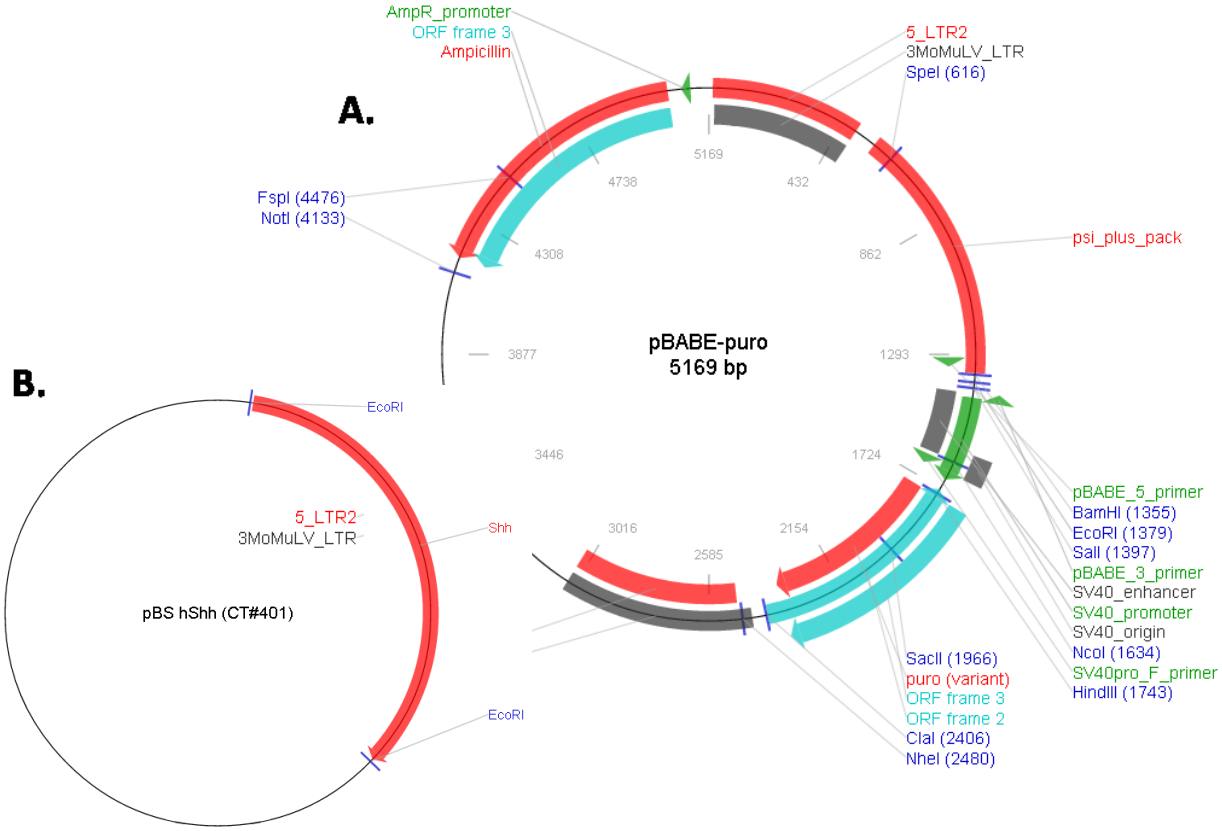


Figure 2. 1 Plasmid Maps of (A) pBABE-puro target backbone and (B) pBS hShh

EcoRI, HindIII enzymatic sites utilised in the generation of pBABE-puro hShh are located either side of the SV promoter (green arrow). SHH gene insert can be extracted by EcoRI restriction enzyme digestion (figures from addgene.org/13996/ and addgene.org/1764/).

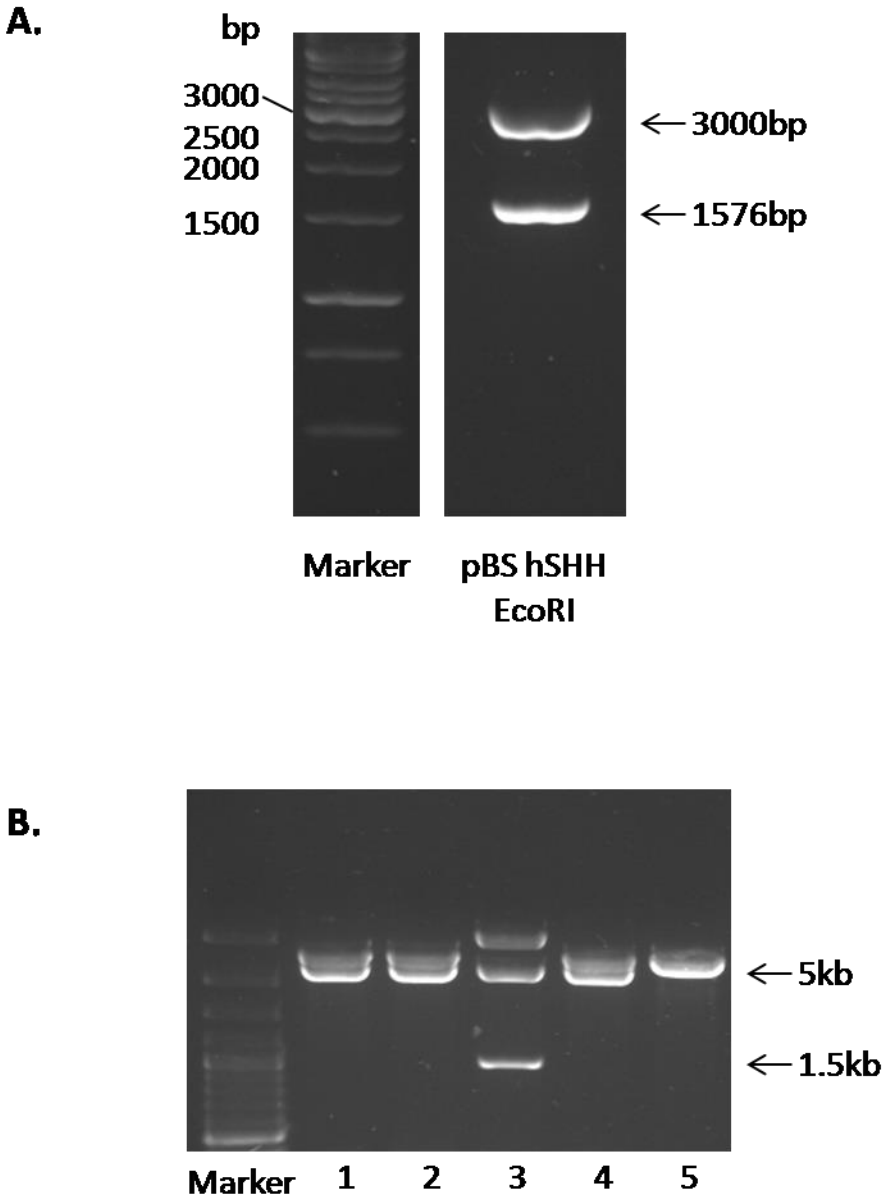


Figure 2. 2 Generation of the SHH expression plasmid
(A) Digestion of Addgene plasmid 13996: pBS hShh with EcoRI to generate 1576bp fragment for excision. (B) BglII and HindIII diagnostic digestion of DNA isolated from five bacterial colonies grown on ampicillin plates. Colony three contains the desired fragment sizes.

2.6 Luciferase reporter assay

Dual-Luciferase Reporter Assay System (Promega, US) was used to sequentially measure a single transfection for luciferase activity of firefly (*Photinus pyralis*) for the reporter of interest, and Renilla (*Renilla reniformis*) acting as an internal cell viability and transfection control. Cells were seeded at 0.5-2 x10^5, depending on individual cell proliferative rates, in a 12-well plate and allowed to reach 70% confluence.

Table 2. 3 Preparation of transfection media

Reagent (Supplier)	**Control plasmid transfection (µl/well)**	**Reporter plasmid transfection (µl/well)**
Optimem (Life technologies, US)	250	250
Plasmid of interest		0.5
Control plasmid	0.5	
Renilla	0.5	0.5
Plus™ reagent (Invitrogen, US)	1	1
	5 min at RT	
LTX (Invitrogen, US)	3	3
	30 min at RT	
Add 255 µ transfection media per well		

Per well 0.5-1 µg of plasmid DNA was diluted in 250 µl Optimem and complexed with 1 µl Plus™ reagent (Invitrogen, US) for 5 min at room temperature to increase transformation efficiency. Lipofectamine™ LTX reagent (Invitrogen, US) was added to the DNA complex and left for a further 30 min whilst the medium on the cells was replaced with 745 µl complete growth medium, without the addition of antibiotics. 255 µl transfection media was added to each well (Table 2. 1). Cells were incubated for 6-12 hr, and lysed in 500 µl 1x Passive Lysis Buffer for 30 s with agitation prior to freezing at -20 ^{o}C. Plasmids used for luciferase are summarised in Table 2. 2.

Table 2. 4 Plasmids utilised in this thesis

Plasmid	Control	Source
8xGLI-BS-Luc	pGL2-basic	Kindly provided by Dr. Steven Cheng, Nanjing Medical University, China
pSHH	pBABE-puro	Generated in this thesis, see Materials and Methods, section 2.5 and Chapter 3, section 3.6.1

2.7 Stimulation and inhibition of cells

A number of cytokines, pharmacological and chemical inhibitors were used in this thesis (Table 2.3). Cells were plated at appropriate densities, in complete media, and allowed to attach, prior to replacement with media containing 0.5% FCS for 12-24 hr. Cytokines and inhibitors were added at appropriate concentrations in 0.5% serum media and incubated for an assay dependent length of time.

Table 2. 5 Cytokines and inhibitors utilised in this thesis

Reagent (supplier)	Standard concentration	Nature and target pathway
SHH (Peprotech, US)	0.5 µg/ml	Soluble HH pathway ligand
5E1 (Developmental Studies Hybridoma Bank, University of Iowa)	10 µg/ml	Anti-SHH antibody
Cyclopamine (Miltenyi Biotech, Germany)	25 µM	HH pathway agonist SMO inhibitor
GANT58 (Tocris, US)	25 µM	HH transcription factor GLI1 chemical inhibitor (Lauth et al., 2007)
GANT61 (Tocris, US)	12.5 µM	HH transcription factor GLI1 and GLI2 chemical inhibitor (Lauth et al., 2007).

2.8 5E1 and conditioned media

5E1 (anti-SHH mAb) or C651.6 (anti Notch2 mAb) hybridoma cells were grown to 80% confluence and then switched to medium (RPMI1640) containing 0.5% FCS and allowed to condition the medium for 48 hr. The medium was aspirated and

centrifuged to remove cells, prior to filtration through a 0.2 micron filter and storage at 4^{o}C. In SHH stimulation/inhibition experiments, conditioned medium was diluted 1:1 with complete growth medium prior to incubation.

2.9 Molecular biology

2.9.1 Isolation of RNA

Cells were plated onto 10 cm^2 dishes and grown to 80% confluence prior to total RNA extraction. 500 ml Trizol Reagent (Invitrogen, US) was used to lyse cells and processed as per manufacturer's instructions. A scraper was used to transfer lysed cells to a 1.5 ml eppendorf and shaken for 2 min to homogenise sample. Cell lysates were frozen overnight to ensure complete cell fragmentation. Defrosted samples were incubated at room temperature for 5 min prior to the addition of 100 µl 100% chloroform. Tubes were shaken vigorously for 15 s and incubated at room temperature for 2-3 min before centrifugation at 3,000 rpm for 30 min at 4 ^oC. The upper phase, containing the RNA, was transferred to a fresh eppendorf being careful to leave the participated interphase, containing the DNA, behind. The interphase and phenol was set aside when protein extracts were also to be isolated.

250 µl 100% isopropanol was added to the upper aqueous phase, inverted to mix, and incubated for 10 min at room temperature. Centrifugation at 3,000 rpm for 30 min at 4 ^oC pelleted the RNA which was washed in 0.5 ml 75% ethanol and incubated overnight at -20 ^oC. Ethanol was removed from the RNA pellet, which was allowed to air dry for 5 min before resuspension in max 200 µl DEPC treated water in a fresh eppendorf. Complete dissolution was insured by incubation at 55 ^oC for 10 min. RNA concentration and quality was analysed using a spectrophotometer (Nanodrop, Thermo Scientific, US) and diluted to 0.5 µg/µl with DEPC treated water then stored at -80 ^oC.

2.9.2 cDNA synthesis

First strand cDNA synthesis was achieved using SuperScript III Reverse Transcriptase (Invitrogen, US) as per manufacturer's instructions. Table 2. 6 lists the reagents and volumes used.

Table 2. 6 Reaction mixture A for initial cDNA synthesis reaction

Reagent	Volume (µl)
Random Primers (250 ng/µl)	1
dNTP mix (10 mM)	1
RNA (0.5 µg/µl)	2
SDW	9

The reaction mix A was heated to 65^{o}C for 5 min and subsequently kept on ice for 5 min. Reaction mixture B, in Table 2. 7, was added to each reaction followed by incubation at 50 ^{o}C for 1 hr then 72 ^{o}C for 15 min. cDNA was stored at -20 ^{o}C.

Table 2. 7 Reaction mixture B added to initial cDNA synthesis reaction

Reagent	Volume (µl)
First strand buffer (5x)	4
DTT (0.1 M)	1
RNaseOUT (40 U/µl)	1
SuperScript III RT (200 U/µl)	1

2.9.3 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out using cDNA generated as described above. The reaction mixture for the amplification of a known gene is listed Table 2. 8

Table 2. 8 RT-PCR reaction mixture

Reagent	Volume (µl)
DreamTaq (Thermo Scientific, US)	25
5' Forward Primer (100 pmol/µl)	1
3' Reverse Primer (100 pmol/µl)	1
DEPC-water	22
cDNA	1

The general thermal cycler conditions used are depicted in Table 2. 9. The number of cycles and annealing temperature were optimised for the specific primer set presented (Sigma Aldrich, US).

Table 2. 9 General PCR thermal cycler conditions

Step	Temperature	Time (min)
1.	95 ^{o}C	5
2.	95 ^{o}C	1
3.	Annealing Temperature	0.5
4.	72 ^{o}C	2
Repeat step 2 to 4 for specified cycle no.		
5.	72 ^{o}C	5

Table 2. 10 Primer DNA sequences

Gene	Primers	Annealing Temp (^{o}C)	No. of Cycles	Product Size
SHH	***FWD:*** GCGGACAGGCTGATGACTC ***REV:*** CGAGTTCTCTGCTTTCACCG	62	40	248
IHH	***FWD:*** CTGAACTCGCTGGCTATCTC ***REV:*** CCCACTCTCCAGGCGTAC	57	35	305
GLI1	***FWD:*** GTGTGGGGACAGAAGGAC ***REV:*** GAGATGACCGTAGGAGCC	60	40	248
GLI2	***FWD:*** GTGTCCCGTTTCTCCAGC ***REV:*** GCTTGTTCTGGTTGGTGTCA	58	40	424
GLI3	***FWD:*** CACTACCACCCTCCTCATC ***REV:*** GTATTCTGCTGGCTGAC	56	35	337
SUFU	***FWD:*** GCGGACAGTGCCTATTGCT ***REV:*** GAGGGTTGATTGGTGGAAGG	60	30	320
SFRP1	***FWD:*** AGCGAGTTTGCACTGAGGAT ***REV:*** GGGCACTCATGGTTTTTCAT	58	35	307
STK36	***FWD:*** GAGCAAGAGGACAAGACCAGC ***REV:*** GCAGGATGTGGGAAGCAC	60	35	444
PTCH1	***FWD:*** CACTGTGGTCCATCCCGA ***REV:*** GTGATGGGCTGGCAGTAGC	62	40	306
SMO	***FWD:*** CCCTGGTCTCCAACCCATTC ***REV:*** ACCACAAGCTCTGCATACCC	62	40	481
FOXM1	***FWD:*** CCACTCAGCCTCCAGGACTC ***REV:*** AACCTGTCGCTGCTCCAG	59	30	308
WNT5A	***FWD:*** TGGCTTTGGCCATATTTTTC ***REV:*** CCGATGTACTGCATGTGGTC	57	35	220
BMP2	***FWD:*** CCTGAAACAGAGACCCACC ***REV:*** GCATTCTGATTCACCAACCT	56	56	417
BCL2	***FWD:*** CGGGAGATAGTGATGAAGTAC ***REV:*** AGCGGCGGGAGAAGTCGTCG	62	35	288
SNAI1	***FWD:*** CCACATCAGCCCCACA ***REV:*** CATAGTTAGTCACACCACGT	58	35	321
SIP1	***FWD:*** GCAAACAAGCCAATCCCAGG ***REV:*** TGCATTCTTCACTGGACCATCT	58	35	299
CD133	***FWD:*** CGTAGCAGGTATCAAAAGGG ***REV:*** AGTGCCGTAAGTGCCTCTATT	56	40	583
P75NTR	***FWD:*** CGAGTGCGAGGAGATCCC ***REV:*** ACAACCACAGCAGCCAGG	56	40	234
LRIG1	***FWD:*** GACCTGCCCTCCTGGAC ***REV:*** CAAATGCTCCCAACTCCA	58	35	358
EZH2	***FWD:*** TAGGGCGGTGGAAGATGAA ***REV:*** TTTAGTTCTTCTGCTGTGCC	58	40	355
BMI1	***FWD:*** CATTCCTTCTGTAAAACGTGTATTTA ***REV:*** TCATCTGCAACCTCTCCTC	56	30	254
SOX2	***FWD:*** AGAACCCCAAGATGCACAAC ***REV:*** ATGTAGGTCTGCAGCTGGT	61	40	455
CD44v6	***FWD:*** TCCAGGCAACTCCTAGTAGT ***REV:*** CAGCTGTCCCTGTTGTCGAA	60	32	129
OCT4	***FWD:*** CCTGGGGGTTCTATTTGGG ***REV:*** GCCTCTCACTCGGTTTCG	60	35	219
NESTIN	***FWD:*** CTGGAAGGTCGGCAGCAG ***REV:*** CACCCTGTGTCTGGAGCAG	56	35	415

NANOG	***FWD:*** CAGAGAAGAGTGTCGCAAAAA ***REV:*** ATCCCTGCGTCACACCATT	55	38	267
cMYC	***FWD:*** AACCAGAGTTTCATCCGACCCG ***REV:*** TTGTGCTGATGTGTGGAGACGTGG	62	35	591
CXCR4	***FWD:*** GGTGGTCTATGTTGGCGTCT ***REV:*** TGGCGTGTGACAGCTTGGAG	60	40	228
EPCAM	***FWD:*** GAATGTGTCTGTGAAAACTAC ***REV:*** CAGTGTCCTTGTCTGTTCTTC	55	30	318
EBNA1	***FWD:*** GATGAGCGTTTGGGAGAGCTGATTCTG ***REV:*** TCCTCGTCCATGGTTATCAC	56	40	320
LMP1	***FWD:*** CCCCCACTCTGCTCTCAAA ***REV:*** CCGTGGGGGTCGTCATCAT	56	32	488
GAPDH	***FWD:*** GCCTCCTGCACCACCAACTG ***REV:*** CGACGCCTGCTTCACCACCTTCT	52	25	351

EBV primers used for characterisation of HONE1 cell lines

EBNA1 Qp	***FWD:*** GTGCGCTACCGGATGGCG ***REV:*** CATTTCCAGGTCCTGTTACCT	52	35	236
EBNA1 Wp	***FWD:*** TGGCGTGTGACGTGGTGCAA ***REV:*** CATTTCCAGGTCCTGTTACCT	53	35	265
LMP1 2.8Kb	***FWD:*** ACACACTGCCCTGAGGATGG ***REV:*** ATACCTAAGACAAGTAAGCA	45	35	381
LMP1 3.7Kb	***FWD:*** GCGTTACTCTGACGTAGCCG ***REV:*** ATACCTAAGACAAGTAAGCA	45	35	460
LMP2A	***FWD:*** ATGACTCATCTCAACACATA ***REV:*** CATGTTAGGCAAATTGCAAA	44	35	280
LMP2B	***FWD:*** CAGTGTAATCTGCACAAAGA ***REV:*** CATGTTAGGCAAATTGCAAA	46	35	324
BamH1 A1/2	***FWD:*** CCTTCGATATCGAGTGTCTG ***REV:*** ATGGCCGGAGCTCGTCGACG	50	35	163
BamH1 A3/4	***FWD:*** AGAGACCAGGCTGCTAAACA ***REV:*** AAGCAGCTTTCCTTTCCGAG	53	35	232
EBER1	***FWD:*** AGGACCTACGCTGCCCTAGA ***REV:*** AAAACATGCGGACCACCAGC	55	35	166
EBER2	***FWD:*** GCCGTTGCCCTAGTGGTTT ***REV:*** GGGATTAGAGAATCCTGACTT	50	35	130
BZLF1	***FWD:*** ATTGCACCTTGCCGCCACCTTTG ***REV:*** CGGCATTTTCTGGAAGCCACCCGA	46	35	731
BARF1	***FWD:*** GGCTGTCACCGCTTTCTTGG ***REV:*** AGGTGTTGGCACTTCTGTGG	56	35	202
BHRF1	***FWD:*** GTCAAGGTTTCGTCTGTGTG ***REV:*** TTCTCTTGCTGCTAGCTCCA	51	35	650

2.9.4 Agarose gel electrophoresis

Gel electrophoresis was used to separate PCR product dependent on the size of the DNA fragment. Electrophoresis gel at 0.5% - 2.0%, dependant on fragment size, was made by dissolving ultrapure DNA grade agarose (Sigma Aldrich, US) in 1x TBE buffer (containing 10.8 g Tris, 4.5 g orthoboric acid and 0.74 g EDTA per 1 litre SDW) with aid of a microwave oven. The solution was poured into a casting gel around an appropriate gel comb and allowed to solidify at room temperature. Amplified PCR produces were loaded into the gel in a gel electrophoresis tank containing 1x TBE alongside a molecular weight ladder (1 Kb and 100 bp ladder, Invitrogen, US). DNA was visualised and photographed with a GeneFlash bio imaging system (GeneFlow, UK).

2.10 Real-time quantitative PCR (QPCR)

Expression of viral mRNAs was performed on cDNA generated as in section 2.9.2. TaqMan® primer and probe sets for EBNA1, LMP1 and LMP2A, Table 2. 11; sequences from Bell at al., (2006), were labelled with a 6-carboxyfluorescein phosphormaidite (FAM™) reporter dye at the 5’ end and 6-carboxytetramethylrhodamin (TAMRA™) at the 3’ end. Expression of mRNA of interest was normalised against a commercially available huGAPDH primer/probe set containing a VIC™ labelled probe (4310884E, Applied Biosciences, US) as an internal control. The reaction mixture is described in Table 2. 12.

Table 2. 11 TaqMan primer/probe sequences for EBV transcript detection
Sequences from Bell et al., (2006)

Gene	Oligonucleotide sequence	EBV coordinates
EBNA1	**5'** GTGCGCTACCGGATGGC	62440-62456
	3' CATGATTCACACTTAAAGGAGACGG	107952-107941
	Probe TCCTCTGGAGCCTGACCTGTGATCG	67563-67587
LMP1	**5'** AATTGCACGGACAGGCATT	169102–169083
	3' AAGGCCAAAAGCTGCCAGAT	168893–168912
	Probe TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT	168951–168965
LMP2A	**5'** CGGGATGACTCATCTCAACACATA	166870–166893
	3' GGCGGTCACAACGGTACTAACT	166870–166893
	Probe CAGTATGCCTGCCTGTAATTGTTGCGC	66–92

Table 2. 12 QPCR reaction mixture

Reagent	Volume (µl)
Gene of interest primer and probe (10 pmol primer, 5 pmol probe)	1
huGAPDH primer and probe	0.5
1x TaqMan universal mastermix (Applied Biosystems, US)	10
DEPC-water	3.5
cDNA (5 ng/µl)	5

Thermal-cycling conditions were based on those described in Bell et al., (2006). All reactions were performed in triplicate and analysed on an ABI 7500 Fast RT-PCR machine (Applied Biosystems, US) using the $2^{-\Delta\Delta Ct}$ method described in Livak and Schmittgen (2001). Cycle threshold (Ct) values were determined by identifying the value at which amplification exceeded background levels for each reaction, Primers for the gene of interest were then normalised to the internal GAPDH control giving a ΔCt value which were averaged to produce a mean ΔCt value for each gene of interest. Normalisation of all value to the control cell line resulted in a ΔΔCt value from which fold changes relative to the control could be calculated using the $2^{-\Delta\Delta Ct}$ formula.

2.11 Immunoblotting

2.11.1 Solutions and buffers

RIPA Buffer 50 mM: Tris-HCl (pH 8.0), 150 mM NACl, 1% NP40, 0.5% deoxycholate, 0.1% SDS. 40 µl/ml complete protease inhibitors (Roche, Switzerland), 0.5 mM sodium orthovanadate (S6508, Sigma Aldrich, US) and 1 mM PMSF (P7626, Sigma Aldrich, US).

3x SDS sample buffer: 197.5 mM Tris-HCl (pH 8.0), 6% w/v SDS, 30% glycerol, 0.03% w/v bromophenol blue.

*Resolving Gel Buffer***:** 500 ml solution of 1.5 M Tris-HCl (pH 8.8) made by dissolving 90.86 g Tris in SDW containing 0.4% w/v SDS and 0.24% w/v TEMED.

Stacking Gel Buffer: 500ml solution of 0.25 M Tris-HCl (pH 6.8) made by dissolving 15.14 g Tris in SDW containing 0.2% w/v SDS and 0.12% w/v TEMED.

1x Running Buffer: 10x solution made up of 30g Tris-HCl, 144 g glycine, and 10 g SDS. Made up to 10 litres with SDW. Diluted 1 in 10 with SDW before use.

1x Transfer Buffer: Solution made up of 30g Tris and 144g glycine in 8 litres of SDW and 2 litres of 100% methanol.

10x Tris Buffered Saline-Tween (TBS-T): Stock solution made up of 60.5 g Tris-HCl, 200 g NaCl, 25 ml Tween-20 made up to 10 litres with SDW. Diluted 1 in 10 with SDW before use.

Blocking Buffer: 5% non-fat milk powder in 1% TBS-T

Stripping Buffer: Solution made up of 100 mM Tris-HCl pH 6.8, 2% w/v SDS, 50 mM 2-mercaptoethanol, in 500 ml SDW

Ammonium Persulphate (APS): 20% stock made up by dissolving 2 g APS in 10 ml and aliquoted at 1 ml in 1.5 ml eppendorfs.

2.11.2 Preparation of whole cell extracts

Cells were plated onto 10cm^2 dishes and grown to 80% confluence prior to protein extraction. Cells were washed once in PBS and lysed with 50ml ice-cold RIPA supplemented with a complete protease inhibitor tablet (Roche, Switzerland). A scraper was used to transfer lysed cells to a 1.5 ml eppendorf and sonication for 10 s on a 50% setting. Cellular debris was removed by centrifuging at 13,000 rpm for 5 min at 4 ^{o}C and the transferring the supernatant to a new eppendorf.

The DC protein assay kit (Bio-Rad, US) was used to determine protein concentration of lysates as per manufacturer's instructions. 5 µl of protein standard and sample lysates were aliquoted in triplicate into a 96 well plate and to this added 25 µl of reagent A and 200 µl of reagent B per well. The plate was placed on a plate shaker at 200 rpm for 3 min prior to reading on a microplate automated plate reader (Biotek, US) at 620 nm. A standard curve of the BSA standards allowed for protein concentration to be calculated.

Equal amounts of cell sample lysates were suspended in 3x sodium dodecyl sulphate sample buffer and incubated to 100 ^{o}C for 5 min. Boiled lysates were returned to ice prior to loading on appropriate percentage poly-acrylamide gel next to PageRuler protein standard marker (Fermentas, Fisher Scientific, US).

2.11.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Mini-PROTEAN 3 electrophoresis kit (Biorad, US) was used to cast an appropriate concentration of SDS-polyacrylamide gel. Table 2. 13 shows the reagents used to make up a single 10% polyacrylamide gel.

Table 2. 13 Reagents for 10ml of 10% resolving gel

Reagent	Volume (ml)
SDW	4.0
30% acrylamide mix	3.3
Resolving Buffer	2.5
20% APS	0.1
0.8% N,N-bis acrylamide (v/v)	0.004

To assist with a level gel being cast, once poured the gel was overlaid with 100% ethanol and allowed to polymerise at room temperature. The ethanol was then poured off and the gel washed with SDW. Stacking gel, detailed in Table 2. 14, was then poured on top, a gel comb inserted and allowed to polymerise.

Table 2. 14 Reagents for 5ml of 10% stacking gel

Reagent	Volume (ml)
SDW	3.2
30% acrylamide mix	0.83
Resolving Buffer	0.84
20% APS	0.05
0.8% N,N-bis acrylamide (v/v)	0.005

Prior to loading the wells were washed with SDW. Equal amounts of protein samples were loaded into the wells and accompanied by a full range molecular weight marker (10,000-250,000 bp) (Amersham Biosciences, UK). Electrophoresis was carried out, cooled by a constant cold water flow, at 90 V for 90 min or until the loading dye had reached the bottom of the running gel.

2.11.4 Western blot analysis

Membranes were blocked in 5% milk powder in Tris buffered saline-Tween 20 (TBST) for a minimum of 30 min and incubated in primary antibody overnight on a nutator at 4 ^{o}C. Antibodies used are shown in Table 2.13.

Following three 5 min washes in TBST, the membranes were incubated in horseradish peroxidise-tagged secondary antibody (1:1,000; Dako, US) for 1 hr at room temperature on a nutator. After three 5 min washes in TBST, membrane were incubated with enhanced chemiliuminescence (ECL) detection reagents (Amersham International, UK) for 5 min, rinsed with TBST and exposed to X-ray film (Kodak, US).

Following electrophoresis, the resolved soluble proteins were transferred onto a nitrocellulose membrane (Geneflow, UK). The gel and membrane were sandwiched between two layers of 3MM filter paper (Whatman, UK) which were pre-soaked in transfer buffer. Proteins were transferred from the gel onto the membrane using the Mini-transblot apparatus (Biorad, UK), at 90 V for 90 min on ice. Non-specific binding sites on the membrane were blocked in blocking buffer for 1 hr at room temperature with gentle agitation. The membrane was then placed into blocked buffer containing the relevant primary antibody (Table 2. 15) and incubated overnight at 4 ^{o}C with gentle agitation. The membrane was rinsed in TBS-R followed by 3 x 5 min washes in TBS-T and subsequently incubated with the appropriate horse radish peroxidise (HRP) conjugated secondary antibody (Dako, US) at room temperature for 1 hr with gentle agitation. Following incubation with the secondary antibody, the membrane was rinsed in TBS-T followed by 3 x 5 min washes in TBS-T. The complex bound to the protein of choice was detected and visualised by enhanced chemiliuminescence

(ECL) detection reagents (Amersham International, UK). Briefly, the membrane was incubated in a 1:1 solution of reagent A and reagent B for 5 min at room temperature with no agitation. The membrane was rinsed in TBS-T to removed excess ECL reagent and covered in saran wrap. The membrane was exposed to X-ray film (Kodak, US) for different time periods depending on the strength of the chemiluminescent signal. X-ray films were developed in a compact automatic film processor x2 (Xograph Ltd., UK).

Table 2. 15 Antibodies and conditions used for western blot analysis, immunofluorescence staining, and FACS analysis
(Abbreviations: ab; Abcam, SC; Santa Cruz, C; Cell Signalling, M; Miltenyi, AC; Merck Millipore, Sig; Sigma, -MM; mouse monoclonal, RM; rabbit monoclonal, RP; rabbit polyclonal, H; from human serum, WB; western blot analysis, IHC, immunohistochemistry, IF; immunofluorescence, FACS; flow activated cell sorting)

Protein	Antibody	Nature	WB	IHC	IF/FACS
SHH	ab53281	RM	1:500	1:200	1:100
PTCH	ab53715	RP	1:500	1:400	1:200
SMO	ab38686	RP	1:1000	1:50	1:50
GLI1	ab49314	RP	1:400	-	1:100
GLI1	SC20687	RP	-	1:50	-
GLI2	ab26065	RM	1:250	1:100	1:50
GLI3	ab6050	RP	1:500	1:800	1:50
FOXM1	SC500	RP	1:250	-	1:50
FOXM1	ab83907	RP	-	1:25	-
SUFU	ab52913	RM	1:1000	1:50	-
BMP2	ab14933	RP	1:200	-	-
WNT5A	ab30436	MM	1:1000	-	-
CD133	C24B9[1]	RM	-	-	1:10
CD133macs	M293C3pure	MM	-	-	1:50
P75NTR	ab8874	RM	-	-	1:100
CD44v6	ab30436	MM	1:1000	-	1:100
CD44	ab51037	RM	1:5000	-	-
NESTIN	ab22035	MM	1:1000	-	-
BMI1	SC10745	RP	1:200	-	-
EZH2	AC22	MM	1:1000	-	-
SIP1	ab25837	RM	1:500	-	-
SNAIL1	SC28199	RM	-	-	1:50
SNAIL1	ab17732[1]	RM	1:500	-	-
BZLF1	BZ1[2]	H	1:500	-	-
EBNA1	R4[3]	H	1:5000	-	1:1000
β-Actin	ab3280	MM	1:10000	-	-

[1]Discontinued
[2]Monoclonal human antibody provided in house, acknowledgements to Dr. Martin Rowe
[3]A kind gift from Prof. L. Frappier

2.12 *In situ* immunofluorescence staining

2.12.1 Immunofluorescence staining solutions

4% Paraformaldehyde (PFA): 20 g PFA was dissolved in 500 ml SDW, dissolved by stirring overnight at 42 ^{o}C. Once dissolved, five Dulbecco A tablets (Sigma Aldrich, US) were added and the bottle was covered in tin foil and stored at room temperature.

20% HINGS: Heat inactivated normal goat serum diluted to 20% in PBS and stored at -20 ^{o}C.

0.5% Triton X-100: 2.5 ml Triton X-100 dissolved in 497.5 ml 1 x PBS and stored at 4 ^{o}C

DABCO: 90 ml 100% glycerol was mixed with 10 ml 1x PBS and 2.5 mg DABCO powder (Sigma Aldrich, US). The solution was adjusted to pH 8.6, covered in silver foil, and stored at room temperature.

2.12.2 *in –situ* immunofluorescence staining procedure

Cells of interest were seeded on to Teflon coated microdot slide (Henley, UK) at 1 x10^{4} cells in 50µl complete growth medium. Slides were kept in a sterile petri dish with 2ml PBS, and incubated overnight at 37 ^{o}C in a humidified incubator containing 5% CO_2 to allow the cells to adhere. Two staining protocols were used.

Protocol 1: Cells were washed and fixed in 4% PFA for 10 min and permeabilised in 0.5% Triton X-100 for 5 min for the detection of intracellular proteins. Non-specific binding sites were blocked by incubation with 50 µl 20% HINGS in PBS for 1 hr in a wet box at room temperature. Slides were incubated for 1 hr with primary antibody (see table 2. 13) diluted in 20% HINGS, in a wet box at room temperature. Following

3 x 5 min washes in PBS, cells were incubated for 1 hr in a wet box at room temperature with secondary antibody (1:1000 in 20% HINGS) containing DAPI nuclear stain (1:1000) for 1 hr. Slides were washed again 3 x 5 min prior to mounting with DABCO anti-fading agent. Slides were stored at -20 ^{o}C until being viewed on a fluorescence microscope.

Protocol 2 (for the detection of cell surface markers without fixation): Cells were washed and maintained at 4 ^{o}C throughout this protocol. Cells were blocked in 50 µl 20% HINGS in PBS with the addition of 0.1% sodium azide (from a stock solution of 10% sodium azide) for 30 min. Primary antibody (20% HINGS, 0.1% sodium azide in PBS) was incubated for 1 hr, cells washed trice for 5 min in PBS, and secondary antibody (20% HINGS, 0.1% sodium azide in PDBS) incubated for a further hr. Slides were washed a further 3 times prior to fixation in 4% PFA for 10 min.

2.13 FACS analysis

Cells harvested using accutase reagent (Innovative Cell Technologies, US) to maintain cell surface protein integrity, plated into a v-bottomed 96 well plate at 5 x10^{5} cell per well and maintained at 4 ^{o}C on ice for the staining procedure. Cells were spun down prior to addition of primary antibody (see table 2. 13) diluted in 20% HINGS for 1 hr. Cells were washed with 1% FCS three times by resuspension and re-centrifugation, prior to addition of appropriate secondary antibody Alexa Flour 488 (Invitrogen, US) for 60 min. Following three further washes, cells were fixed in 1% PFA and analysed on XL-MCL Flow Cytometer (Beckman Coulter's Epics, US) or Accuri C6 (BD Biosciences, US).

2.14 IHC staining

2.14.1 IHC staining solutions

EDTA buffer/Tween: 10x solution supplied by Abcam, UK (ab64216) Diluted 1:10 prior to use

2.14.2 IHC pre-treatment and staining procedure

Formalin-fixed, paraffin-embedded blocks of NPC and normal tonsil tissue were obtained from the Pathology Department of the Cancer Centre at Sun Yat-Sen University (Guangzhou, Guangdong, China) in 2007 were sectioned, heated at 60 ^{o}C for 60 min, and pre-treated by sequentially placing sides into xylene, 100% IMS, and 0.3% hydrogen peroxide in water for 5 min.

The ALTER (Agitated Low Temperature Epitope Retrieval) method was used prior to staining by heating slides to 65 ^{o}C in 1 litre of diluted pH 8 EDTA buffer/Tween, and leaving stirring for 16 hr. EDTA/Tween buffer was removed from the hotplate and placed under a running tap until the liquid reached room temperature.

Slides were washed in PBS and non-specific antibody binding blocked by applying serum for 10 min, with use of a wax pen, prior to staining. Primary antibody was added at serial dilutions in PBS for 1.5 hr, and slides washed with PBS/Tween prior to the addition of secondary antibody for 30 min. DAB chromagen was used to visualise antibody as a brown stain for a defined time (10 s to 2 min). Slides were washed prior to counter stain with Haematoxylin for 40 s, washed again in warm tap water for 5 min, de-hydrated in IMS for 5 min, washed in xylene, and left to dry before mounting in DPX mountant (Sigma Aldrich, UK).

Following optimisation, staining for proteins of interest was performed on tissue arrays, containing cores from 50 NPC tumour samples and 20 normal counterparts, using the optimised antibody concentrations and conditions. Scores for protein expression (values 0-9) were obtained using a semi-quantitative scoring system by multiplying staining intensity (negative = 0, weak = 1, moderate = 2, or strong = 3) by the proportion of positive cells (< 30% = 1, 30-70% = 2, > 70% = 3) (Hu et al., 2012).

2.15 Cell proliferation ELISA, BRDU (colorimetric)

This colorimetric immunoassay was purchased as a kit from Roche, Switzerland (catalogue no. 11 647 229 001) and used according to manufacturer's instructions. Briefly cells, in exponential grown phase, in a 96 well plate were incubated with diluted BDRU labelling reagent for 4-6 hr, depending on proliferative rate of the individual cell lines. Cells were fixed with FixDenat solution according to instructions, and anti-BRDU-POD solution applied, to bind incorporated BRDU, prior to washing. Anti-BRDU-POD was detected using a tetrametyl-benzidine colorimetric substrate reaction allowing for spectrophotometric detection on a Victor™ plate reader (PerkinElmer, US).

2.16 Anchorage independent growth assay

2.16.1 Anchorage independent growth assay solutions

2x RPM1 acquired from Fisher Scientific, US.

10% agarose: made up with dH_2O and autoclaved for sterilisation. Agarose was re-liquefied prior to use by briefly heating in a microwave and allowing to cool to

37 ^{o}C in a water bath. All solutions made with agarose were kept at 37 ^{o}C to prevent their solidification.

2.16.2 Anchorage independent growth assay protocol

This assay measures proliferative ability by assessing the tendency of a single cell suspension to form colonies. Cells were plated out at 1 x10^{2}-10^{3} cells per well in full complement 5% FCS media containing 0.3% agarose. This was achieved by first layering the bottom of a 12 well plate with 0.5ml of 5% FCS/1% RPMI/1% agarose and allowing the agarose to set at 4 ^{o}C, before transferring briefly to -20 ^{o}C. A cell line dependent number of cells were seeded in 1ml per well 5% FCS/1%RPMI/0.3% agarose, and allowed to set in pre-layered and cooled plates before transferring to the incubator. GANT treatment was applied at 25-50 µM total volume of well in 50 µl every 3-4 days for 2-4 weeks. All colonies greater of equal to 25 µm in size were counted and four representative images of each well, in triplicate, were taken to allow scoring of maximal diameter using calibrated AxioVision software (Zeiss, Germany).

2.17 Sphere forming assay

2.17.1 Sphere forming assay solutions

Assay as with colony forming replacing FCS with restricted supplements as follows: N2® supplement (Invitrogen, US); used at 1% total agarose containing media, B27® supplement (Invitrogen, US); used at 2% total agarose containing media, FGF (10 ng/ml) and EGF (100 ng/ml).

2.17.2 Sphere forming assay protocol

Cell set up as with the colony forming assay in restricted supplement 1% RPM1/0.3% agarose on a layer of restricted supplement 1% RPM1/1% agarose. Cells seeded at a higher concentration 5 $x10^2$-10^3 depending on proliferative potential of the cell line. GANT treatment was applied at 25-50 µM total volume of well in 50 µl every 3-4 days for 2-4 weeks. All colonies greater of equal to 25 µm in size were counted and three representative images of each well, in triplicate, were taken to allow scoring of maximal diameter using calibrated AxioVision software (Zeiss, Germany).

2.18 Statistics

Unless otherwise stated, statistical significance was determined using a Student's t-test preceded by an f-test to establish the equality of variance.

CHAPTER THREE: DYSREGULATION OF THE HEDGEHOG SIGNALLING PATHWAY AND ITS RELATION TO STEMNESS IN NASOPHARYNGEAL CARCINOMA.

"Science and art belong to the whole world, and the barriers of nationality vanish before them."

– Johann Wolfgang Goethe

Remark to a German historian, 1813. From the series Great Ideas of Western Man.

3.1 Introduction

A number of signalling pathways have been found to be consistently dysregulated in NPC. These include the TGFβ, WNT, EGF, MAPK, PI3K/Akt, NOTCH and NFκB signalling pathways (Sriuranpong et al., 2004, Zeng et al., 2007b, Shi et al., 2006, Morrison et al., 2004, Lee et al., 2007b, Liu et al., 2009, Zhang et al., 2009a). Whilst these pathways function to regulate various aspects of cellular growth and differentiation, they also play an essential role in the growth, renewal and cell fate commitment of both ESC and adult stem cells (Rochon et al., 2006, Ramalho-Santos et al., 2002). Adult stem cells, like ESC, have properties of limitless self-renewal and multi-lineage differentiation, while residing life-long in adult organs and tissues. These specialised cells have the role of generating transit amplifying (TA) cells and terminally differentiated cells of the tissue in which they reside during normal growth and under situations of wound healing or tissue repair (Prockop et al., 2003, Lehrer et al., 1998, Alison et al., 2002).

Many cancers, including NPC, exhibit dysregulation of one or more signalling pathways implicated in stem cell maintenance. Additionally, certain malignancies contain small populations of cells that share characteristic properties with stem cell populations such as limitless self-renewal and multiple lineage differentiation capacity (Reya et al., 2001). Furthermore, isolation of cell sub-populations has provided evidence that these “stem-like” cells display increased tumour forming properties compared to non “stem-like” cells (Wang et al., 2007). The ability of cells to form tumours upon serial transplantation is the gold standard for identification of CSCs (Visvader and Lindeman, 2008).

Two opposing theories exist as to the process of CSC generation. In the first scenario, CSCs are produced through acquired mutations in normal stem cells; the second proposes that stem cell-like characteristics are acquired in more committed cells through genetic and/or epigenetic changes. Regardless of their mechanism of generation, it is now recognised that most, if not all, solid tumours and haematological malignancies possess a sub-population of CSCs. These undifferentiated cells are responsible for generating more committed malignant cells that make up the bulk of the tumour (Tu et al., 2002, Bjerkvig et al., 2005). The presence of CSCs, which are inherently more resistant to chemotherapy and radiotherapy (Costello et al., 2000, Dean et al., 2005, Guzman et al., 2002), are believed to be at least partly responsible for the return of malignancies following periods of regression. Evidence for this comes from the finding that tumour responses to chemotherapeutic drugs, as measured by shrinkage of the tumour mass rather than the eradication of the CSC population, does not always correlate with increased survival (Reya et al., 2001, Al-Hajj et al., 2004, Wicha et al., 2006). This is true of head and neck malignancies, which often respond poorly to chemotherapy, resulting in high rates of recurrence (Prince and Ailles, 2008). In comparison, drug targeting of CSC populations in addition to conventional therapies are often more effective therapies (Tozer et al., 2005). Whilst CSC targeted therapies may not induce rapid tumour shrinkage, these therapies can increase tumour remission times and overall survival (Hirsch et al., 2009, Beier et al., 2008).

Like many other solid tumours sub-populations of cells displaying CSC-like characteristics have been isolated from NPC. One means by which NPC CSCs have been isolated is based on their ability to exclude Hoechst DNA binding dye through

upregulation of multidrug transport proteins (Wang et al., 2007). Both normal stem cell and CSC populations express high levels of multidrug transporters, which act to exclude potentially damaging chemicals that could impinge on the stability of the cellular genome. Expulsion of drugs protects these cell populations allowing them to exist stably for periods of time greater than that of more differentiated cells and the expression of these multidrug transporters is associated with increased chemotherapy resistance (Doyle and Ross, 2003). Hoechst dye expulsion used in conjunction with rhodamine 123 (Wolf et al., 1993) or detected with dual fluorescence emission (Goodell et al., 1996) has facilitated the purification of “side population” (SP) cells from numerous tissues (reviewed by Hadnagy et al., 2006). SP sorted cells contain populations which will undergo long-term repopulation, and differentiate to regenerate the lineages of cells of the tissue from which they were generated. Tumour-derived SP cells will additionally demonstrate increased tumour forming potential compared to non SP cells and may show abnormal cell maintenance pathway activity such as NOTCH, PI3K/Akt, and HH (Dontu et al., 2004, Fan et al., 2006, Bleau et al., 2009, Bar et al., 2007).

Hoechst dye has been used to identify sub populations of cells displaying increased expression of these multidrug transporters from the EBV-negative NPC cell line CNE2. Cells isolated through their ability to exclude Hoechst dye, displayed increased proliferative potential, could differentiate to recapitulate the whole cell population from which they were isolated, and showed increased tumour formation in non-SCID mice. This sub population also demonstrated greater resistance to a number of drugs, X-rays and radiation. However, sensitivity to radiation could be restored in response to cyclopamine treatment, a HH pathway inhibitor. These

abilities suggest that the Hoechst dye excluding cells were, or included, a stem cell population that was, at least partially, dependent on HH signalling pathway activity (Wang et al., 2007).

Tissue specific CSC markers have been used to identify populations of tumour-initiating cells from a variety of tumours. The isolation of cells expressing putative CSC markers has also been performed from NPC cell lines. CD44 positive cells (CD44+), isolated from the EBV negative NPC cell line SUNE1, displayed increased proliferative capacity compared to CD44- or unsorted counterparts. CD44+ cells also displayed increased expression of stem cell-associated genes, BMI1 and OCT4, and were less sensitive to radiation and the chemotherapy reagent cisplatin. This is consistent with the theory that CSCs are refractory to genotoxic assault in response to chemo or radiotherapy and repopulate the tumour following remission (Su et al., 2011). Further studies used small interfering RNAs (siRNAs) to inhibit CD44 expression, were found to significantly impact on tumour volume by inhibiting progression from G0/G1 to S phase in the EBV negative NPC cell line CNE-2L2 (Shi et al., 2007).

CD44 has many splice variants; different isoforms have been identified in many epithelial carcinomas (Gunthert et al., 1991, Ghatak et al., 2010, da Cunha et al., 2010). In breast carcinoma, isoform switching has been shown to be essential for an EMT (Brown et al., 2011) and also correlates with tumour subtypes and CSC marker expression (Olsson et al., 2011). CD44 expression has also been shown to influence cell phenotype in head and neck squamous carcinoma, whereas CD44 variant

expression, including variant 6 (CD44v6), correlated with tumour grade, migration, proliferation and cisplatin sensitivity (Wang et al., 2009).

The expression of stem cell maintenance proteins has also been demonstrated in NPC. The BMI1 protein is required from stem cell maintenance and self-renewal in a variety of tissue types (Park et al., 2004) and is frequently upregulated in a wide variety of epithelial and even haematological malignancies (Kim et al., 2004, Bhattacharya et al., 2009, Bea et al., 2001). Expression of BMI1 in NPC is associated with poor prognosis (Song et al., 2006), and down regulation of BMI1 assists in growth inhibition (Wu et al., 2011). Another stem cell maintenance protein, EZH2, is often overexpressed in recurrent NPC, highlighting a correlation between an increase in the population of cells that express this marker and increased resistance to therapy (Alajez et al., 2010).

Work presented in this chapter investigates the expression of putative stem cell markers, and stem cell associated proteins in NPC, and investigates the status of HH signalling in this disease.

3.1.1 Hedgehog (HH) signalling pathway

The HH signalling pathway was described in detail in section 1.11. Briefly, HH signal transduction involves HH ligand, of which there are three (SHH, DHH and IHH), binding to the membrane receptor PTCH. In the absence of HH ligand, PTCH inhibits the activity of SMO, a constitutively active serpentine receptor (Denef et al., 2000). HH ligand binding relieves PTCH inhibition of SMO (Ingham, 1998), which localises

to the membrane cilium and activates the GLI transcription factors (GLI1, 2 and 3) and the transcription of downstream targets.

The HH signalling pathway is critical for stem cell maintenance; it plays an essential role in development, morphogenesis, and patterning (Ruiz i Altaba et al., 2002, Goodrich and Scott, 1998, Ingham and McMahon, 2001, Fournier-Thibault et al., 2009, Huycke et al., 2012, Huang et al., 2010a, Dessaud et al., 2008). HH signalling is operational in both the embryonic stem cell and adult stem cell compartments (Lai et al., 2003, Palma and Ruiz i Altaba, 2004) as well as being implicated in the cancers including basal cell carcinoma (BCC) (Kasper et al., 2012), Glioblastoma Multiforme (GBM) (Braun et al., 2012), meduloblastoma (Berman et al., 2002), lung (Watkins et al., 2003), gastric (Berman et al., 2003), prostate (Karhadkar et al., 2004) and pancreatic carcinomas (Thayer et al., 2003). In common with primordial BCC, NPC WHO III displays an undifferentiated phenotype, raising the possibility that NPC is derived from less differentiated stem cell progenitors (Brennan, 2006). Activating mutations in the HH pathway, such as SMO, when targeted to the epidermis of transgenic murine models, is sufficient to induce BCC formation (Aszterbaum et al., 1999, Mancuso et al., 2004), as is overexpression of the GLI2 transcription factor (Grachtchouk et al., 2000). Indeed the vast majority of BCC are the result of mutations most commonly in PTCH and less often in SMO (Johnson et al., 1996, Xie et al., 1998).

The HH signalling pathway engages many downstream targets that intersect with other signalling pathways. These include WNT5A, which feeds into the WNT pathway, NOTCH, ACTIVIN and the BMPs, members of the TGFβ family of proteins,

which serve to modulate HH signalling and promote differentiation. Induction of the HH signalling pathway could, therefore, have a widespread and significant impact on a cellular signalling network responsible for stem cell maintenance, renewal and differentiation. In addition, the HH signalling pathway is itself a downstream target of other cell signalling pathways such as TGFβ/Activin/BMP, WNT and NOTCH, which are also dysregulated in NPC (Zeng et al., 2007a, Zeng et al., 2007b, Wang et al., 2011, Man et al., 2012).

Previous work in this laboratory has utilised gene expression profiling of 4 normal nasopharynx and 15 microdissected NPC biopsies along with the C666.1 cell line, to identify cell signalling pathways whose dysregulation is a feature of this disease. Whilst other NPC xenografts have been isolated C666.1 is the only authentic EBV positive NPC cell line that can be propagated *in vitro*. Derived from an NPC xenograft originating in Southern China, the C666.1 cell line is unique as it maintains the EBV genome. These data were interrogated for dysregulation of signalling pathways involved in stem cell maintenance and confirmed previous studies demonstrating that the TGFβ, BMP, ACTIVIN and WNT signalling pathways were indeed dysregulated in NPC (Zeng et al., 2007b, Zeng et al., 2007a, Wang et al., 2011, Man et al., 2012). Further interrogation has been performed on this data, which will be presented in this thesis and was indeed the initial finding which sparked further investigation into the condition of the HH signalling pathway in NPC.

3.2 Primary nasopharyngeal carcinoma and the C666.1 cell line are enriched for basal cell and putative stem cell markers

3.2.1 Microarray expression data reveals increased expression of basal cell and putative stem cell markers in nasopharyngeal carcinoma tumour biopsies

Initially, microarray expression data obtained from microdissected NPC tumours was mined for the expression of basal/stem cell markers and HH pathway components. Normal age-matched control nasopharyngeal epithelium and tonsil epithelium served as controls. Whilst the microarray analysis was not performed as part of this project, some understanding of the methodologies involving data collection, sample correlation, the methods of analysis employed, and results generated from this study should be mentioned.

Global gene expression profiling was performed on total RNA extracted from laser microdissected purified cells (LMPCs) from 3 normal and 1 tonsil epithelium biopsies, along with 14 NPC tumours samples and the C666.1 cell line using Affymetrix Human Genome U133Plus 2.0 chips. Previously, a correlation heatmap of the four normal samples (MSTA, MBEZ, MHAU and T3) and 15 tumours (14 biopsies and C666.1 prototype NPC cell line) established that geographical and gender differences were not significant between samples and that C666.1 constitutes a good tumour model for NPC. It was also established that tonsil epithelium closely resembles nasopharyngeal epithelium, allowing for the inclusion of this tissue in subsequent immunohistochemical (IHC) validation. Additional gene expression profiling between the C666.1 cell line and the four normal biopsies demonstrated fold

changes in the same direction as those observed between the NPC tumours and normal biopsies, and established the C666.1 cell line as a good model for NPC (Hu, 2010).

Microarray data previous obtained in our laboratory (Hu, 2010) was interrogated for genes relating to epithelial cell 'stemness'. The array data has been deposited in the GEO database, http://www.ncbi.nlm.nih.gov/geo, with accession number GSE34573 (Hu et al., 2012). Initially the gene expression array data generated from the 16 NPC samples and 4 normal samples (three normal nasopharynx and one tonsil epithelium) were normalised and processed using Robust Multi-array Average (RMA) and Rank Product (RP) analyses to identify differentially expressed genes and the fold change in their expression. Affymetrix GeneChip Operating Software (GCOS) analysis was also performed allowing the present/absent status of the gene expression above background levels to be taken into account.

Interrogation of the microarray data from the four normal nasopharyngeal/tonsil tissue samples, the 14 microdissected NPC samples and C666.1 cell line, identified increased expression of squamous epithelial cell basal cell markers in NPC tumours, consistent with the undifferentiated nature of NPC tumours. In addition, a number of putative stem cell markers were found to be upregulated in NPC specimens relative to control tissue. Table 3.1 shows the fold changes and statistical significance, from RP analysis on expression array data, of basal cell and putative stem cell markers identified from the literature. Genes have been included if present in 8 out of 16 NPC samples and/or present in 2 of 4 normal nasopharyngeal epithelium by GCOS analysis, or otherwise believed to be of key importance.

Epithelial basal cell markers upregulated in NPC included the β1 and α6 integrins, and ΔNp63 (designated as TP63). Integrins play a key role in establishing cell-matrix interactions but also facilitate cell-cell adhesion. A number of integrins are often overexpressed in carcinomas, where they increase cell survival and promote angiogenesis, invasion and metastasis (Jin and Varner, 2004, Orimoto et al., 2008). Additionally, high integrin levels, typically β1 and α6, have been used to isolate putative epidermal stem cells from normal squamous epithelium (Jones and Watt, 1993, Seery and Watt, 2000). ΔNp63 is also required for cell adhesion and its expression in stratified epithelium modulates cell survival and stem cell maintenance (Carroll et al., 2006). Markers that have been used to identify putative stem cell populations in other epithelial cell types were also found to be highly expressed in NPC tumour biopsies compared to normal epithelium, along with proteins known to have a role in stem cell maintenance.

SC markers upregulated in NPC include LRIG1; an inter-follicular stem cell marker with a role in maintaining quiescence (Jensen et al., 2009); SOX2, a transcription factor required to maintain self-renewal that has the capacity to induce pluripotent stem cells when combined with OCT4 (Huangfu et al., 2008), and EZH2 and BMI1; polycomb family members controlling proliferative potential and self-renewal (Ezhkova et al., 2009, Park et al., 2004). LRIG1, SOX2, EZH2 and BMI1 were found to be upregulated in 87.5%, 75%, 93.5% and 81.25% of NPC samples respectively, relative to control tissues, with fold changes of 2.79, 3.15, 5.05, and 2.12. In addition, two cell surface stem cell and/or CSC markers: CD44, a receptor for glycosaminoglycan hyaluronate with a role in tumour metastasis (Gunthert et al., 1991), and p75NTR, an oesophageal and oral keratinocyte stem cell marker

(Okumura et al., 2003, Nakamura et al., 2007a), were found to be upregulated in 81.25%, and 50% of NPC samples, relative to control tissues, by 2.50 and 1.98 fold respectively.

A heat map showing the relative expression of basal cell and stem cell markers in the NPC tumours compared to control tissue (normal nasopharyngeal/tonsil epithelium) is shown in Figure 3. 1. From the heatmap it can be seen than no one tumour sample shows upregulation of all markers but there is a general consensus that NPC tumours are enriched for expression of various basal and stem cell markers compared to control epithelium. It can also be seen that the C666.1 cell line appears to correlate with the NPC tumours displaying a comparable stem cell marker expression profile.

Table 3.1 Expression of basal cell and putative stem cell markers genes in micro-dissected NPC tumour tissue verses normal nasopharyngeal/tonsillar epithelium by microarray analysis

Gene Accession	Gene Symbol	Name	p value (t)	FC (overall)	Present In NPC (normal)	up in (RMA)	up in % (RMA)
	Basal cell marker						
1553678_a_at	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.6E-06***	9.37	16/16 (1/4)	16	100
201656_at	ITGA6	Integrin, alpha 6	0.0010**	3.17	16/16 (4/4)	15	93.75
201983_s_at	EGFR	Epidermal growth factor receptor (erythroblastic leukaemia viral (v-erb-b) oncogene homolog, avian)	NS	1.72	13/16 (3/4)	9	56.25
212567_s_at	MAP4	Microtubule-associated protein 4	NS	1.44	13/16 (0/4)	12	75
207382_at	TP73L	Tumour protein p73-like	NS	1.33	10/16 (0/4)	10	62.5
203110_at	PTK2B	PTK2B protein tyrosine kinase 2 beta	NS	1.32	8/16 (0/4)	13	81.25
	Stem cell markers						
203685_at	BCL2	B-cell CLL/lymphoma 2	0.0035**	37.02	16/16 (2/4)	16	100
213880_at	LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5	3.2E-05***	30.89	13/16 (0/4)	15	93.75
212588_at	PTPRC	Protein tyrosine phosphatase, receptor type, C	0.0016**	13.14	15/16 (2/4)	14	87.5
217901_at	DSG2	Desmoglein 2	0.0039**	11.31	16/16 (2/4)	16	100
208783_s_at	CD46	CD46 molecule, complement regulatory protein	0.016*	6.33	16/16 (4/4)	15	93.75
212097_at	CAV1	Caveolin 1, caveolae protein, 22kDa	0.021*	5.79	14/16 (2/4)	14	87.5
203358_s_at	EZH2	Enhancer of zeste homolog 2 (Drosophila)	0.018*	5.05	15/16 (0/4)	15	93.75
200999_s_at	CKAP4	Cytoskeleton-associated protein 4	0.00015***	4.40	16/16 (4/4)	16	100
204457_s_at	GAS1	Growth arrest-specific 1	0.18	3.34	6/16 (0/4)	9	56.25
228038_at	SOX2	SRY (sex determining region Y)-box 2	0.085	3.15	16/16 (0/4)	12	75
208691_at	TFRC	Transferrin receptor (p90, CD71)	0.017*	2.97	16/16 (4/4)	15	93.75
211596_s_at	LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1	0.137	2.79	15/16 (2/4)	14	87.5
202935_s_at	SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	0.112	2.76	12/16 (2/4)	12	75
203196_at	ABCC4	ATP-binding cassette sub-family C member 4	0.056	2.64	15/16 (1/4)	15	93.75
208476_s_at	FRMD4A	FERM domain containing 4A	0.0024*	2.62	10/15 (4/4)	13	81.25
212063_at	CD44	CD44 molecule (Indian blood group)	0.013*	2.50	16/16 (4/4)	13	81.25
204343_at	ABCA3	ATP-binding cassette, sub-family A (ABC1), member 3	0.00016***	2.48	9/16 (0/4)	16	100
213353_at	ABCA5	ATP-binding cassette, sub-family A (ABC1), member 5	NS	2.26	9/16 (0/4)	13	81.25
226774_at	FAM120B	Family with sequence similarity 120B	NS	2.19	11/16 (1/4)	12	75

Gene Accession	Gene Symbol	Name	p value (t)	FC (overall)	Present In NPC (normal)	up in (RMA)	up in % (RMA)
202265_at	BMI1	BMI1 polycomb ring finger oncogene	0.205	2.12	14/16 (4/4)	13	81.25
204531_s_at	BRCA1	Breast cancer 1, early onset	0.021*	2.06	16/16 (1/4)	16	100
205858_at	NGFR	Nerve growth factor receptor (TNFR superfamily, member 16)	NS	1.98	7/16 (0/4)	8	50
204405_x_at	DIMT1L	DIM1 dimethyladenosine transferase 1-like (S. cerevisiae)	0.259	1.92	14/16 (4/4)	14	87.5
201278_at	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	NS	1.81	4/16 (0/4)	9	56.25
220184_at	NANOG	Nanog homeobox	NS	1.79	3/16 (0/4)	5	31.25
209439_s_at	PHKA2	Phosphorylase kinase, alpha 2 (liver)	NS	1.40	6/16 (0/4)	12	75
	EMT markers						
202351_at	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	0.00029***	21.97	16/16 (2/4)	16	100
225664_at	COL12A1	Collagen, type XII, alpha 1	0.0015**	15.74	10/16 (0/4)	12	75
201852_x_at	COL3A1	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	0.016*	14.37	14/16 (3/4)	13	81.25
211980_at	COL4A1	Collagen, type IV, alpha 1	0.0013**	13.66	16/16 (3/4)	16	100
210495_x_at	FN1	Fibronectin 1	0.016*	11.11	15/ 16 (0/4)	14	87.5
202403_s_at	COL1A2	Collagen, type I, alpha 2	0.0030**	8.49	14/16 (1/4)	14	87.5
201426_s_at	VIM	Vimentin	0.019*	4.39	14/16 (3/4)	14	87.5
208083_s_at	ITGB6	Integrin, beta 6	0.164	3.93	12/16 (4/4)	11	68.75
203936_s_at	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	0.0044**	2.79	16/16 (2/4)	13	81.25
213425_at	WNT5A	Wingless-type MMTV integration site family, member 5A	0.067	2.51	15/16 (3/4)	11	68.75
200606_at	DSP	Desmoplakin	0.0071**	2.29	16/16 (4/4)	13	81.25
201131_s_at	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	NS	2.03	16/ 16 (4/4)	13	81.25
213139_at	SNAI2	Snail homolog 2 (Drosophila)	NS	1.57	7/16 (2/4)	6	37.5
213943_at	TWIST1	Twist homolog 1 (acrocephalosyndactyly 3; Saethre-Chotzen syndrome) (Drosophila)	NS	1.46	8/16 (0/4)	11	68.75
211453_s_at	AKT2	V-akt murine thymoma viral oncogene homolog 2	NS	-1.83	8/16 (3/4)	2	12.5

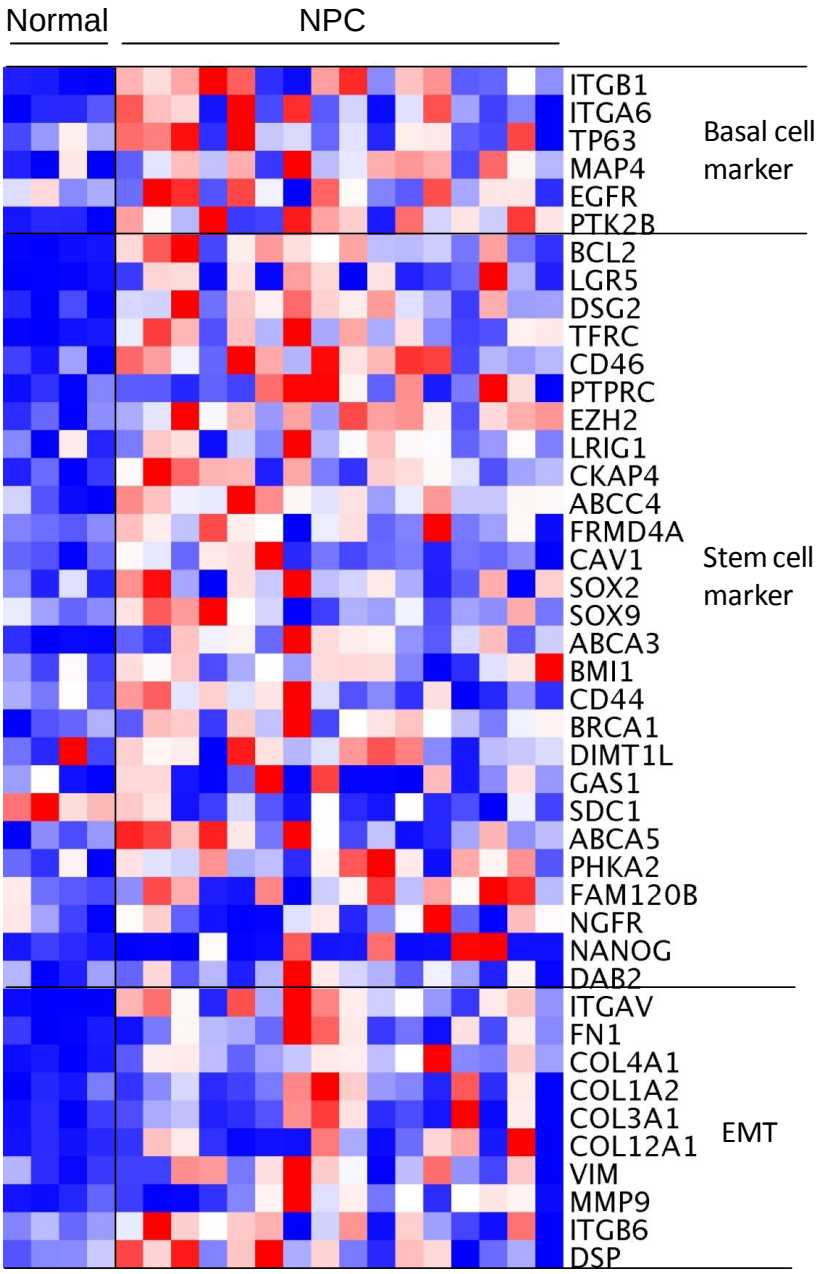


Figure 3. 1. Microarray analysis demonstrating an enrichment of basal and stem cell marker expression in NPC tumours
RP analysis of 4 normal epithelial tissue samples compared to 15 micro-dissected NPC biopsies and the C666.1 NPC cell line identified increased expression of basal and stem cell markers in the NPC tissue. This was accompanied by increased expression of epithelial to mesenchymal transition (EMT) markers.

3.2.2 IHC validation of microarray expression profiling on tumour biopsies

Validation of the microarray expression data was performed on an NPC tissue array containing both NPC tumour and normal nasopharyngeal mucosa. Expression of proteins of interest was assessed using standard IHC staining protocols, on approximately 20 normal mucosa and 50 NPC biopsy specimens and scored using a semi quantitative system (Hu et al., 2012). Representative images are shown in Figure 3. 2. Additionally, GCOS graphs of microarray data showing normalised array intensities (i.e. relative expression) of the gene of interest in the 4 normal nasopharynx (grey), 15 NPC tumour biopsies (red) and the C666.1 cell line (yellow) have been included for comparison.

IHC staining for BMI1, P75NTR, SOX2, EZH2 and CD44 demonstrated localised expression of stem cell markers in the basal cell layers of normal nasopharynx and higher, more uniform expression in the majority of NPC tumour cells. Expression of NANOG and NESTIN was very weak in both the NPC and normal samples and was positive in isolated cells of both the normal and NPC tissue. GCOS analysis of the highest expressing probe set for each gene of interest demonstrates upregulation of all these stem cell/CSC markers in the majority of NPC tumour biopsies compared to normal nasopharynx.

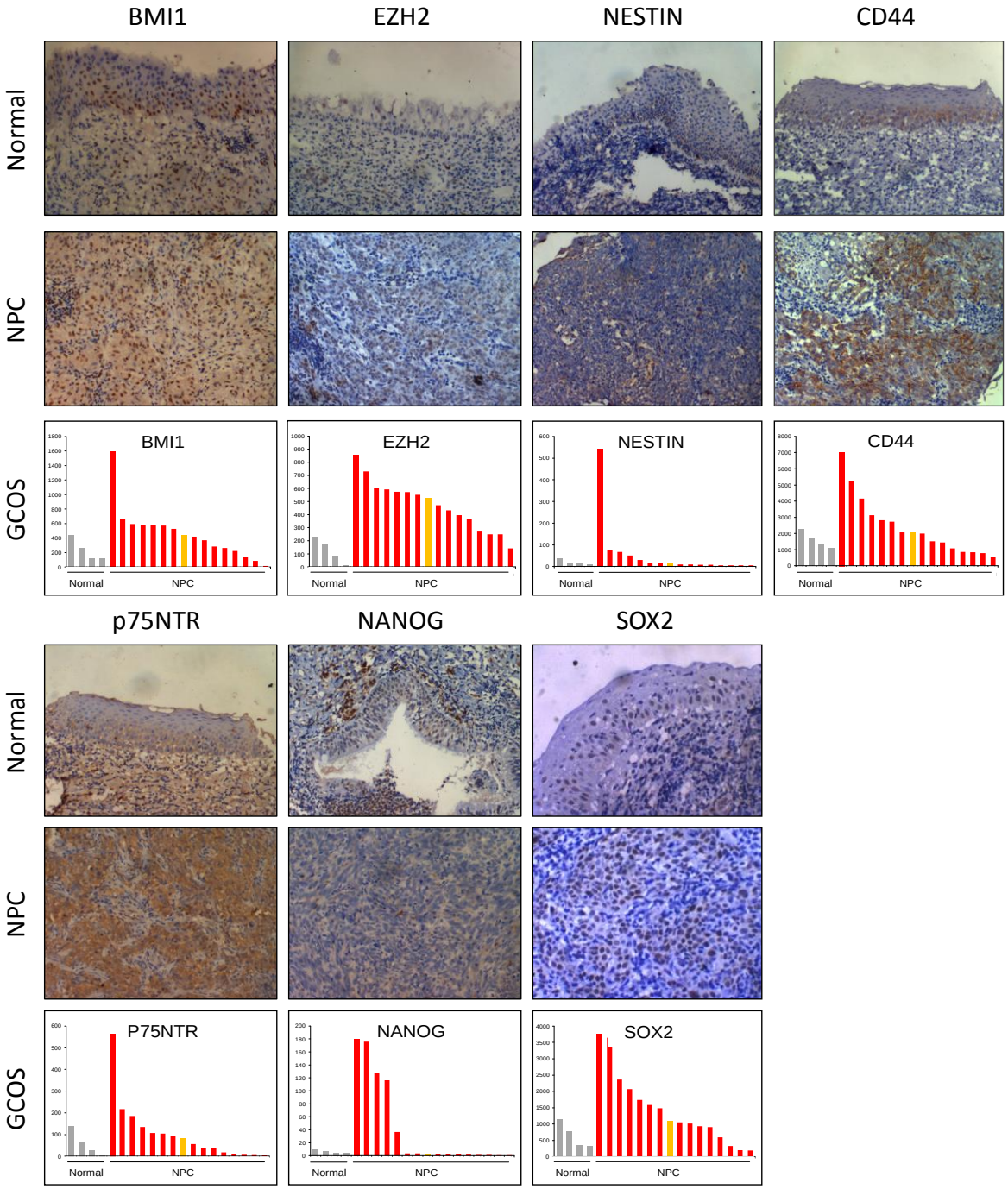


Figure 3. 2. IHC validation of microarray expression data demonstrates increased expression of stem cell markers in primary NPC tissue compared to normal nasopharynx

Representative IHC staining of normal and NPC sections, and associated gene GCOS analysis, for a number of stem cell/CSC markers. GCOS graphs show gene expression in the 4 normal nasopharygeal samples (grey) compared to the 16 NPC samples (red), with the C666.1 cell line highlighted in yellow.

A semi quantitative scoring system (0; negative, 9; positive in a high proportion of cells) was used to evaluate the IHC staining (see materials and methods, section 2.13.2). Average scores, standard distribution and statistical probabilities for staining of interest on NPC biopsies compared to normal nasopharynx are presented in Figure 3. 3. Expression of BMI1, P75NTR and SOX2 was upregulated in 87%, 80%, and 94% respectively. Scoring the staining for NANOG and NESTIN demonstrated no significant difference between the NPC and normal tissue samples.

Whilst representative staining was performed on both normal and NPC tissue, staining for EZH2 and CD44 has previously been scored on the tissue array and has not been repeated here (Hu et al., 2012). Hu et al., (2012) demonstrated upregulation of EZH2 and CD44 in 75% and 50% of NPC case respectively.

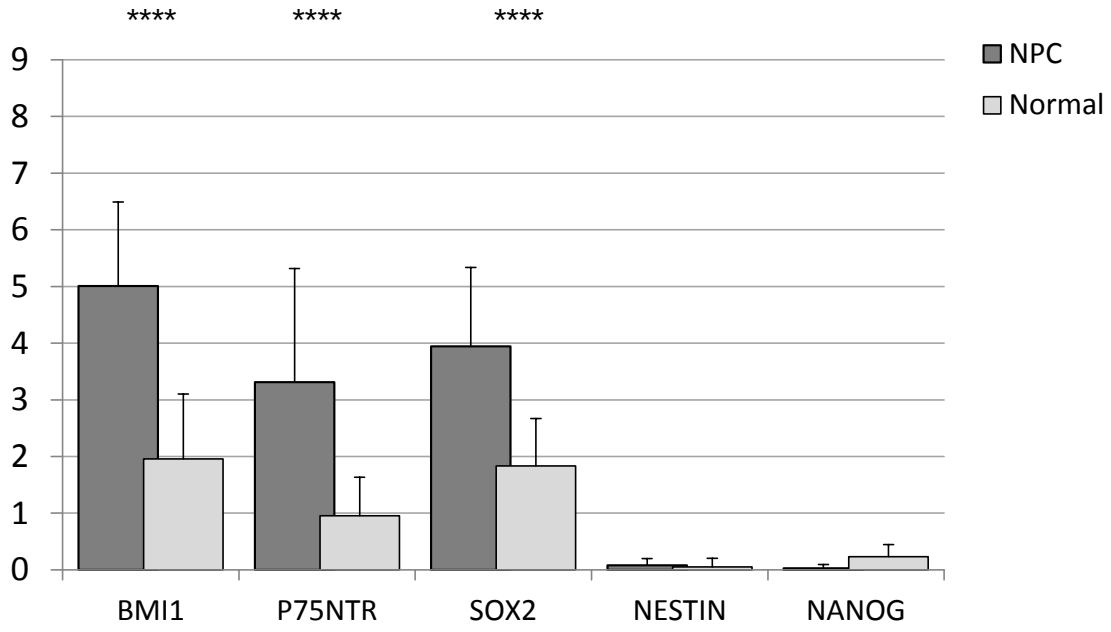


Figure 3. 3 Scoring for IHC staining for stem cell markers in NPC tissue compared to normal nasopharynx

Staining was scored on a normalised scale of 0 to 9, where 0 represents no staining present. Average scores and are standard deviations presented for NPC tissue biopsies (dark grey) and normal nasopharyngeal samples (light grey). *; P<0.05, **; P<0.001, ***; P<0.001, ****, P<0.0001.

3.3 The C666.1 cell line shows increased expression of stem cell markers compared to normal primary oral keratinocytes

Having confirmed increased expression of a number of putative stem cell markers in primary NPC tumour specimens, the expression of selected stem cell markers in the authentic EBV positive NPC cell line, C666.1, was explored. Expression profiling was preformed against primary human oral keratinocytes (HOK) and the OKF6 cell line, a hTert-immortalised normal human oral keratinocyte cell line. HOK have previously been established as a good model for normal nasopharynx and served as a valid reference to control for the effects of *in vitro* cultivation (Hu, 2010, Yanai et al., 2008). Further microarray analysis interrogated the expression of stem cell markers in total RNA samples isolated from C666.1 and normal HOK (Figure 3. 4A) (GSE39826). Many tissue dependant markers have previously been described as upregulated in putative stem cell populations; the markers seen to be upregulated in the C666.1 cell line relative to HOK included SOX2, BMI1, LRIG1, EZH2 and p75NTR. Other putative stem cell markers were found to be reduced in this cell line compared to HOK and OKF6, including the ABC transporter gene ABCG2, NANOG, a homeobox protein essential for ESC renewal, and CD44, of which the variant 6 is most often associated as a CSC marker.

Whilst HOK are a valid model for normal nasopharyngeal epithelium, the lack of availability of primary tissue, coupled with the limited lifespan of primary oral keratinocytes in culture, prompted us to search for a suitable replacement. The hTert-immortalised oral keratinocyte cell line OKF6 was chosen as a normal control for C666.1 (Cheung et al., 1999). To establish that the OKF6 cell line was a valid replacement for HOK, RT-PCR analysis was performed to compare the expression of

a number of stem cell markers in OKF6, HOK and C666.1 (Figure 3. 4B). This also acted to validate the microarray work as the genes investigated by RT-PCR demonstrated the same upregulation in the C666.1 cell line compared to the normal epithelial counterparts as seen in the microarray analysis. In addition, RT-PCR analysis for the expression of CD44 variant 6 (CD44v6) showed that this gene appeared to be upregulated, whereas microarray analysis for the non-variant CD44 demonstrated downregulation in the C666.1 cell line.

The level of CD44v6, CD133 and SOX2 mRNA in the HOK and OKF6 cells were too low to be detected by RT-PCR; however, mRNA could be detected in the C666.1 cell line. The stem cell markers p75NTR, LRIG1 and BMI1 were expressed in the OKF6 cell line and HOK cells, mRNA expression was similar between the primary and hTert-immortalised cells, however marker expression was significantly increased in the C666.1 cell line. This would suggest OKF6 cells are a suitable substitute for HOK in experiments where continued culture of the cell line would be preferable.

Further investigation into the expression of cell surface marker expression in C666.1 and OKF6 cells was performed by flow cytometric analysis (Figure 3. 4C). Staining was performed on viable cells with a rabbit polyclonal antibody specific for CD44v6 and a mouse monoclonal antibody to CD133. C666.1 cells displayed a 4.6 fold increase in CD44v6 expression ($P=0.039$, $n=3$), and an 11.6 fold increase in CD133 expression ($P<0.05$, $n=3$) compared to the OKF6 cell line. In addition, flow cytometric analysis demonstrated that this increased expression was observed in a subset of cells rather than as a population shift, indicating the C666.1 cell line possesses a sub population of cells which may possess stem cell like characteristics. This sub-

population, had it been isolated, would be expected to possess increased tumourigenicity compared to an unsorted population of cells.

This increased expression of stem cell markers in C666.1 cell line mirrors that observed in the NPC tumour biopsy microarray suggesting C666.1 can be used as a model to investigate the activity of cellular signalling implicated in the maintenance of a CSC-like phenotype.

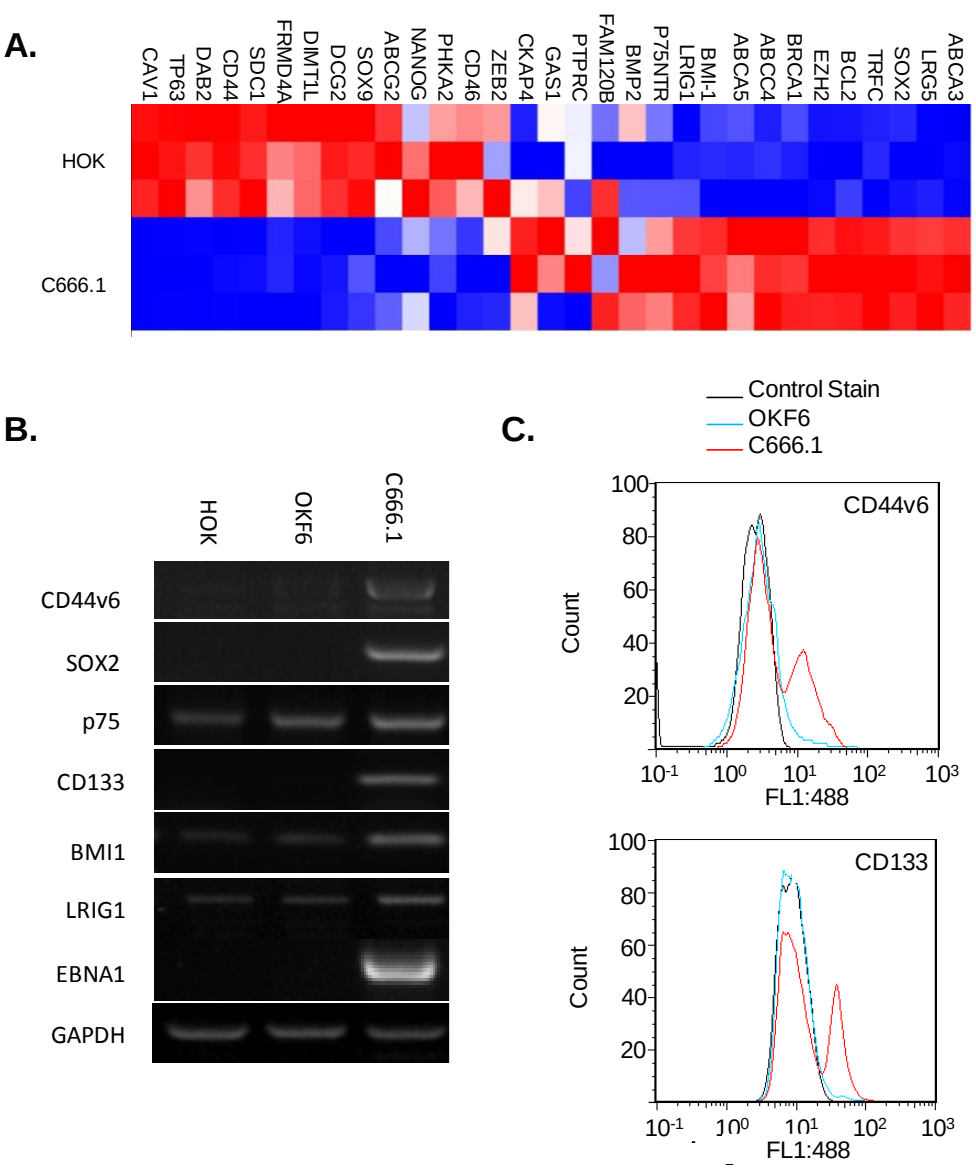


Figure 3. 4 The C666.1 cell line expresses high levels of stem cell/CSC markers
(A) Microarray analysis of 3 C666.1 total RNA samples compared to normal primary tonsil epithelium. (B) Validation of dysregulated HH signalling in the C666.1 cell line compared to normal tonsil epithelium. (C) FACS analysis showing increased expression of cell surface stem cell markers CD44v6 and CD133 in C666.1 cell line compared to OKF6. C666.1 displays a 4.6 fold increase (P=0.039, n=3) in expression of CD44v6 and an 11.4 fold increase (P=0.040, n=3) in CD133 expression.

3.4 Primary NPC tumours display aberrant HH signalling activity – the link to stemness

To date, studies have identified dysregulation of a number of stem cell signalling pathways in NPC. These include the NOTCH, WNT, TGFβ, BMP and JAK/STAT pathways. Previous work in this laboratory supports published findings demonstrating that a number of these pathways are dysregulated in NPC (Hu, 2010, Yanai et al., 2008). Studies into signalling dysregulation in NPC has thus far not been extended to interrogate the HH signalling pathway, a pathway commonly dysregulated in other carcinomas, such as BCC, which arise from stem cell or early progenitor cells. Subtypes of BCC maintain an undifferentiated “basal cell” phenotype and are dependent on sustained HH signalling for proliferation (Hutchin et al., 2005). Additional interest in the HH signalling pathway arose from finding that BMI1, a stem cell marker consistently upregulated in NPC tumours and the C666.1 cell line, is regulated by, and required for, HH pathway driven tumourgenesis in meduloblastoma and mammary gland carcinomas (Wang et al., 2012b, Michael et al., 2008). Furthermore BCL2, a downstream target of the GLI transcription factors (Regl et al., 2004, Bigelow et al., 2004), is also known to be upregulated in NPC (Vera-Sempere et al., 1997). Previous studies analysing the expression of BCL2 in NPC tumours has identified overexpression, by IHC staining and QPCR, in the region of 80% of type I tumours (Low et al., 2012), and inhibition of the BCL2 family of proteins induces apoptosis and reduces tumour growth in NPC xenographs (Hu et al., 2008).

The finding that a number of HH targets are consistently upregulated in NPC combined with the finding that other tumours displaying increased stem cell marker expression show aberrant HH signalling, such as that of the CD44+, CD24+, ESA+,

SHH ligand expressing CSC population of pancreatic tumours (Li et al., 2007), highlighted a plausible role for HH signalling dysregulation in NPC pathogenesis.

3.4.1 Microarray analysis reveals perturbations in the expression of HH signalling pathway in nasopharyngeal carcinoma tumours

Data obtained from the previous microarray analysis was interrogated to examine the status of the HH signalling pathway in this cohort of NPC samples. Table 3. 2 shows the fold changes and statistical significance, from RP analysis on expression array data, of individual genes of the HH pathway and downstream target genes. An extensive list of genes potentially involved in, or downstream of, the HH signalling pathway exists in the literature. Again, genes have been included if present in 8 out of 16 NPC samples or downregulated and present in at least 2 of 4 normal tonsil epithelium samples. However, some of key components of the pathway where absent from the microarray study by this criteria but have been included in table 3.2 for completeness (HH ligands, GLI1 and PTCH2). Genes that are not present in this table include HhAT, HhIP, PTCHD1, PTCHD2, NPC1L1, DISP1, CDO1, BOC, LRP1, LRP2, OTX2, FKBP8, FGF9, CDON and BMP4.

Table 3. 2 Expression of HH pathway associated genes in microdissected NPC tumour tissue verses normal tissue by microarray analysis

Gene Accession	Gene Symbol	Name	p value (t)	FC (overall)	Present in NPC (normal)	up in (RMA)	up in % (RMA)
	Hh ligand						
236263_at	SHH	Sonic hedgehog homolog (Drosophila)	-	-	1/16 (0/4)	-	-
1552730_at	DHH	Desert hedgehog homolog (Drosophila)	-	-	0/20	-	-
215420_at	IHH	Indian hedgehog homolog (Drosophila)	-	-	0/20	-	-
	Hh receptor						
209815_at	PTCH1	Patched homolog 1 (Drosophila)	0.013*	3.79	16/16 (2/4)	14	87.5
221292_at	PTCH2	Patched homolog 2 (Drosophila)	-	-	0/20	-	-
218629_at	SMO	Smoothened homolog (Drosophila)	NS	-	5/16 (0/4)	2	12.5
	Hh antagonists						
224203_at	SUFU	Suppressor of fused homolog (Drosophila)	NS	-1.57	11/16 (3/4)	0	0
209456_s_at	FBXW11	F-box and WD-40 domain protein 11	NS	-1.54	13/16 (4/4)	2	12.5
	Protein kinases						
202742_s_at	PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	0.0046**	5.00	14/16 (2/4)	12	75
200605_s_at	PPKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	0.059	3.10	15/16 (4/4)	13	81.25
235464_at	CSNK1A1	Casein kinase 1, alpha 1	0.049*	2.44	13/16 (1/4)	14	87.5
213331_s_at	NEK1	NIMA (never in mitosis gene a)-related kinase 1	0.142	2.29	14/16 (1/4)	11	68.75
	Signalling mediators						
212097_at	CAV1	Caveolin 1, caveolae protein	0.021*	5.79	14/16 (3/4)	12	75
223642_at	ZIC2	Zic family member 2 (odd-paired homolog, Drosophila)	0.00024***	3.90	11/16 (0/4)	13	81.25
223463_at	RAB23	RAB23, member RAS oncogene family	0.0011**	3.53	12/16 (1/4)	8	50
204457_s_at	GAS1	Growth arrest-specific 1	0.18	3.43	6/16 (0/4)	14	87.5
226364_at	HIP1	Huntingtin interacting protein 1	0.0004***	2.36	15/16 (2/4)	15	93.75
228680_at	KIF3A	Kinesin family member 3A	NS	2.28	14/16 (4/4)	8	50
226166_x_at	STK36	Serine/threonine kinase 36, fused homolog (Drosophila)	NS	1.34	13/16 (1/4)	10	62.5
224471_s_at	BTRC	Beta-transducin repeat containing	NS	-1.38	12/16 (4/4)	0	0
204522_at	DZIP1	DAZ interacting protein 1	NS	-1.43	13/16 (3/4)	10	62.5

Gene Accession	Gene Symbol	Name	p value (t)	FC (overall)	Present in NPC (normal)	up in (RMA)	up in % (RMA)
	Transcription factors						
206646_at	GLI1	GLI-Kruppel family member GLI1	-	-	0/20	-	-
228537_at	GLI2	GLI-Kruppel family member GLI2	NS	-1.36	6/16 (3/4)	1	6.25
227376_at	GLI3	GLI-Kruppel family member GLI3	0.025*	2.19	14/16 (2/4)	13	81.25
	HH targets						
203685_at	BCL2	B-cell CLL/lymphoma 2	3.3E-06***	37.02	16/16 (2/4)	16	100
239629_at	CFLAR	CASP8 and FADD-like apoptosis regulator	0.0037**	5.94	14/16 (1/4)	13	81.25
202037_s_at	SFRP1	Secreted frizzled-related protein 1	0.199	5.68	14/16 (2/4)	8	50
200951_s_at	CCND2	Cyclin D2	0.00024***	5.35	11/16 (0/4)	16	100
209784_s_at	JAG2	Jagged 2	0.0054**	4.81	16/16 (3/4)	16	100
218469_at	GREM1	Gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	0.0030**	4.13	10/16 (0/4)	12	75
203131_at	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide		3.91	12/16 (1/4)	12	75
228964_at	PRDM1	PR domain containing 1, with ZNF domain	0.155	2.78	11/16 (2/4)	12	75
202935_s_at	SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	0.112	2.76	13/16 (2/4)	12	75
213425_at	WNT5A	Wingless-type MMTV integration site family member 5A	0.067	2.51	15/16 (3/4)	11	68.75
209757_s_at	MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	NS	2.42	9/16 (1/4)	9	56.25
202580_x_at	FOXM1	Forkhead box M1	0.05	2.31	12/16 (1/4)	14	87.5
206104_at	ISL1	ISL1 transcription factor, LIM/homeodomain, (islet-1)	NS	2.11	7/16 (2/4)	6	37.5
208712_at	CCND1	Cyclin D1	NS	1.28	16/16/ (4/4)	7	43.75
208606_s_at	WNT4	Wingless-type MMTV integration site family, member 4	NS	-1.63	5/16 (1/4)	2	12.5
	HH EMT and stem cell targets						
213880_at	LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5	3.2E-05***	30.89	13/16 (4/4)	15	93.75
205290_s_at	BMP2	Bone morphogenetic protein 2	0.0014**	3.42	11/16 (0/4)	12	75
203603_s_at	ZEB2	Zinc finger E-box binding homeobox 2	0.0076**	3.05	9/16 (0/4)	14	87.5
212063_at	CD44	CD44 molecule (Indian blood group)	0.013*	2.50	16/16 (4/4)	13	81.25
202265_at	BMI1	BMI1 polycomb ring finger oncogene	0.205	2.12	14/16 (4/4)	13	81.25

To illustrate the extent of HH pathway dysregulation in NPC, a KEGG map, Figure 3. 5, is shown to highlight statistically significant upregulated and downregulated genes. Upregulated genes, represented in red, are defined here as genes that presented with a GCOS normalised expression level greater or equal to two times the mean of the normal samples, and were found to be significantly upregulated by RP analysis. Downregulated genes, none of which exist under this definition, would be defined as significantly downregulated by RP analysis and have a normalised expression of less than or equal to two times less than the mean of the normal samples. Genes that did not meet either of these definitions are unchanged and remain white.

Dysregulation in the HH signalling pathway, presented in Table 3.2, can also be visualised as a heatmap illustrating the expression of HH pathway components in the 4 normal control and 15 microdissected tumour samples, in addition to the C666.1 cell line (Figure 3. 6). Expression of genes, such as DHH, IHH, PTCH2, SMO, GLI1, GLI2, and ISL1, was minimal; therefore, it is not possible to compare the expression of these genes in the NPC tumour biopsies and normal control tissue. SHH is also absent; its expression was so low as to be classified absent from all but one sample; the NPC biopsy XY3. Its absence may allude to a ligand independent mechanism for chronic HH signalling activation in NPC tumours or simply be due to a lack of sensitivity of the probe sets present in the Affymetryx gene chips.

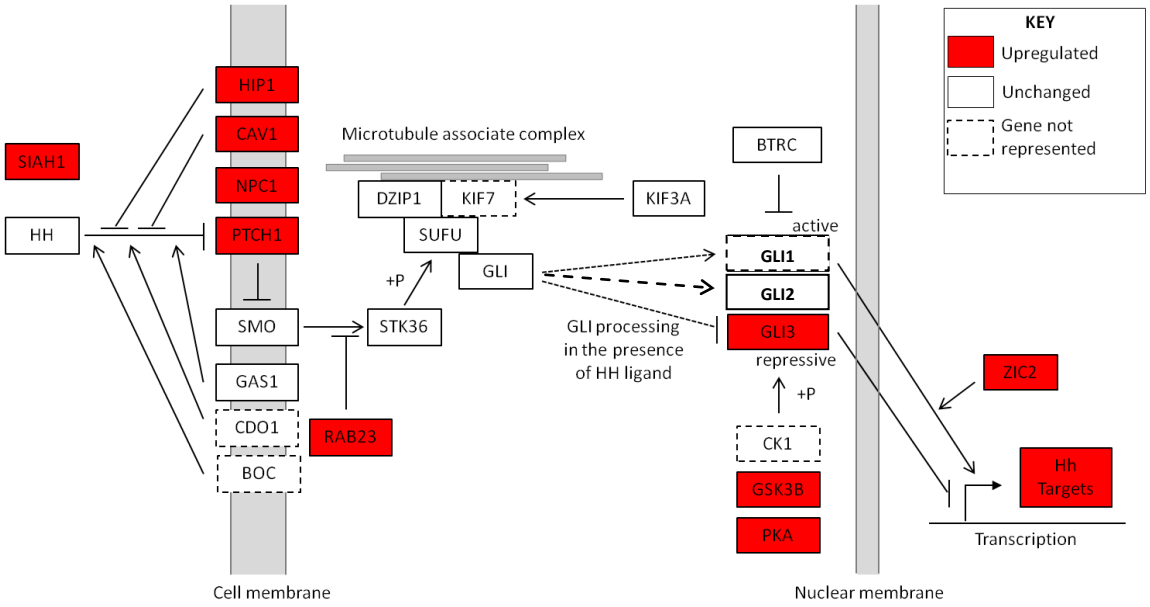


Figure 3. 5 KEGG map of the HH pathway genes differentially expressed in NPC
Here the differentially expressed HH genes are imposed on signalling pathway in the condition of activated SMO and the processing of GLI into its active form. Genes in red are significantly upregulated by microarray analysis in the tumour samples compared to the four normal epithelial samples with a fold change ≥ 2 and significantly increased by RP analysis. No components of the HH signalling pathway were found to be significantly downregulated (fold change ≥-2).

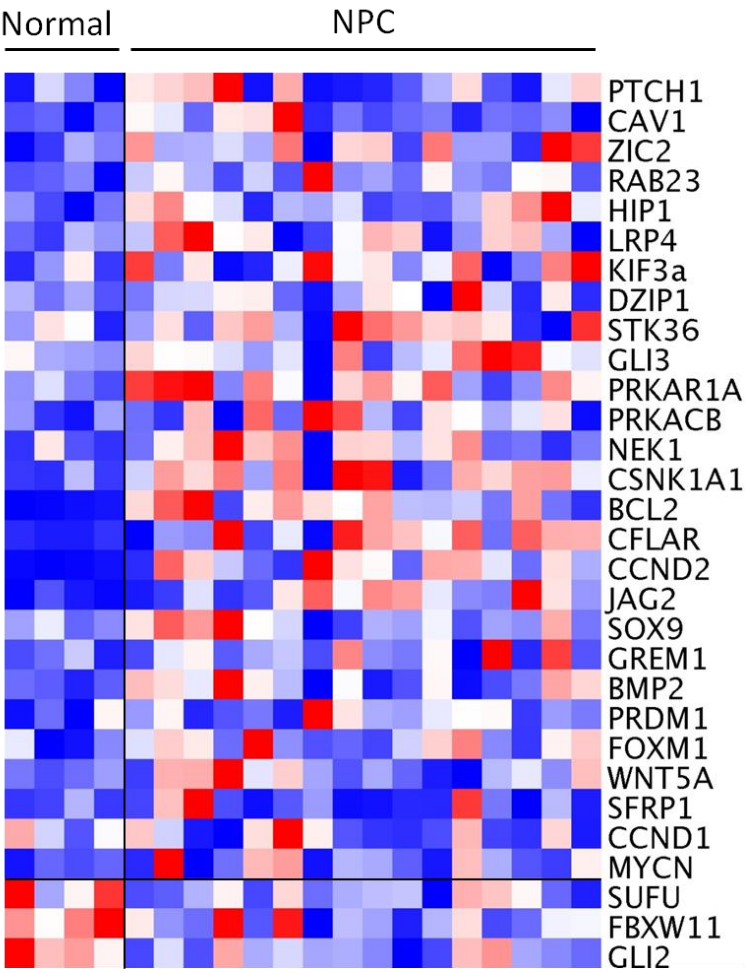


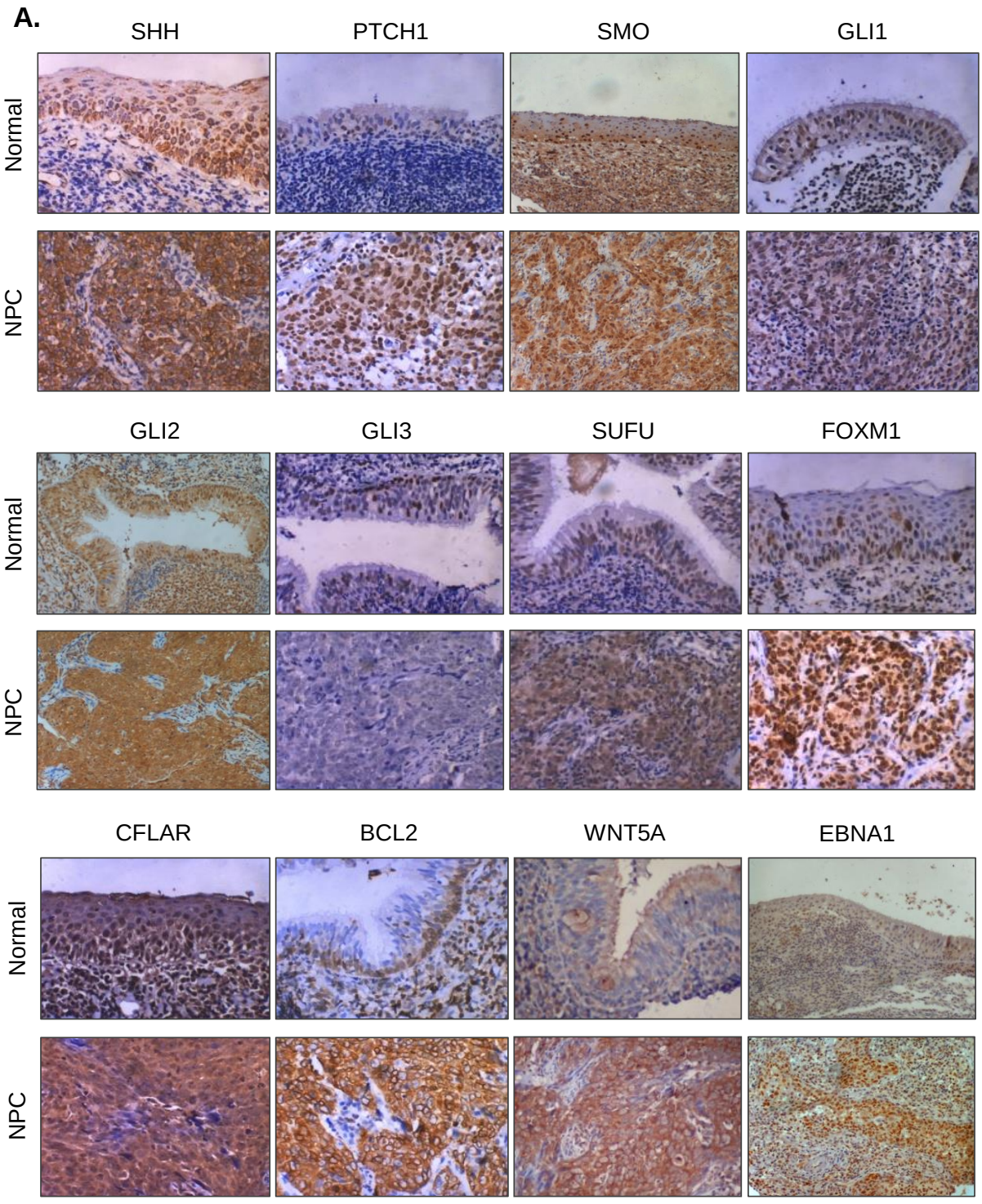
Figure 3. 6 Heat map showing dysregulated HH pathway genes in the 16 NPC samples compared to normal nasopharyngeal and tonsil epithelium
HH pathway components and targets presented are either upregulated in NPC and present in ≥8/16 micro-dissected tumour samples, or downregulated in NPC and present in ≥2/4 normal tonsil tissue samples.

The microarray data identified a number of HH pathway genes that were significantly upregulated in NPC tumour specimens relative to normal control tissue. These included the HH receptor, and putative target gene PTCH1 (Marigo and Tabin, 1996), the HH transcription factor GLI3, and additional HH target genes (FOXM1, CCND2, WNT5A, SOX9, CFLAR, PDGFR, and BCL2) (Katoh and Katoh, 2009a). Other HH pathway signalling components were found to be significantly upregulated in NPC relative to normal epithelium, which included positive regulators of HH signalling CAV1, NPC1, and ZIC2, (Capozza et al., 2012, Bidet et al., 2011, Chan et al., 2011) and negative regulators such as PKA, GSK3B, HIP1, and RAB23 (Wen et al., 2010, Yang et al., 2010, Chuang and McMahon, 1999). ZIC2 acts to maintain activated GLI transcription factors in the nucleus facilitating downstream HH target transcription (Mizugishi et al., 2001, Chan et al., 2011), whereas HIP1 negatively regulates HH signalling by competing with PTCH to bind SHH (Chuang and McMahon, 1999). CAV1 is reportedly required for the intracellular transport of SHH ligand to the cell membrane and targeted deletion of CAV1 in zebrafish, who possess only one CAV protein compared to the three mammalian homologs, produces a SHH deficient-like phenotype (Mao et al., 2009). RAB23 acts downstream of SMO (Yang et al., 2010) and may have a role in inhibiting the production of the activated form of GLI2 (GLI2A) and promoting processing of GLI3 into repressor forms (GLI3R) (Eggenschwiler et al., 2006). Inhibitory protein kinases, PKA, CK1 and GSK3B, phosphorylate GLI2 and GLI3 in the absence of SHH facilitating the processing of GLI transcription factors into repressor forms (GLIR) (Wen et al., 2010). Other pathway components not significantly changed included SKT36, GAS1, DZIP1, SUFU, KIF3A and BTRC. Taken as a whole, the microarray analysis identified abundant but varied expression of a number of HH pathway components and targets in NPC specimens relative to

normal control tissue, revealing the general presence of aberrant or HH signalling in this tumour.

3.4.2 IHC validation of microarray expression profiling on tumour biopsies.

Validation of the microarray data was performed for HH pathway components and targets by IHC, as previously described for stem cell markers (Chapter 3, section 3.2.2), using an NPC tissue array containing both NPC tumour and normal nasopharyngeal mucosa. IHC staining for HH pathway components (SHH, SMO, PTCH1, GLI1, GLI2, GLI3 and SUFU), downstream targets (FOXM1, CFLAR, BCL2 and WNT5A) and the EBV latent gene EBNA1 was performed on approximately fifty primary NPC tumour sections and twenty normal nasopharyngeal epithelium biopsies, dependent on tumour array section quality. Representative IHC staining for individual proteins of interest are shown in Figure 3. 7A, and GCOS analysis of microarray data from the highest expressing probe set for each of these genes is also presented (Figure 3. 7B). GLI1, is absent from Figure 3. 8B as expression of this gene was not detected in any samples.


A.
SHH
PTCH1
SMO
GLI1
Normal
NPC
GLI2
GLI3
SUFU
FOXM1
Normal
NPC
CFLAR
BCL2
WNT5A
EBNA1
Normal
NPC

B.

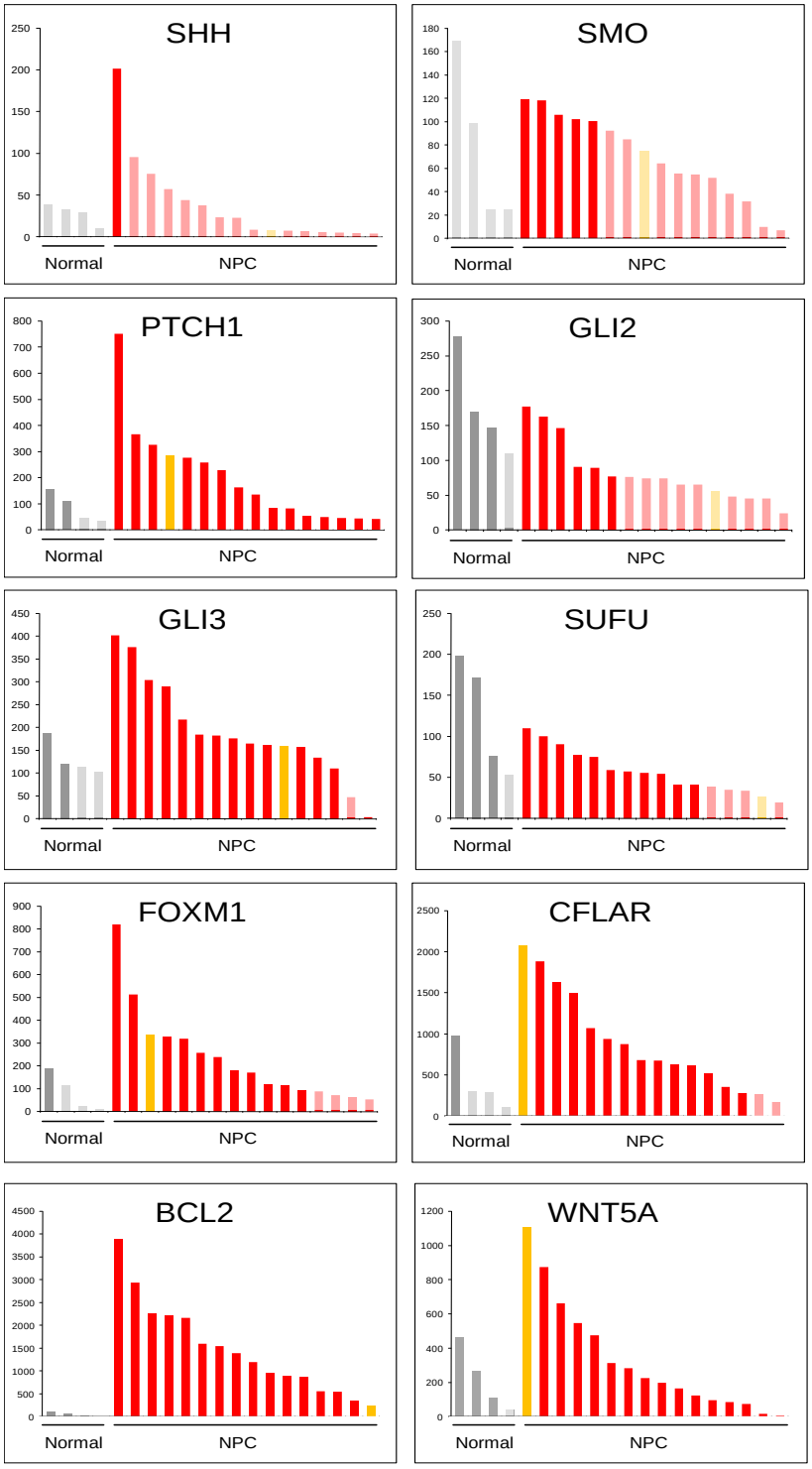


Figure 3. 7 IHC validation of microarray data confirms activation of the HH pathway in NPC

(A) Representative staining of normal and NPC sections for these HH pathway components and targets. (B) Corresponding GCOS maps of gene expression in the 4 normal nasopharyngeal samples (grey) compared to the 16 NPC samples (red), including the C666.1 cell line (yellow). Samples that were not present above background by RP analysis are presented as faded samples.

IHC staining revealed increased expression of pathway components SHH, SMO, PTCH, GLI1 and GLI2 in 60%, 96%, 96%, 90% and 84% of NPC cases compared to normal nasopharyngeal epithelium respectively, whilst GLI3 expression was reduced in 76% of NPC samples. Additionally, expression of the negative regulator SUFU (Kogerman et al., 1999, Jia et al., 2009) was reduced in 6/50 (12%) of tumour samples.

A number of downstream targets of the HH pathway were also interrogated by IHC. Overexpression of the homeobox protein FOXM1 has been demonstrated in basal cell carcinoma as a downstream target of GLI1 (Teh et al., 2002). Homeobox proteins have roles in the regulation of genes involved in cellular proliferation, growth and differentiation. Activation of the HH target FOXM1 is implicated in the transformation of keratinocytes (Gemenetzidis et al., 2009, Teh et al., 2010). In this study, FOXM1 was found to be upregulated in 98% of NPC biopsies by IHC. Other downstream HH targets; BCL2 and CFLAR were found to be upregulated in 48/50 96% and 82% of tumour samples respectively, while WNT5A was found to be upregulated in representative samples stained in this study. Quantitation of WNT5A expression has previously been presented by Hu (2012), a study which utilised the same tissue array and demonstrated upregulation of this protein in 39/50 (78%) of NPC samples. A summary of the IHC staining can is shown in Figure 3. 8; average scores and standard deviations for NPC (dark grey) and normal nasopharyngeal epithelium (light grey) are presented for each of the antibody columns.

GCOS analysis is also presented here to allow a comparison of these genes at the RNA and protein level (Figure 3. 7B). Whilst SHH, SMO and GLI2 have been

included they were scored as absent in the majority of samples by microarray analysis as the levels of expression were very low. GCOS analysis demonstrated upregulation of pathway component PTCH in 69% of NPC biopsies compared with the average expression of this protein in the four control samples. Upregulation of downstream HH targets was also observed; FOXM1 was overexpressed in 87.5% of NPC tumours with a fold change of 2.3, whilst BCL2, CFLAR and WNT5A were upregulated in 100%, 63% and 75% of NPC biopsies respectively.

Overall, the IHC study supports findings from the microarray analysis demonstrating differential expression of HH pathway components and upregulation of key downstream targets. In addition, SHH ligand and GLI1, which were absent from the microarray gene expression profile, were found to be upregulated in a significant number of primary NPC tumour specimens. This is significant as in meduloblastoma there is a link between the expression of SHH ligand, and the induction of the HH signalling pathway, and acquisition of “stemness”; in this disease SHH can induce the expression of stem cell maintenance associated gene BMI1 through direct binding of GLI1 to the BMI1 promoter (Wang et al., 2012b).

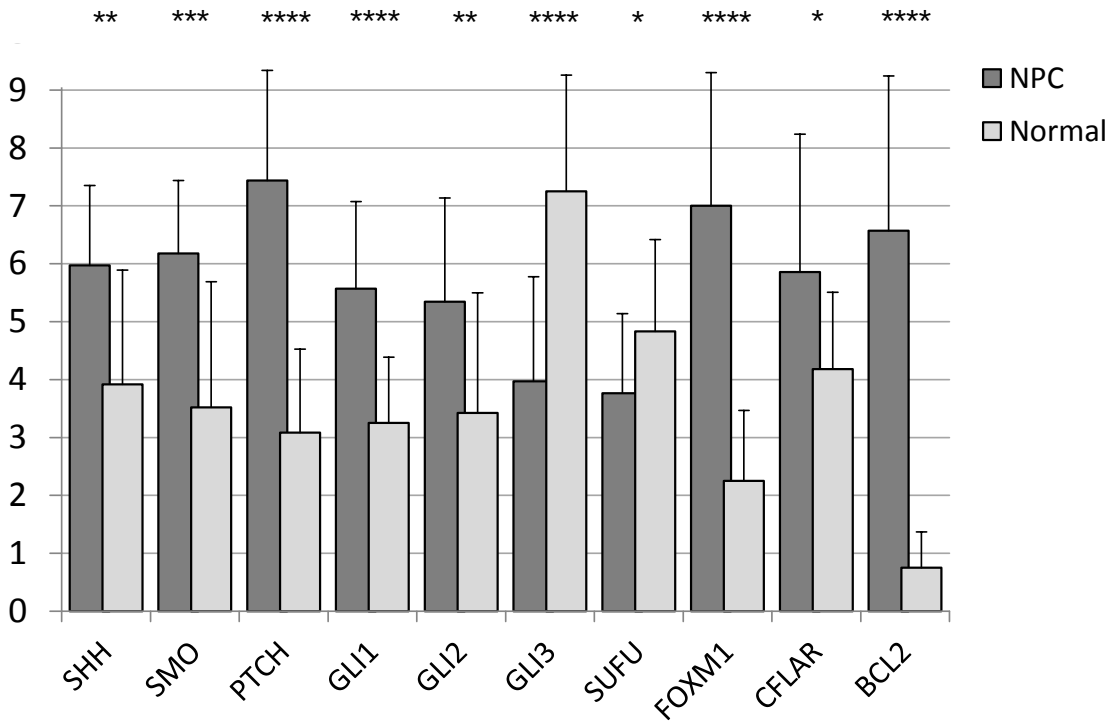


Figure 3. 8 Scoring for IHC staining for HH pathway components and targets in primary NPC tissue specimens compared to normal nasopharynx
Staining was scored on a normalised scale of 0 to 9 where 0 represents no staining present. Average scores and standard deviations presented for 50 NPC tissue biopsies (dark grey) and 20 normal nasopharynx samples (light grey). *; P<0.05, **; P<0.001, ***; P<0.001, ****, P<0.0001.

3.5 Dysregulation of HH signalling in the C666.1 cell line

3.5.1 Microarray analysis reveals an activated HH pathway in the C666.1 cell line compared to normal primary keratinocytes

Having obtained evidence for HH pathway dysregulation in authentic primary NPC specimens, the extent of this dysregulation was examined in more detail by comparing C666.1 cells with HOK cultured *in vitro*. Total RNA isolated from C666.1 and normal primary tonsillar epithelial cells was analysed for the expression of HH ligand, HH pathway components and HH target genes.

Gene expression profiling revealed elevated HH pathway activity in C666.1 cells compared to HOK. This dysregulation is apparent when normalised array intensities for individual HH pathway components are shown in the form of a heatmap (Figure 3. 9A). Compared to HOK, C666.1 cells showed increased expression of pathway components and downstream targets including PTCH1, FOXM1 and BCL2, consistent with activated HH signalling in NPC. While a number of negative inhibitors of HH signalling (BTRC, DZIP and RAB23) were found to be downregulated in NPC, expression of positive regulator SKT36 (FUSED), was found to be upregulated. The expression of downstream targets including FOXM1, BCL2, CFLAR and WNT5A was seen to be increased in the C666.1 cell line, whilst the expression of other HH targets; CCND2 and SOX9, were found to be downregulated in NPC tumours by microarray analysis.

The expression of all three GLI transcription factors in the microarray was highly variable. As a downstream target of HH signalling, GLI1 is used as an indicator of HH

activation. The GLI transcription factors influence the state of HH pathway activity and are subject to control by post translational modification into repressive or activator forms, and location of the resulting protein, which cannot be analysed by techniques looking at RNA levels.

RT-PCR analysis was once again performed to confirm the predictions of the microarray analysis the expression of key pathway components. SHH ligand expression (Figure 3. 9B), whilst very low, could be detected in the C666.1 cell line by RT-PCR but was again not detected above background levels in the microarray analysis. All other genes shown were defined as present in the microarray analysis and showed the same directional change, except GLI2, which was found to be upregulated by RT-PCR but downregulated in authentic tumours relative to normal nasopharyngeal epithelium.

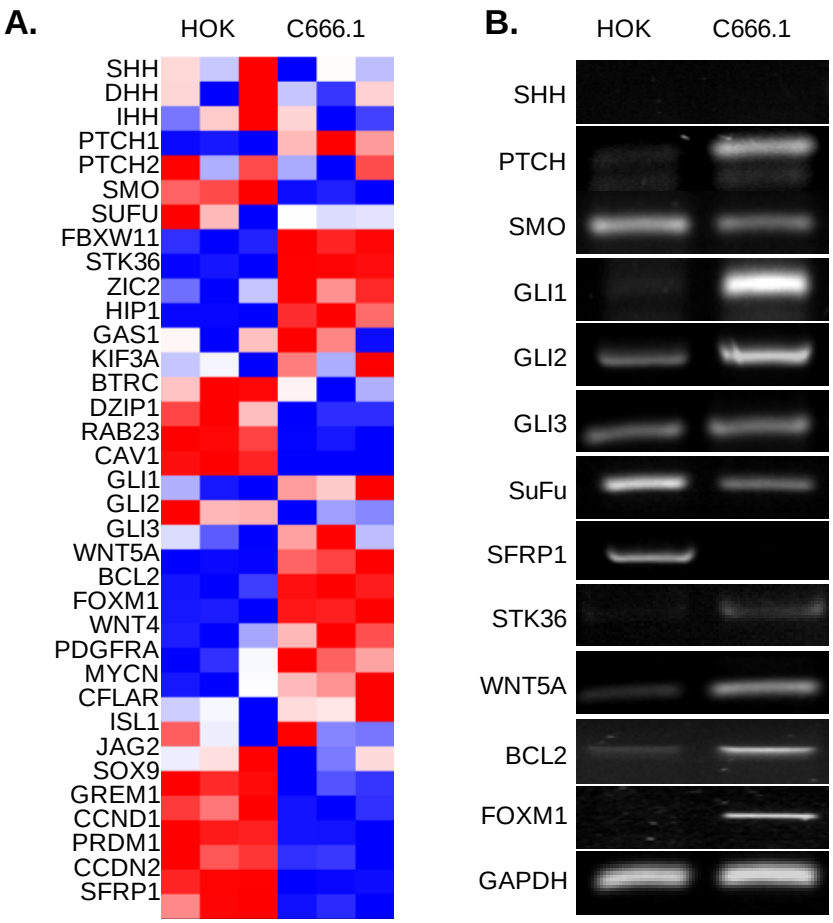


Figure 3. 9 The C666.1 cell line displays aberrant HH signalling compared to normal HOKs

(A) Microarray analysis of 3 C666.1 total RNA samples compared to normal primary keratinocytes display significant differences in the expression of HH pathway components consistent with an activated HH signalling pathway. (B) Validation of microarray data by RT-PCR supports dysregulated HH signalling in the C666.1 cell line compared to normal tonsil epithelium.

3.5.2 Validation of HH expression in the C666.1 cell line, HOK and OKF6 cell lines

The expression of HH pathway components and downstream targets was interrogated in C666.1, HOK and OKF6 cell lines to validate the OKF6 cells as a viable replacement for HOK (Figure 3. 10A). RT-PCR analysis confirmed increased expression of SHH, PTCH1, GLI1 and GLI2 in C666.1 cells compared to both HOK and OKF6. Discrepancies arose in the expression of SMO between HOK and OKF6. Expression of SMO was significantly greater in HOK compared to OKF6 or C666.1 cells, while all other pathway components and downstream targets showed comparable expression between OKF6 and HOK cells and, more importantly, showed the same directional change with respect to C666.1. This analysis established the OKF6 cell line as a suitable model for normal nasopharyngeal epithelial cells.

The expression of HH pathway components in the OKF6 and C666.1 cell lines was interrogated at the protein level by western blot analysis (Figure 3. 10B). Protein expression of all pathway components and targets closely resembled that observed by RT-PCR; SHH, PTCH, GLI1, GLI2 and FOXM1 were significantly upregulated whereas SUFU was highly expressed in OKF6 cells but much lower in the C666.1 cell line. There was little difference in the expression of GLI3 between these two cell lines. Western blot analysis supported findings from the microarray analysis and RT-PCR demonstrating that C666.1 show activated HH signalling when compared to normal HOK and the hTert-immortalised oral keratinocyte cell line, OKF6.

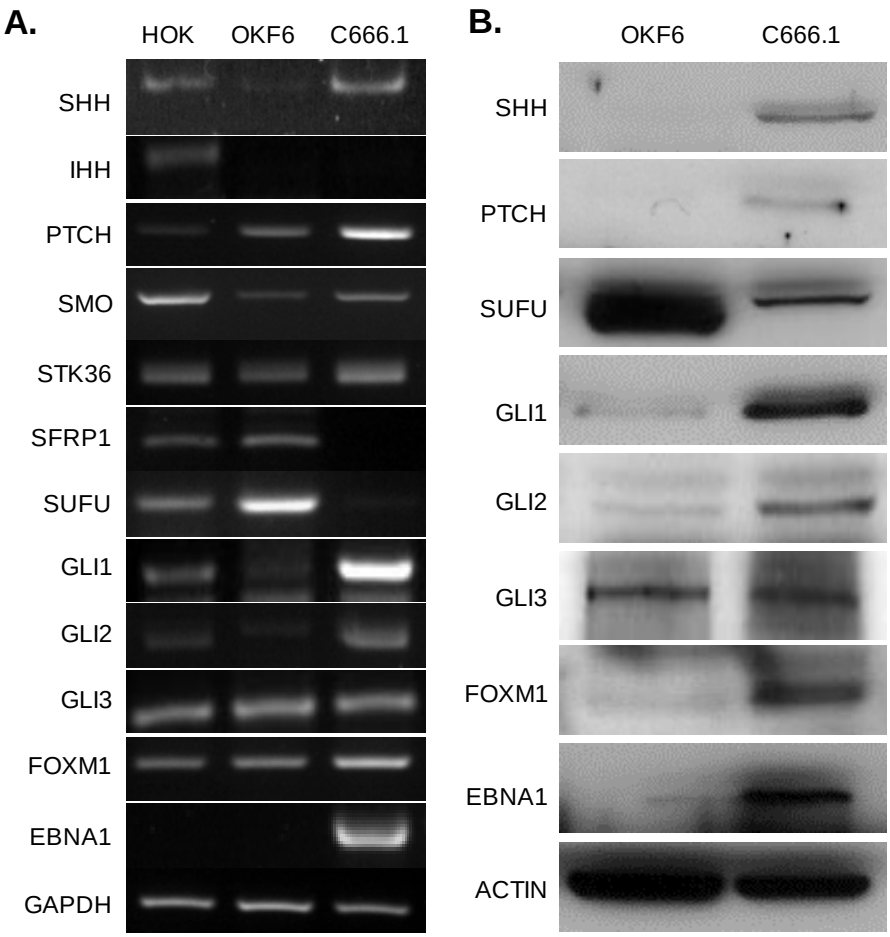


Figure 3. 10 C666.1 cells express higher levels of HH pathway components and targets compared to normal HOK and OKF6 counterparts
(A) Validation of OKF6 as a model for HOK. RT-PCR analysis comparing the expression of HH pathway components and downstream targets in HOK cells, the t hTert-immortalised normal human oral keratinocyte cell line (OKF6) and the C666.1 NPC cell line. (B) Western analysis reveals increased expression of HH pathway components and downstream targets in the C666.1 cell line compared to OKF6, normal human keratinocyte cells. C666.1 cells display chronic activation of the HH pathway expression demonstrated by increased expression of PTCH, GLI1 transcription factor and downstream HH targets.

3.5.3 Validation of the hTert-immortalised normal nasopharyngeal and oral cell lines, NP460 and OKF6, and comparison to C666.1

Further validation of the OKF6 cell line became possible when an hTert-immortalised normal nasopharyngeal cell line, NP460, became available towards the end of this study. While HOK have previously been shown to be a suitable control for normal nasopharyngeal tissue by microarray analysis, the need for a cell line with a longer replicative lifespan necessitated the use of the hTert-immortalised oral and nasopharyngeal epithelial cell lines. RT-PCR analysis was performed to compare the status of the HH signalling pathway and the expression of stemness-associated markers in OKF6 and NP460 cell lines relative to C666.1 (Fig 3. 11). As predicted, both OKF6 and NP460 displayed similar expression profiles with regard to both the HH signalling pathway and putative stem cell marker expression. Expression of downstream HH targets GLI1, PTCH and FOXM1, and all stem cell markers, were found to be expressed at low levels in the OKF6 and NP460 cells relative to C666.1 cells. However, there was variation in the expression of other GLI transcription factors between the OKF6 and NP460 cell lines; GLI3 was found to be expressed at higher levels in NP460 relative to OKF6, while expression of GLI2 was higher in the OKF6. Overall the expression profile was comparable between these two cell lines justifying use of OKF6 as a suitable model for normal nasopharynx in earlier experiments.

Immunofluorescence staining (Figure 3. 12) was also performed to compare the expression of HH pathway components and downstream target FOXM1 in OKF6, the newly acquired NP460, and C666.1 cell lines at the single cell level. C666.1 shows

increased nuclear expression of GLI1, which correlates with the increased expression of ZIC2 (as seen in the C666.1 cell line by microarray analysis in Figure 3. 6) that acts to maintain nuclear localisation of activated GLI transcription factors. Additionally, the NPC cell line demonstrates increased SMO and FOXM1 expression, and increased and redistributed PTCH expression compared to OKF6 cells

OKF6 and NP460 display a similar HH expression pattern however subtle differences exist; NP460 cells display increased expression of GLI2 and SHH, and decreased expression of cytoplasmic GLI3 compared to the oral keratinocyte cell line. These data support the RT-PCR analysis and demonstrate the presence of a dysregulated HH signalling pathway in the C666.1 cell line at the protein level, and further validate the OKF6 cell line as an adequate model for normal nasopharyngeal cells.

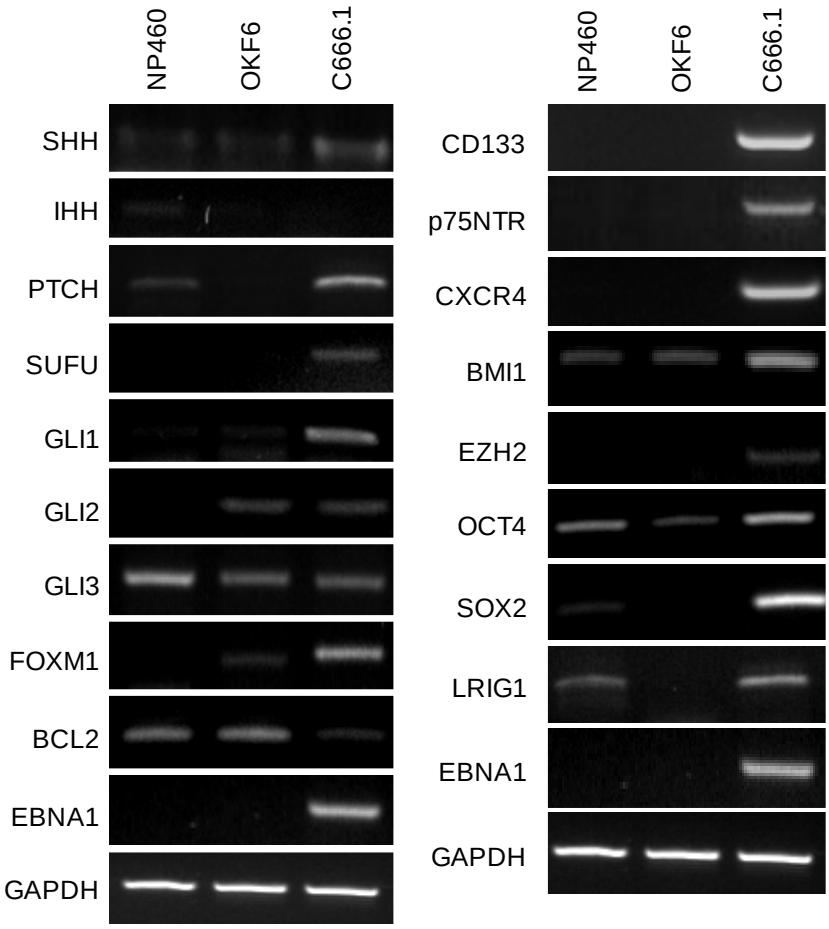


Figure 3. 11 Expression of HH pathway components and stem cell markers in the hTert-immortalised OKF6 and NP460 cell lines compared to C666.1
Expression of HH pathway components and targets (left), and stem cell/CSC markers (tight) was compared by RT-PCR in the NP460 and OKF6 normal cell lines compared to the C666.1 NPC cell line.

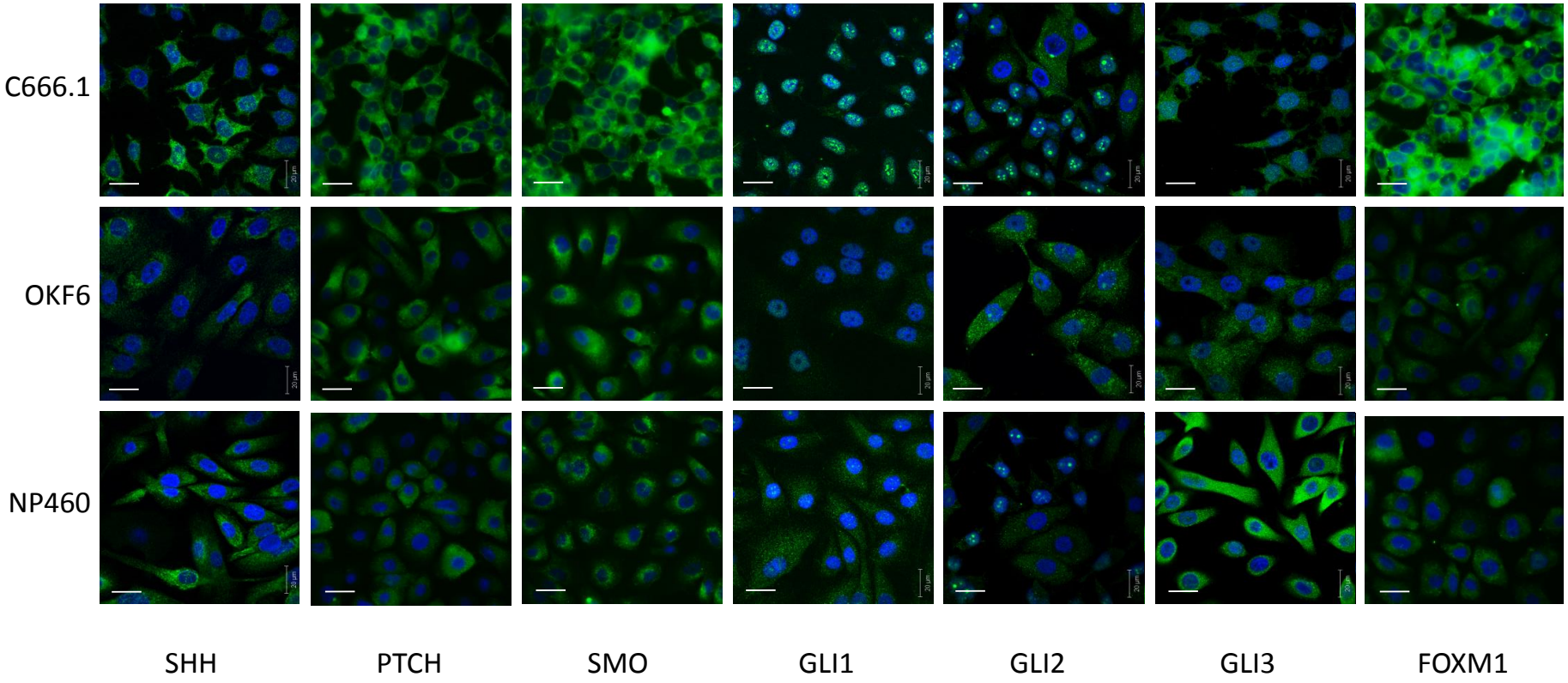


Figure 3. 12 Immunofluorescence staining of HH pathway components in C666.1, OKF6 and NP460 cell lines
Overexpression and redistribution of HH pathway components (SHH, PTCH, SMO), increased nuclear expression of transcription factors GLI1 and GLI3, and increased FOXM1 HH target expression in the C666.1 cell line compared to the hTert-immortalised normal oral keratinocyte cell line OKF6 and normal Chinese nasopharyngeal cell line NP460. Bar = 20 µm.

3.6 HH signalling pathway integrity in C666.1 and OKF6 cell lines

It was important to establish the integrity of the HH signalling pathway in OKF6 and C666.1 cells, and to demonstrate that both cell lines were responsive to SHH pathway stimulation.

3.6.1 The responsiveness of OKF6 and C666.1 cells to exogenous SHH ligand stimulation

The responsiveness of OKF6 and C666.1 cells to SHH ligand was examined by transfecting pBABE SHH, or a control empty vector (pBABE puro), along with an 8xGLI-BS-Luc, a reporter construct containing eight tandem copies of a consensus GLI responsive element upstream of the luciferase gene (Sasaki et al., 1997), to assay HH pathway activity (Figure 3. 15).

A point two-fold stimulation in GLI-luciferase reporter activity was observed in response to 0.5 µg SHH plasmid transfection in both OKF6 and C666.1 cells. 5E1 is a rat monoclonal antibody that inhibits the HH pathway by binding HH ligand at a pseudo-active site (Peterson and Bogenmann, 2003). Treatment of the OKF6 and C666.1 cell lines with 5E1 conditioned media (used at a 1:1 ratio with full complement growth media and compared to 1:1 control conditioned media) was sufficient to inhibit increased 8xGLI-BS-Luc reporter activity in SHH stimulated cells. This demonstrates that both the C666.1 and OKF6 cells have an intact HH pathway and are capable of further stimulation with SHH plasmid over 48 hr. Whilst the C666.1 cell line demonstrated increased HH signalling, the basal level of reporter activity in this cell line was substantially lower than that of the OKF6 cells. This is

possibly due to the slow proliferative nature of the C666.1 cell line or a differential susceptibility of the cell line to the transfection protocol.

3.6.2 Cell line responsiveness to HH pathway stimulation and inhibition

The consequence of HH pathway stimulation was examined by measuring the expression of HH target genes: PTCH, GLI1 and FOXM1, in response to SHH plasmid transfection. The consequences of HH pathway inhibition was then examined using 5E1, a neutralising SHH antibody, and treatment with GANT58 and/or GANT61, two chemical inhibitors that act on the GLI transcription factors (Lauth et al., 2007). Representative experiments for OKF6 and C666.1 are shown in Figure 3.16 and Figure 3.17 respectively.

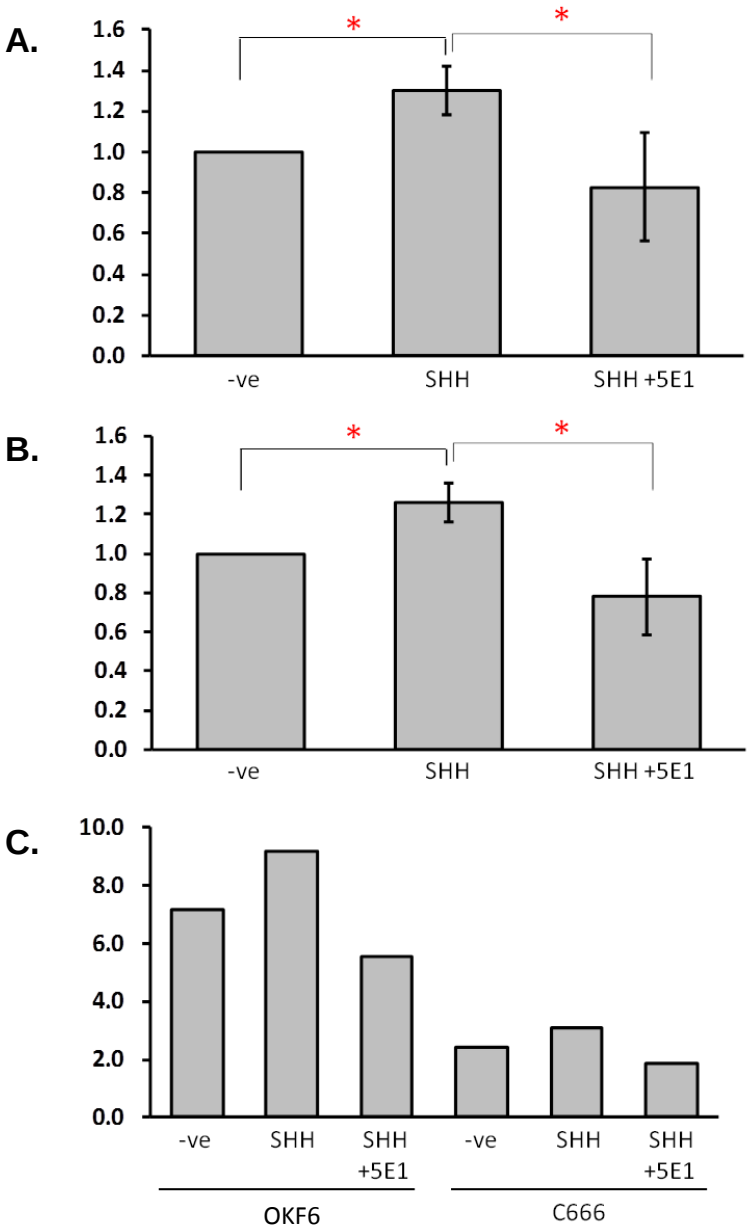


Figure 3. 13 Transient transfection of a SHH expressing plasmid induces 8xGLI-BS-Luc activity in both OKF6 and C666.1 cells

(A) OKF6 and (B) C666.1 cell lines normalised to control transfection. Cells were transfected with SHH and grown in 1:1 conditioned control media or 1:1 5E1 expressing media. SHH transfection induced a greater than 0.25 fold increase in 8xGLI-BS-Luc activity in both cell lines (P=0.011 in both cell lines, n=3).Treatment with 5E1 containing media reduced 8xGLI-BS-Luc activation to 0.63 in the OKF6 and 0.62 fold in the C666.1 cells (P=0.047 and 0.019 respectively, n=3). (C) Shows the average raw values normalised to pGL2 activity to demonstrate significantly reduced 8xGLI-BS-Luc basal activity in the C666.1 cell line. *, p<0.05; ** p<0.01; *** p<0.001.

mRNA was collected at 0, 24, and 48 hr from OKF6 and C666.1 cells transfected with 0.5 µg of SHH expression vector, or treated with a 1:1 dilution of 5E1-conditioned media, 25 µM GANT58 or 12.5 µM GANT61. GANT61 was used at half the concentration of GANT58 due to observations that it is toxic at higher concentrations possibly due to its nature to target both GLI1 and GLI2. Cells will proliferate readily in 12.5 µM of GANT 61 and 25 µM GANT58.

Whilst OKF6 cells do not produce significant amounts of SHH ligand they are capable of responding to SHH ligand induction of the HH pathway. SHH ligand was seen to induce the expression of PTCH, GLI1 and FOXM1 at both the protein and RNA level in both cell lines after 24 hr. While both cell lines showed decreased expression of HH pathway components and targets upon 5E1 treatment, they demonstrated differential sensitivity to the GLI transcription factor inhibitors. GANT61 inhibition decreased the expression of these HH targets significantly after 48 hr in the C666.1 cell line but only marginally in the OKF6. GANT58 also significantly inhibited the expression of PTCH and FOXM1 in the C666.1 cell line but had little effect on the OKF6 cells. Inhibition of the GLI1 transcription factor with GANT58 did not reduce the expression of GLI1 in either cell lines over 24 hr possibly due to the uninhibited GLI2 transcription factor, which is known to induce GLI1 by binding to its promoter (Ikram et al., 2004).

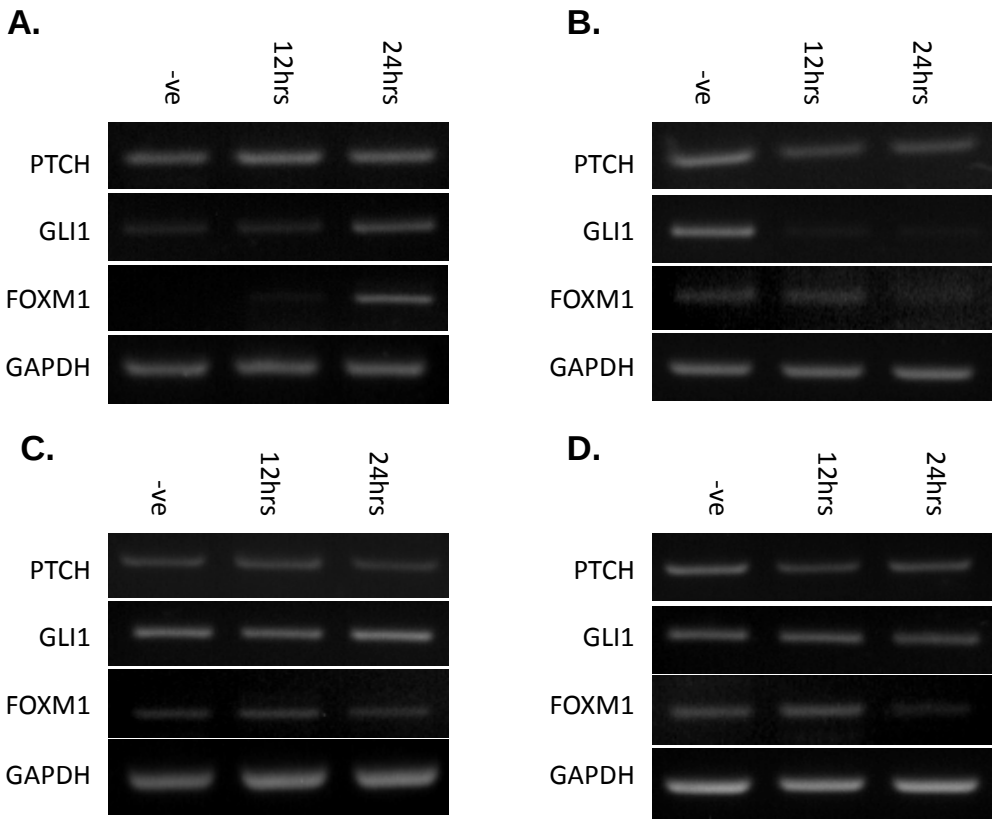


Figure 3. 14 The effect of stimulation and inhibition of the HH signalling pathway in the OKF6 cell line
The OKF6 cell line responds to stimulation with (A) SHH ligand (0.5µg), and inhibition of the HH pathway with (B) 5E1 (1:1 dilution of 5E1 conditioned media), (C) GANT58 (25 µM), and (D) GANT61 (12.5 µM) over 48 hr.

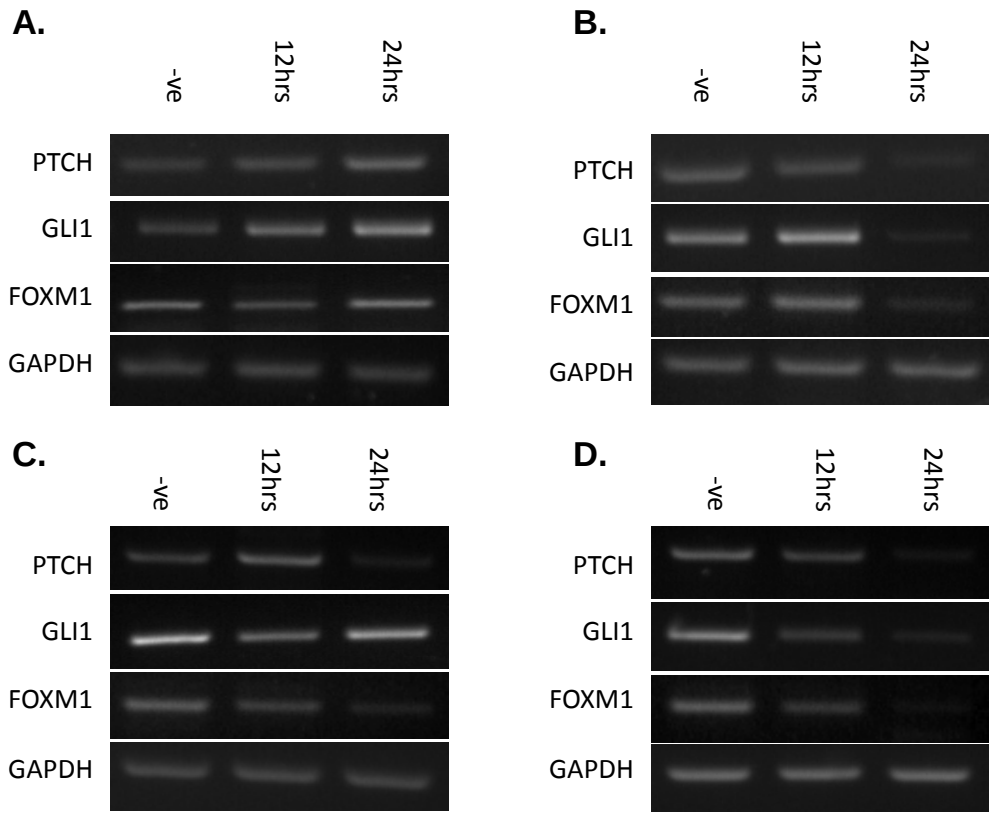


Figure 3. 15 The effect of stimulation and inhibition of the HH signalling pathway in the C666.1 cell line

The C666.1 cell line responds to stimulation with (A) SHH ligand (0.5µg/ml), and inhibition of the HH pathway with (B) 5E1 (1:1 dilution of 5E1 conditioned media), (C) GANT58 (25 µM), and (D) GANT61 (12.5 µM) over 48 hr.

3.7 The expression of stem cell markers and the maintenance of a stem cell like phenotype are dependent on HH signalling

3.7.1 Inhibition of the HH signalling pathway influences stem cell marker expression

The requirement of HH signalling for stem cell marker expression in the C666.1 cell line was investigated by inhibiting the HH pathway. HH pathway inhibition, with the GLI1/2 inhibitors GANT58 and GANT61 over 48 hr, was found to reduce the expression of putative stem cell markers by RT-PCR (Figure 3. 18A). Significant downregulation of the stem cell markers: CD44v6, SOX2, BMI1, CD133, EZH2 and NESTIN, was observed in response to GANT61 treatment, which targets both GLI1 and GLI2, whilst marginal effects were observed on p75NTR, CXCR4 and LRIG1 expression.

FACS analysis provided evidence for the dependence of CD133 expression on HH signalling as CD133 expression was decreased following GANT58 treatment in a dose dependant manner (Figure 3. 19). The CD133 positive sub population of C666.1 cells was decreased from 29% to 25% by 25 µM GANT58 treatment in low serum medium over 72 hr. 12.5 µM GANT61 treatment was sufficient to reduce the CD133 positive sub population from 29% to 23%. HH inhibition did not, however, alter cell surface CD44v6 expression (Figure 3. 20) despite evidence that HH inhibition can decrease cellular CD44v6 by RT-PCR and western analysis.

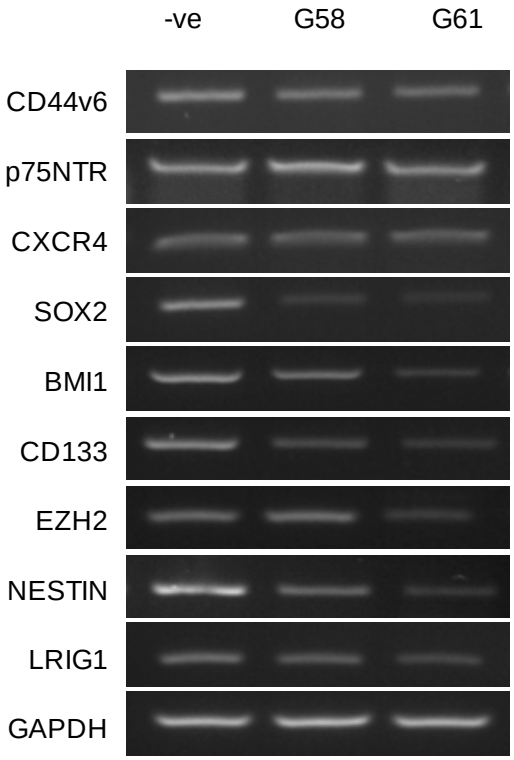


Figure 3. 16 Expression of stem cell markers in C666.1 on inhibition of the HH pathway with GANT58 and GANT61
RT-PCR demonstrating inhibition of stem cell markers CD44v6, SOX2, BMI1, CD133, EZH2 and NESTIN upon treatment with 25μM GANT58 (G58) or 12.5μM GANT61 (G61) over 48 hr. Expression of P75NTR, CXCR4 and LRIG was not significantly altered.

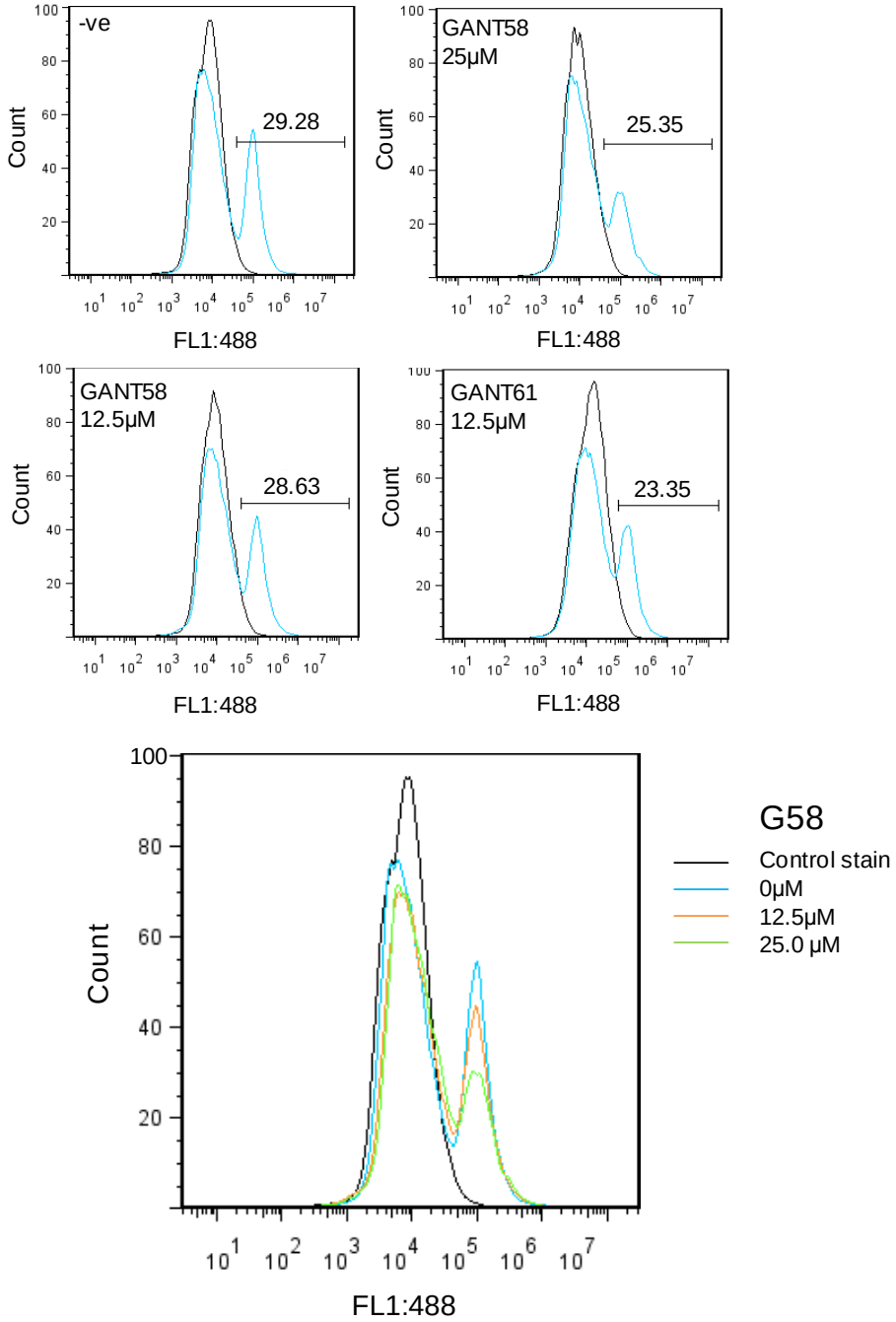


Figure 3. 17 FACS analysis of CD133 expression in the C666.1 cell line treated with HH inhibitors

Representative FACS analysis demonstrating the expression of stem cell marker CD133 is dependent on HH signalling and responsive to hedgehog inhibition with GANT58 (12.5 μM and 25 μM) and GANT61 (12.5 μM). GANT58 (G58) treatment reduces cell surface CD133 marker expression in a dose dependent manner. GANT61 treatment produced more substantial inhibition of CD133 expression than GANT58. CD133 antibody = C24B9.

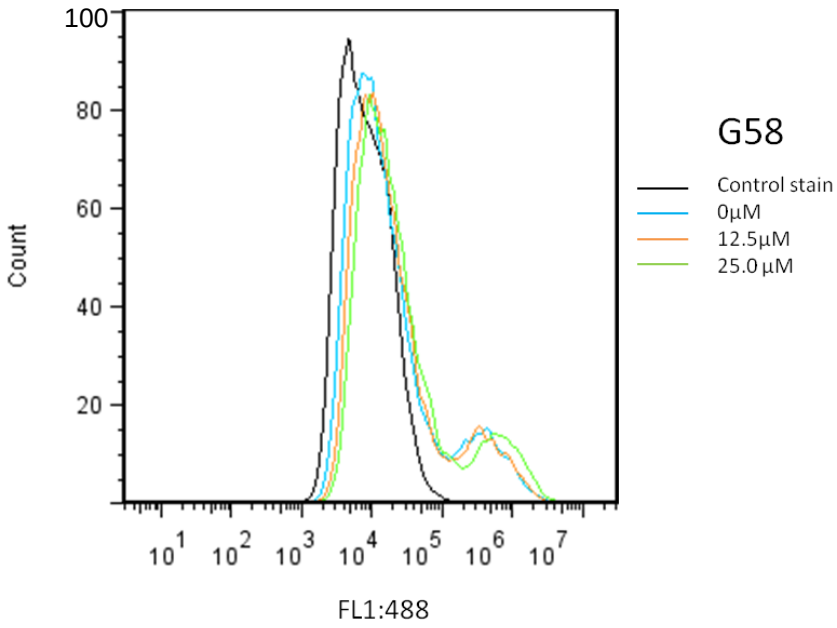


Figure 3. 18 FACS analysis of CD44v6 expression in the C666.1 cell line treated with HH inhibitor
GANT58 (G58) treatment (0-25µM) does not significantly alter cell surface CD44v6 expression.

3.8 DISCUSSION

Current theories regarding the origin of head and neck cancer (HNSCC) propose that these malignancies arise from populations of tumour-initiating CSCs. As the origin and phenotype of CSCs in many tumours remains unclear, the molecular characteristics of CSCs are largely unknown. Although there is concern that stem cells and CSCs can only be defined operationally, and that a single marker may not be sufficient to identify true stem cells or CSCs, recent studies on oral, gastric and breast cancer-derived cell lines show that the hyaluronic receptor CD44 "marks" a heterogeneous population of cells which retain stem cell/CSC-like properties. Other studies have shown that cells expressing high levels of CD44 ($CD44^{high}$) overlap phenotypically with SP-cells, identified on the basis of their ability to exclude the DNA-labelling dye Hoechst 33342. Interestingly, both $CD44^{high}$ and SP-cell populations display many characteristics ascribed to *bona fide* CSCs; they contain cells that are resistant to the cytotoxic effects of DNA-damaging drugs, have an ability to seed new tumours at low cell density in SCID mice, and are enriched for a variety of stem cell markers. As with HNSCC, previous studies have identified tumourigenic sub-populations of cells in NPC cell lines marked by the CSC marker CD44 (Su et al., 2011), or through increased Hoechst DNA binding dye exclusion (Wang et al., 2007). We sought to use previously collected microarray data to identify markers of interest from the many putative stem cell/CSC markers that are proposed to identify CSC containing sub populations of cell in NPC, or other tumours of head and neck carcinoma origin, to determine if there was selective enrichment of CSCs in NPC tumours.

Analysis of microarray data comparing NPC tumour biopsies to normal nasopharyngeal epithelium revealed increased expression of basal cell markers and a number of putative stem cell/CSC markers, a select few of which were validated on a NPC tissue array by IHC.

The cell surface receptor CD44 has been used to isolate subpopulations of tumour cells with stem cell-like characteristics from two EBV-negative NPC cell lines: CNE1 and SUNE1 (Couve-Privat et al., 2004), and the authentic EBV-positive NPC cell line C666.1 (Cheung et al., 1999). Although CD44 and was not found to be significantly upregulated in the microarray analysis, it was found to be consistently over expressed in primary NPC tumours by IHC, and by RT-PCR and western blot analysis in the C666.1 cell line. This may highlight technical problems associated with the RNA amplification protocol used in the microarray analysis, or a fault in the probe sets which may have been unable to efficiently recognise CD44-derived transcripts. Unlike CD44, BMI1, a stem cell-associated marker and putative downstream target of HH signalling, along with EZH2 and SOX2, were found to be overexpressed in the NPC microarray. Additionally, these genes were found to be over expressed at the protein level in primary NPC relative to normal nasopharyngeal tissue and in C666.1 cells relative to HOK and OKF6.

While a number of putative stem cell-associated markers were found to be upregulated in the microarray analysis, a few notable genes were absent. These included ABCG2 and CD133. ABCG2 is an ABC transporter gene that is reportedly upregulated in CSC populations isolated from a variety of tumours, including NPC (Hu et al., 2008, Wang et al., 2013), and CD133, a marker that has been used to

isolate stem cell populations from various tissues and malignant tumours including pancreatic, brain, liver, colorectal and lung (Singh et al., 2003, Suetsugu et al., 2006, Bertolini et al., 2009, Choi et al., 2009). The absence of putative stem cell markers may be due, in part, to the relatively small size of the sample population examined, as CSC populations expressing this marker may be localised or constitute too small a population to be detected by microarray analysis of total amplified RNA. However, additional research into the expression of CD133 suggests that expression is downregulated during metastatic progression such that tumour from a late stage biopsy would be expected to have low CD133 expression (Shmelkov et al., 2008). CD133 could not be detected on the array and was only upregulated in the C666.1 cell line, which may allude to cell line specific expression of this marker or an expression profile that is an artefact of *in vitro* culture.

Upregulation of p75NTR was also not observed by microarray analysis, again possibly due to problems associated with the RNA amplification protocol or probe sets used. However, IHC analysis revealed consistent overexpression of p75NTR in the majority of primary NPC biopsies. p75NTR was also expressed at high levels in C666.1 cells *in vitro*, and may constitute a useful marker in the identification of NPC-derived CSCs as it has been used to isolate both normal and squamous cell carcinoma stem cells from the head and neck (Okumura et al., 2003, Okumura et al., 2006, Huang et al., 2009).

With a key concern about the relative size of CSC populations in authentic NPC tumour hindering the identification of markers by microarray analysis it may be regarded as a bit of an oddity that IHC validation for the CD44, EZH2, BMI1 and

SOX2 demonstrated marker expression in the vast majority of tumour biopsies and the majority of the cells therein.

Expression of CD44 and CD44 splice variants has been interrogated in HNSCC; Prince et al., showed CD44 positivity in fewer than 10% of HNSCC tumour cells which also demonstrated increased BMI1 expression (Ruat et al., 2012), whilst in another studies CD44 positivity in HNSCC reportedly ranges from less than 5% to 95%, with established cell lines all demonstrating over 90% positivity (Lauth et al., 2007, Louvi and Grove, 2011). A study into the CD44 and CD44v6 expression of the epithelium of the head and neck demonstrated positivity of these two markers in 50-80% of cells with only moderate increases in differentiated carcinomas, questioning the selectivity of these proteins as CSC markers (Liao et al., 2010). When CD44 was used to identify cells with CSC-like properties from NPC derived C666.1 and SUNE1 cells the marker was seen to be expressed in a distinct subpopulation comprising 45.3% and 52.5% of the cell lines respectively (Janisiewicz et al., 2012, Su et al., 2011). CD44 positivity was also associated with poor clinical prognosis, increased clone forming ability and increased drug sensitivity in the SUNE1 cell line. Additionally a recent paper demonstrated that the CD44+ population constituted the tumour initiating population of C666.1 (Lun et al., 2012). Our findings identify a substantial increase in CD44 marker expression during NPC pathogenesis, highlighting a potential role of CSC expansion in disease progression.

Another stem cell maker interrogated in this study was EZH2, which was found to be consistently upregulated in the tumour microarray and in primary NPC tumours analysed by IHC. Studies have found over expression of EZH2 in numerous cancers;

however, analysis of this polycomb group protein demonstrates varied expression in regard to the number of positive cells within different tumour types. In prostate cancer, EZH2 expression did not exceed 10% of the tumour cell population, with the majority of tumours (75%) demonstrating positivity in less than 1% of malignant cells (van Leenders et al., 2007). A study of GC detected positive EZH2 staining in greater than 10% of cells and reported this in 78.8% of cases (Mattioli et al., 2007). Staining of bronchiole SCC tissue demonstrates an increased expression of this marker compared to normal epithelium, where EZH2 staining is present in isolated cells near the basal layer. Positivity appears to be present in greater than 10% of cells, although percentages were not presented, and EZH2 did not correlate with BMI1 expression which was widely expressed in normal tissue (Breuer et al., 2004).

The expression of stem cell/CSC markers was further interrogated in the C666.1 cell line, the only available EBV positive NPC cell line that is amenable to growth *in vitro*. Normal oral keratinocytes were used as a model for normal nasopharyngeal keratinocytes, having previously been validated for this purpose by microarray analysis (Hu, 2010).

While the microarray analysis of the C666.1 cell line showed increased expression of many stem cell/CSC markers of interest, a number of markers were seen to be downregulated relative to HOK. This downregulation is not surprising as analysis of stem cell marker expression in tissues and tumour biopsies demonstrate that stem cell marker expression is tumour dependent and, as such, not all of the putative markers would be expected to be upregulated in a single NPC cell line. Some markers may also be dependent on tumour grade, such as CD133 expression which

is proposed to decrease with tumour grade, and/ or mechanism of genetic modification, as it is clear from the heterogeneity of marker expression in the tumour biopsies that the mechanism by which pathogenesis may occur could vary between individual occurrences of NPC. The diversity of marker expressed highlights the need for multiple marker identification to distinguish between true CSCs and more differentiated progeny.

FACS analysis of the C666.1 cells compared to OKF6, an hTert-immortalised oral keratinocyte cell line that was validated as a model for normal nasopharyngeal epithelial cells, demonstrated increased cell surface marker expression of CD133 and CD44v6 in the NPC cell line. It is interesting to note the high proportions of cells within the cell line that show expression of these putative CSC marker; on average 57% of C666.1 cells show CD44v6 positivity, which coincides with the expression seen in previous studies of NPC and HNSCC, and 48% show CD133 positivity

These markers may identify CSCs in some tissue types; CD44v6 expression in p53/PTCH double heterogeneous mice tumours is found in less than 10% of cells and is induced upon GBM neurosphere formation (LaBarge and Bissell, 2008), and CD133 is reported to be expressed in only 3% of human laryngeal tumour cell line Hep-2, identifying cells with CSC-like properties (Zhang et al., 2003). However, other studies have shown the potential of these markers to widely identify non-CSC populations; CD44v6 was downregulated in poorly differentiated SSC tumours compared to normal tissue and only induced in the formation of some tumours following injection into SCID-mice (Shmelkov et al., 2005), and in the colon, the tumour initiating population is not restricted to a CD133+ population although this

population may give rise to a highly tumourigenic CD133-/ CD44+ population (Qazi et al., 2011).

Microarray analysis and IHC staining also revealed aberrant expression of the HH pathway in NPC; pathway component and downstream target PTCH was found to be upregulated along with other positive regulators of the HH signalling pathway. Additionally, negative regulators of the pathway were found to be downregulated and a number of HH targets were induced in the NPC tumours compared to normal nasopharynx. Having pathway components such as PTCH and GLI1 as downstream targets allows for feedback into the pathway. Increased expression of GLI1 is reported to be an accurate measure of HH signalling activity and activation of the GLI transcription factors is known to influence cell differentiation, proliferation, motility and cause an EMT (Kasper et al., 2006, Kar et al., 2012). However key components, including SHH, GLI1 and GLI2, were scored as absent from the microarray analysis. SHH ligand was found to be expressed in only one of the NPC tumour biopsies by microarray analysis but was later found to be upregulated in 60% of NPC tumour samples by IHC. Similarly GLI1 and SMO could also be detected by IHC and were found to be upregulated in approximately 90% and 96% of NPC tumours respectively compared to normal tissue. The expression of these genes analysed by microarray analysis was too low to be perceived as present above background in the sample RNA and may reflect problems with the RNA amplification protocol or probe sets used for detection rather than low expression in the samples.

The third HH transcription factor, GLI3, was found to be overexpressed in the NPC tumours by microarray analysis. GLI3 is often referred to as a negative regulator of transcription having both a repressor and activator form (Wang et al., 2000). This is also true of GLI2; however, GLI2 is believed to exist primarily as a transcriptional activator (Simpson et al., 2009). Microarray analysis does not identify the form of GLI3 expressed in NPC tumours; however, it is interesting to note that the production of the repressive form requires SUFU, whose expression appeared to be downregulated in a number of primary NPC tumours. In the absence of HH signalling SUFU is required to bind full length GLI3 in the cytoplasm where it is cleaved into its repressive form (Kise et al., 2009). When HH signalling is activated GLI3 is maintained in its activated form and allowed to translocate to the nucleus (Humke et al., 2010). In a situation where SUFU is absent, as is possibly the case in NPC, GLI3 is not efficiently processed to its repressive form, therefore would not be phosphorylated by GSK3β and PKA, and could act primarily as a transcriptional activator.

Analysis of the HH signalling pathway in primary NPC tumours revealed varying patterns of expression of HH ligand and HH pathway components, with no tumour displaying increased expression of all pathway components. However, expression of HH pathway targets appeared to be a consistent feature in this disease. This suggests that HH pathway dysregulation may be achieved through any one of a number of mechanisms that may involve mutations in PTCH1 or SMO, overexpression of SHH ligand, or decreased expression of SUFU. A dysregulated HH signalling pathway has been shown to be sufficient to induce tumourgenesis in neural precursor cells, and is suggestive of an undifferentiated cell origin in neural

tumours (Schüller et al., 2008, Yang et al., 2008b). Due to the undifferentiated nature of NPC and the abundant expression of putative stem cell markers, it is possible that the origin of the NPC tumour is an undifferentiated cell. EBV infection in an undifferentiated background may favour stable infection, and precedence for this theory exists in persistent stable HPV infection of undifferentiated cervical cells giving rise to cervical cancer (Martens et al., 2004, Bodily and Laimins, 2011). This theory, that the origin of epithelial carcinogenesis is a stem cell, was first proposed in 1974 (Pierce, 1974, Potten and Loeffler, 1990) and has yet to be disproved, although evidence implicates more committed cells in a number of tumours such as unipotent progenitor cells in meduloblastoma, luminal progenitors in BRAC1-associated breast cancer and interfolicular epidermal progenitors in BCC (Visvader, 2011). Identification of the cellular origin of NPC may help identify high risk individuals and has implications for treatment.

Microarray analysis allowed comparison of the C666.1 cell line to human primary keratinocytes and identified upregulation of HH pathway components consistent with activation of the HH pathway in this tumour cell line. Expression of GLI1 and FOXM1 was confirmed by RT-PCR and immunofluorescence staining. PTCH2 and SMO expression were seen to be downregulated in the microarray of C666.1 and HOK, and SHH expression was so low as to be barely detectable above the background. Upon RT-PCR and western analysis SHH could once again be detected in the C666.1 cell line however the expression of SHH ligand was very low in the RNA samples.

Inconsistencies in HH downstream target expression by microarray analysis were seen; some HH targets were found to be upregulated (FOXM1 BCL2, CFLAR and WNT5A) others where downregulated (CCND2 and SOX9), this may be due to competing signalling pathways. For instance CCDN2 expression can also be regulated by other signalling pathway including a STAT5/BCL6-dependent mechanism, and the MYC/MAX/MAD network (Fernandez de Mattos et al., 2004, Bouchard et al., 2001), and SOX9 is regulated by WNT signalling in the intestinal crypt (Blache et al., 2004). Additionally, the decreased expression of cell surface receptors PTCH2 and SMO seen in this microarray, and the absence of SHH expression, may point towards ligand independent activation of the HH pathway downstream of these receptors, leading to increased PTCH1 and other downstream target expression. Ligand independent activation of the HH pathway can be demonstrated in a few cell lines in which the HH signalling pathway has become uncoupled. For instance, RPMI 8226 cells lack SMO expression and exhibit low GLI responsive luciferase activity in response to the addition of HH ligand (ShhNp) (Peacock et al., 2007).

Activation of the HH pathway in NPC, in the absence of SHH ligand, could be achieved by inadequate repression of the pathway, through mechanisms including the epigenetic silencing of SUFU, downregulation of HH receptor antagonists such as HHIP and GAS1 or through non-canonical signalling. Furthermore, crosstalk between the HH signalling pathway and other stem cell maintenance pathways has been proposed where the PI3K/ATK and KRAS pathways have been demonstrated to positively regulate HH signalling, and HH independent activation of GLI can occur

through the TGFβ and TNF-α signalling pathways (reviewed in Chung and Fu, 2013) and via mutant KRAS (Collins et al., 2012).

Alternatively, as mentioned previously, activation of the HH signalling pathway could be due to activating mutations in proteins such as SMO or loss-of-function mutations in PTCH1. Oncogenic transformation of receptor proteins is seen in multiple carcinomas including BCC, where a constitutively active form of the SMO protein is sufficient to induce oncogenic transformation in transgenic mouse models when directed to the skin. Additional ligand–independent mechanisms of pathway activation also exist in carcinomas such as squamous cell carcinoma (SCC), where N-cadherin mediated cell-cell contacts and CD44 can activate EGFR signalling in the absence of EGF, leading to downstream MAPK pathway activation (Shen and Kramer, 2004).

Immunofluorescence staining for HH pathway components and downstream targets identified a redistribution of the PTCH1 protein, possibly due to increased endocytosis of PTCH1 due to chronic HH pathway stimulation. Further studies are required to interrogate the function of PTCH1 in this system or if this redistribution is caused by some other factor which may impact on HH signalling activity. PTCH is normally localised in the primary cilium, maintaining SMO in vesicles and preventing its activation (Ruat et al., 2012). It would thus seem that the distribution of PTCH could have significant consequences on HH signalling; for instance redistribution of the receptor into vesicles away from the cilia could allow for activation of the pathway in a ligand independent manner. Additionally constitutive SMO expression has been shown to activate the HH pathway (Xie et al., 1998). Evidence from a variety of

techniques used to measure expression of HH pathway components and downstream target expression, at both the RNA and protein level, reveal an activated HH signalling pathway in the C666.1 cell line which, when combined with the evidence that NPC tumours demonstrate the same deregulation, highlights the importance of this pathway in the pathogenesis of EBV-positive NPC.

Inhibition of the HH pathway with cyclopamine has been shown to impact on stem cell marker expression and limits the growth of CSC populations in pancreatic xenograph models (Jimeno et al., 2009). Work presented here shows that inhibition of the GLI transcription factors with GANT58 and GANT61 attenuates HH signalling and reduce expression of key stem cell/CSC markers in the C666.1 cell line. GANT61 was found to be effective at inhibiting stem cell marker expression, consistent with evidence that GANT61 is a more efficient HH inhibitor, targeting both GLI1 and GLI2 (Lauth et al., 2007).

Whilst the expression of multiple stem cell markers, including CD44v6 and CD133, was found to be dependent on HH signalling, only cell surface CD133 expression was reduced in response to HH pathway inhibition, as there was no effect on cell surface CD44v6 expression in response to HH pathway inhibition. One could postulate that multiple cell signalling pathways are responsible for the expression of CD44; indeed the WNT and TGFβ signalling pathways have been shown to influence the expression of this stem cell marker among other canonical pathways (Wielenga et al., 1999, Shipitsin et al., 2007). As such, inhibition of the HH pathway alone may not be sufficient to reduce the expression of CD44v6. Further work is required to

better understand the interaction between these stem cell maintenance pathways in the background of signalling dysregulation present in the NPC tumour.

CHAPTER FOUR: EBV INDUCES THE EXPRESSION OF STEM CELL MARKERS BY DIRECTLY ENGAGING THE HEDGHOG SIGNALLING PATHWAY.

"Truth in science can be defined as the working hypothesis best suited to open the way to the next better one."

— Konrad Lorenz

In On Aggression (1966, 2002), 279.

4.1 Introduction

In NPC, viral gene expression is restricted to EBNA1, the non-coding EBER1/2 RNAs, the *Bam*HI1A-derived miRNAs, BARF1, and variable expression of LMP1 and LMP2 (Raab-Traub, 2002, Port et al., 2013). These latent gene products influence epithelial cell behaviour, modulating the activity of various cell signalling pathways to effect changes in cell growth, differentiation, motility, invasion, apoptosis and angiogenesis (Young and Rickinson, 2004, Lung, 2012). In relation to stem cell maintenance in epithelial cells, LMP2A has been shown to transform epithelial cells, to block epithelial cell differentiation, and to enhance epithelial cell adhesion and motility. Key roles have been identified for Syk, PI3K/Akt and ERK-MAPK signalling in these responses (Fotheringham et al., 2012, Scholle et al., 2000, Dawson et al., 2008). Similarly LMP2A has been shown to inhibit differentiation and to enhance colony formation and tumorigenic potential through activation of the WNT/β-catenin pathway, mediated in part through PI3K/Akt inactivation of GSK3 (Morrison et al., 2003). Crosstalk between the HH and WNT signalling pathways is well documented and HH activation can induce the expression of a number of WNT proteins (Mullor et al., 2001, Cohen, 2003, Cohen, 2010). LMP2A induction of WNT/β-catenin signalling may impact on the HH signalling pathway; activation of β-catenin decreases SHH expression in ventral midbrain precursors and mouse embryonic stem cells (Tang et al., 2010) while in developing hair buds, WNT is proposed to precede and be required for a tumourigenic response to activated HH signalling (Yang et al., 2008a). The tissue dependent nature of the relationship between WNT and HH is further demonstrated in the developing tooth, which relies on a WNT/HH/FGF/BMP signalling network, where the HH and WNT signalling pathways are mutually inhibitory (Nakashima and Reddi, 2003, Ahn et al., 2010).

Like LMP2A, LMP1 also engages a plethora of cellular signalling pathways which exert a profound effect on epithelial cells. LMP1 has been shown to impact on mTOR signalling in the HONE1 and 6-10B NPC cell lines (Chen et al., 2010), and can also increase levels of nuclear β-catenin (You et al., 2011). Studies have reported variable expression and detection of LMP1 in NPC tissue, leading to discussion over its importance is NPC pathogenesis. However, LMP1 detection is associated with the sensitivity of the technique used. Whilst RT-PCR testing of NPC biopsies has demonstrated LMP1 expression in a higher percentage of tumours than previously described, when NPC cell lines are infected with type EBV LMP1 can only be detected on early passage and is often lost on establishment of a stable cell line (Jia, J and Dawson, C.D. unpublished observation). The low level LMP1 expression, which persists in the NPC tumour following the initial high level expression upon infection (Strong et al., 2013), may suggest that LMP1 dysregulation of the cellular signalling background occurs during initial infection. If LMP1 is responsible for the dysregulation of HH signalling seen in the C666.1 cell line, which does not express LMP1 but continues to display aberrant HH signalling, either LMP1 was important in the initial dysregulation of this signalling pathway, which is maintained without LMP1 expression, or another latent protein is continuing to act on the pathway, or both.

Latent EBV infection has also been shown to activate STAT3 and NFκB and to induce the expression of downstream targets such as c-Myc, VEGF and COX-2, whilst suppressing the p38-MAPK signalling pathway (Lo et al., 2006). More recently studies have interrogated the role of viral-encoded miRNAs on cell signalling pathway activity and their impact on cellular phenotype (Lung et al., 2013). While this

research is on-going it is likely that it will determine that these miRNAs have a significant role in modulating cell signalling and therefore cellular phenotype.

In addition to influencing stem cell maintenance pathways, EBV latent proteins have previously been associated with the expression of stem cell markers in NPC. One study looked at the effect of LMP1 positivity on CD44v6 expression, a CD44 variant highly expressed in certain GCs and non-Hodgkin's lymphoma, in tumour biopsies from NPC of various stages. CD44 variant expression correlated neither to LMP1 expression or stage; however, it was not determined if other EBV latent genes could affect CD44 expression (Brooks et al., 1995). Whilst LMP1 was not found to influence CD44 variant expression in this study there is much evidence of the transforming ability of this oncoprotein in epithelial cells (Dawson et al., 2012). Indeed in the context of "stemness" LMP1 can induce the expression of TWIST and the transition of cells from an epithelial to mesenchymal phenotype (Horikawa et al., 2007).

Prior to this study, EBV had not been shown to engage the HH pathway. However, a number of HH downstream targets had previously been demonstrated to be upregulated in EBV positive NPC. BCL2, a target of HH signalling that possesses the GACCACCCA motif to which active GLI transcription factors bind (Katoh and Katoh, 2009a), is over expressed in EBV positive tumours, including NPC, compared to EBV negative counterparts (Yang et al., 2001, Yip et al., 2006). BCL2 expression in NPC is correlated with histological type and mean survival of patients, and has been proposed as a prognostic marker (Vera-Sempere et al., 1997). Studies investigating the role of EBV latent protein in the expression of this protein have found that LMP1 silencing does not affect BCL2 expression, suggesting an LMP1 independent

mechanism for BCL2 upregulation (Mei et al., 2007). However both BCL2 and LMP1 proteins have been demonstrated to act synergistically to promote cell growth (Sheu et al., 2004).

BMI1 is another HH target found to be upregulated in NPC biopsies. BMI1 is a prognostic marker in NPC, where expression correlates with disease stage and can be used to predict survival (Song et al., 2006). Downregulation of BMI1 inhibits the growth of nasopharyngeal cells (Wu et al., 2011) and increases NPC cell sensitivity to cytotoxic drugs resulting in increased apoptosis (Qin et al., 2008). BMI1 expression has also been shown to immortalise primary nasopharyngeal epithelial cells *in vitro* through the induction of telomerase and inactivation of $p16^{INK4a}$ (Song et al., 2006), and can induce an EMT via activation of the PI3K/Akt pathway and epigenetic silencing of PTEN (Song et al., 2009). Again EBV may have a role in the induction of this HH target protein as LMP1 has been shown to induce the expression of BMI1 in HL cells (Dutton et al., 2007). LMP2A has also been demonstrated to induce an EMT, and the dedifferentiation of cells towards a stem cell like phenotype, which could play a role in establishing the undifferentiated phenotype seen in NPC tumours (Kong et al., 2010). Further phenotypic consequences of an activated HH pathway, including increased motility, proliferation and survival can be also induced by individual EBV viral proteins LMP1 and LMP2A (Dawson et al., 2012).

This chapter investigates the possible contribution of EBV infection to the activation of the HH signalling pathway, expression of putative stem cell markers and acquisition of a stem cell-like phenotype as observed in the C666.1 cell line. Finally,

some preliminary data as to the role of individual EBV latent genes in the activation of the HH signalling pathway will be presented.

4.2 EBV infection drives the expression of putative stem cell markers in latently infected epithelial cells: the role of the HH pathway

4.2.1 Authentic NPC and gastric cell lines derived from NPC or EBVa-GC express higher levels of putative stem cell markers than EBV negative cell lines

To obtain an overall impression of stem cell marker gene expression in epithelial cell lines derived from EBV-positive and EBV-negative carcinomas, RT-PCR profiling was performed on EBV positive; C666.1 and gastric derived YCCEL1, and EBV negative cell lines; SUNE-1, A549, CNE2, and OKF6 (Figure 4. 1). This analysis identified differences in the expression of a number of putative stem cell markers between EBV positive and EBV negative epithelial cell lines. It was found that C666.1 and YCCEL1 expressed significantly higher levels of the cell surface markers CD133 and CD44v6, and stem cell maintenance genes; SOX2, BMI1 and LRIG1. p75NTR was also expressed at higher levels in the C666.1 cell line but was not expressed in YCCEL1. Expression of epithelial cell adhesion marker (EPCAM), a cell surface receptor overexpressed in a variety of cancers, was expressed in OKF6 cells and the EBV negative carcinoma-derived cell lines and expressed at slightly higher levels in C666.1 and YCCEL1 cells.

From these data it was postulated that EBV infection might induce the expression of a number of putative stem cell markers by activating cell signalling pathways involved

in stem cell maintenance. Although dysregulation of a number of stem cell signalling pathways has been described in NPC, for example TGFβ, WNT/β-catenin and NOTCH, the contribution of EBV infection to this phenomenon is currently unknown. Whilst NPC tumour biopsies and the authentic EBV positive C666.1 cell line display increased expression of HH pathway components, as demonstrated in Chapter 3 and by Yue et al., (2012), the contribution of this particular pathway to the induction of stem cell marker expression and the role EBV infection plays in this phenomenon is unknown.

With this in mind, the effect of EBV infection on HH signalling and its contribution to stem cell marker expression and the acquisition of stem cell-like characteristics were investigated.

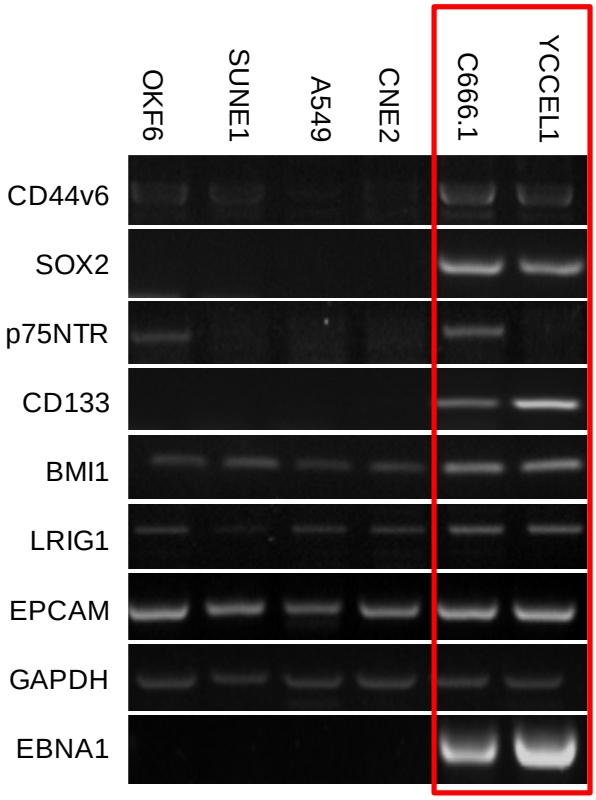


Figure 4. 1 Increased expression of stem cell markers in EBV positive C666.1 and YCCEL1 cell lines compared to EBV negative cell lines
Increased stem cell marker expression is seen in the EBV positive NPC cell line C666.1 compared to EBV negative NPC cell line CNE2, A549 and SUNE1, and the OKF6 normal nasopharynx model. Additionally, increased expression of the same markers is seen in the YCCEL1 EBV positive gastric carcinoma cell line.

4.2.2 The generation of rEBV infected epithelial cell lines

EBV infected epithelial cell lines were generated after co-cultivation with Akata BL cells induced into lytic cycle as described in the Materials and Methods (section 2. 3) (Stewart et al., 2004). The NPC-derived cell lines: CNE1, CNE2, HONE1 and SUNE1, along with the non-small cell lung cancer (NSCLC) cell line, A549, were chosen for study; EBV-infected AGS cells had been developed in a previous study (Stewart et al., 2004). Stably infected clones were selected and maintained as polyclonal populations under neomycin selection.

Once established, the pattern of EBV gene expression in stable rEBV infected cell lines was analysed by RT-PCR (Figure 4. 2). For comparison, the C666.1 cell line was included as a reference. The pattern of EBV gene expression in the C666.1 cell line was similar to that presented by Lo et al., 2006 (Lo et al., 2006) with detection of Qp-driven EBNA1, EBER1/2, BamH1A transcripts (1/2, 3/4) and BARF1, low level expression of LMP1 and BZFL1, and little if any LMP2A and LMP2B. An examination of the freshly derived rEBV-infected epithelial cell lines revealed patterns of EBV gene expression that were broadly similar to C666.1. All cell lines were found to express Qp-driven EBNA1 and the EBER1/2 RNAs; however, EBER2 expression in the AGS cell line was particularly low and may allude to the propensity for this cell line to lose EBV upon serial propagation (Feng et al., 2007). Low levels of LMP1 expression were observed in all cell lines, and variable levels of LMP2A, LMP2B, *BamH1*A–derived transcripts and BARF1 were observed in seven of the eight EBV positive cell lines; in AGS all four of these EBV latent genes were undetectable. With the exception of ADAH and HONE1, the majority of cell lines displayed low or

undetectable levels of the immediate early (IE) gene BZLF1. Little correlation was observed between expression of BZLF1 and the other IE gene BHRF1, where expression was restricted to CNE2, CNE1, ADAH and HONE1.

From the panel of EBV infected epithelial cell lines generated, two were chosen to further investigate the role of EBV infection on the induction of stem cell marker gene expression. RT-PCR analysis was performed to profile stable EBV infected A549 and CNE2 cell lines for EBV gene expression (Figure 4. 3). Both cell lines displayed an EBV gene expression profile that closely resembled that observed in the authentic EBV positive NPC cell line, C666.1. EBV gene expression profiling was repeated to include the EBV negative parental cell lines, an EBV transformed LCL, X50/7; the Akata BL cell line; and the EBV negative BL cell line, DG75 (Figure 4. 3). Again both rEBV-infected CNE2 and A549 cell lines displayed a viral gene expression profile that closely resembled the C666.1 and Akata BL cell lines, expressing EBNA1 from the Qp rather than the Cp/Wp promoters, high levels of EBER1/2 and BamH1A-derived transcripts (BARTs), and BARF1. Whilst very low levels of LMP1 were observed, rEBV-infected CNE2 cells were found to express levels of LMP2A mRNA that closely approximated those observed in X50/7. The lack of BZFL1 expression established that both rEBV infected cell lines had adopted a latency type II infection programme.

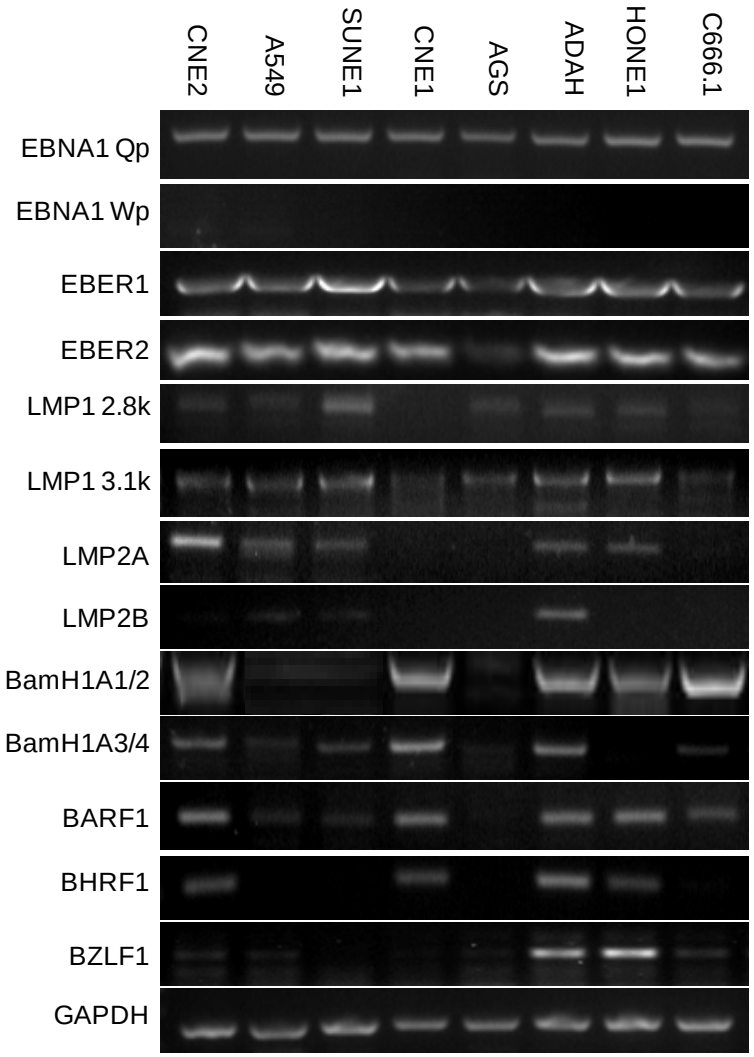


Figure 4. 2 RT-PCR expression profiling of NPC cell lines stable infected with rEBV

Multiple rEBV infected carcinoma cell lines were analysed for expression of latency II expression products; EBNA1 Qp, EBER1, EBER2, LMP1, LMP2A/2B, the BamH1 transcripts and BARF1, as well as immediate-early genes BHRF1 and BZLF1. Expression profiles were compared to the EBV positive C666.1 prototype cell line.

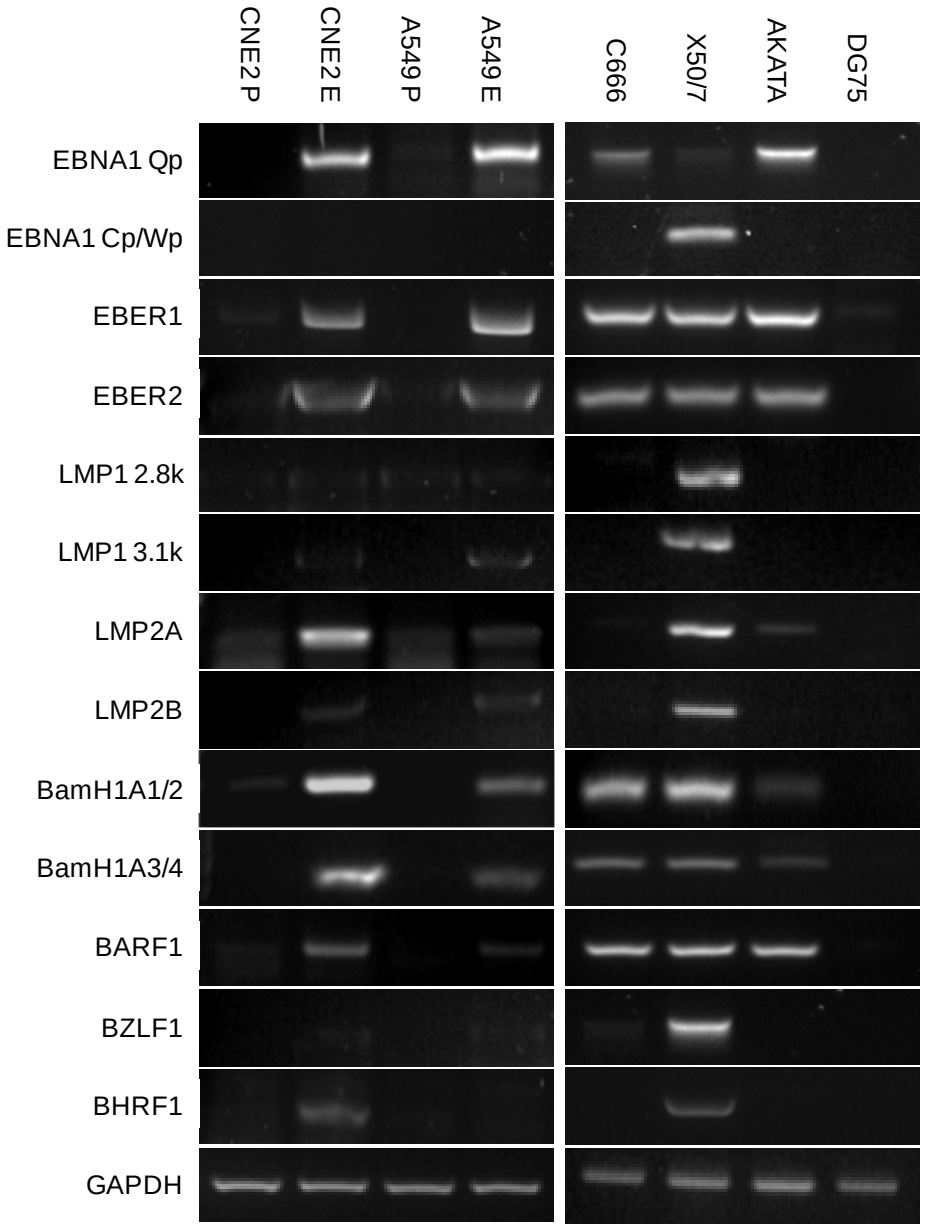


Figure 4. 3 Generation of EBV positive CNE2 and A549 cell lines
RT-PCR comparing the expression of EBV latent genes (EBNA1, EBERs, LMP1, LMP2A, LMP2B) and lytic genes (BZLF1 and BHRF1) demonstrates that the rEBV infected CNE2 and A549 cell lines display an expression pattern similar to that seen in the EBV positive C666.1 cell line. Additional comparison with EBV positive, X50-7, and EBV negative Burkitt's lymphoma cell line, DG75, and EBV positive Akata B-cell line.

Further interrogation into the expression of EBV latent genes in the A549, CNE2 and C666.1 cell lines was achieved by QPCR analysis (acknowledgements go to Lauren Lupino for the LCL cell line data). Expression of EBNA1, LMP1 and LMP2A was analysed using RNA from a BL cell line (Rael BL) as a positive control to which all scores where normalised. Figure 4. 4 shows the relative expression of these EBV latent genes. EBNA1 was found to be expressed in all EBV positive cell lines but was substantially less than that observed in the BL cell line. LMP1 was not detected by QPCR in any of the carcinoma cell lines. However LMP2A was found to be expressed at higher levels in the CNE2 and A549 cell lines than in the BL sample but could not be detected in the C666.1 cell line. Interestingly, A549 demonstrated a six fold increase in LMP2A expression compared to BL, and the relative level of EBNA1 in CNE2 was only half that found in the A549 cell line.

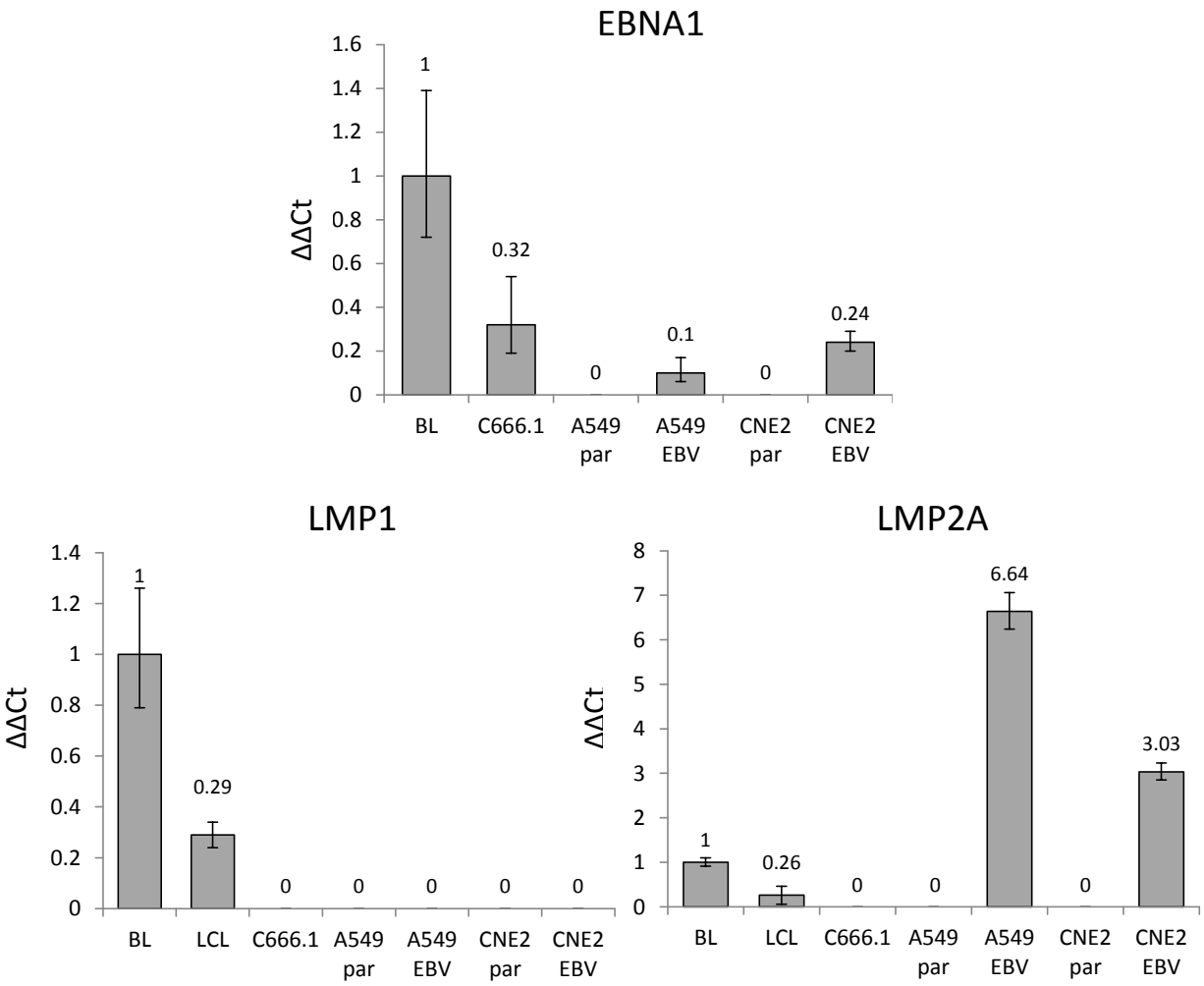


Figure 4. 4 QPCR analysis of the relative expression of EBNA1, LMP1 and LMP2A in C666.1 and EBV infected CNE2 and A549 cell lines
QPCR analysis of the relative expression of EBNA1, LMP1 and LMP2A in BL, C666.1 and the A549 and CNE2 parental and EBV infected cell lines, normalised to the BL positive control.

Immunofluorescence staining of rEBV infected CNE2 and A549 cells for expression of the EBV genome maintenance protein EBNA1 was performed using an EBNA1 specific polyclonal rabbit serum (R4), subsequently detected with Alexa-Fluor® 488-conjugated goat anti-rabbit immunoglobulin. Counterstaining with DAPI was performed to identify cell nuclei. Representative fluorescence microscopy images, Figure 4.4, confirmed the presence of EBV in all viable cells in both rEBV infected CNE2 and A549 cell lines and demonstrated variability in the staining such that around 10% of cells in each cell line demonstrated higher EBNA1 expression. Immunofluorescence staining was repeated through the course of the experiments presented in this thesis to check the EBV status of stably infected cell lines; no change was seen in expression throughout.

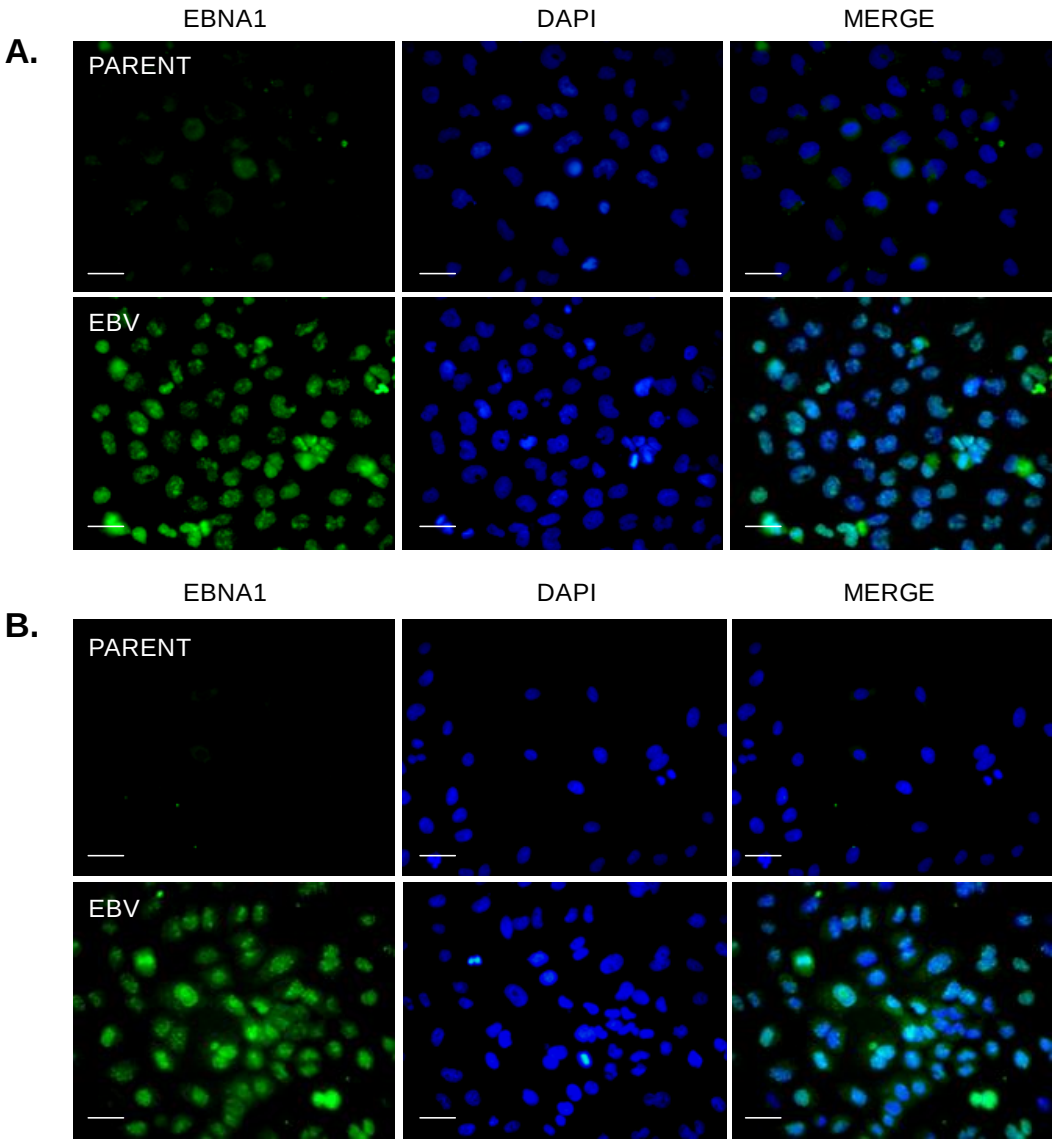


Figure 4. 5 Immunofluorescence staining demonstrating EBNA1 antibody expression
(A) CNE2 and (B) A549 parental and stable rEBV infected cell lines stained with EBNA1 antibody, R4 (green), demonstrates positivity in all 100% of cells of the rEBV infected cell lines. Positivity was not seen in the EBV negative parental counterparts.

4.2.3 Stable EBV infected CNE2 and A549 cells express higher levels of putative stem cell markers

While a number of individual EBV latent genes, notably LMP1 and LMP2A, have been shown to activate signalling pathways which can impact on stem cell marker gene expression (described in Chapter 1. 9), it is unclear whether EBV infection, with biologically relevant levels of latent gene expression, can do the same. This is particularly relevant given that most studies have been performed on cell lines engineered to overexpress the EBV latent gene of interest, with levels of expression that far exceed those found in authentic NPC specimens (Kong et al., 2010, Kondo et al., 2011). As such, they have not taken into account potential cooperative or modulatory mechanisms that occur when epithelial cells are infected with whole virus, where expression of these latent genes occurs at physiological levels.

Experimental infection of epithelial cells with rEBV in vitro has demonstrated an ability of the virus to alter epithelial cell behaviour. For example, EBV infection of the GC-derived cell line, AGS, resulted in anchorage-independent growth (Marquitz et al., 2012). In NPC-derived cell lines, EBV infection can induce cell growth through the induction of insulin-like growth factor 1 (Iwakiri et al., 2005), whilst EBV infection of the hTert-immortalised normal nasopharyngeal cell line, NP460, induced anchorage independent growth, and increased invasive properties and survival in growth factor and nutrient deficient medium (Tsang et al., 2010).

To explore the possibility that stable EBV infection is associated with the induction of stem cell/CSC marker genes, control uninfected and stable rEBV infected cell lines were analysed for the expression of a number of putative stem cell/CSC marker

genes, as it was hypothesised that the EBV-infected cells might show increased expression of stem cell/CSC markers. As a guide, expression of CD133, CD44v6, SOX2, BMI1 and others were chosen, as the expression of these had been found to be increased in the authentic EBV positive C666.1 and YCCEL1 cell lines.

RT-PCR analysis demonstrated that rEBV infected CNE2 and A549 cell lines both displayed increased expression of the putative CSC markers: CD133, CD44v6 and p75NTR, and the stem cell maintenance genes: SOX2, LRIG1, NANOG and BMI1 compared to their uninfected counterparts (Figure 4. 5A). Additionally, rEBV infected CNE2 cells also showed increased expression of EZH2 compared to their uninfected counterparts; however, this upregulation was not observed in rEBV infected A549 cell line. Expression of the genome maintenance protein EBNA1, confirmed that rEBV infected cell lines were latently infected with EBV.

Western blot analysis confirmed findings made from the RT-PCR analysis, supporting EBV driven stem cell marker gene expression, with significant increases of CD44, NESTIN, BMI1 and EZH2 in the EBV infected CNE2 and A549 cell lines. Both CD44 and NESTIN expression in the parental cell lines was very low prior to EBV infection, BMI1 and EZH2 showed increases expression above the basal level observed in parental cell lines (Figure 4. 5B).

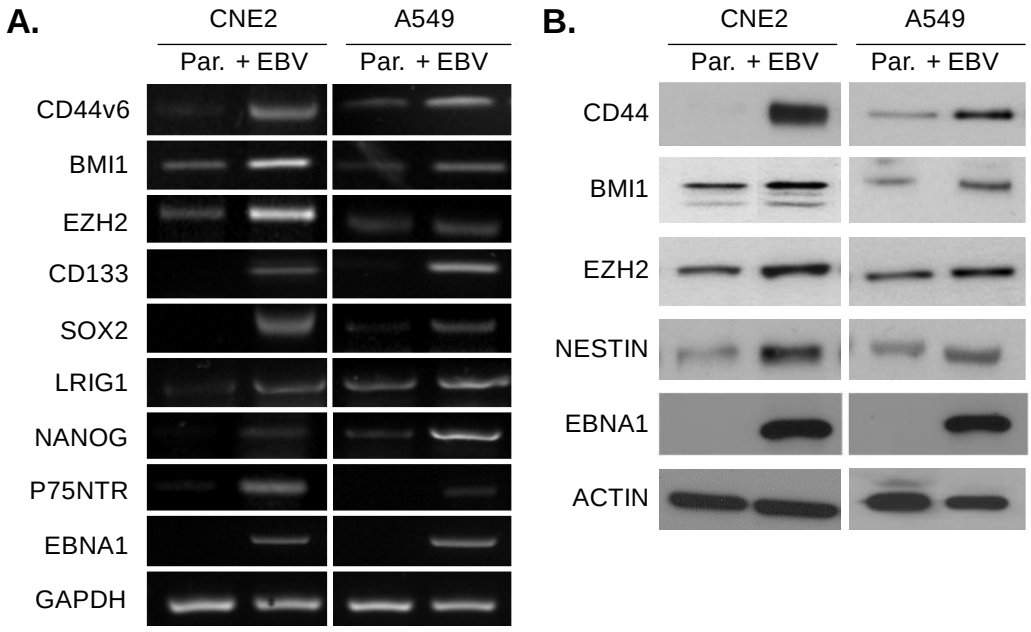


Figure 4. 6 EBV expression induces the expression of a number of putative stem cell markers in both CNE2 and A549 cell lines
(A) RT-PCR and (B) western analysis for putative stem cell markers in CNE2 and A549 cells and rEBV infected counterparts.

FACS analysis of the CNE2 (Figure 4. 7) and A549 (Figure. 4. 8) cell lines demonstrated increased expression of cell surface stem cell markers CD133 and CD44v6 on rEBV infected cell lines. During the course of these experiments the CD133 antibody (Cat. No. CS24B9) from Cell Signalling Technology, Danvers, USA, was discontinued and data for this antibody can only be presented on the CNE2 cell line.

Like the C666.1 cell line, CNE2 cells contained a subpopulation displaying high levels of CD133 expression, which was increased in response to EBV infection (FC=3.6, P=0.041, n=3). Similarly CD44v6 was also found to be increased in a subpopulation of cells (FC=2.2, P=0.021, n=3). However, the replacement CD133 MACS (293C3) antibody displayed a population shift demonstrating increased expression of this marker in the EBV positive CNE2 cells as an increase in cell surface marker rather than an increase in a sub-population of positive cells (FC=1.6, P=0.041, n=3). The increase in expression of CD133 MACS in the A549 EBV infected cell line was greater than that seen in the CNE2 cell line (FC=3.1, P=0.007, n=3). Basal CD44v6 expression in the parental A549 cell lines was greater than that of the CNE2 parental cell lines, and EBV infection of the A549 cells produced a fold change of 1.8 (P=0.015, n=3). An additional cell surface marker was used to replace CD133 (C24B9) to analyse marker expression in the A549 cell line; p75NTR was seen to increase 4.6 fold on EBV infection (P=0.048, n=3) and produced a separate population peak.

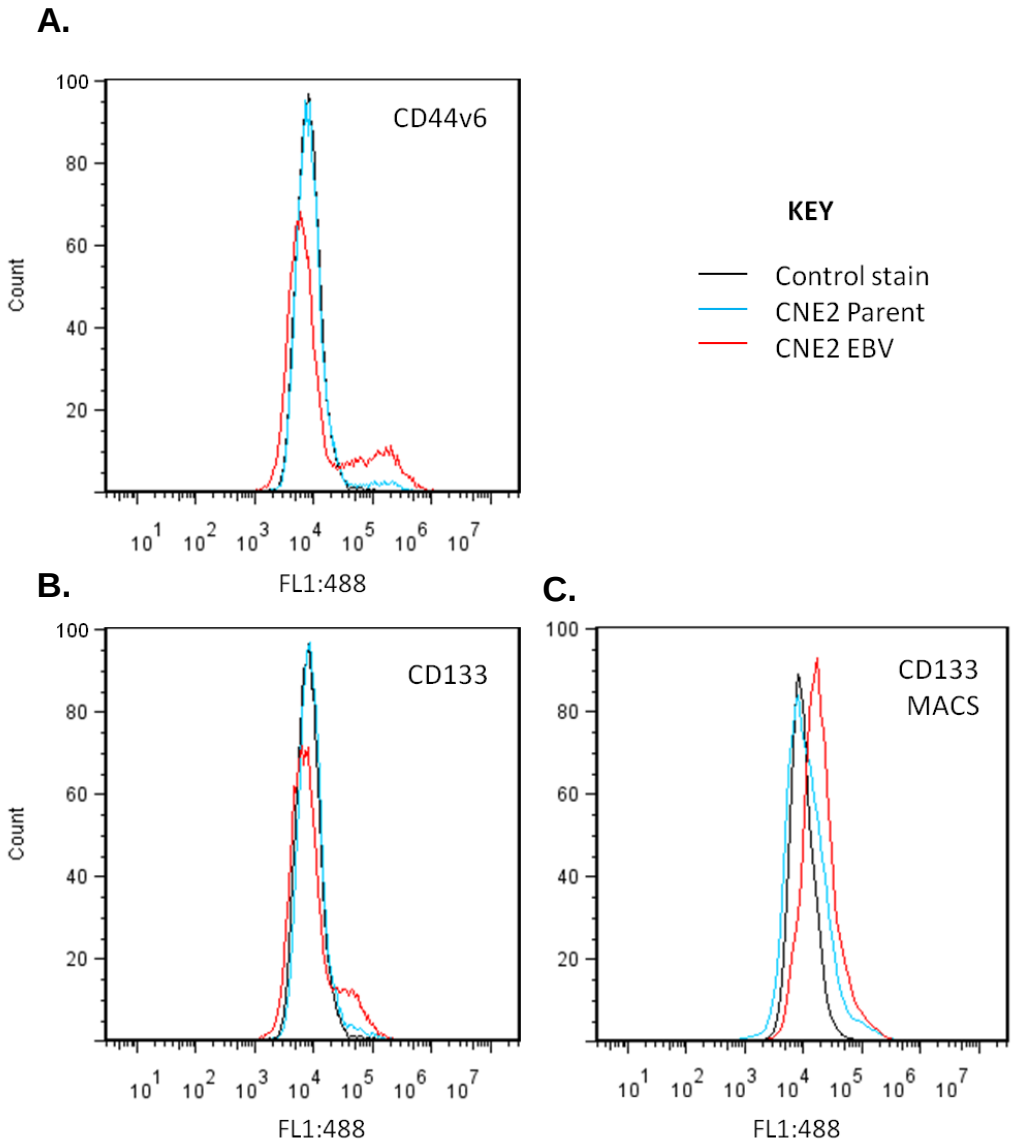


Figure 4. 7 FACS analysis for stem cell markers in CNE2 cells stably infected with EBV compared to their uninfected counterparts
(A) CNE2 EBV cell line (red) displays a 2.2 fold induction of cell surface CD44v6 (P=0.021, n=3), (B) 3.6 fold induction of cell surface CD133 (P=0.041, n=3), and (C) 1.6 fold induction of cell surface CD133 (MACS antibody) (P=0.041, n=3) compared to CNE2 parental cell line (blue).

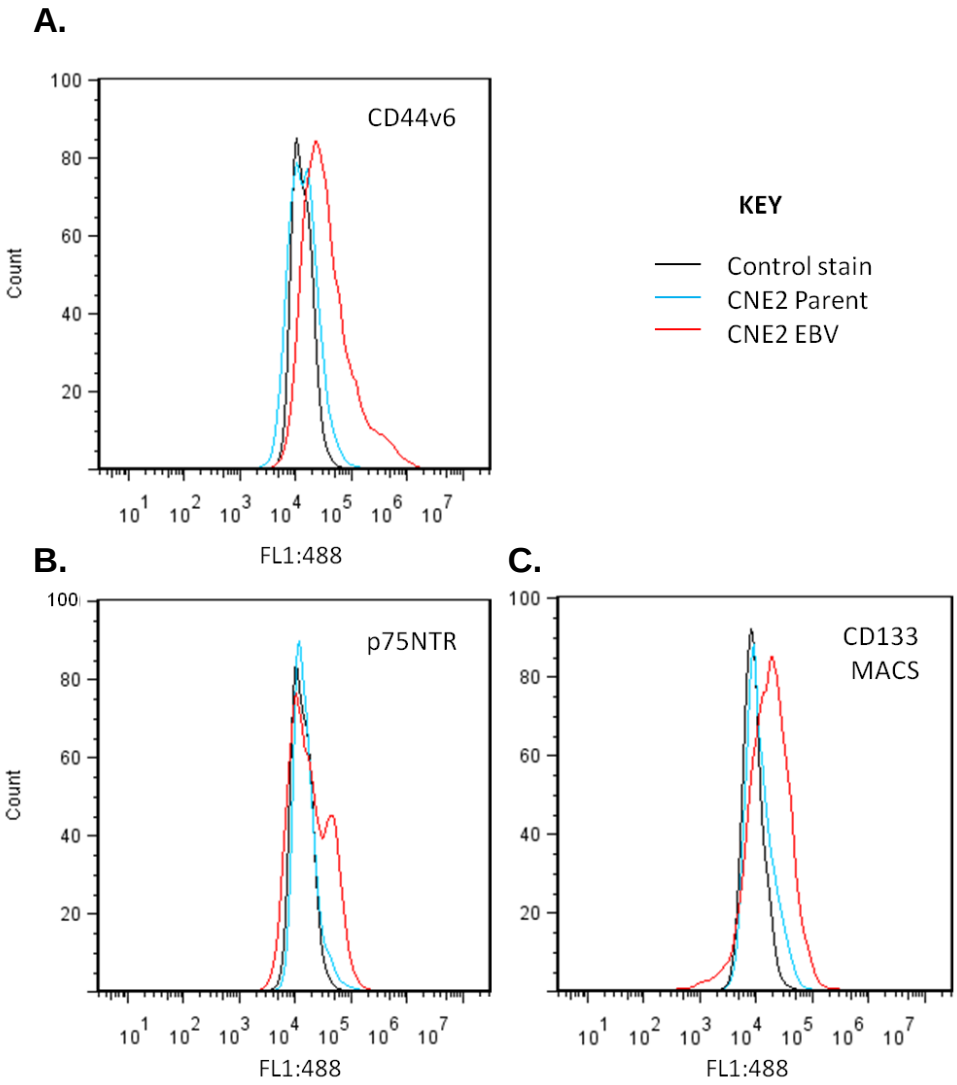


Figure 4. 8 FACS analysis from stem cell markers in A549 cell line stably infected with EBV compared to their uninfected counterparts
(A) A549 EBV cell line (red) displays a 1.8 fold induction of cell surface CD44v6 (P=0.015, n=3), (B) 4.6 fold induction of cell surface p75NTR (P=0.048, n=3), and (C) 3.1 fold induction of cell surface CD133 (MACS antibody) (P=0.007, n=3) compared to A549 parental cell line (blue).

The average positivity for the CD44v6 and CD133 MACS markers, and standard deviation over multiple experiments, are shown in Figure 4. 9. The basal percentage positivity for both antibodies was highest in the A549 cell line, at CD133macs; 58.5%, and CD44v6; 55.2%, and lowest in the OKF6 cell line CD133macs; 13.0%, CD44v6; 13.9%. All three cell lines demonstrated significant increases in percentage positivity, with greatest fold changes being recorded in the C666.1 cell line; CD133 MACS; FC=3.8 (P=0.042, n=3), CD44v6; FC=4.6 (P=0.039, n=3). These data demonstrate that EBV infection induces an increase in putative stem cell/CSC marker expression in accordance with the enhanced marker expression demonstrated in naturally EBV positive carcinoma cell lines.

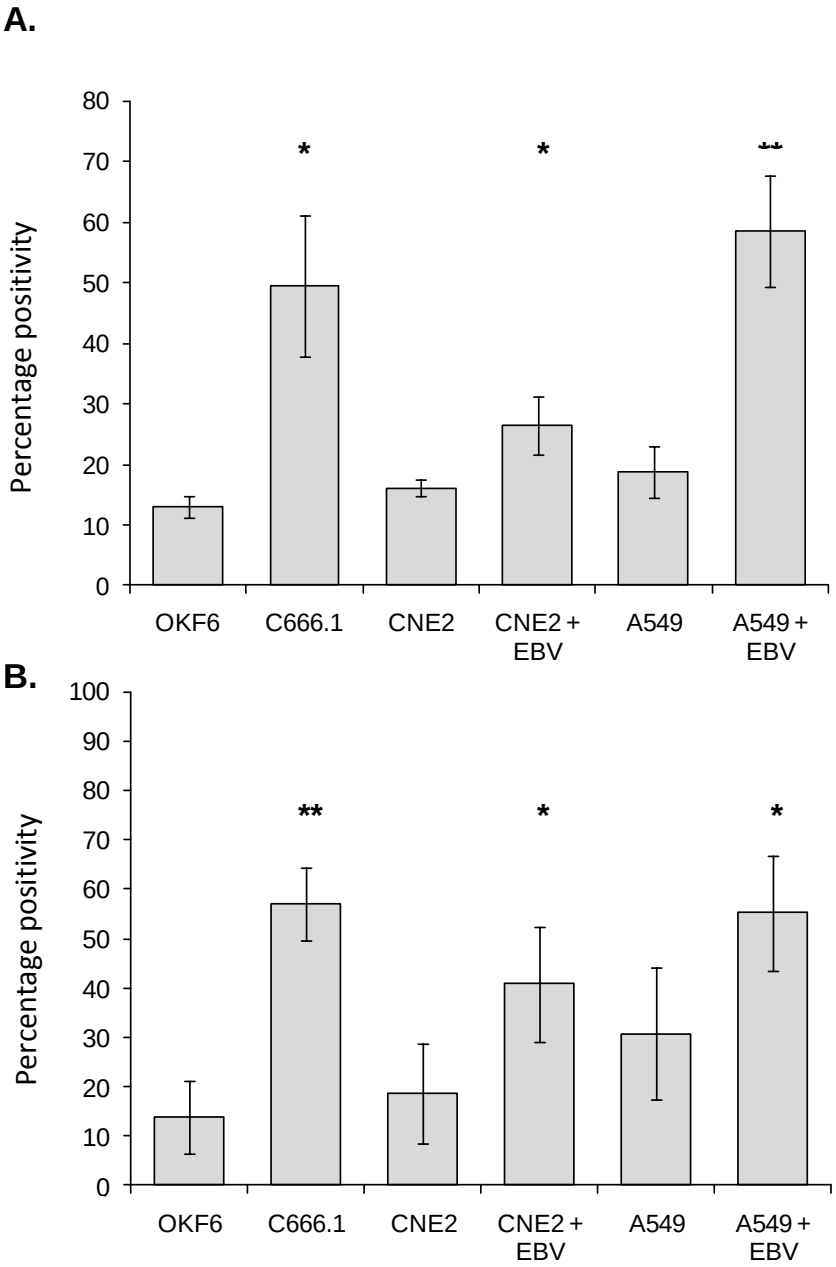


Figure 4. 9 Expression of (A) CD133 and (B) CD44v6 cell surface markers in OKF6, CD133, CNE2, CNE2 + EBV, A549 and A549 + EBV cell lines
Average expression and standard deviation of CD133 (MACS) and CD44v6 by FACS analysis. Fold changes for CD133 in the C666.1, CNE2 EBV and A549 EBV to respective control was 3.8 (0.042), 1.6 (P=0.041), 3.1 (P=0.007). Fold change for CD44v6 in the C666.1, CNE2 EBV and A549 EBV to respective control was 4.6 (P=0.039), 2.2 (P=0.021), 1.8 (P=0.015). All n=3, *, p<0.05; ** p<0.01; *** p<0.001.

4.3 EBV infection engages the HH pathway

EBV latency programs have been shown to influence a number of cell signalling pathways that could alter stem cell marker expression; however, whilst aberrant HH signalling has been demonstrated in NPC (Yue et al., 2012) the role of EBV in the dysregulation of this pathway is unknown. Following findings that HH signalling can directly influence the expression of stem cell markers in the C666.1 cell line, it was hypothesised that EBV infection may directly influence the status of HH signalling in NPC cell lines resulting in increased stem cell marker expression in EBV positive carcinomas.

4.3.1 rEBV infection induces SHH ligand expression in multiple carcinoma cell lines.

Induction of hedgehog ligand SHH due to mutation is rare, and is seen in less than 2% of cases of BCC (Daya-Grosjean and Couve-Privat, 2005, Couve-Privat et al., 2002). The vast majority of BCC cases are caused by mutations in the PTCH or SMO membrane proteins, these “common” basal cell carcinomas do not display increased SHH expression (Rubin and de Sauvage, 2006). Whilst most HH driven cancers appear to be regulated by mutations downstream of SHH ligand, SHH is found to be overexpressed in a subset of gliomas where its expression is associated with tumour grade (Becher et al., 2008); in breast cancer, SHH ligand expression is correlated with increased risk of metastasis, and SHH is upregulated in 70% of primary pancreatic adenocarcinomas (Thayer et al., 2003). Additionally, whilst direct gene mutation may not be responsible for its induction, SHH overexpression has a clear role in HH pathway dysregulation and tumour formation in BCC (Fan et al., 1997).

The expression of SHH ligand in multiple rEBV-infected NPC cell lines, the NSCLC-derived A549 cell line and the AGS gastric carcinoma cell line, was interrogated by RT-PCR (Figure 4. 10A) and western blot analysis (Figure 4. 10B). The highest basal level of SHH expression was observed in the HONE1 and CNE1 cell lines, and the lowest in the CNE2 and AGS cell lines. Increased expression of SHH ligand was demonstrated at both the RNA and protein level in all rEBV infected cell lines compared to their uninfected counterparts.

The expression of SHH in the rEBV infected cells was further interrogated in the CNE2 and A549 cell lines. Immunofluorescence staining was performed to examine the expression of SHH ligand at the single cell level, using an antiserum specific for SHH, subsequently detected with Alexa-Fluor® 488-conjugated goat anti-rabbit immunoglobulin, and the DAPI nuclear stain (Figure 4. 11A). Representative staining shows CNE2 parental cells demonstrate lower basal SHH expression than A549 cell lines, whilst EBV infected cell lines show similar levels of expression. Additionally, immunofluorescence staining highlighted variable SHH expression between different cells; overall more cells of the rEBV-infected cell lines showed positivity and, in those SHH positive cells, expression was greater. Supporting densitometry readings from RT-PCR data recorded a 14.8 fold relative increased in the rEBV-infected CNE2 cells and a 5.7 fold increase in the rEBV-infected A549 cells which, on average, demonstrated higher basal SHH expression (Figure 4. 11B).

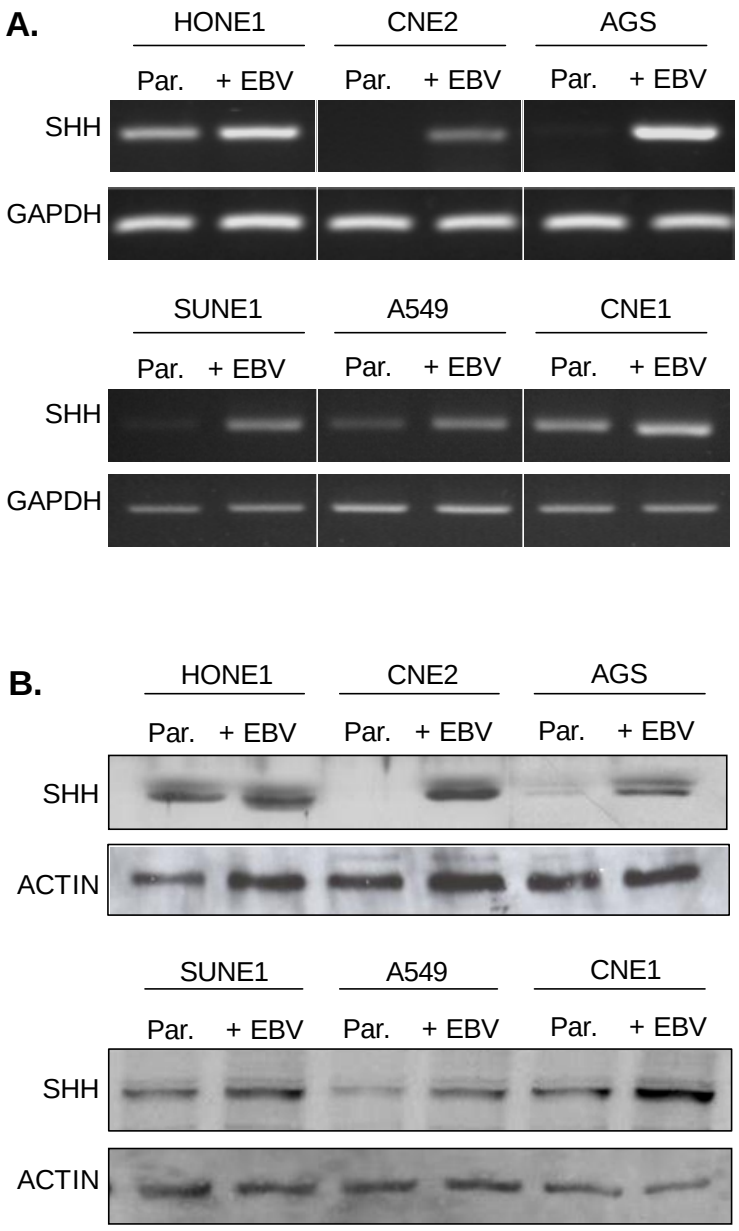


Figure 4. 10 Stable EBV infection induces the expression of HH ligand in multiple carcinoma cell lines
(A) RT-PCR and (B) western blot analysis demonstrates EBV infection in multiple carcinoma cell lines of NPC, EBV-aGC and NSCLC origin increases the expression of SHH ligand at both the RNA and protein level.

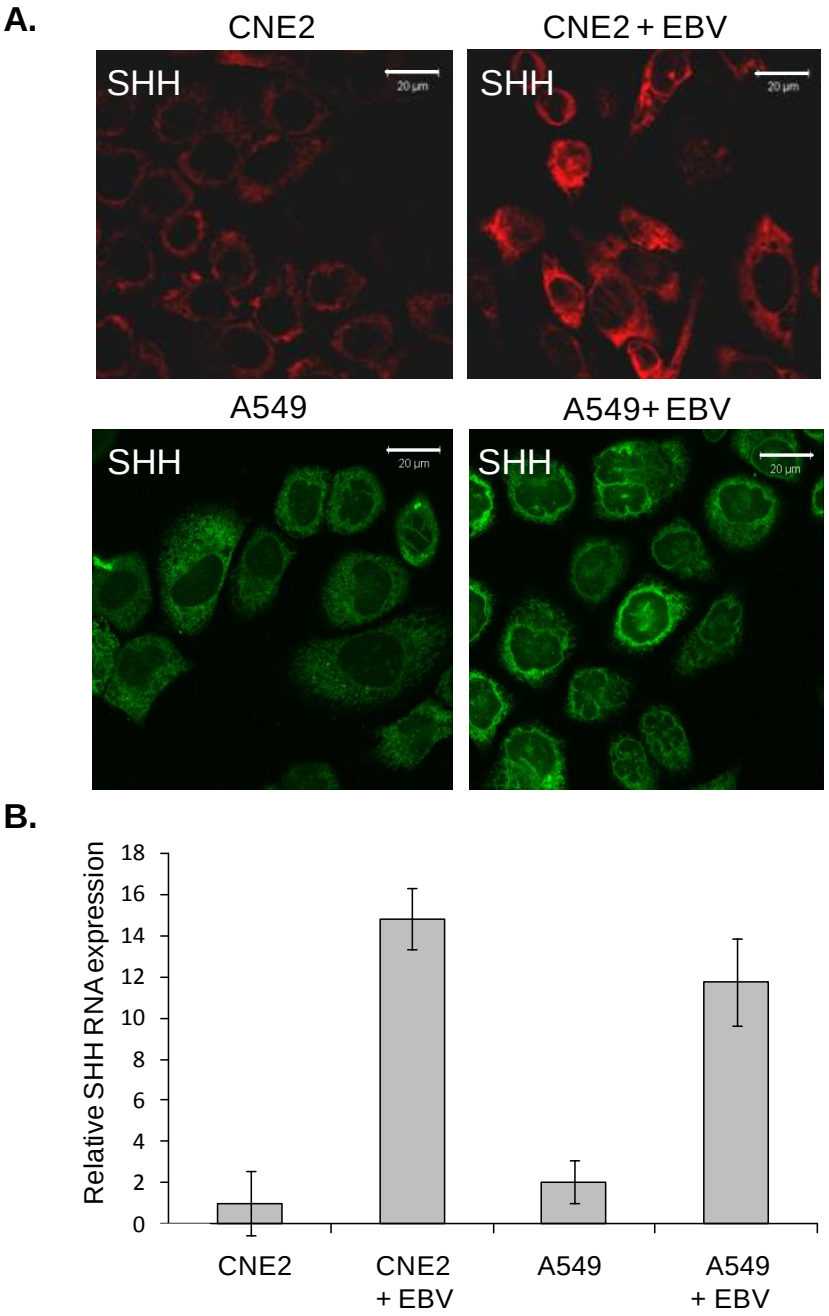


Figure 4. 11 Stable EBV infection induces SHH ligand expression in the CNE2 and A549 NPC cell lines

(A) Immunofluorescence and (B) RT PCR demonstrates EBV infection of CNE2 cells induces SHH expression. Densitometry of RT-PCR data shows EBV infection increases relative SHH gene expression 14.8 fold in CNE2 cells (P=0.0033) and 5.7 fold in A549 cells (P= 0.0040) (n=3). Bar =20 µm.

4.3.2 Activation of the HH pathway in rEBV-infected NPC cell lines is mediated through autocrine induction of SHH ligand

The induction of SHH ligand is sufficient to activate the HH pathway and to promote cell proliferation in NSCLC, pancreatic, prostate, colorectal and ovarian cancers, which require pathway activity for tumour growth (Kasper et al., 2006, Teglund and Toftgard, 2010). Constitutive activation of the HH pathway promotes tumourgenesis through the induction of a number of proteins that influence cellular phenotype (Kar et al., 2012) as outlined in Chapter 1.

To investigate the impact of EBV infection, and associated increase in SHH ligand expression on the activity of the HH signalling pathway, RT-PCR and western blot analysis were enlisted to interrogate the relative expression of HH pathway components and downstream targets in control uninfected and rEBV infected CNE2 and A549 cell lines (Figure 4. 12). Both cell lines showed increased expression of GLI1 and PTCH1 in response to stable rEBV infection, indicative of an activated HH signalling pathway and highlighting the stimulation of this stem cell maintenance pathway by EBV infection. Furthermore, stably infected CNE2 cells also demonstrated upregulation of GLI2 and downstream HH pathway targets FOXM1, WNT5A and BMP2; however, similar downstream target induction was not observed in the A549 cell line.

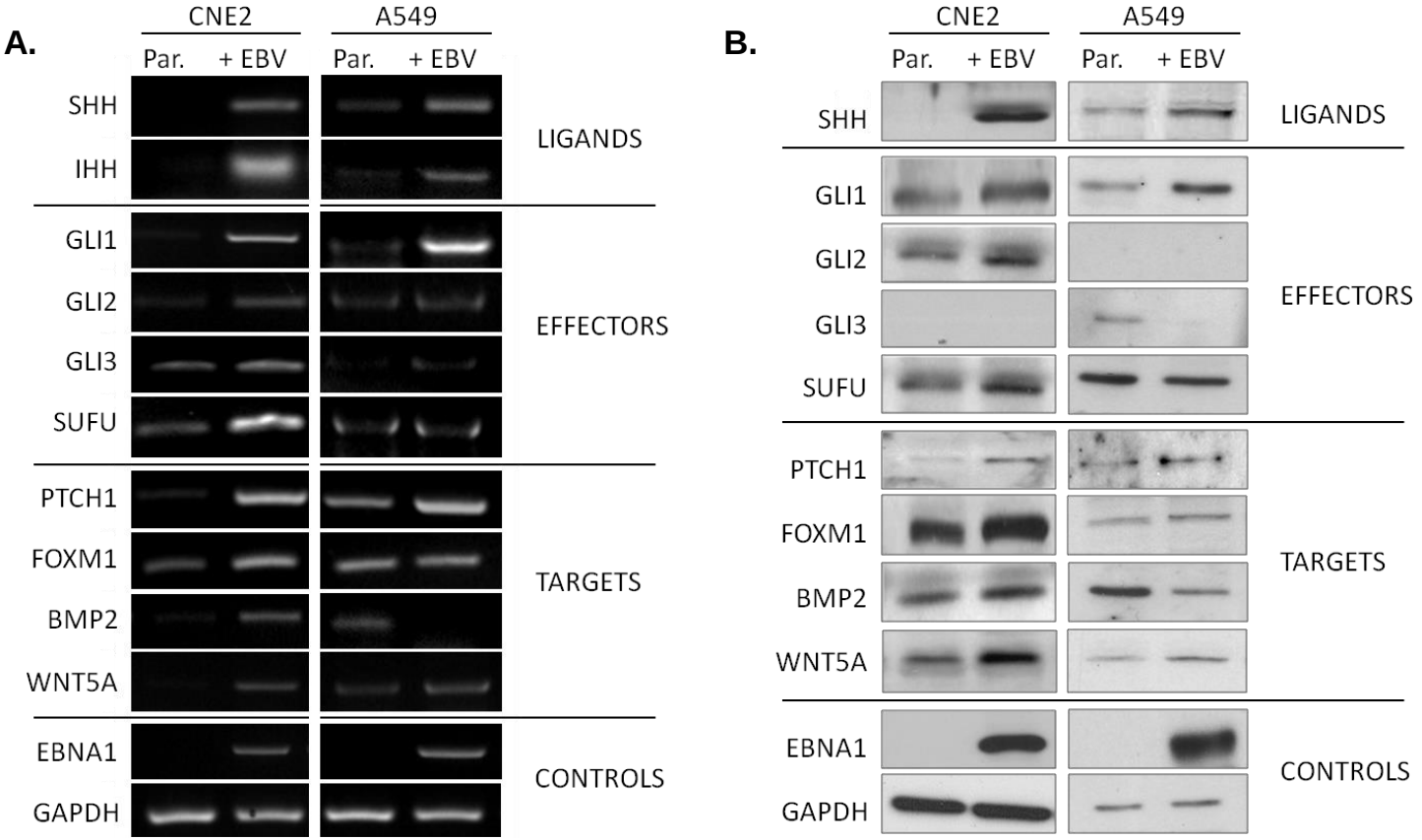


Figure 4. 12 Stable EBV infection activates the HH pathway in CNE2 and A549 cells
(A) RT-PCR and (B) western analysis demonstrates increased expression of HH ligands; SHH and IHH, effectors; PTCH1 and GLI1, and targets; FOXM1, BMP2, and WNT5A in CNE2 and A549 cells stable infected with rEBV.

Immunofluorescence staining further supported RT-PCR and western blot analysis, confirming activation of the HH signalling pathway in response to EBV infection. rEBV infected CNE2 and A549 cells showed increased expression of SHH ligand, pathway components, SMO and PTCH, and downstream targets: GLI1, and FOXM1 (Figure 4. 13). Additionally, the expression of GLI1 in the rEBV infected A549 cell line was found to be more nuclear, suggesting the presence of an active form of this transcription factor. Increased expression of GLI1 in the CNE2 cell line appeared to occur in a subset of cells that demonstrated increased nuclear and cytoplasmic staining. Increased nuclear expression of GLI2 was observed in the rEBV infected CNE2 cell line; however, GLI2 expression in the A549 cell line remained low following EBV infection. In the CNE2 cell line redistribution of the SMO and PTCH proteins towards the cell membrane occurred upon EBV infection, which, as highlighted in the discussion of Chapter 3, may impact on HH signalling. This redistribution in PTCH and SMO is less apparent in the A549 cell line, which showed significantly increased expression of these membrane proteins.

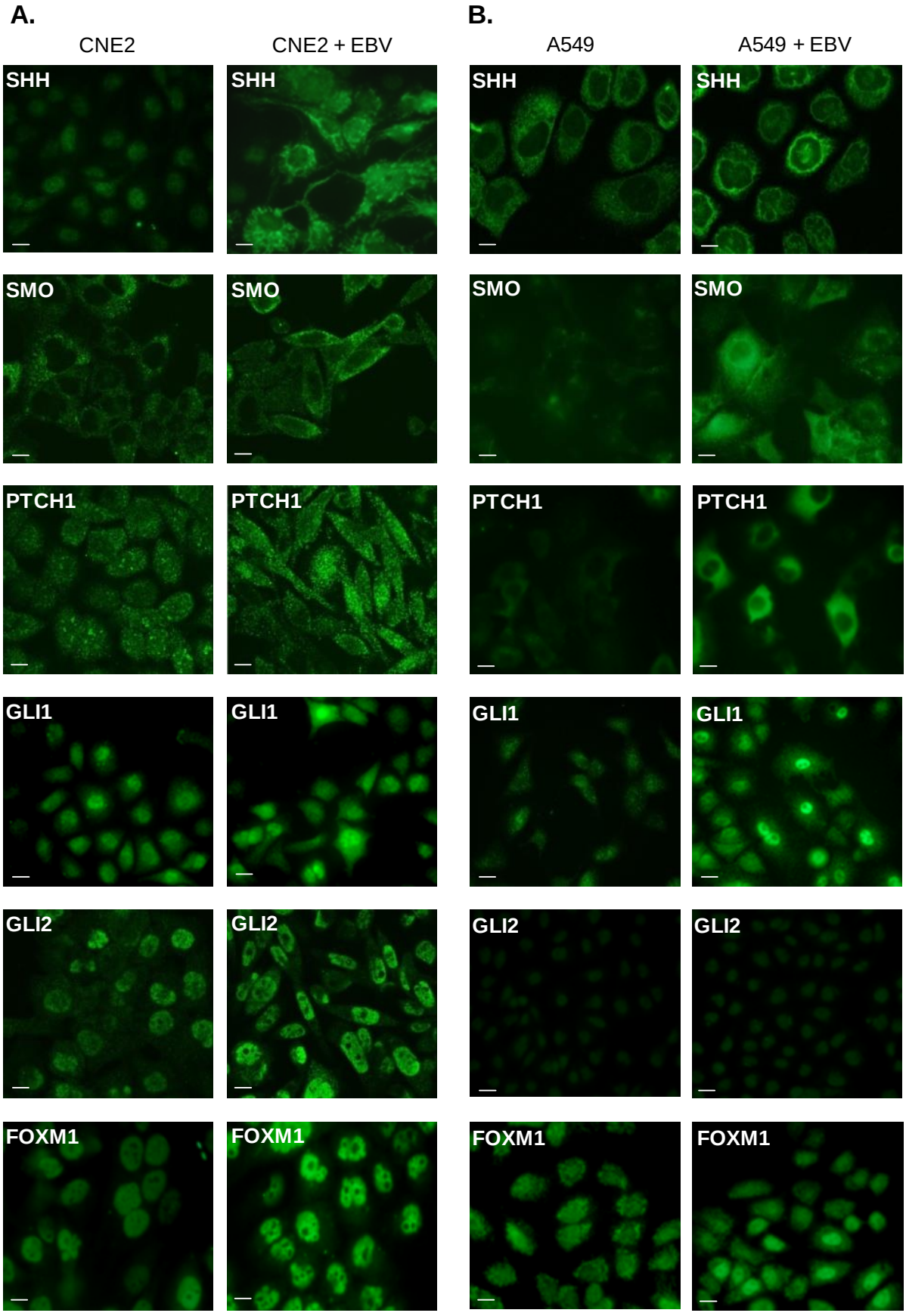

A.
CNE2
CNE2 + EBV
B.
A549
A549 + EBV
SHH
SMO
PTCH1
GLI1
GLI2
FOXM1

Figure 4. 13 Immunofluorescence staining demonstrating increased expression of a number of HH signalling pathway components and FOXM1, a downstream target, in CNE2 cells stably infected with wild type recombinant EBV
Representative immunofluorescence staining for SHH ligand, pathway components (PTCH1, GLI1, GLI2) and downstream target FOXM1 in uninfected and stable EBV-infected CNE2 (A) and A549 (B) cell lines. Bar =10 μm.

4.4 The integrity of the HH signalling pathway in CNE2 and A549 epithelial cell lines

It was important to ascertain the nature of HH pathway activation in response to rEBV infection. HH signalling can be activated in both a ligand dependent and independent manner. Ligand independent activation occurs upon chronic activation of the pathway through SMO and PTCH mutation as previously described as the main mutations driving basal cell carcinoma (Rubin and de Sauvage, 2006), and can also occur through non-canonical pathway activation and the loss of negative pathway regulators. For instance, loss of function of SUFU accounted for 5% of sporadic basal cell carcinomas in a study by Reifenberger et al., (2005), and has also been implicated in the development of meduloblastoma (Wildeman et al., 2012, Mei et al., 2007, Yue et al., 2012).

Ligand dependent activation can occur through both autocrine and paracrine mechanisms; the latter of which involves stromal cells which can either produce HH ligand or react to ligand produced by the tumour. Stromal cells that react to tumour produced HH ligand activate the tumour cell through VEGF, IGF and WNT signalling. The various mechanisms involved in HH pathway activation are reviewed by Scales and Sauvage (Scales and de Sauvage, 2009).

Whilst SHH expression is induced in EBV infected cells lines the activity of this ligand, and therefore its ability to stimulate the HH pathway, has yet to be verified. It was first established that the CNE2 and A549 cell lines possessed an intact HH pathway and are able to respond to ligand stimulation.

4.4.1 Transfection of CNE2 and A549 cell lines with SHH expression plasmid.

To examine the responsiveness of CNE2 and A549 cells to SHH ligand, and to establish the integrity of the HH pathway in these cell lines, transient luciferase reporter assays were performed using the 8xGLI-BS-Luc reporter construct. CNE2 and A549 cell were cotransfected with a SHH expression plasmid (see material and methods, section 2. 5) and a Renilla plasmid to control for transfection efficiency (Figure 4. 14). 8xGLI-BS-Luc activity, normalised to pGL2, was seen to increase in a dose dependant manner upon SHH plasmid transfection. Both cell lines responded to transfection with 0.5 µM SHH plasmid inducing a 2 fold increase in basal 8xGLI-BS-Luc activity in the A549 cell line, and 1.2 fold increase in the CNE2 cell line. As such, both cell lines appear to have an intact HH signalling pathway and are likely to respond to increased SHH ligand expression induced by EBV infection.

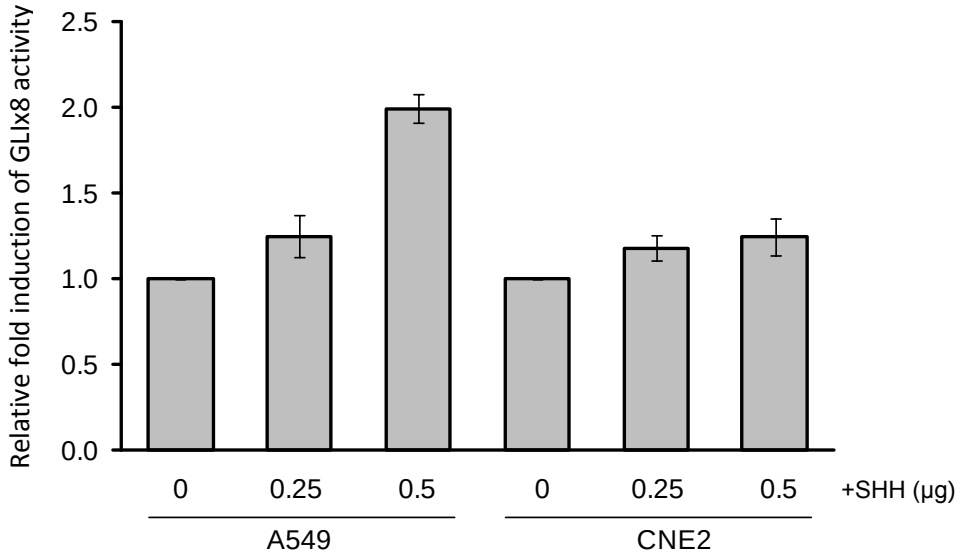


Figure 4. 14 Relative fold induction 8xGLI-BS-Luc activity in A549 and CNE2 cell lines

Induction of 8xGLI-BS-Luc activity, relative to pGL2, upon co-transfection with increasing concentrations (µg) of SHH plasmid in the A549 and CNE2 parental cell lines (n=3).

4.4.2 The effect of HH stimulation and HH pathway inhibition on the expression of HH pathway components and downstream targets.

RT-PCR and western blotting analysis showed increased expression of HH pathway components and targets in CNE2 cells in response to 48 hr treatment with SHH ligand, and a reduction in expression in response to cyclopamine treatment (Figure 4. 15). The expression of HH pathway components and targets could also be inhibited in response to 5E1 and GANT58 treatment in the rEBV infected CNE2 (Figure 4. 16) and A549 cell lines (Figure 4. 17). A 1:1 ratio of 5E1 containing supernatant-to-serum free medium was used for both the CNE2 and A549 cell lines, with conditioned media being used as a control. While a concentration of 50 µM GANT58 in conditioned media was tolerated by CNE2 cells, this concentration resulted in noticeable cell death in the A549 cell line after 48 hr. A concentration of 25 µM GANT58 was therefore chosen to treat the A549 cell line in this assay. The CNE2 cell line seemed more responsive to 5E1 inhibition than the A549 cell line, with more significant reduction in the expression of GLI1 and FOXM1, whilst treatment with GANT58 was roughly comparable.

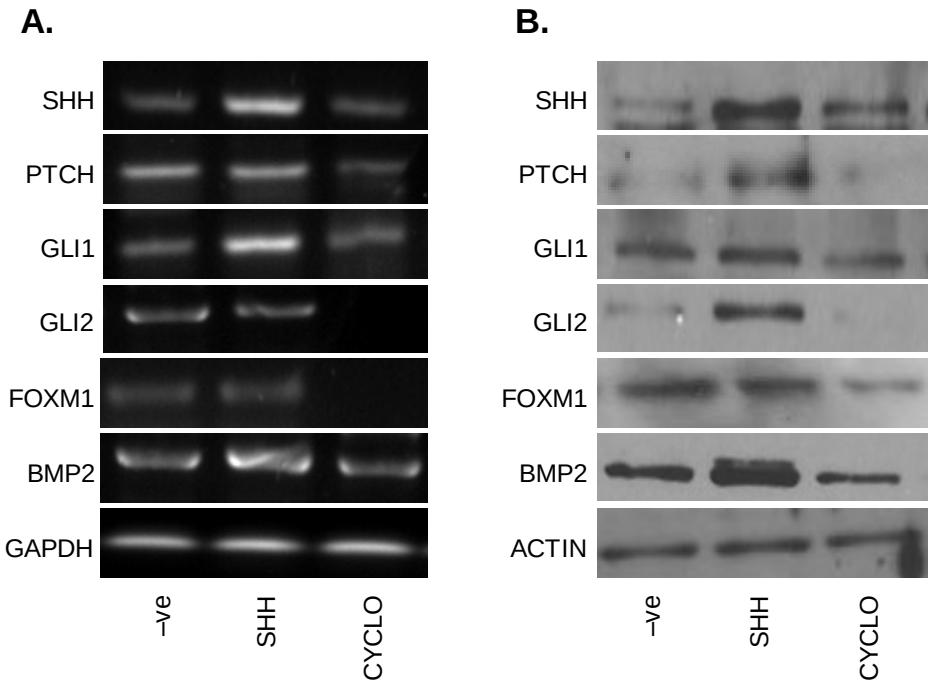


Figure 4. 15 The expression of HH pathway components and target genes in the CNE2 cell line is responsive to SHH ligand stimulation and cyclopamine inhibition

The CNE2 parental cell line was stimulated with 25 µM SHH ligand and inhibited with 25 µM cyclopamine (CYCLO) over 48 hr. (A) RT-PCR and (B) western analysis demonstrates the CNE2 cell line possess an intact, responsive HH signalling pathway.

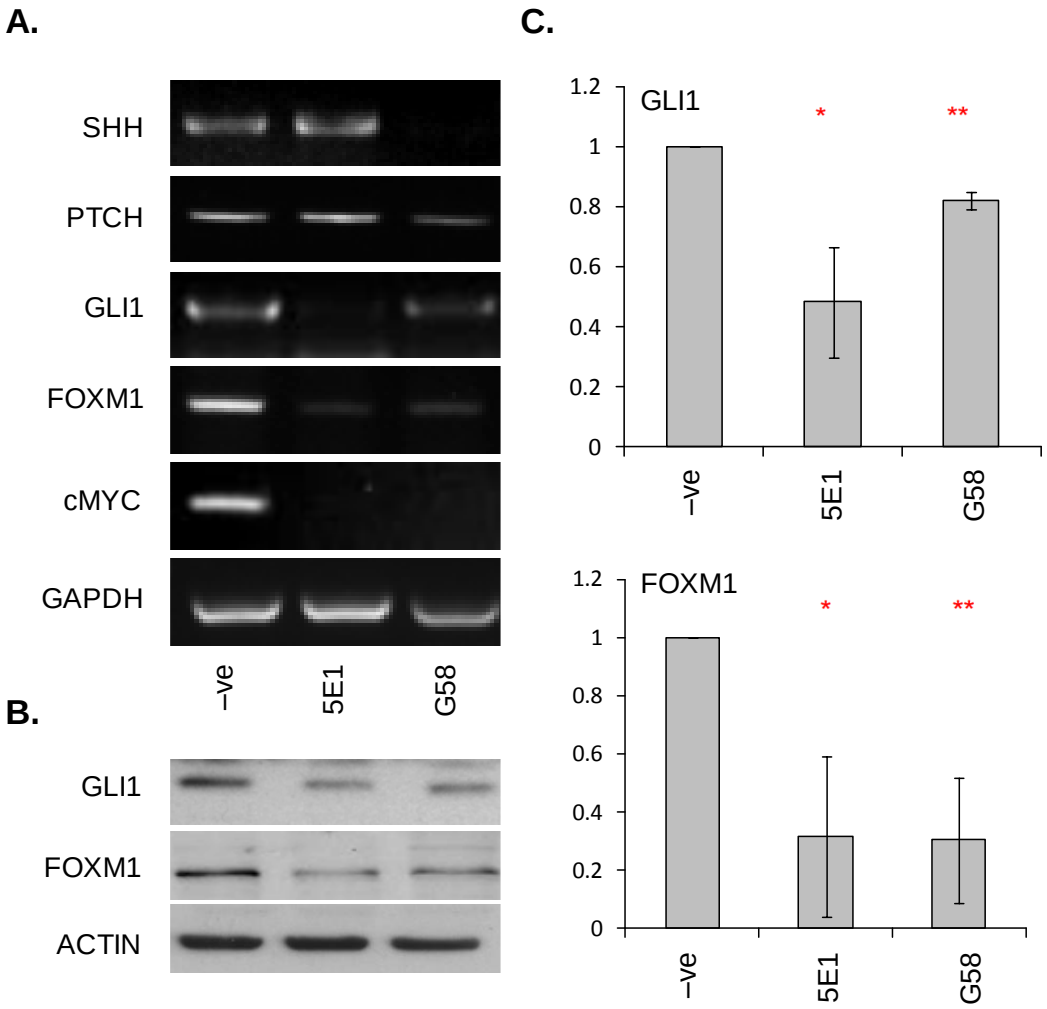


Figure 4. 16 HH pathway component and target expression in CNE2 rEBV infected cell line is responsive to HH signalling pathway inhibitors 5E1 and G58 (A) RT-PCR, (B) western analysis and (C) semi-quantitative RT-PCR (n=3) demonstrates inhibition of the HH pathway, with 10µl/ml 5E1 (Developmental Studies Hybridoma Bank, University of Iowa) and 50 µM GANT58 (G58) impacts on the expression of pathway components and downstream targets. *, $p<0.05$; ** $p<0.01$; *** $p<0.001$

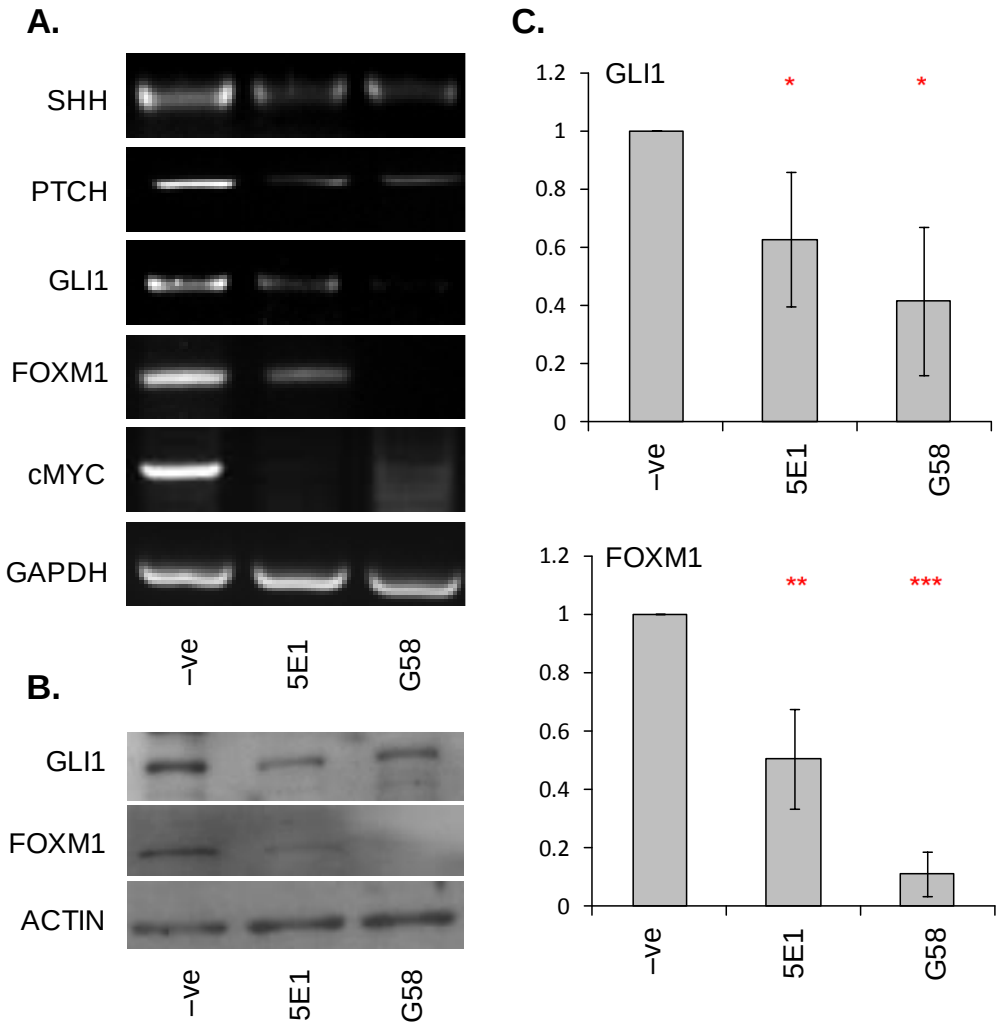


Figure 4. 17 HH pathway component and target expression in A549 rEBV infected cell line is responsive to HH signalling pathway inhibitors 5E1 and G58 (A) RT-PCR, (B) Western analysis and (C) semi-quantitative RT-PCR (n=3) demonstrates inhibition of the HH pathway, with 10µl/ml 5E1 (Developmental Studies Hybridoma Bank, University of Iowa) and 25 µM GANT58 (G58) impacts on the expression of pathway components and downstream targets. *, $p<0.05$; ** $p<0.01$; *** $p<0.001$

4.4.3 The expression of stem cell/CSC markers in rEBV infected cell lines is dependent on HH signalling

The established CSC markers: CD133, CD44v6, BMI1, and LRIG1 were found to be upregulated in response to stable EBV infection (Figure 4. 18A). The effect of HH pathway inhibition on the expression of these stem cell/CSC markers was subsequently investigated using the GLI1 inhibitor GANT58. Treatment of cells with GANT58 for 48 hr was found to inhibit the expression of these markers to varying extents in both the rEBV infected CNE2 and A549 cell lines.

RT-PCR analysis established that the levels of CD133, BMI1, NANOG and LRIG1 expression were significantly reduced upon treatment of rEBV infected CNE2 cells with 25 µM GANT58. Whilst expression of CD44v6 and CXCR4 were also reduced, the change was not as marked. A similar inhibition of these stem cell/CSC markers was observed in the A549 cell line, although the reduction in marker gene expression was not as substantial as that observed in the CNE2 cell line. Expression of LRIG1 in the A549 cell line was reduced only marginally by GANT58 treatment. Western blot analysis supported findings that GANT58 treatment is sufficient to inhibit the expression of stem cell markers (Figure 4. 18B). In both the A549 and CNE2 cell lines expression of CD44, BMI1 and NESTIN were reduced after 48 hr treatment with GANT58.

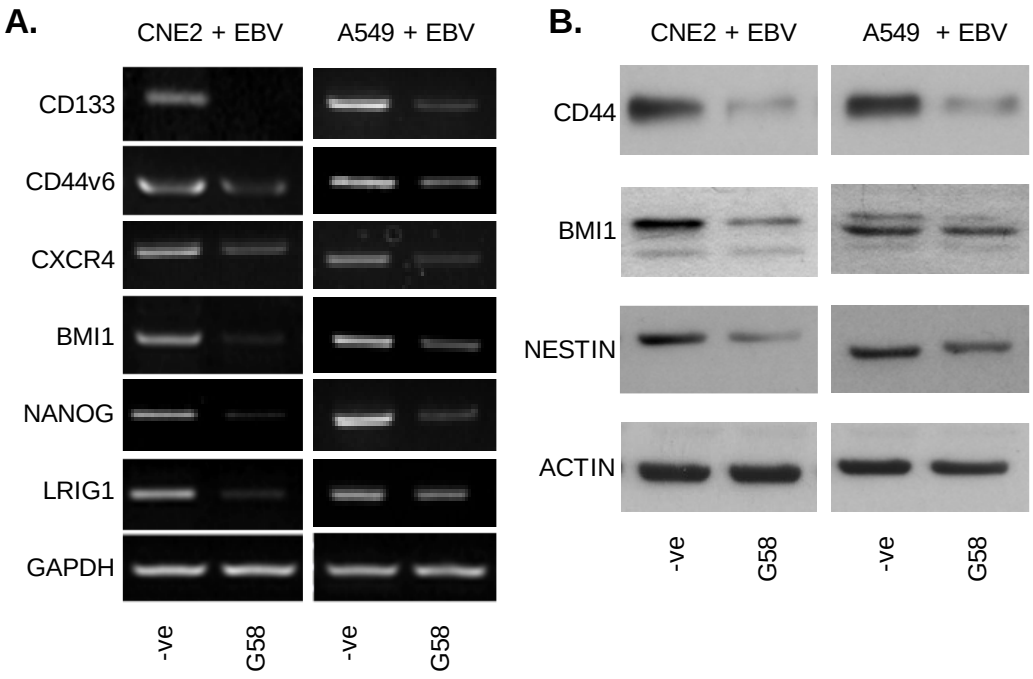


Figure 4. 18 Expression of stem cell markers in rEBV infected epithelial cells is dependent on HH signalling

CNE2 and A549 rEBV Cell lines were cultured in the presence or absence of GANT58 (G58; CNE5 50 µM, A549 25 µM, 48 hr). (A) RT-PCR and (B) western analysis demonstrates that inhibition of the HH signalling pathway decreases the expression of stem cell marker genes.

4.5 Identification of EBV genes required for SHH ligand induction, engagement of the Hedgehog pathway, and induction of a stem cell like phenotype.

Following the identification of dysregulated HH signalling in rEBV infected CNE2 and A549 cell lines, the HH pathway was interrogated further using the authentic EBV negative HONE1 cell line; HONE1 cells were originally infected with EBV but lost the virus after propagation in vitro (Yao et al., 1990). The availability of stable transfected cell lines expressing a number of individual EBV genes provided an opportunity to identify the EBV latent gene(s) responsible for HH pathway activation.

4.5.1 The generation of HONE1 cell lines expressing individual EBV latent genes

HONE1 cells engineered to express EBNA1, LMP1, LMP2A and a neomycin resistant gene, together with a stable rEBV infected cell line were made available for study. RT-PCR, western blotting and immunofluorescence staining confirmed expression of individual EBV genes in the respective cell lines (Figure 4. 19).

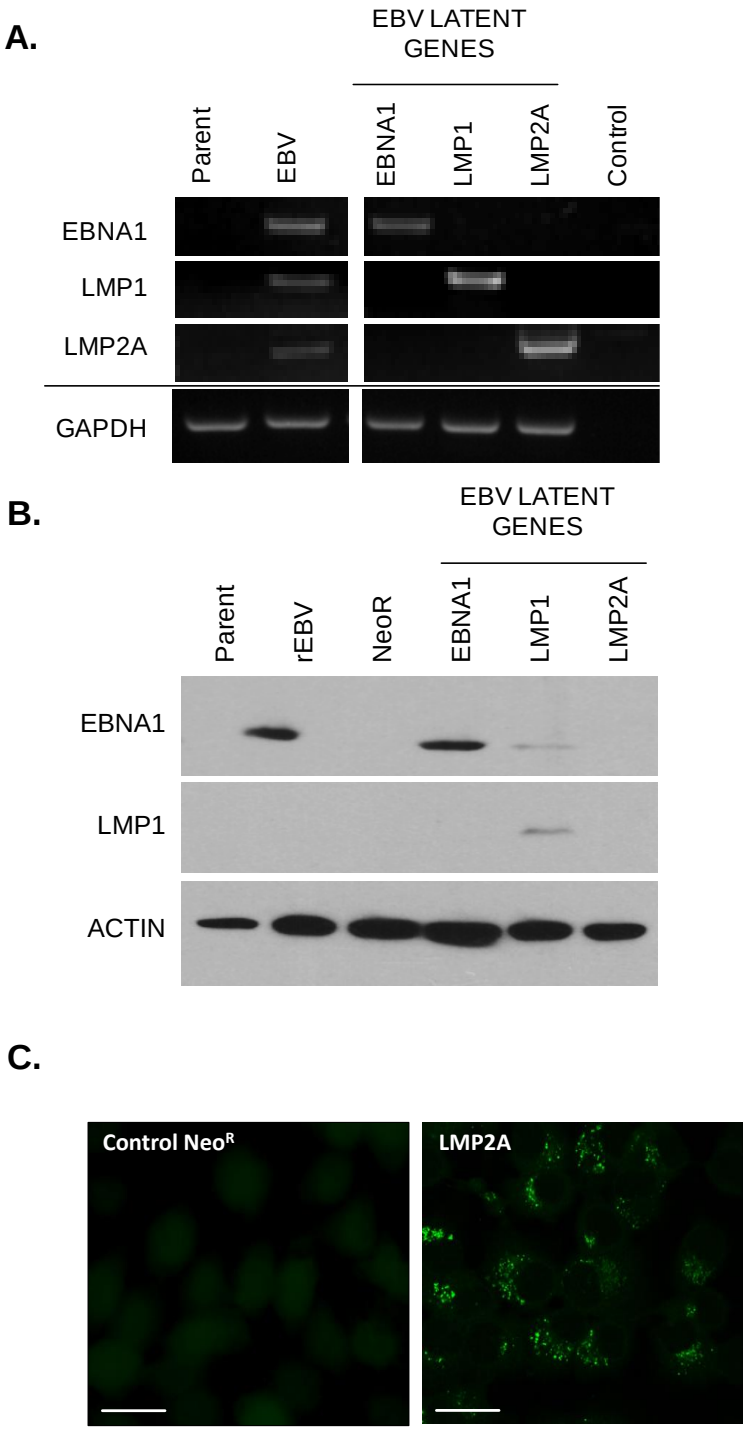


Figure 4. 19 Expression profiling of HONE1 single gene transfectant cell lines
The expression of EBV latent genes was interrogated in the single gene transfectant cell lines by (A) RT-PCR analysis, (B) western analysis and (C) immunofluorescence. Bar = 20 µm.

4.5.2 The role of EBNA1, LMP1 and LMP2 in the expression of HH pathway components.

To examine the contribution of individual EBV latent genes to the expression and activity of the HH pathway in epithelial cells, RT-PCR analysis was performed to examine the expression of key HH pathway components, effectors and validated target genes. As shown in Figure 4. 19, all three viral genes (EBNA1, LMP1 and LMP2A) induced expression of SHH and IHH ligand to levels that were comparable to rEBV infected cells. Expression of the HH receptor and putative HH target gene PTCH1 was also increased in all three cell lines, as was expression of the key HH effector proteins GLI1 and GLI2. However, whilst GLI1 and GLI2 were increased to variable levels in all three cell lines, they were more strongly induced in LMP1 expressing cells. The expression of SMO appeared unaltered in all cell lines. Expression of the HH effector protein GLI1 was also induced in all three cell lines, but more strongly so in the LMP1 expressing cell line. Similar results were found for GLI2, although expression was increased in EBNA1 and LMP1 expressing cell lines but not in LMP2A expressing cells. Expression of the HH target genes BCL2 and FOXM1 were also increased to varying extents in all three cell lines.

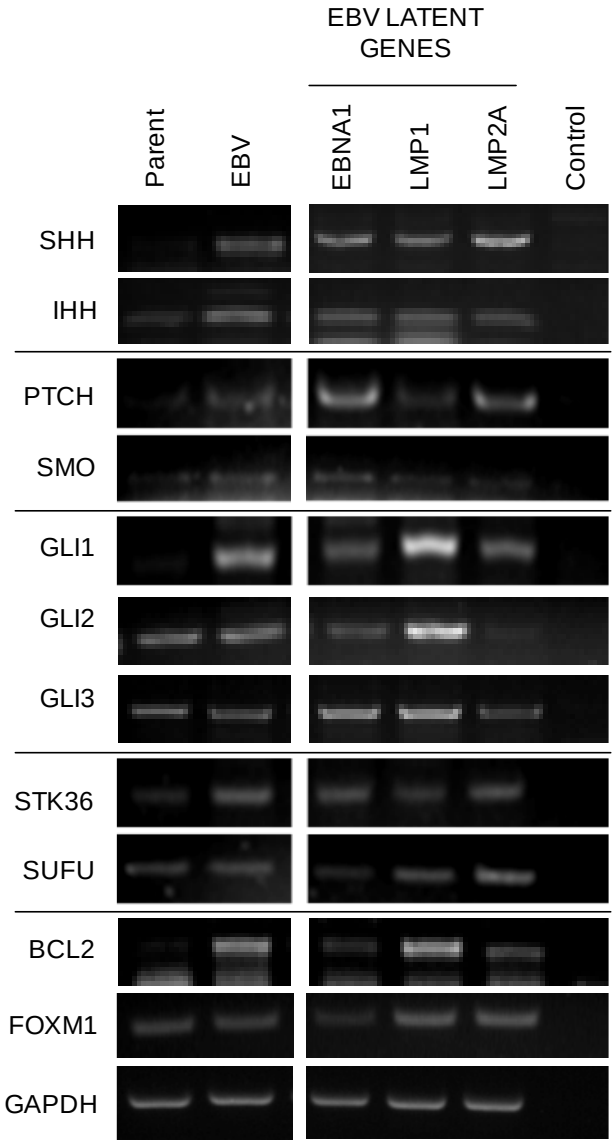


Figure 4. 20 Expression of HH pathway components in the single latent gene HONE1 cell line transfectants.
The state of the HH signalling pathway was investigated by RT-PCR in the HONE1 EBNA1, LMP1, and LMP2 cell lines compared with HONE1 parental and rEBV counterparts.

4.5.3 The role of EBNA1, LMP1 and LMP2 in the expression of stem cell markers

The EBV-encoded latent proteins LMP1 and LMP2A have previously been found to induce the expression of stem cell markers and to impose stem-like characteristics on NPC derived cell lines *in vitro* (Kong et al., 2010, Kondo et al., 2011). LMP2A has been shown to increase the expression of stem cell markers (ABCG2, BMI1, NANOG and SOX2) in the CNE2 and SUNE1 cell lines, an effect that was linked to the induction of an EMT, and an increase in the number of cells displaying a side population (SP)-like phenotype, a state associated with increased proliferative and tumour initiating potential. In addition, LMP2A infected cells demonstrated increased activity of the Akt pathway, which when inhibited, significantly decreased the size of cell populations with SP-like features (Kong et al., 2010). However, this work failed to establish levels of LMP2A expression that were representative to that observed in NPC, relying on the over expression of LMP2A in cell lines to achieve cellular consequences. As such, it does not extend to establish the effect of whole viral infection on stem cell marker expression or the establishment of a side population of cells. In addition, LMP1 has been shown to induce and EMT and to induce a stem-like phenotype in nasopharyngeal epithelial cells (Horikawa et al., 2011). Individual EBV genes were seen to induce the expression of stem cell markers in the HONE1 cell line (Figure 4. 20). A number of markers were seen to be responsive to a single EBV latent gene; CD44v6 and P75NTR were upregulated in response to LMP1 expression, whereas CXCR4 and weak induction of CD133 was seen in the LMP2A expressing HONE1 cell line.

Little change was observed in BMI1 and OCT4 expression upon either rEBV infection or expression of individual EBV latent genes, whereas SOX2 was strongly induced by rEBV and found to be upregulated in the EBNA1, LMP1 and LMP2A expressing HONE1 cell lines compared to the parental control. LRIG1 appeared to be responsive to both LMP1 and LMP2A expression, and EZH2 was down regulated in the rEBV infected cell line and most strongly upregulated in the LMP2A expressing cell line. These finding demonstrate the different ability for these viral proteins to engage stem cell marker expression. While LMP1 appears to most strongly engage the HH signalling pathway, both LMP1 and LMP2A can induce stem cell marker expression.

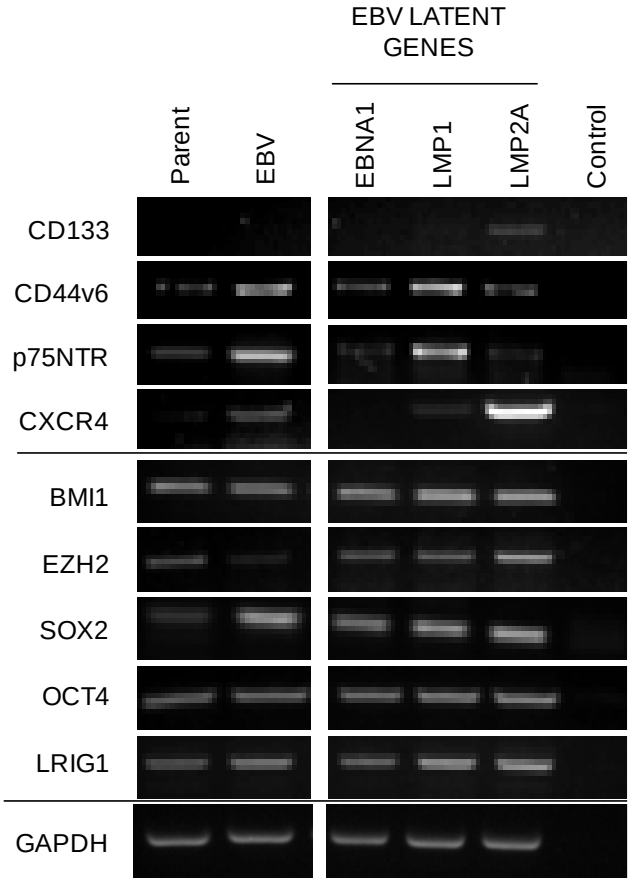


Figure 4. 21 Expression of putative stem cell markers in HONE1 cells expressing individual EBV latent genes
The state of the HH signalling pathway was investigated by RT-PCR in the HONE1 EBNA1, LMP1, and LMP2 cell lines compared with HONE1 parental rEBV counterparts.

4.6 DISCUSSION

The rationale for investigating the effect of EBV infection on the induction of stem cell/CSC marker genes and activation of stem cell maintenance pathways in epithelial cells stemmed from the observation that the authentic EBV positive cell lines, C666.1 and YCCEL1, were found to express higher levels of stem cell/CSC markers compared to their EBV negative counterparts. Using two EBV infected epithelial cell lines, which displayed an EBV gene expression profile closely resembling that of the authentic EBV positive C666.1 cell line: CNE2-EBV and A549-EBV, the role of rEBV infection on the induction of stem cell/CSC markers and stem cell maintenance pathways was investigated.

Stable EBV infection was associated with the induction of a number of established stem cell/CSC markers (CD44v6, BMI1 and EZH2) that were also found to be overexpressed in the authentic EBV-positive NPC cell line C666.1 and a high proportion of primary NPC tumour biopsies. Whilst the induction of these genes may have widespread effects on epithelial cell biology, their involvement in promoting a “stem-cell-like” phenotype was of particular interest. Previous studies investigating the role of CD44 in CSC formation and maintenance have identified a role for this protein in mediating intercellular reactive oxygen species that promote the aggressive phenotype observed in NPC tumours (Lin et al., 2013). Furthermore, in a study looking at polymorphisms in this gene a CD44 rs13347C>T polymorphism was found to affect NPC development by increasing CD44 expression (Xiao et al., 2013), while CD44 shRNA gene therapy has been demonstrated to suppress breast cancer tumours in NOD/SCID mice (Van Pham et al., 2012).

While both epithelial cell lines demonstrated a clear increase in expression of a number of stem cell/CSC markers in response to EBV infection, differential upregulation of NANOG, p75NTR and EZH2 was observed between the CNE2 and A549 cell lines. Additionally, the induction of stem cell/CSC marker gene expression was more substantial in the CNE2 cell line. Although the reasons for this are presently unclear, it may reflect inherent differences in the plasticity of this cell line to respond to signalling pathway modulation, and/or the ability of EBV to modulate cell signalling on the background of pre-existing signalling pathway dysregulation. EBV may have a role in reprogramming the cell in which it resided such that it becomes more compatible with continued latent infection, which does not normally occur in normal non-transformed epithelial cells. Expression of these proteins, that either maintain an undifferentiated cell or reprogram a more committed cell to dedifferentiate, may have a role in the establishment of stable latent infection and, therefore, initiation of NPC.

Additional investigations into EBV induced expression of stem cell markers CD133 and CD44v6 were performed by FACS analysis. It was observed that, in some instances, increased stem cell/CSC marker gene expression occurred as a population shift demonstrating an increase in marker expression in all cells causing a rightward shift of the FACS curve, whilst others demonstrated an increase in the positive population, without a shift in the remaining negative population. Perhaps more surprising is that detecting the same protein with different antibodies might produce two very different results as was the case with CD133. EBV infection of epithelial cell lines was found to increase the expression of the CD133 MACS antibody (C293C3) whereas the cell signalling CD133 antibody (CS24B9) displayed

an increase in the positive population, in the CNE2 cell line. Similarly, the same antibody, CD44v6, in different cell lines produced more of a population shift demonstrating an increased expression of this marker in the A549 cell line that was not observed in the CNE2 cell line.

Further refinement of the molecular marker expression of CSC populations in NPC may isolate subpopulations of cells demonstrating marker positivity, when using antibodies with subpopulation peaks, or the gating of cells demonstrating the highest levels of stem cell/CSC marker expression. For instance, the CD44 positive fraction of cells isolated from NPC cell lines has been reported to contain the CSC population (Su et al., 2011), whereas integrin β1 positive human epidermal stem cells are contained in the highest integrin expressing sub-population (Watt, 2002). Many different markers have been utilised to isolate sub-populations of NPC cells which display “stem cell-like” properties and increased tumourigenic potential, it is likely that a combination of markers is required to isolate a true CSC population.

In addition to inducing stem cell marker expression, findings presented in this study show that stable latent infection of epithelial cells with EBV is associated with the induction of SHH ligand. EBV induced SHH ligand expression was found in six cell lines of NPC, GC and NSCLC origin, demonstrating that this ability is not cell type specific. Furthermore, *in vitro* studies utilising the CNE2 and A549 cell lines demonstrated that rEBV induces SHH ligand, and activates the HH pathway through autocrine signalling as demonstrated by increased expression of HH pathway components and downstream targets by RT-PCR, western blot analysis and immunofluorescence staining. While these observations are not supported by data

obtained from the microarray analysis of NPC tumours described in Chapter 3, the IHC based study did confirm overexpression of SHH ligand in primary NPC tumours. These data highlight technical difficulties associated with RNA amplification or the probes used to detect SHH ligand, which may be inadequate for the detection of this gene. Taken together, results presented here demonstrate the aberrant HH signature displayed in NPC tumours is likely due to an autocrine mechanism for EBV engagement of the HH signalling pathway but does not rule out additional paracrine mechanisms to this aberrant HH activity.

Previous studies have shown that both the CNE2 and the A549 cell lines display elevated HH signalling activity and are dependent on HH signalling for survival and proliferation (Yue et al., 2012, Bermudez et al., 2013). Furthermore a study by Yuan et al., (2006) demonstrates the A549 cell line harbours a constitutively active HH signalling pathway due to the overexpression of GLI1, and reports that this cell line is refractory to cyclopamine-mediated SMO inhibition. This highlights possible intrinsic differences in the HH signalling pathway between these two cell lines which could explain the differences in the level of pathway activation in the CNE2 and A549 cell line upon EBV induced SHH induction and supports findings demonstrating that the CNE2 cell line is more responsive to HH activation upon induction with SHH ligand. Results presented here demonstrate that HH signalling in the A549 cell line can be inhibited by the 5E1 anti-SHH ligand antibody and can respond to SHH plasmid induction of the 8xGLI-BS-Luc reporter demonstrating that the A549 cell line, whilst displaying aberrant HH signalling, is still responsive to SHH ligand stimulation. A more recent study by Ali et al., (2011) demonstrates that TGFβ can induce the expression of SHH and induction of the HH signalling pathway in this cell line. This

activation was responsible for the induction of an EMT and an aggressive CSC phenotype demonstrating increased growth, motility and invasion. Similarly Bermudez et al., (2013) demonstrated that A549 cells do respond to cyclopamine but that exogenous SHH only induced modest increases in GLI1 and PTCH1 expression. While this work does not extend to investigate the differences in activation of the HH signalling pathway between cell lines it does demonstrate the ability for EBV to induce SHH expression which can impact on HH signalling activation in responsive cell lines. Future studies may wish to investigate the ligand responsiveness of EBV-associated tumours and cell lines to assess possible paracrine involvement of other pathways.

While a number of tumour types are dependent on HH-mediated paracrine signalling, both the A549 and CNE2 cell lines appear to show autocrine responsiveness to SHH production, as inhibition of SHH ligand with 5E1 attenuated HH pathway activity. This does not rule out a paracrine mechanism for HH signalling in the tumour where EBV would function to activate HH signalling in a subset of tumour cells that are then responsible for inducing cell signalling in the bulk of the tumour. Additionally, HH signalling may act in a paracrine manner to influence surrounding stroma or the lymphoid infiltrate which assist in the maintenance of NPC tumours. Further work should investigate the contribution of the stroma to tumour growth and interrogate HH activity in the stromal cells compared to tumour cells. Understanding the implications of tumour micro-environment have significant implications for therapy, as such animal models should be utilised to investigate the adequacy of cell line models which cannot replicate this micro-environment.

The discovery that EBV infected cell lines expressed higher levels of stem cell markers than their uninfected counterparts invited an investigation into which EBV-encoded gene(s) was responsible for stimulating their expression. Previous work investigating the EBV gene responsible for the induction of “CSC-like” characteristics has failed to establish biologically relevant expression of latent proteins.

This study set out to establish the effect of viral infection on stem cell marker expression, and the relationship between pathway activity and the expression of these markers. Characterisation of rEBV infected cell lines for EBV latent gene expression revealed that EBNA1 was expressed in all EBV positive cell lines; the low level of expression in the EBV infected AGS cell line is likely due to the lytic nature of this cell line and loss of EBV upon serial passage. Very low levels of LMP1 were detected in all EBV-infected cell lines, apart from SUNE1, which were of an earlier passage, possibly demonstrating an inverse correlation with passage number following EBV infection and LMP1 expression. The expression of LMP2A was also low in all cell lines with the exception of CNE2, which showed high levels of expression.

Zhang et al., (2010) demonstrated that overexpression of LMP2A in the CNE2 and SUNE1 cell lines increased the size of cell populations displaying a “side population”-like phenotype, which express the ABCG2 drug transporter protein and hence export Hoechst dye. This study also found that tumours could be initiated by smaller numbers of LMP2A expressing cells, indicating increased tumourigenic potential of these cells. These results may explain the undifferentiated phenotype of EBV positive NPC and GC tumours and points towards the possibility that stable

latent EBV infection of a differentiated cell induces cellular reprogramming, with cells acquiring a less differentiated or CSC-like phenotype. However, it does not rule out the possibility that infection of undifferentiated stem progenitor cells occurs, and stable infection drives the proliferation of these cells. Whether the EMT phenomenon will remain true upon the expression of physiological levels of LMP2A, and in the presence of other viral latent proteins, remains to be assessed. Indeed, Kong et al., (2010) did note that in the C666.1 cell line, which expresses low levels of LMP2A, no Hoechst dye effluxing putative stem cells where found. This could indicate that Hoechst dye efflux is not sufficient to identify a side population of cells, at least in this cell line, and would support the characterisation of other markers of “stemness” in NPC and the characterization of cell isolated from side population studies.

In addition to inducing an EMT, LMP2A has also been reported to drive stem cell marker expression inducing the expression of stem cell markers including BMI1, SOX2, NANOG and ABC transporter ABCG2 in CNE2 and SUNE1 cell lines over-expressing LMP2A (Kong et a., 2010). In this study, increased expression of NANOG was not readily observed in rEBV infected CNE2 cells, which, of all cell lines examined, expressed the highest levels of LMP2A. The effects observed may occur as a result of over-expression, with high levels of LMP2A forcing a phenotypic change in the CNE2 cell line that is not demonstrative of a natural stable infection in this epithelial background. Furthermore the effect of other EBV viral proteins, which may act in concert with or counter LMP2A-mediated effects, was not addressed. The ability of LMP2A to establish a stem cell phenotype may be restricted to cells possessing very high LMP2 expression, which might make up a sub-population of a

cell line or indeed a tumour, or occur in cell lines that are more sensitive to cellular reprogramming.

Work presented here also investigated the expression of individual EBV latent proteins in the HONE1 cell line and demonstrated that different stem cell markers were induced in response to the expression of individual EBV genes. SOX2 was found to be upregulated in response to EBNA1, LMP1 and LMP2A expression, whereas LRIG1 was upregulated by LMP1 and LMP2A. Interestingly, LMP1 alone was found to strongly induce the expression of CD44 and p75NTR, whereas LMP2A alone induced the expression of CD133 and CXCR4. SHH ligand and HH targets were found to be induced by all three individual EBV latent genes, suggesting each of these viral proteins can modulate HH signalling, but HH activation was most noticeable in the LMP1 expressing cell line. This may suggest that LMP1 induced markers may be HH dependent while the expression of markers such as CD133 and CXCR4 may be related to other cell signalling pathways that may act in concert with the HH signalling pathway to induce a CSC phenotype in EBV infected epithelial cells. The role that EBV plays in the growth and maintenance of stem cell/CSCs and the viral gene products required for this effect is still under investigation and further interrogation is required to tease out the complexities relating to multiple signalling pathway interactions.

Aberrant activation of a number of stem cell/CSC maintenance pathways (WNT, NOTCH, FGF and HH), have been identified in many cancers. While this may lead to the aberrant induction of stem cell/CSC markers and the acquisition of stem-like properties in more committed or differentiated cells, in most instances it is associated

with the growth of CSC subpopulations, which are responsible for generating the bulk of the tumour. Dysregulation of a number of stem cell maintenance pathways (WNT/β-catenin, TGFβ, and NOTCH) have been identified in NPC, the role that these pathways play in the growth and maintenance of stem cell/CSC populations in this cancer is unclear. A recent study has not only shown that NOTCH3 is overexpressed in primary NPC tumours, but that tumour initiating subpopulations marked by the CD44 antigen require NOTCH signalling for maintenance (Man et al, 2012). Although still preliminary, these findings suggest that NPC may arise from an EBV infected stem cell or CSC populations.

With regard to the origin of NPC-derived CSCs at least two possibilities are proposed. In the first scenario, EBV infects an undifferentiated early progenitor cell or a stem cell which already display high levels of stem cell markers and heightened HH signalling and drives the proliferation of these cells through sustained HH pathway activation. Alternatively, EBV infects a precancerous cell of unknown differentiation status that is amenable to latent infection, and imposes a CSC-like phenotype through sustained engagement of the HH pathway. In this scenario, EBV becomes “trapped” in a latency program due to a mistake in cell programming or an inability to alter cell signalling in a mutated or undifferentiated background.

CHAPTER FIVE: THE CONSEQUENCES OF DYSREGULATED HEDGEHOG SIGNALLING PATHWAY IN EBV-INFECTED EPITHELIAL CELLS.

"It is often claimed that knowledge multiplies so rapidly that nobody can follow it. I believe this is incorrect. At least in science it is not true. The main purpose of science is simplicity and as we understand more things, everything is becoming simpler. This, of course, goes contrary to what everyone accepts."

— Edward Teller

Edward Teller, Wendy Teller, Wilson Talley, Conversations on the Dark Secrets of Physics (1991, 2002), 2.

5.1 Introduction

Dysregulation of the HH pathway is frequently associated with oncogenic transformation. As outlined in Chapter 1 section 1.12, loss of function mutations in PTCH or gain of function mutations in SMO, that cause constitutive activation of the HH pathway, are sufficient to induce basal cell carcinomas in mouse models (Rubin and de Sauvage, 2006). Whilst aberrant activation of the HH pathway plays an essential role in the renweal and maintenance of CSC populations, the HH pathway also impacts on other aspects of cell behaviour, influencing cell proliferation, invasion, metastasis, angiogenesis and differentiation (see Chapter 1, Figure 1. 8). It is estimated that dysregulated HH signalling is implicated in the aetiology of approximately 20-25% of all cancers (Briscoe and Therond, 2005). As such, it has become apparent that this pathway constitutes a viable therapeutic target in many cancers, particularly those whose aetiology is linked to the presence of CSC populations. For example, in hepatocellular carcinoma, HH pathway component overexpression is correlated with migration and invasion (Chen et al., 2013). Similarly, ectopic overexpression of GLI1 in ovarian cells has been shown to promote migration, proliferation and invasion, whilst inhibition of this pathway with cyclopamine, suppressed growth, migration, invasion and motility, and induced apoptosis (Liao et al., 2009).

In recent years, the HH pathway has been identified as a viable therapeutic target in a range of solid and lymphoid tumours. In pancreatic cancer, which also displays dysregulated HH signalling, transient GLI1 overexpression can induce proliferation, and cyclopamine treatment reduced anchorage independent growth in soft agarose and inhibited cancer invasion and metastasis (Feldmann et al., 2007). Cyclopamine

treatment was also able to inhibit anchorage-independent growth and proliferation in both ER+ and ER- breast cancer cell lines (Zhang et al., 2009b), and reduced the growth of gastric cancer cells *in vitro* (Yanai et al., 2007). HH may even have a role in the transition of breast cancer towards estrogen-independent growth, as stable GLI1 expression was seen to induce estrogen-independent growth, promote G1/S phase transition and downregulate estrogen receptor α (Zhao et al., 2010). Whilst cellular consequences of dysregulated HH signalling have been demonstrated in numerous cancers of epithelial origin including those of the head and neck, its role in the pathogenesis of NPC remains uncertain. A recent study by Yue et al., (2012) demonstrated that HH pathways component PTCH was seen to be upregulated by RT-QPCR in NPC tissue compared to inflamed nasopharyngeal tissue and normal tissue, whilst GLI1 was seen to be upregulated in NPC by both PCR and IHC compared to these control. Additionally, they found that inhibition of the HH pathway was sufficient to decrease proliferation and induce apoptosis in three NPC derived cell lines including CNE2 and this was accompanied by decreased GLI1 and PTCH, and altered cyclin D and E expression, which may impact on cell cycle regulation (Yue et al., 2012).

The HH signalling pathway can influence differentiation and self-renewal by activating a variety of downstream target genes. For instance, GLI2 is able to inhibit the differentiation of squamous epithelial cells, a necessary requirement for the maintenance of an undifferentiated stem cell, through negative regulation of differentiation-associated genes: Keratin 1 and 10 (KRT1/10), involucrin (IVL) and loricrin (LOR), and additionally cooperates with GLI1 in the promotion of cell proliferation though upregulation of cyclin-dependent kinase 1 (CDK1) and CCND2

(Regl et al., 2003). GLI2 is also able to influence cell survival through transcription of BCL2 (Regl et al., 2004).

HH activation is known induce cellular migration and invasion through an epithelial to mesenchymal transition (EMT), and it has been shown that GLI1 can upregulate the expression of EMT markers such as SNAI1 and SIP1 (Li et al., 2005, Katoh and Katoh, 2009a). It has previously been noted that EBV can induce an EMT through expression of EBV latent protein LMP1 and LMP2A (Dawson et al., 2012), indeed previous studies using rEBV infected CNE2 cell lines have documented the morphological transition that accompanies an EMT upon EBV infection (Kong et al., 2010).

EMT describes the process by which differentiated epithelial cells lose their differentiated characteristic, such as cellular adhesion and polarity, and adopt a more mesenchymal phenotype displaying increased motility, invasiveness and apoptosis resistance (Thiery and Sleeman, 2006). It has been demonstrated that, in a number of cellular backgrounds, an EMT can lead to the acquisition of stem cell and malignant traits (Brabletz et al., 2005, Blick et al., 2008). EMT occurs in embryogenesis as a transient state, being required for development past the blastula stage (Thiery and Sleeman, 2006) and is followed by the reverse process, an mesenchymal to epithelial transition (MET) (Duband et al., 1995). MET allows somatogenesis and is essential for the development of epithelial organs such as the kidneys, coelomic-cavity, gastrointestinal tract, lungs, skin and neural crest (Christ and Ordahl, 1995, Duband et al., 1995, Davies, 1996, Funayama et al., 1999, Locascio and Nieto, 2001, Gonzalez-Mariscal et al., 2007). During the EMT process

epithelial cells show decreased expression of junctional proteins (epithelial (E)-cadherin, tight junction and desmosomal proteins) and cytokeratin-rich intermediate filaments, and gain motility and mesenchymal markers such as mesenchymal (N)-cadherins and vimentin-rich intermediate filaments (Thiery and Sleeman, 2006).

As well as an essential function in embryogenesis, EMT has a role in the maintenance of adult tissues during tissue regeneration and wound healing (Kalluri, 2009). Signalling pathways are critical in the control of EMT and disruption has been associated with disease including cancer (Thiery and Sleeman, 2006, Hugo et al., 2007, Baum et al., 2008, Yang and Weinberg, 2008), where induction of an EMT is associated with poor prognosis (Sabbah et al., 2008). Reduced cellular adhesion, induced by E-cadherin disruption, has been demonstrated to be involved in the malignant transformation of non-invasive tumours (Birchmeier and Behrens, 1994, Perl et al., 1998).

During tumour metastasis cells acquire the ability for self-renewal, allowing tumour initiation. As EMT is required for the dissemination process, it has been hypothesised that an EMT could also bestow cells with a CSC-like phenotype (Kondo et al., 2003, Mani et al., 2008). Mani et al., (2008) isolated cells on the basis of $CD44^{high}/CD24^{low}$ expression, which characterises the stem cell population of normal and cancerous mammary tissue, and demonstrated that these cells displayed an EMT phenotype. Additionally, this population displayed enhanced cell survival, and resistance to anoikis and chemotherapeutic drugs. They also demonstrated that human mammary cells induced to undergo EMT acquire these stem cell characteristics and display increased malignant potential. Similarly, in colon cancer, loss of epithelial features

accompanied by downregulation of E-cadherin and increased vimentin expression is seen at the invasive front (Brabletz et al., 2005). Recently, a study by Luo et al., (2013) demonstrated increased expression of a SOX2 and OCT4 stem cell markers at the invasive front of NPC tumours and found a correlation between high levels of OCT4, and high N-cadherin/low E-cadherin expression. Expression of SOX2 and OCT4 at the invasive front was also associated with poor overall survival.

Progress has been made into defining the embryonic transcription factors critical for embryogenesis due to the induction of an EMT. These transcription factors, when expressed in neoplastic cells, confer a malignant phenotype by inducing motility, invasiveness, and resistance to apoptosis (reviewed by Sánchez-Tilló, et al., 2012). Transcription factors that inhibit E-cadherin through the E-box promoter element act to induce an EMT. These same transcription factors are often commandeered during cancer progression. For instance, SLUG and SNAIL transcription factors are regulators of EMT and may contribute to ovarian and pancreatic cancer progression (Elloul et al., 2005, Yoshida et al., 2009, Hotz et al., 2007).

Within the stem cell niche the microenvironment is believed to play a large part in the maintenance and regulation of the stem cell population. Stromal interactions play an important role in the maintenance of cellular phenotype in regard to characteristics such as tumour initiation. Furthermore, stromal interactions may regulate an EMT as the tumour microenvironment is implicated in the induction of dysregulated cellular signalling. Studies have shown an induction of EMT at the tumour stromal interface in both human and animal models (Brabletz et al., 2001, Franci et al., 2006, Sheehan et al., 2007). In this regard the tumour microenvironment may have a role in maintaining

the cancer stem cell population and tumour characteristics much in the same way that the adult stem cell niche is responsible for the maintenance of this sub population of cells.

A mesenchymal to epithelial transition, the reverse of an EMT, also has a role in cancer progression as it is involved in metastasis, possibly acting to re-establish the epithelial characteristics of distant metastases in the absence of EMT-inducing signals (Polyak and Weinberg, 2009). MET may also be dependent on tumour micro environment as a study by Yates et al., (2007) showed that epithelial marker E-cadherin was upregulated when prostate cancers were co-cultivated with hepatocytes. However it has been unresolved whether an epithelial state is the default phenotype in the absence of an EMT inducing signal, such that tumours without activated stromal influences will reverse back to an epithelial phenotype via an MET.

Following the identification of a dysregulated HH signalling pathway in NPC the phenotypic consequences of HH signalling activation on the proliferation, anchorage independent growth and induction of “stemness”, were investigated in the authentic EBV positive C666.1 cell line. Given that EBV infection is capable of inducing HH dependent stem cell marker expression, the phenotypic consequences of stable latent infection on EMT, cell proliferation, anchorage independent growth and sphere formation, and the role of HH signalling in these responses were investigated.

5.1.1 Authentic EBV positive carcinoma cell lines require sustained HH signaling for cell proliferation

The incorporation of 5-bromodeoxyuridine (BrdU) into DNA is used to assess cell proliferation both *in vivo* and *in vitro*. Transient pulsing of cells or tissues with BrdU results in incorporation of the label in cells undergoing DNA replication. Cells replicating DNA demonstrate nuclear staining when probed with antibodies specific for BrdU, allowing quantitation of cells undergoing DNA synthesis. BrdU label retention has also been used to identify putative stem cell populations; in the intestine staining over 24 hr is sufficient to stain all the crypt base columnar cells where BrdU is retained in the slow cycling cells but rapidly lost from actively proliferating cells (Barker et al., 2007, Barker et al., 2008). In other populations staining is required for significantly longer periods to ensure all cells are labelled prior to wash out; for instance breast cancer cell lines are pulsed with BrdU for 10 days (Fillmore and Kuperwasser, 2008). However the use of this assay for the identification of stem cells/CSC has been questioned as not all label retaining cells are true stem cells/CSC, indeed terminally differentiated cells retain BrdU (Barker et al., 2007), and this assay has been shown to be non-specific in the identification of haematopoietic stem cells (Kiel et al., 2007). More recently label retaining cells have been identified by the expression of histone 2B-green fluorescent protein (H2B-GFP) transgene under the control of a tetracycline-regulatable enhancer element (Fuchs, 2009b).

While stem cells are usually regarded as slow cycling label retaining cells, increased BrdU incorporation following transient pulsing, which measures proliferation, has been associated with stem cell properties such as self-renewal and the expression of

CSC markers. For instance, in the central nervous system stem cells, neural progenitors derived from $BMI1^{-/-}$ mice display decreased BrdU incorporation compared to WT cells, an effect that was associated with the inability of these cells to undergo self-renewal (Molofsky et al., 2003). In another study Ki-67, a marker of proliferation, was increased in CD133 positive, tumour sphere forming populations of glioblastoma cells. Proliferation of this CD133 positive population of cells was demonstrated to be more sensitive to NOTCH inhibition than the CD133 negative population (Fan et al., 2010).

The requirement of HH signalling for sustained cell growth in authentic EBV positive cell lines was investigated using an ELISA-based assay which measures BrdU incorporation. The effects of GANT58 and GANT61 treatment on BrdU incorporation in the C666.1 and EBV positive gastric carcinoma cell line YCCEL1 were investigated. Inhibition of the HH signalling pathway was shown to impact on the proliferation of C666.1 and YCCEL1 cells in a dose dependant manner (Figure 5. 1). GANT61 was used at half the concentration of GANT58 due to inherent toxicity when used at higher concentrations, perhaps reflecting the fact that GANT61 targets both GLI1 and GLI2. Proliferation was significantly inhibited by 25 µM GANT61 and 50 µM GANT58 in both cell lines highlighting the potential importance of sustained HH signalling for cell proliferation in these unsorted EBV positive carcinoma-derived cell lines.

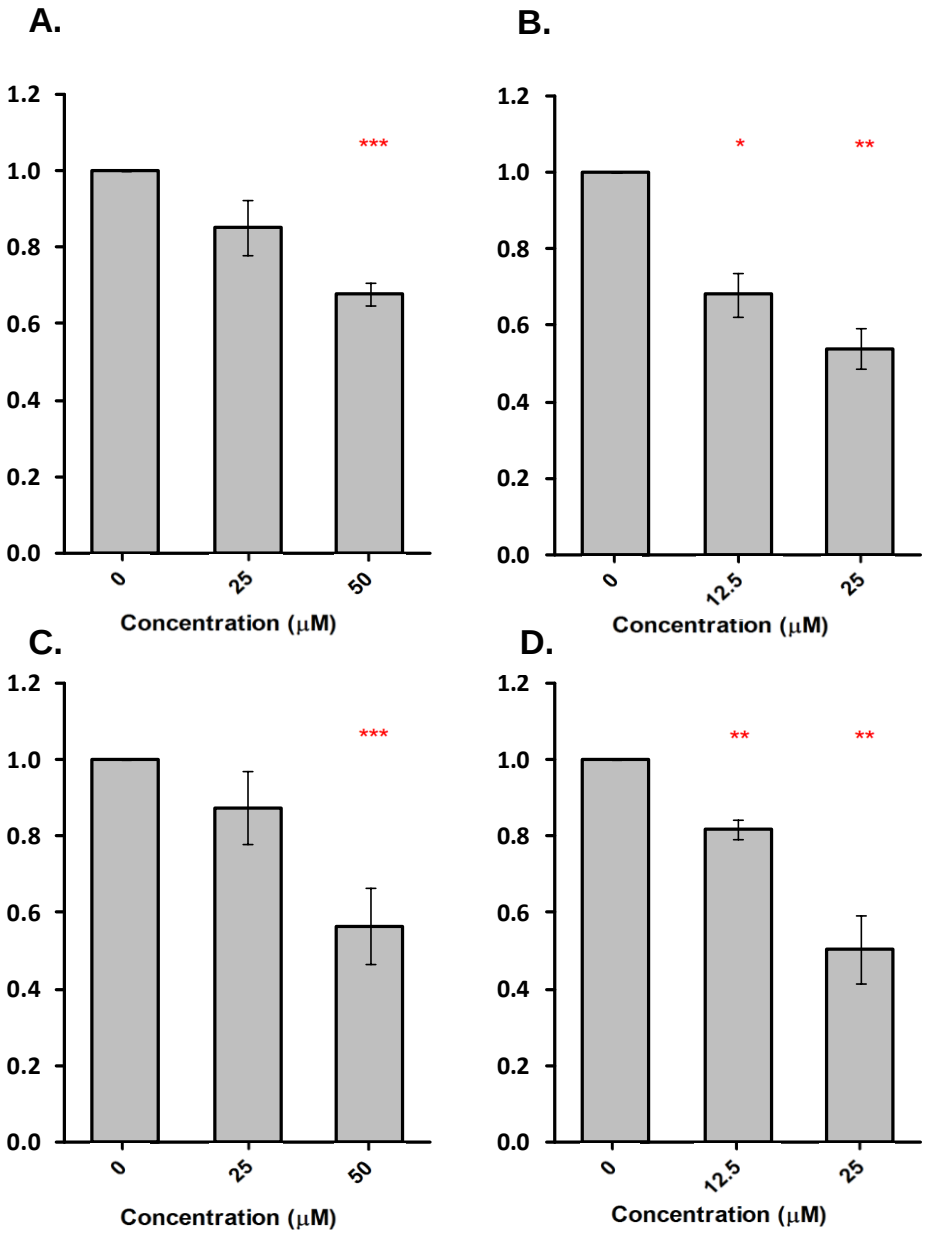


Figure 5. 1 Normalised BrdU incorporation in C666.1 (A, B) and YCCEL1 (C, D) cell lines.
Cells were plated at 2.5 x10^4 and allowed to settle for 24 hr prior to treatment with either 25 μM GANT58 (A, C) or 12.5 μM GANT61 (B, D) for 48 hr. BrdU incorporation was performed in accordance with the manufactures' instructions. BrdU was pulsed for 6-8 hr.

5.1.2 Inhibition of the HH pathway impacts on anchorage-independent growth in the C666.1 cell line

A uniform characteristic of transformed cells is their ability to proliferate in the absence of substrate attachment; so called “anchorage-independent growth” (reviewed by Wang, 2004). The ability of cells to proliferate in suspension differs from the formation of tumourspheres, which utilises special culture conditions which favour the growth of CSCs or cells with SC-like properties.

To examine the “transformed” properties of C666.1 cells *in vitro*, and to identify a role for HH signalling in this response, C666.1 cells were plated out in normal growth media containing 0.3% agarose and overlaid on a base of 1% LMP agarose. Cells, cultured in the presence or absence of GAN58 or GANT61 were allowed to form colonies over a 3-4 week period, after which time they were photographed and scored for colony number and size. As shown in Figure 5. 2 and Figure 5. 3, both GANT 58 (Figure 5. 2) and GANT61 (Figure 5. 3) significantly reduced the ability of C666.1 cells to form colonies, and reduced the size of those spheres that formed, in a dose dependent manner. Significant inhibition of both colony size and number ($P<0.005$) was achieved with 25 µM of GANT58 or GANT61.

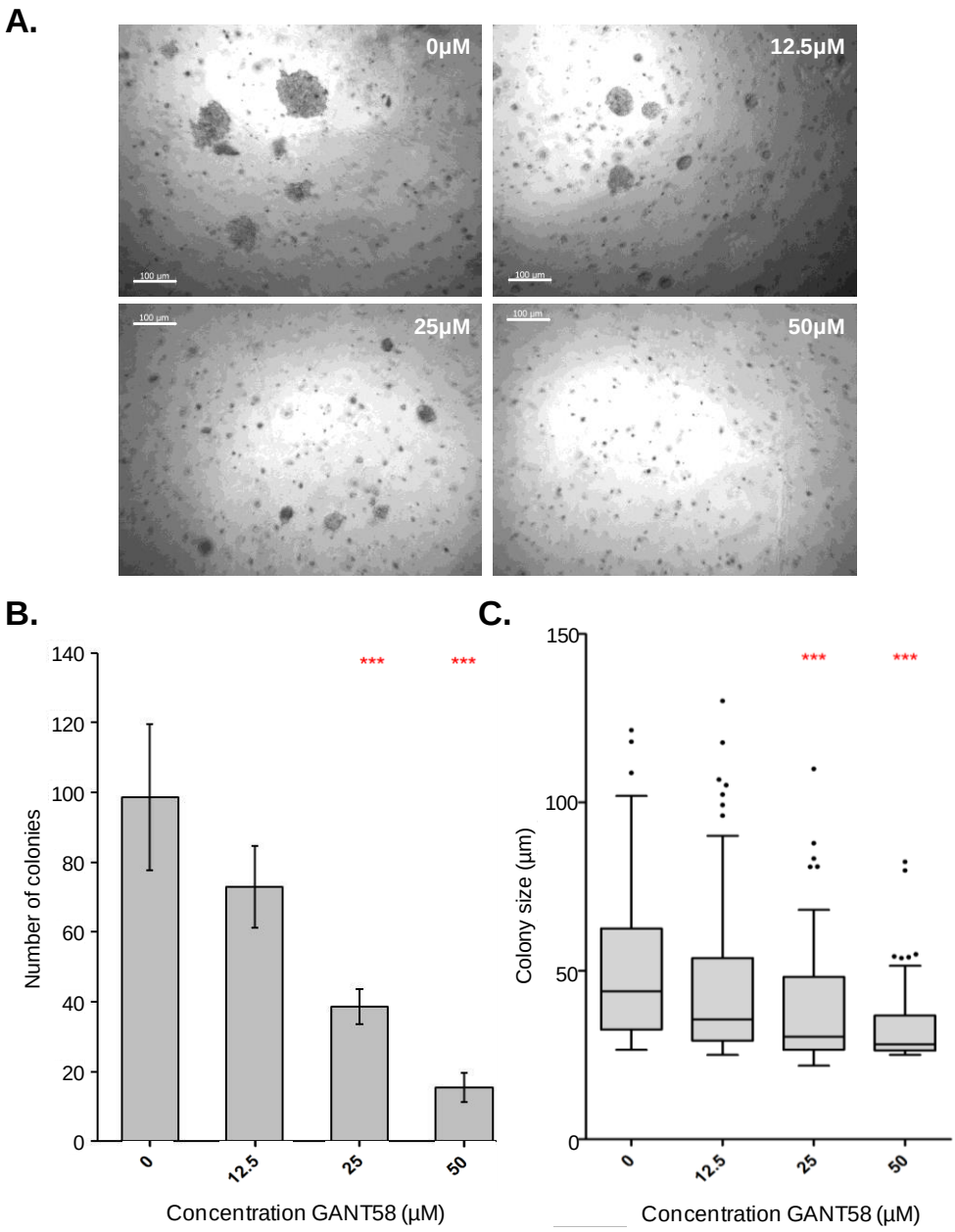


Figure 5. 2 Colony formation assay in the presence of HH inhibitor GANT58
C666.1 cells plated out in triplicate at 5 x10^3 cells per well in a 12 well plate in 0.33% agarose solution containing 10% FCS and 1% RPMI, on a base of 1% agarose/ 1% RPM1/ 10% FCS. GANT58 used at increasing concentrations per total well volume, in 10% B-cell serum RPMI. Cells were allowed to form colonies for 2-3 weeks, or until around 100 colonies/well were present in the control wells. Four, non-overlapping, images were taken per well with an x10 objective. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted using a box and whisker plot (P relative to control, n=3). Bar = 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, p<0.05; ** p<0.01; *** p<0.001

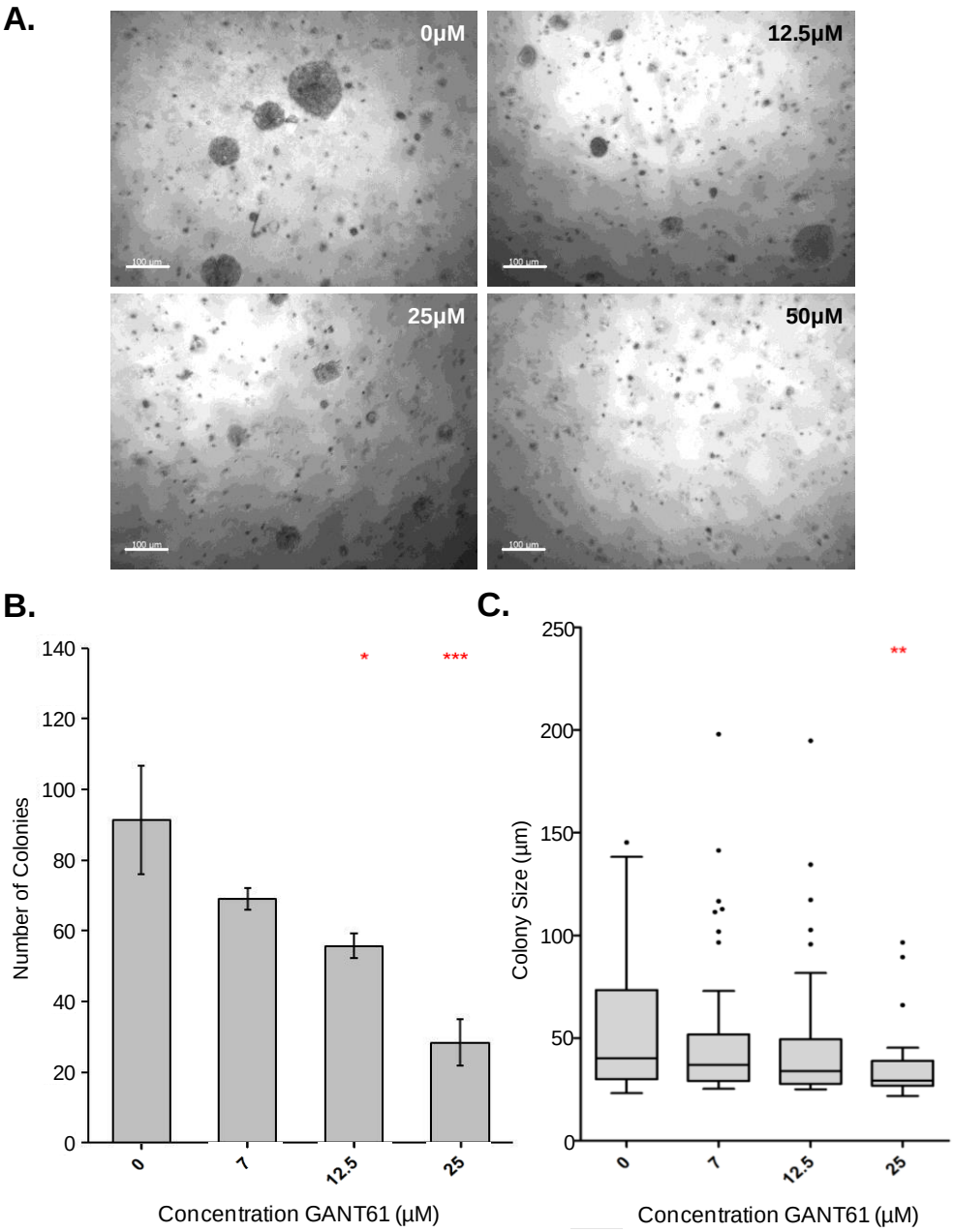


Figure 5. 3 C666.1 colony formation in the presence of HH inhibitor GANT61
C666.1 cells plated out in triplicate at 5 x10^3 cells per well in a 12 well plate in 0.33% agarose solution containing 10% FCS and 1% RPMI, on a base of 1% agarose/ 1% RPM1/ 10% FCS. GANT61 used at an increasing concentrations per total well volume, in 10% B-cell serum RPMI. Cells were allowed to form colonies for 2-3 weeks, or until around 100 colonies/well were present in the control wells. Four, non-overlapping, images were taken per well with an x10 objective. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted using a box and whisker plot (P relative to control, n=3). Bar = 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, p<0.05; ** p<0.01; *** p<0.001

5.1.3 Inhibition of the Hedgehog pathway impacts on sphere formation capacity in the C666.1 cell line

It is now widely appreciated that CSCs, isolated on the basis of unique cell surface marker signatures, express higher levels of stemness-associated transcription factors and display a higher tumour-initiating potential than the bulk tumour population. Whilst the gold standard for the identification of a CSC is to use a limiting dilution study to assay for tumour formation in nude mice, a number of *in vitro* assays have been developed which select for cells with CSC-like properties. These assays are based on the ability of SC/CSCs to undergo self-renewal under highly selective culture conditions (Pastrana et al., 2011). Since its first use in the isolation of neuronal progenitor cells, allowing characterisation of these "neurosphere" populations *in vitro* (reviewed in Pastrana et al., 2011), sphere formation has been used to isolate and characterise CSC populations from a wide variety of cancers, including those of head and neck, prostate, pancreatic, neuronal and gastric origin (Dyall et al., 2011).

In this study the ability of NPC-derived CSC populations to form spheres was examined using specialised culture conditions. C666.1, OKF6 and NP460 cells were plated at low cell density onto ultra-low attachment plates in serum free media (RPMI 1640) supplemented with epidermal growth factor (EGF), fibroblast growth factor (FGF) and the N2 and B27 growth supplements in the presence or absence of GANT58 and GANT61. The C666.1 cell line formed colonies, defined as 3D spherical structures obtaining a diameter of at least 25 microns after 21 days. Both GANT58 and GANT61 were found to significantly reduce the ability of C666.1 derived CSCs to form colonies and the size of the colonies that formed, Figure 5. 4. Importantly, it was

found that the hTert-immortalised OKF6 and NP460 cell lines were unable to form colonies under these highly selective conditions (Figure 5. 5).

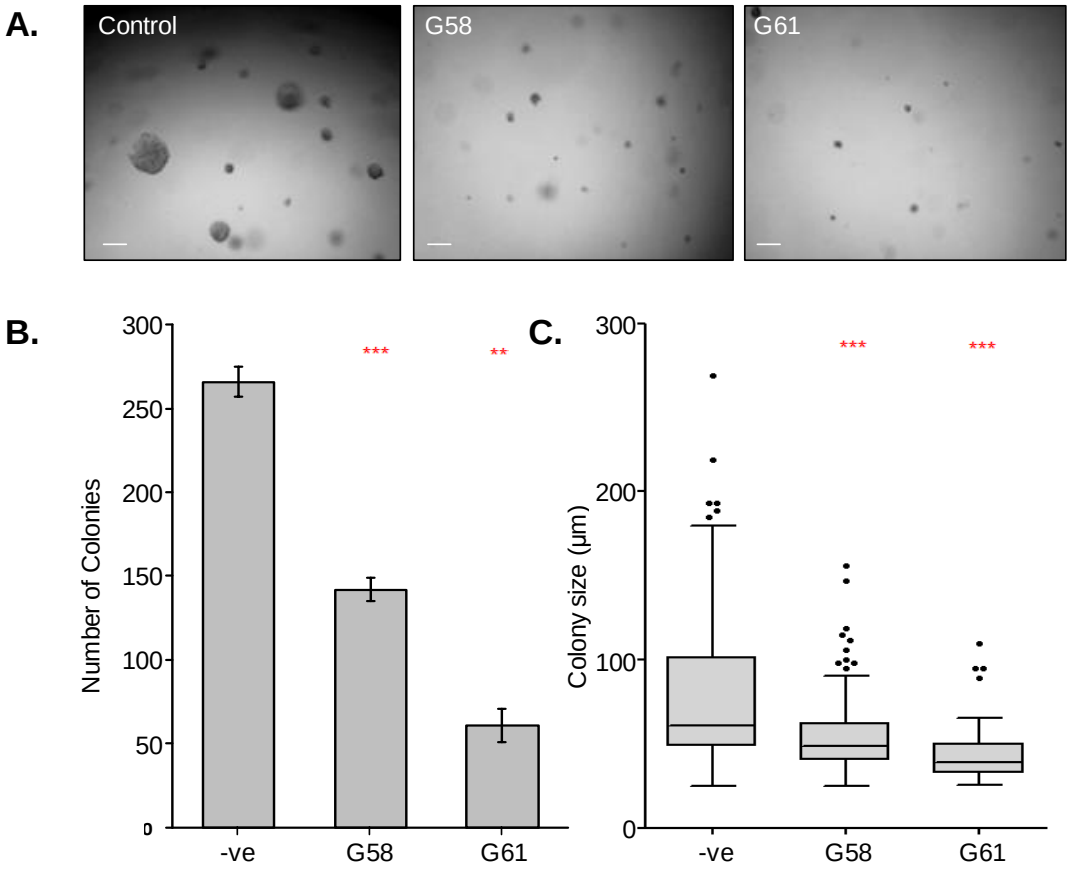


Figure 5. 4 GANT treatment inhibits sphere formation in the C666.1 cell line
Cells were plated in triplicate at 1x10^3 cells per well of a 12 well plate in 0.33% agarose solution containing 1%RPM1 and growth supplements. GANT58 (G58) and GANT61 (G61) used at a concentration of 25 µM and 12.5 µM total well volume respectively. Cells were allowed to form colonies for 3 weeks. Four, non-overlapping, images were taken per well with an x10 objective. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted using a box and whisker plot (P relative to control, n=3). Bar = 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, p<0.05; ** p<0.01; *** p<0.001

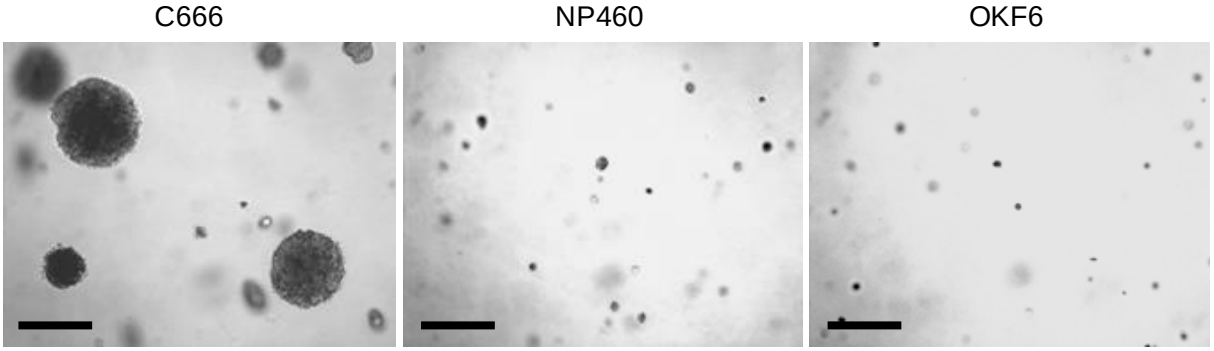


Figure 5. 5 OKF6 and NP460 cell lines were incapable of forming the same spheres seen in the C666.1 cell line
After 3 weeks of incubation, the C666.1 cell line produced spheres with an average diameter of 74 µM. In the absence of any HH inhibitors OKF6 and NP460 did not produce sphere after 3 weeks of incubation. Bar = 100 µm.

5.1.4 EBV infection induces EMT marker expression and a morphological transition in CNE2 but not A549 cells.

In a number of epithelial cell types, EMT is associated with the induction of stem cell markers and the acquisition of stem cell-like characteristics (reviewed by Singh and Settleman, 2010). The status of EMT marker expression in rEBV infected CNE2 and A549 cell lines were investigated. EBV infection was found to induce a morphological transformation, with cells adopting a more mesenchymal or “fibroblastoid” state (Figure 5. 6) in the CNE2 cell line only. RT-PCR analysis confirmed that the morphological transition in rEBV infected CNE2 cell line was accompanied by the expression of mesenchymal markers, such as SIP1, SNAI1 and WNT5A by RT-PCR (Figure 5. 6A), which were not seen to be upregulated in A549 cells (Figure 5. 6B). This was confirmed at the protein level by western blot analysis.

To investigate the contribution of HH signalling to the induction of an EMT, HH signalling was inhibited. Treatment of CNE2 cells with HH inhibitors was unable to revert the mesenchymal phenotype induced by EBV suggesting that other cell signalling pathways may be responsible for the EMT in this cellular background. However, the expression of EMT markers SIP1, SLUG and SNAIL were responsive to HH signalling and seen to be induced upon SHH ligand stimulation and decreased upon treatment with HH inhibitors cyclopamine and GANT58 (Figure 5. 7).

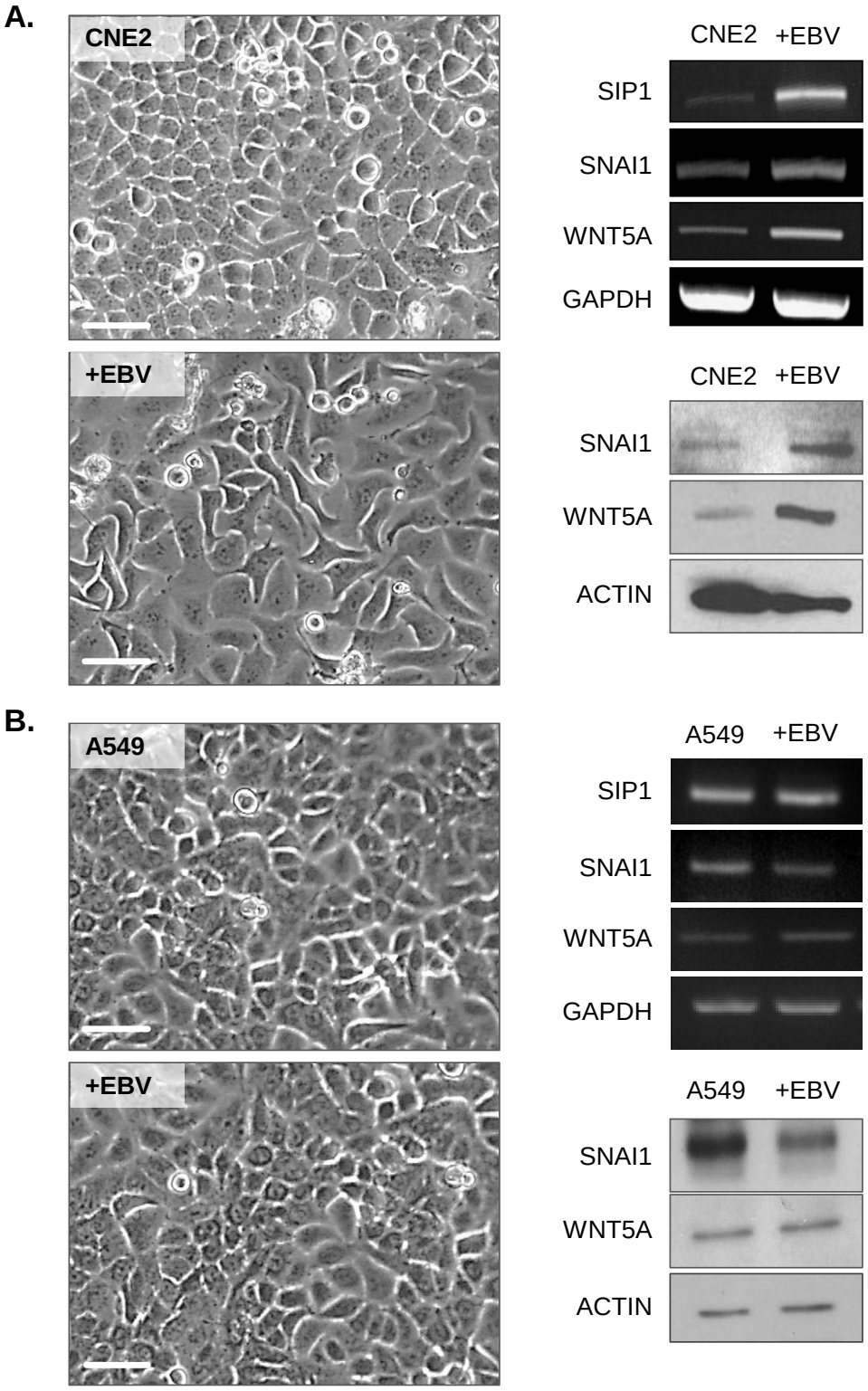


Figure 5. 6 EBV induces an EMT in the CNE2 cell line but not A549 cell line
(A) CNE2 stably infected with rEBV demonstrates a morphological transition to a more mesenchymal state accompanied by increased expression of EMT markers by RT-PCR and western analysis. (B) Stable EBV infection of A549 cells does not induce and EMT. Bar = 50 µm.

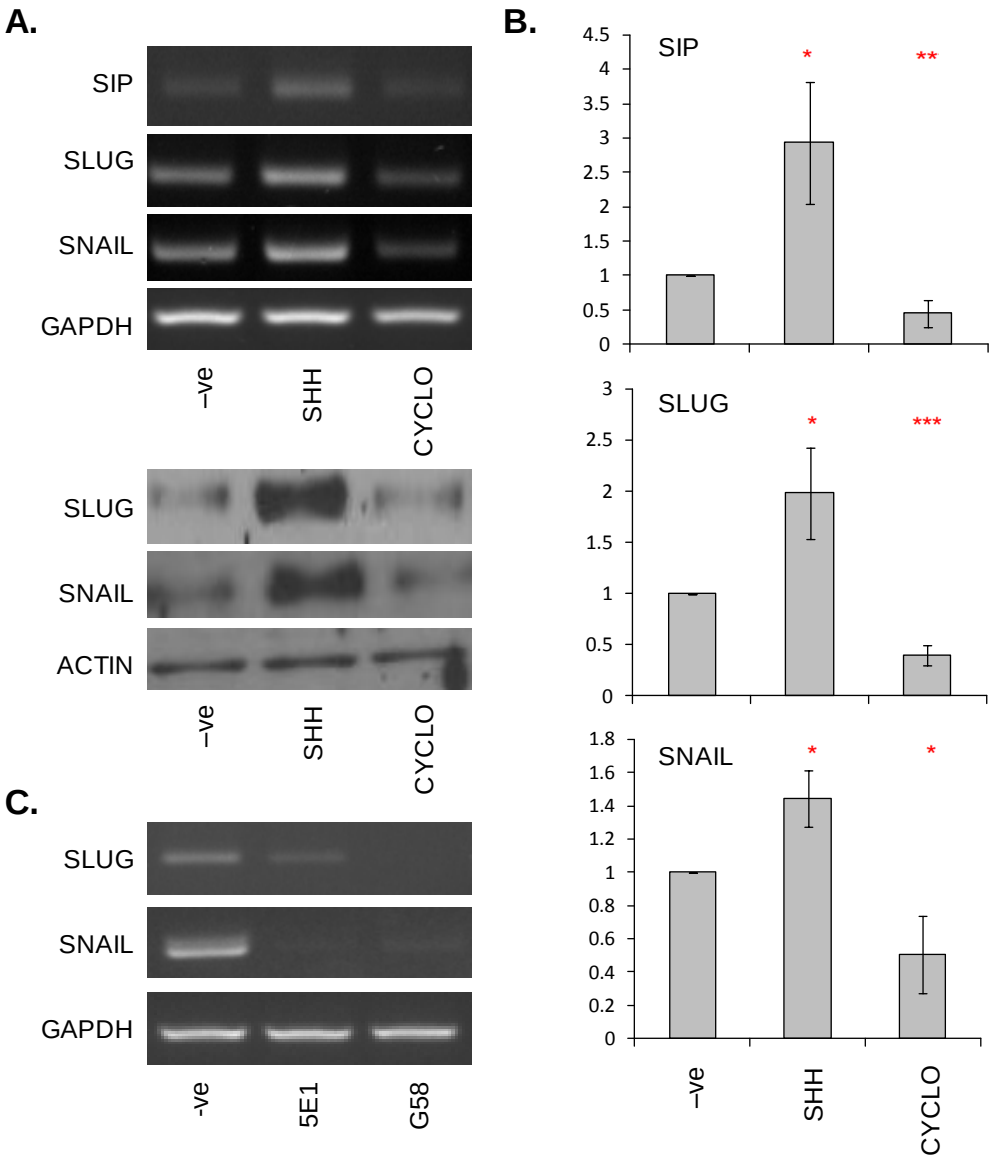


Figure 5. 7 Expression of EMT markers is dependent on HH signalling in CNE2 cells stably infected with EBV

(A) RT-PCR and western analysis demonstrating the expression of EMT markers SMUG, SNAIL and SIP is dependent on HH signalling, following SHH and cyclopamine treatment. (B) Semi quantitative RT-PCR data for the same markers using ImageJ to generate densitometry readings (n=3), SHH (0.5µg/ml) and cyclopamine (25 µM) treatment significantly impact the expression of EMT markers. (C) 5E1 (1:1 with conditioned media) and G58 (25 µM) treatment inhibit EMT markers SLUG and SNAIL expression after 48 hr.

5.2 Phenotypic consequences of an activated HH signalling pathway in CNE2 and A549 cell lines.

5.2.1 EBV-infected CNE2 and A549 cell require sustained HH signaling for cell proliferation.

The role of HH signalling in the proliferation of parental and rEBV-infected CNE2 and A549 cells was investigated by inhibiting the HH signalling pathway with GANT58 and GANT61. As with the C666.1 cell line (Section 5.1.1), HH pathway inhibition resulted in decreased BrdU incorporation in both the A549 and CNE2 cell lines in a dose dependant manner (Figure 5. 8). Again A549, being more sensitive to HH inhibitors, was treated with half the concentration of inhibitors used on the CNE2 cell lines. Despite this both cell lines showed comparable levels of inhibition.

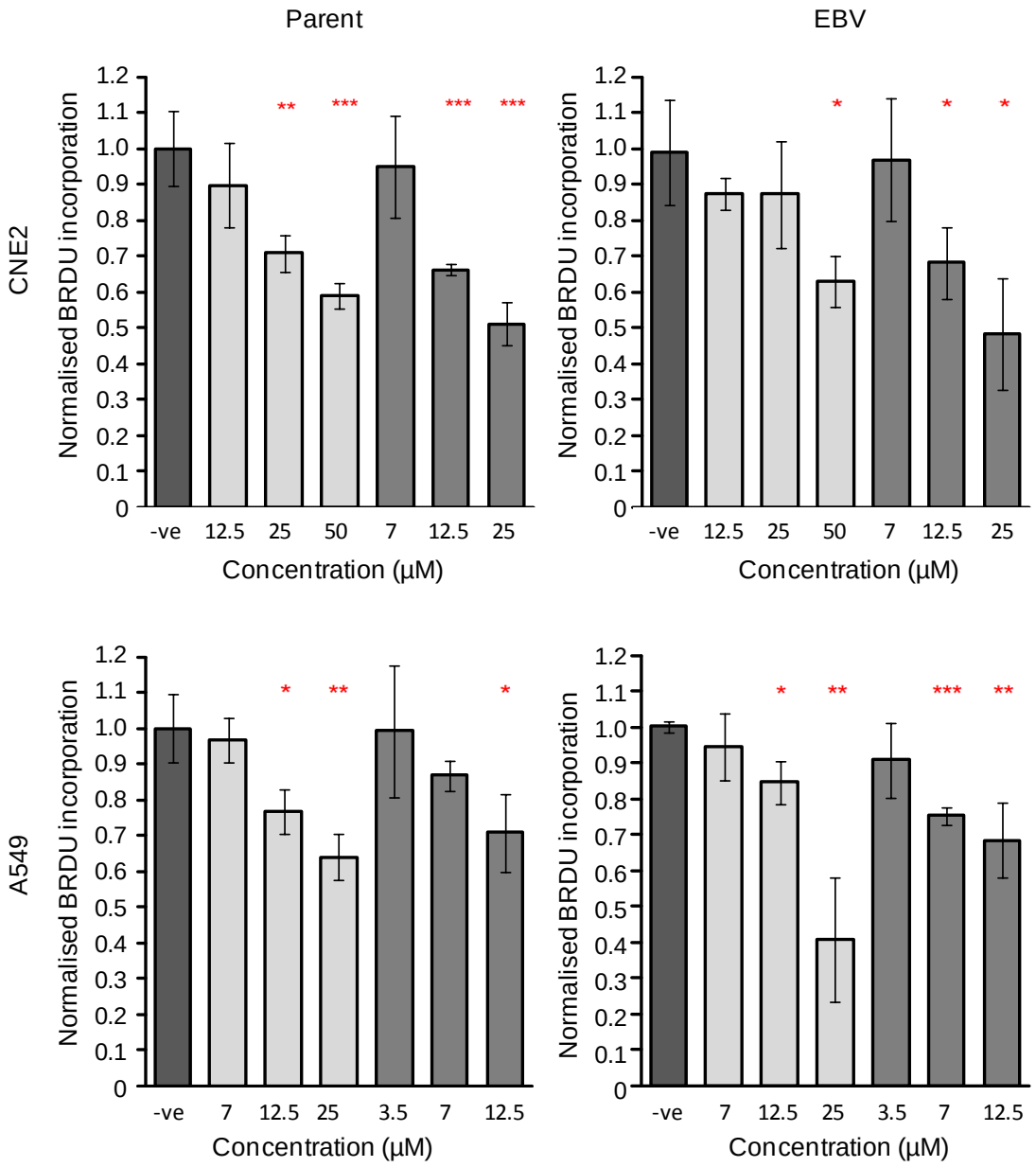


Figure 5. 8 GANT treatment inhibits BrdU proliferation assay
Normalised BrdU incorporation in CNE2 parental (left) and rEBV (right) infected CNE2 (top) and A549 (bottom) cell lines. Cells were plated at 2.5 x10^4 and allowed to settle for 24 hr prior to treatment with either GANT 58 (light grey) or GANT 61 (dark grey) for 48 hr compared to a negative control. BrdU incorporation was performed in accordance with the manufactures' instructions. BrdU was pulsed for 6 hr.*, P<0.05; **, P<0.01; ***, P<0.001.

5.2.2 Anchorage independent growth is enhanced by rEBV infection and dependent on Hedgehog signalling

Having demonstrated an effect of HH pathway inhibition on cell proliferation, the effects of HH pathway inhibition on anchorage-independent growth was investigated in uninfected control and rEBV infected CNE2 (Figure 5. 9) and A549 (Figure 5. 10) cells. Cells were plated out in complete growth medium in the presence of increasing concentrations of GANT58 or GANT61. Compared to control uninfected cells, rEBV infected cell lines formed fewer colonies; however, the colonies that did form were substantially larger. Both GANT58 and GANT61 were found to significantly reduce the ability of both cell lines to form colonies and the size of the colonies that formed. Interestingly, both rEBV infected CNE2 and A549 cell lines were more sensitive to HH pathway inhibition than their uninfected counterparts. The CNE2 cell line was most sensitive to GANT61 treatment, which caused a six fold reduction in the number of colonies that formed. However, the A549 cell lines appeared to be more sensitive to GANT58.

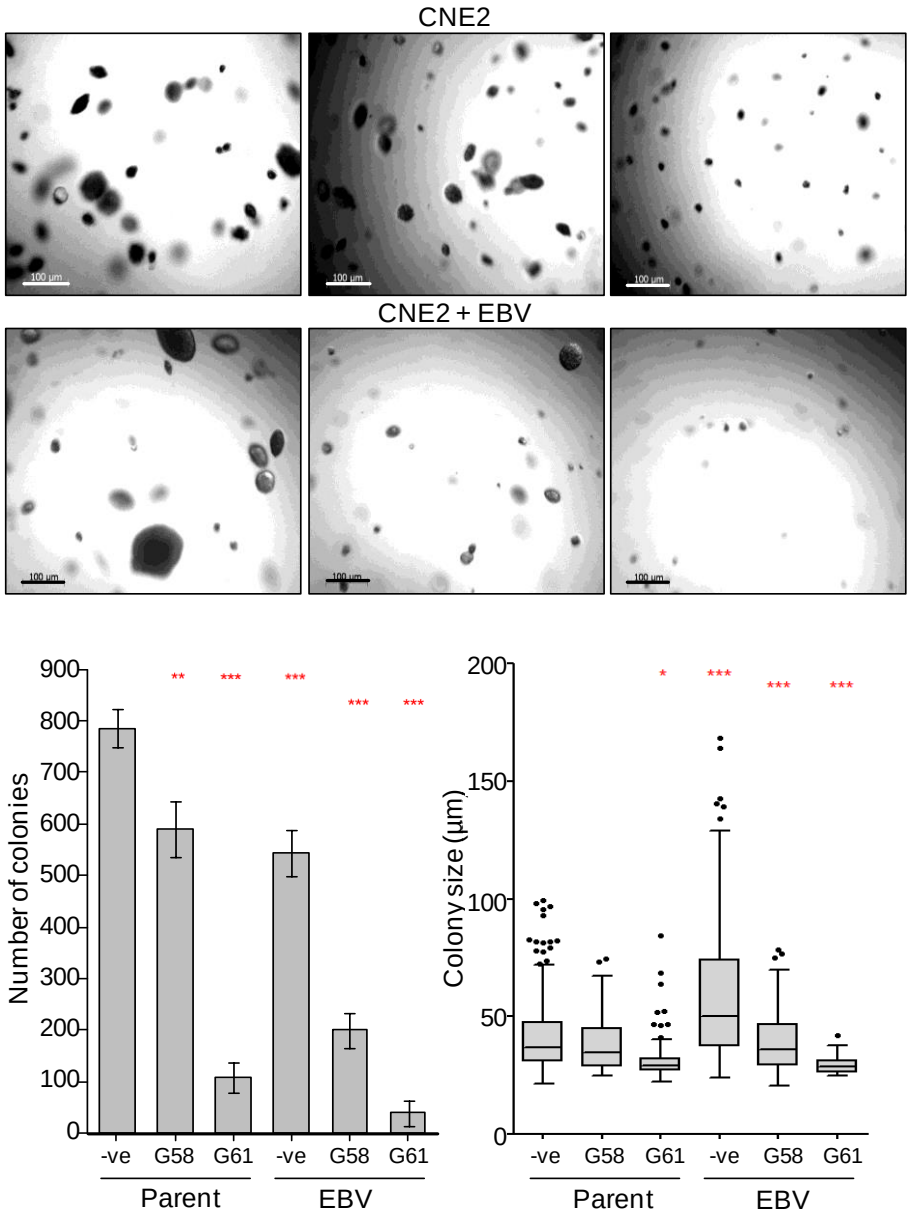


Figure 5. 9 Inhibition of the HH signalling pathway impacts on CNE2 cell anchorage independent growth

Cells plated out in triplicate at 5 x10^3 cells per well in a 12 well plate in 0.33% agarose solution containing 10% FCS and 1% RPMI, on a base of 1% agarose/ 1% RPM1/ 10% FCS. Inhibitors were added to a final concentration of 50 µM of GANT58 (G58) and 25 µM GANT61 (G61). Cells were allowed to form colonies for 2 weeks. Three, non-overlapping, images were taken per well with an x4 objective lens. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted (P relative to control, n=3). Bars= 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, $p<0.05$; ** $p<0.01$; *** $p<0.001$

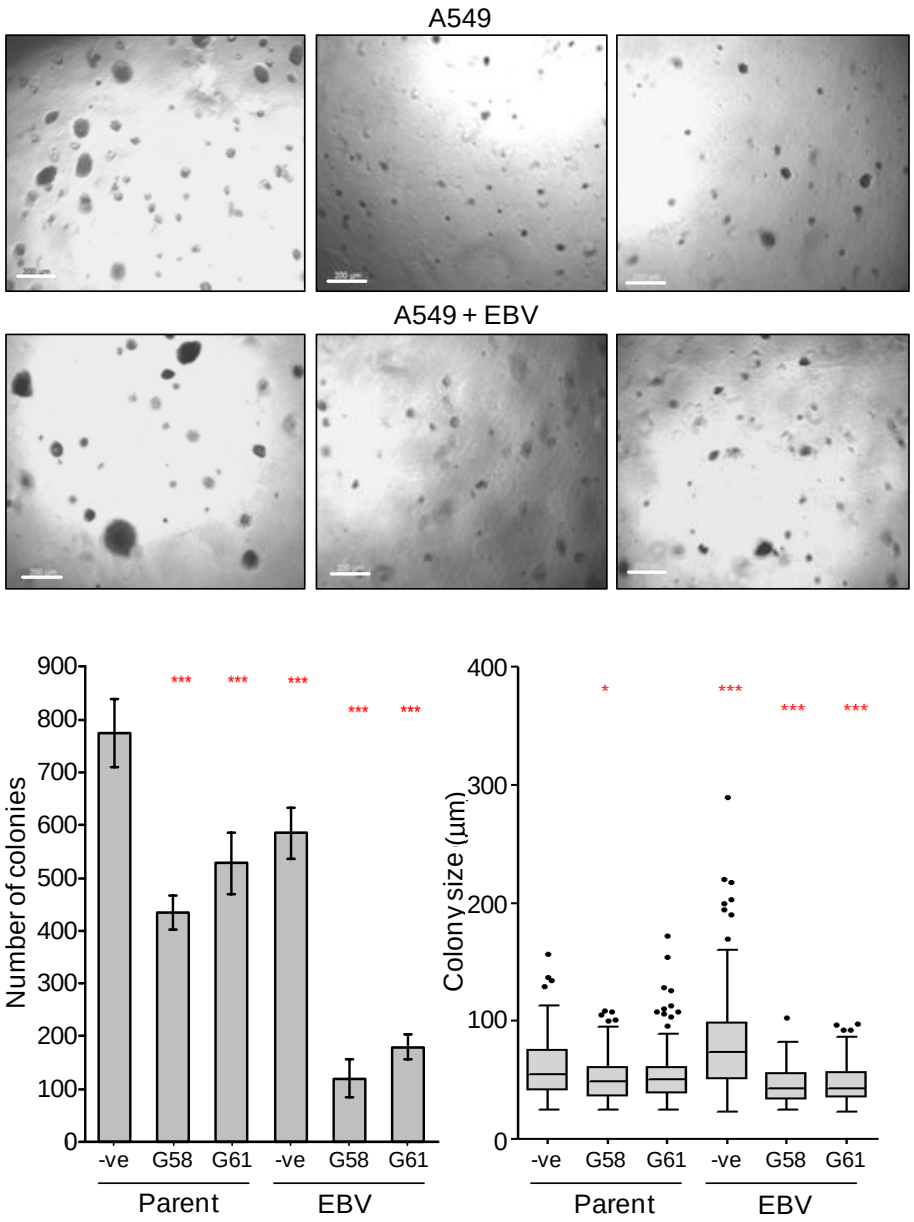


Figure 5. 10 Inhibition of the HH signalling pathway impacts on A549 cell anchorage independent growth

Cells plated out in triplicate at 5 x10^3 cells per well in a 12 well plate in 0.33% agarose solution containing 10% FCS and 1% RPMI, on a base of 1% agarose/ 1% RPM1/ 10% FCS. Inhibitors were added to a final concentration of 25 µM of GANT58 (G58) and 12.5 µM GANT61 (G61). Cells were allowed to form colonies for 2 weeks. Three, non-overlapping, images were taken per well with an x4 objective lens. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted (P relative to control, n=3). Bars= 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, p<0.05; ** p<0.01; *** p<0.001

5.2.3 The ability to form spheres is enhanced by rEBV infection and dependent on HH signalling

As with the C666.1 cell line, it was important to show that EBV induced HH signalling and CSC marker expression correlated with an induction of stem cell-like characteristics. Again the sphere-forming assay was used to assess the tumoursphere forming potential of rEBV infected and control uninfected CNE2 and A549 cells. Both rEBV infected CNE2 (Figure 5. 11) and A549 (Figure 5. 12) cell lines displayed an increased ability to form spheres in this assay, and the spheres that formed were larger than those produced by the parental cell lines demonstrating that these cells, like C666.1, contain cells with CSC-like properties. Furthermore, inhibition of the HH pathway with either GANT58 or GANT61 was sufficient to inhibit tumoursphere formation and growth. Again the CNE2 cell line was highly sensitive to GANT61 treatment, which completely obliterated sphere formation at a concentration of 25 μM, whereas the A549 cell line was more sensitive to GANT58 treatment.

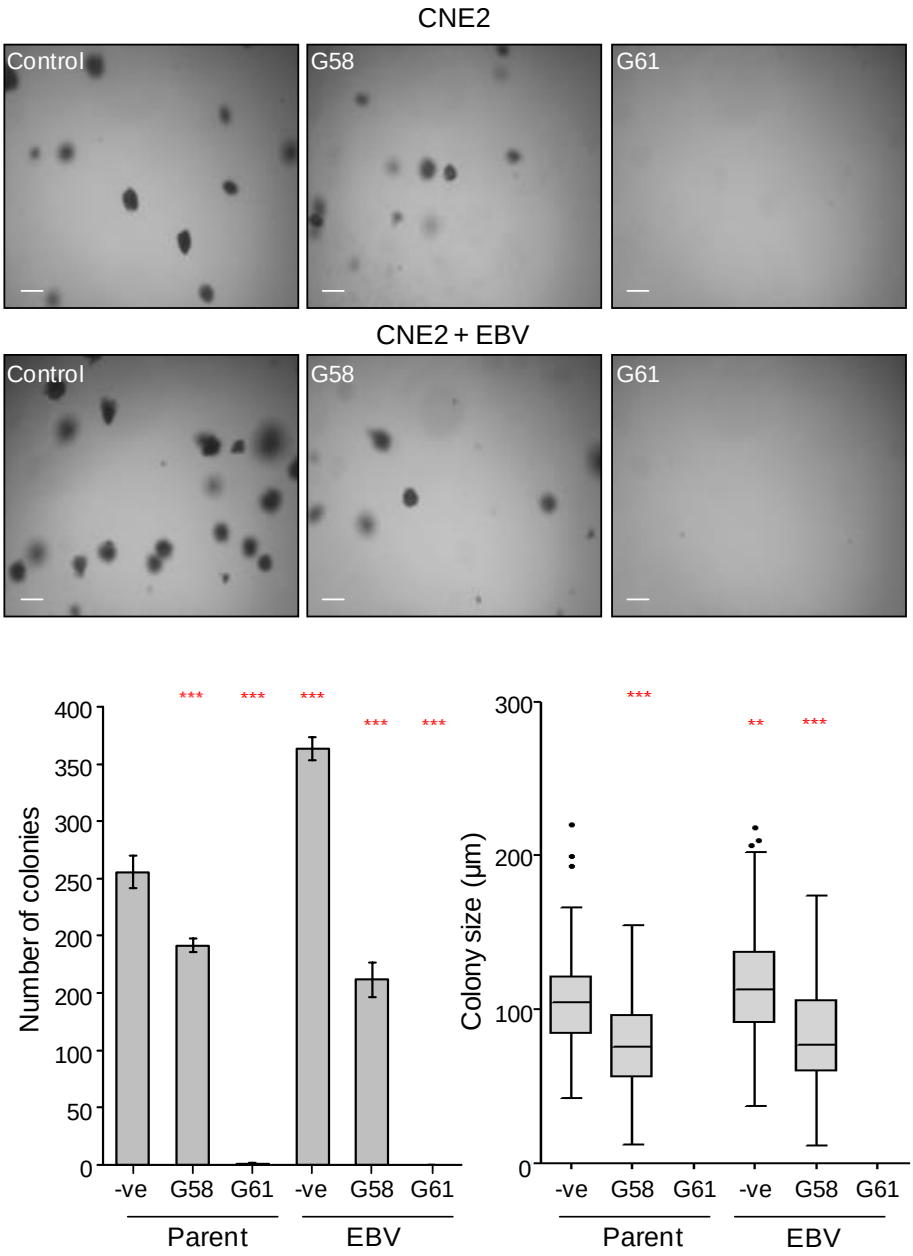


Figure 5. 11 Inhibition of the HH signalling pathway inhibits sphere formation in the CNE2 cell line

Cells were plated in triplicate at 1000 cells per well in 0.33% agarose solution containing 1%RPM1 and growth supplements. GANT58 (G58) and GANT61 (G61) used at a concentration of 50 µM and 25 µM total well volume respectively. Cells were allowed to form colonies for 3 weeks. Four, non-overlapping, images were taken per well with an x10 objective. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted using a box and whisker plot (P relative to control, n=3). Bar = 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, $p<0.05$; ** $p<0.01$; *** $p<0.001$

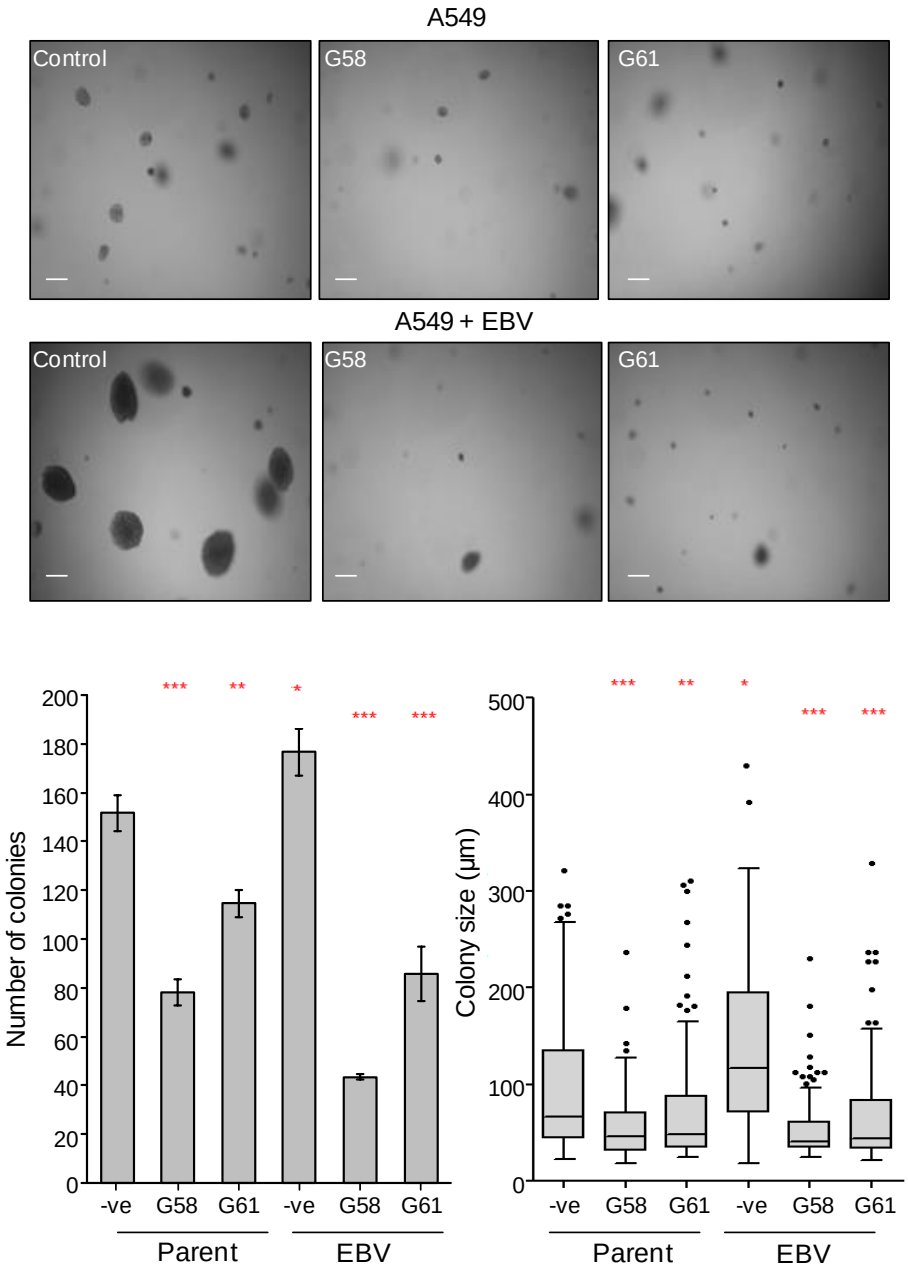


Figure 5. 12 Inhibition of the HH signalling pathway inhibits sphere formation in the A549 cell line

Cells were plated in triplicate at 1000 cells per well in 0.33% agarose solution containing 1%RPM1 and growth supplements. GANT58 (G58) and GANT61 (G61) used at a concentration of 25 µM and 12.5 µM total well volume respectively. Cells were allowed to form colonies for 3 weeks. Four, non-overlapping, images were taken per well with an x10 objective. (A) Representative image of each treatment. (B) Number of colonies (P relative to control, n=3). All colonies of >25 µm were counted for each treatment. (C) The maximum diameter of all colonies within each photographed field of view were measured and plotted using a box and whisker plot (P relative to control, n=3). Bar = 100 µm. Statistical analysis of colony number and size using T-test and one-way ANOVA followed by a Tukey's multiple comparison respectively, where *, p<0.05; ** p<0.01; *** p<0.001

5.3 Hedgehog signalling is required for stable latent infection in the C666.1 cell line

Whilst most of the studies outlined this far focus on the impact of HH signalling on various phenotypic effects in rEBV infected epithelial cells, it was decided to investigate the consequences of HH pathway inhibition on the behaviour of EBV in latently infected epithelial cells. C666.1 cells were cultured in the presence or absence of GANT58 or GANT61, used at 25 µM and 12.5 µM respectively, in combination with 50 µM 12-O-tetradecanoylphorbol-13-acetate (TPA) for 48 hr. RT-PCR and western blot analysis Figure 5. 13A confirmed increased expression of the immediate early (IE) protein, BZLF1, upon treatment with both GANT58 and GANT61 when combined with TPA. BZFL1 was not found to be induced in TPA treated cells; however some induction was seen in the GANT58 and GANT61 treated cells in the absence of TPA. These results indicate the requirement of HH signalling in the maintenance of stable latent infection.

Further interrogation utilised immunofluorescence staining (Figure 5. 13B); treatment of cells with TPA and GANT inhibitors was found to induce the expression of BZLF1 in approximately 5% of cells. This combined effect was greater than the BZLF1 induced by either GANT58 or GANT61 treatment alone, or TPA treatment.

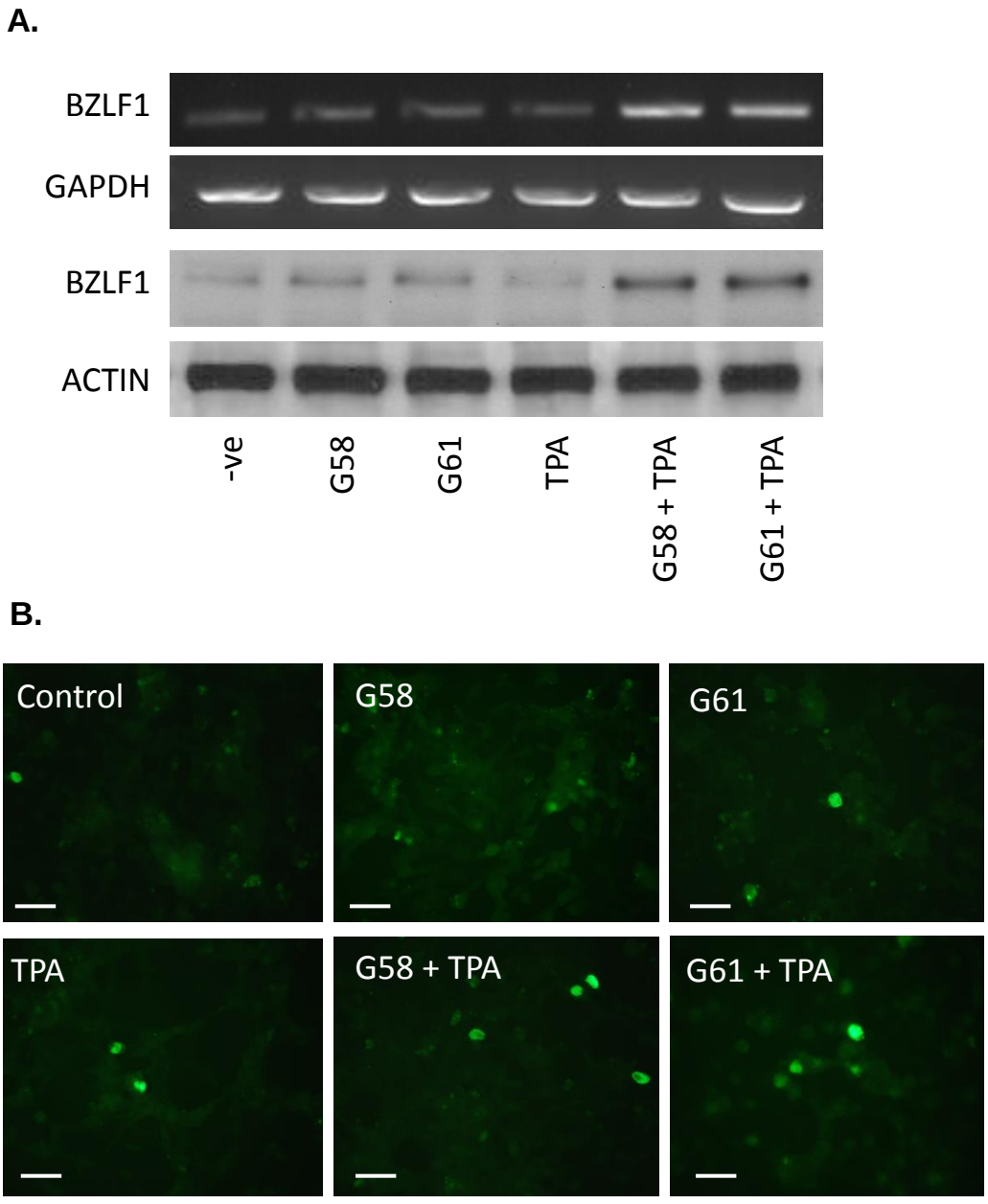


Figure 5. 13 GANT treatment sensitises the C666.1 cell lines to TPA induced lytic cycle
(A) RT-PCR and western analysis, and (B) immunofluorescence demonstrating immediate early antigen BZLF1 induction upon GANT58 or GANT61 treatment in combination with a chemical inducer of differentiation, TPA. Bar = 50 µm.

5.3.1 Hedgehog signalling is required for stable latent infection in the EBV infected epithelial cell lines

The effect of inhibiting the HH signalling pathway on the expression of EBV latent protein expression was investigated. GANT58 and GANT61 inhibitors, used at 25 μM and 12.5 μM respectively, were applied to CNE2 and A549 cell lines stably infected with rEBV for 48 hr. Protein and RNA was collected for analysis by RT-PCR and western analysis (Figure 5. 14).

Induction of BZFL1 was found in the TPA and GANT double treated cells, particularly upon GANT58 and TPA treatment. GANT61 and TPA did not stimulate BZLF1 above the levels seen in the TPA only treated cells suggesting that 12.5 μM of GANT61 was insufficient to inhibit the pathway in this experiment. However, 25 μM GANT58 alone was sufficient to induce BZFL1 in both cell lines.

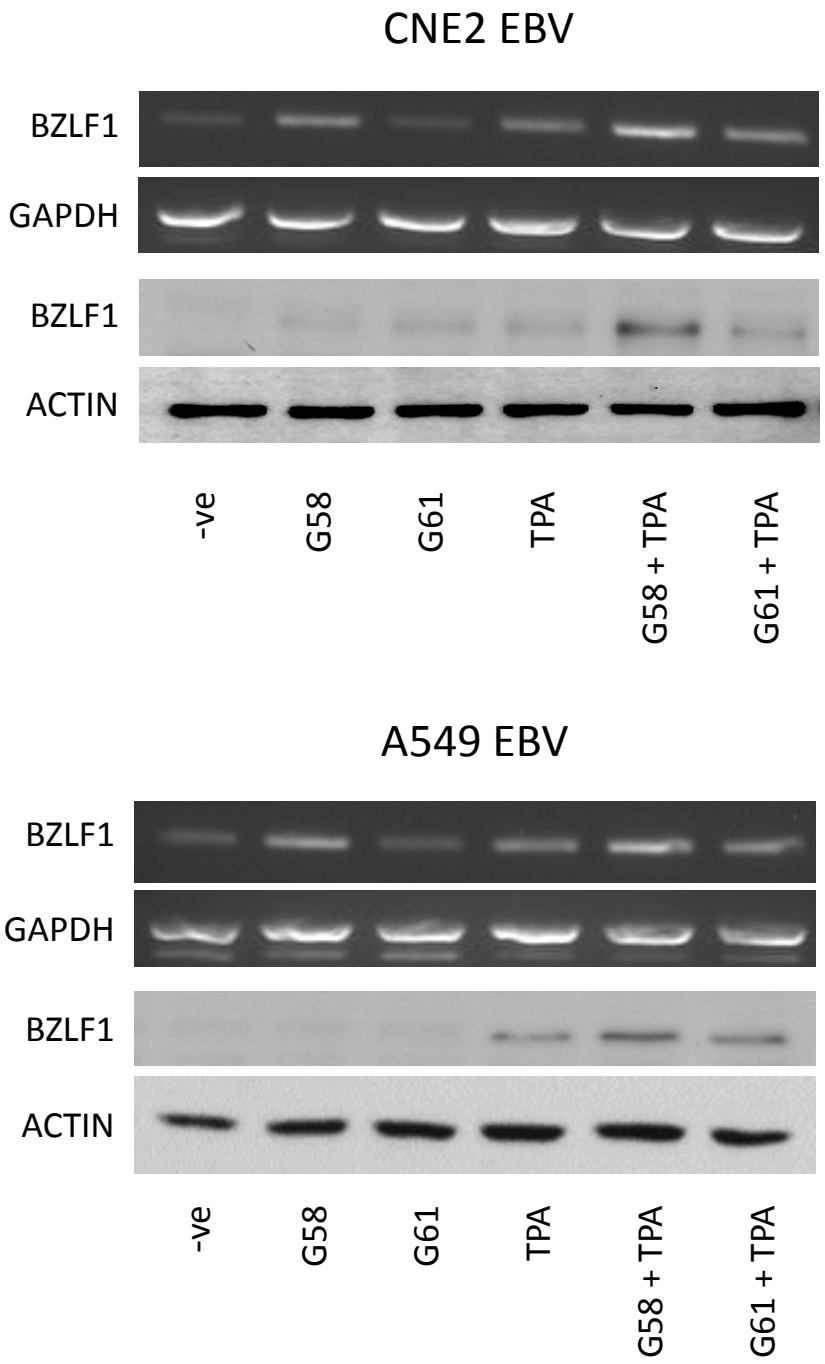


Figure 5. 14 HH inhibition sensitises CNE2 and A549 rEBV infected cell lines to TPA induced lytic cycle

RT-PCR and western analysis of (A) CNE2 rEBV and (B) A549 rEBV cells upon GANT58 (25 µM) or GANT61 (12.5 µM) treatment in combination with a chemical inducer of differentiation, TPA (25 µM)

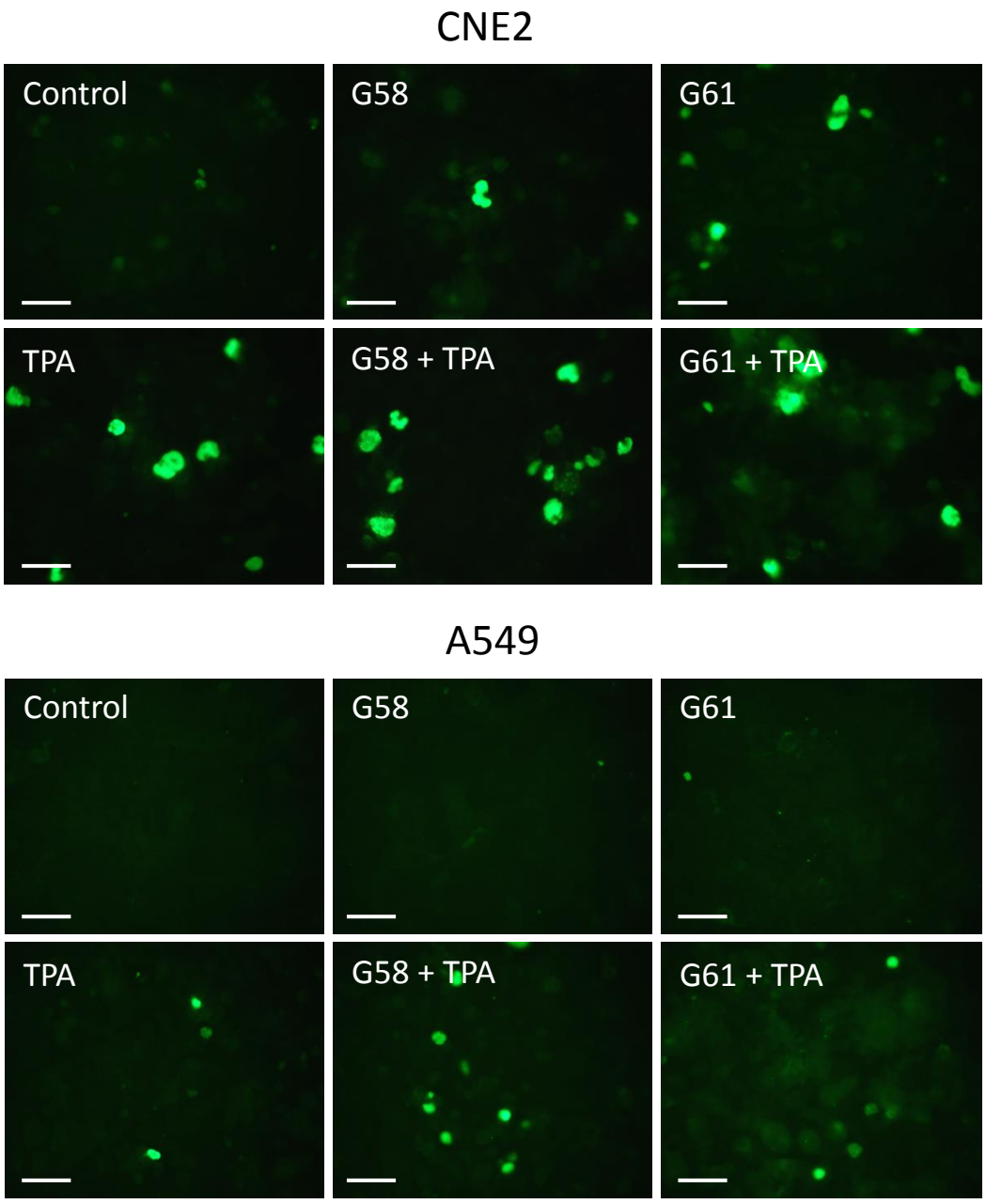


Figure 5. 15 Immunofluorescence staining for BZLF1 in CNE2 and A549 rEBV infected cell lines treated with TPA and HH inhibitors
Immunofluorescence staining demonstrates HH inhibition, using 25 µM GANT58 or 12.5 µM GANT61, sensitises (A) CNE2 rEBV and (B) A549 rEBV cells to TPA induced lytic cycle. Bar = 50 µm.

5.4 DISCUSSION

HH signalling is required for the proliferation of CSC populations in a number of tissue types (Clement et al., 2007, Samarzija and Beard, 2012). Studies on colon cancer epithelial cells have demonstrated that down regulation of both GLI1 and GLI2 expression by RNA interference impacts on BrdU incorporation; supporting findings that GANT61, which targets both GLI1 and GLI2, is more effective at inhibiting proliferation in C666.1 cells than GANT58, which targets GLI1 only (Varnat et al., 2009). HH signalling is also known to influence cell proliferation through CCND1 and CCND2, which have previously been found to be upregulated in NPC gene expression arrays (Shi et al., 2006, Zeng et al., 2007b). Whilst CCND1 expression was present in all tissue samples by microarray analysis, there was no significant upregulation in the NPC tumour samples, and inhibition of the HH signalling pathway in C666.1 produced only marginal effects on CCND1 expression by RT-PCR. This suggests that the HH dependent nature of cell proliferation does not require, or is not solely dependent on, the expression of CCND1. Additionally crosstalk between HH and other stem cell maintenance pathways may be responsible for the maintenance of cell proliferation; for instance NOTCH (VanDussen et al., 2012) and WNT (Kuhnert et al., 2004) can both modulate proliferation in epithelial cells.

Whilst HH inhibition was shown to impact on SC/CSC marker expression in EBV positive carcinoma and rEBV infected epithelial cell lines *in vitro*, as reported in Chapter 4, it was necessary to show that this coincided with an inhibition of CSC-like characteristics in these cells. Stemness *in vitro* can be assessed by the ability of progenitor cells to form colonies in specialised serum-free medium supplemented

with defined growth factors (Song et al., 2011). While so-called “tumourspheres” could be propagated from the C666.1 cell line, indicating the existence of CSC-like cells in this cell line, tumourspheres could not be grown from the hTert immortalised OKTF6 or NP460 cell lines. Importantly, propagation of spheres from the C666.1 cell line was significantly reduced in the presence of GANT58 and GANT61, demonstrating that HH signalling is required for the CSC-like cells residing in the bulk population to proliferate. It is interesting to note that around 300 of 1000 cells formed tumourspheres, which may suggest around 30% of cells have sphere forming capacity. While CSCs are normally considered to represent a minor subpopulation of tumour cells, and exist as a small subpopulation in cancer derived cell lines, stem cell/ CSC profiling of the C666.1 cell line revealed expression of CD133 and CD44v6 in 48% and 57% of cells respectively. This may suggest a role in the dysregulation of cell signalling pathways, including HH, in the expansion of the CSC population in NPC. However, this percentage of sphere forming cells may not provide a true representation of the number of CSC-like cells in a cell line, due to the diffusion of CSC growth factors from true stem cells which may stimulate the growth of surrounding cells.

It is now widely accepted that whilst slow cycling, stem cells and CSCs display increased resistance to chemotherapy or genotoxic agents, anchorage independent growth, cell migration, and self-renewal. The HH dependent nature of anchorage independent growth and cell proliferation were investigated. The C666.1 cell line demonstrated a dose dependent inhibition of both anchorage independent growth and cell proliferation, as assessed by BrdU incorporation, following treatment with GANT58 and GANT61. Whilst the proliferation of CSC-like cells was not interrogated

directly, as these cells were not isolated from the bulk population, this study found that HH signalling is required for the growth of both the C666.1 cell line and parental and rEBV infected A549 and CNE2 cells. This HH dependent proliferation is also demonstrated in BCC; clinical trials targeting the HH signalling pathway have demonstrated that drugs such as Vismodegib can decrease tumour size (Cirrone and Harris, 2012) suggesting these inhibitors may target CSC and non-CSC populations.

Anchorage independent growth is viewed as an important property of CSCs, allowing the migration and metastasis of cells that no longer require attachment to the extracellular matrix to survive, and is correlated with cell tumourigenicity (Shin et al., 1975). In this respect, the targeting of CSC populations within a tumour is an attractive idea and, in the case of NPC, may be achieved through the selective targeting of the HH pathway. Indeed, inhibition of the HH pathway has been shown to impact on metastasis and invasion of pancreatic cancer, which like NPC demonstrates aberrant activation of the HH signalling pathway. Treatment of a metastatic pancreatic cancer cell line with the SMO inhibitor cyclopamine not only decreased cell proliferation, but also induced apoptosis and reduced migration/invasion. Furthermore, whilst HH inhibition did not reduce primary tumour growth in orthotropic xenograft mice models, it significantly reduced the rate of tumour metastasis, an effect that would impact positively on patient survival (Feldmann et al., 2007). NPC is a highly invasive and metastatic disease (Hsu and Tu, 1983), partly due to the late stage of prognosis due to tumour growth in a “silent” anatomical site (Wang et al., 2012a). Distant metastasis is the most important factor impacting on disease survival (Teo et al., 1996) and as such treatment that specifically targets cancer cell metastasis by inhibiting anchorage independent

growth and survival would likely improve prognosis. Anchorage independent growth is also induced in gastric carcinoma tumoursphere cells compared to adherent cells, and these proposed CSC containing tumoursphere cells also demonstrate increased expression of CD44 and CD133, and increased activity of the HH signalling pathway (Song et al., 2011). In this study by Song et al., (2011) blocking the HH pathway was seen to reduce the ability of gastric carcinoma cell lines to form tumourspheres.

The work of Mani et al., (2008) introduced the notion that the induction of an EMT in mammary epithelial cells could result in the acquisition of mesenchymal traits and the upregulation of stem cell-associated markers. Whilst an EMT functions during development to facilitate the migration of stem and early progenitor cells regulating tissue patterning, the aberrant reprogramming of cells and the generation of an EMT endows CSC-like cells with invasive and metastatic properties. rEBV infection of CNE2 cells was associated with the induction of an EMT and the expression of EMT associated markers, the same was not true for A549 cells. However, the fact that stem cell marker expression and the acquisition of CSC-like characteristics was observed in both CNE2 and A549 cells demonstrates that the induction of these characteristics is not dependent on the induction of an EMT in rEBV-infected epithelial cells. Preliminary investigations failed to confirm if the induction of EMT in rEBV infected CNE2 cells was HH pathway dependent, the possibility exists that other onco-developmental pathways may cooperate with HH in this cell line to induce an EMT.

EMT can be induced by numerous cell signalling pathways. As such, it is possible that dysregulation of a single pathway may not be sufficient to induce an EMT and

multiple pathways must act in concert. Members of the TGFβ family of proteins are known to induce an EMT in both normal differentiation and cancer progression (Yang and Weinberg, 2008, Massagué, 2008). It is also known that TGFβ has a role in stem cell maintenance and can act in concert with other signalling pathways, such as the NOTCH, WNT and integrin pathways, to induce an EMT (Polyak and Weinberg, 2009). Furthermore in NSCLC, SHH expression has been found to be regulated by TGFβ, and is associated with the induction of an EMT and acquisition of a highly motile and invasive phenotype (Ali et al., 2011).

Bone morphogenic proteins (BMPs) are members of the TGFβ family of proteins and are expressed in many adult tissues. BMPs have a role in maintenance and renewal (Balemans and Van Hul, 2002, Hogan, 1996, Chen et al., 2004) and disruption of BMP signalling has been observed in a number of cancers leading to decreased apoptosis, increased proliferation and metastasis (Alarmo et al., 2007, Miyazaki et al., 2004, Dai et al., 2008). Additionally, BMP has been associated with an EMT; in ovarian cancer SLUG and SNAIL are induced by BMP4 and the accompanying EMT was found to be dependent on BMP4 as inhibition with Noggin blocked the transition (Thériault et al., 2007).

The WNT signalling pathway, as mentioned above, may act in concert with TGFβ in the induction of EMT and can itself induce this transition. Inhibition of β-catenin degradation, by reduced phosphorylation by glycogen synthase kinase-3β (GSK-3β), allows increased nuclear translocation of β-catenin where it acts to increase the expression of EMT inducing transcription factors. β-catenin overexpression is not sufficient to induce EMT as colorectal carcinomas harbouring an APC mutation, and

so displays increased levels of β-catenin, do not usually display a mesenchymal phenotype.

Recently, a study by Kong et al., (2010) has identified a role for LMP2A in the epithelial mesenchymal transition (EMT). Here overexpression of LMP2A in the CNE2 and SUNE1 cell lines resulted in down regulation of epithelial markers and upregulation of mesenchymal markers. In addition, Kong et al., (2010) also demonstrated a link between EMT transition and the expression of markers expressed by embryonic and adult stem cell populations suggesting that the expression of LMP2A increases the percentage of side population-like and/or CSCs in these epithelial cell lines.

Finally, this study suggests that inhibition of HH signalling may facilitate the reactivation of lytic cycle in C666.1 cells, as demonstrated by increased expression of BZLF1 in approximately 5% of cells. Further studies may wish to investigate lytic reactivation of EBV in cells treated with HH pathway inhibitors, and characterise the expression of other EBV lytic genes and proteins in response to different HH inhibitors. However, the C666.1 cell line may prove to be a flawed model as it is known to carry mutations in genes that are required for efficient virus replication; like the non-producing Raji EBV strain (Polack et al., 1984), it has a BALF2 deletion and cannot replicate its DNA during lytic cycle. Whilst further interrogation into the replication status of EBV in cells treated with HH pathway inhibitors was not undertaken, we postulate that a HH targeted therapy in NPC may not only decrease proliferation, migration/invasion, and induce apoptosis of HH dependent cancer cells, it may have a role in inducing lytic reactivation to assist antiviral therapy.

CHAPTER SIX: GENERAL DISCUSSION

“Nothing in science has any value to society if it is not communicated, and scientists are beginning to learn their social obligations.”

— Anne Roe

The Making of a Scientist (1953), 17.

6.1 Summary of the findings of this thesis

Recent investigations into the aetiology of NPC have identified dysregulation of a number of stem cell maintenance signalling pathways (WNT, NOTCH, TGFβ, ACTIVIN and BMP) implicated in stem cell maintenance. This study, along with the work of Yue et al., (2012), has now identified dysregulation of the HH signalling pathway in this disease. While HH signalling pathway genes have previously been reported to be over expressed in NPC (Yang et al., 2001, Yip et al., 2006, Vera-Sempere et al., 1997, Song et al., 2006), the contribution of this signalling pathway to NPC pathogenesis is unknown. Work outlined in this thesis builds upon recent findings and demonstrates the frequent dysregulation of the HH signalling pathway in primary NPC tumours and the authentic EBV positive NPC cell line C666.1. Whilst work presented here point toward a role for EBV-driven SHH ligand induction as a key factor in HH pathway activation, further work may wish to interrogate NPC tumours for the presence of mutations in pathway proteins such as PTCH, SMO and SUFU. These genes are frequently mutated in cancers that display aberrant HH signalling such as BCC which, like NPC, is characterised by an undifferentiated epithelial phenotype, and mutations would implicate multiple mechanisms of HH dysregulation in NPC.

Activation of the HH signalling pathway was associated with the induction of putative stem cell markers, a number of which have previously been identified in NPC (Alajez et al., 2010, Song et al., 2009, Su et al., 2011, Lun et al., 2012). These included SC/CSC marker genes known to be induced by EBV latent gene expression in EBV negative nasopharyngeal epithelial cells (Kong et al., 2010, Kondo et al., 2011). Work outlined here reveals the HH dependent nature of stem cell marker expression in

EBV-infected carcinoma cell lines *in vitro*, and are supported by the studies of Lun et al., (2012) who demonstrated increased expression of stemness-associated genes and transcription factors in the cancer stem cell populations isolated from the C666.1 cell line.

Data presented in Chapter 4 demonstrate the ability of EBV to directly engage and activate HH signalling through the autocrine induction of SHH ligand and goes on to demonstrate that this activation is associated with the induction of stem cell marker gene expression and acquisition of CSC-like characteristics. Furthermore, preliminary experiments allude to the role of a number of EBV latent genes in the establishment of an activated HH signalling pathway and the expression of these stem cell markers. Interestingly, while EBNA1, LMP1 and LMP2A were all found to induce SHH ligand and activate the HH pathway, only LMP1 and LMP2A were able to induce the expression of stem cell marker genes. However, the fact that LMP1 and LMP2A, unlike EBNA1, were found to induce different stemness-associated marker genes suggests that these oncogenic membrane proteins may engage additional stem cell maintenance programmes that cooperate with the HH pathway to induce stem cell/CSC marker expression and induce CSC-like characteristics. These findings support previous studies showing that the EBV-encoded latent proteins LMP1 and LMP2A can induce the expression of stemness-related genes and impose CSC-like characteristics on NPC-derived cell lines *in vitro* (Kong et al., 2010, Kondo et al., 2011).

The expression of EBV latent protein LMP1 is induced upon the establishment of latency but is detected at low levels in established tumours (Strong et al., 2013).

Whilst only hypothetical, the finding that multiple EBV latent proteins can activate the HH signalling pathway may reflect different functions of these proteins. LMP1 may act to initially dysregulate the HH signalling pathway in premalignant cells, allowing stable latent EBV infection prior to clonal expansion, whereas LMP2A may subsequently maintain this signalling dysregulation. Indeed, LMP1's ability to induce the initial dysregulation of this pathway is supported by the finding that, compared to EBNA1 and LMP2A, this viral protein could most strongly activate HH signalling. This effect may be linked to activation of NFκB and mTOR, which are known to stimulate HH ligand expression and activate GLI1 activity respectively (Kasperczyk et al., 2009, Wang et al., 2012c). Furthermore, activated HH signalling appears to be required for latent infection as HH pathway inhibition, in combination with TPA treatment, an inducer of differentiation, induces the expression of the immediate early lytic protein, BZFL1. The discovery that TPA inhibits HH signalling further supports the importance of HH signalling in the maintenance of EBV latency, presumably by maintaining an undifferentiated cellular phenotype.

Finally, work presented demonstrated the importance of a dysregulated HH signalling pathway on the expression of stem cell/CSC marker expression and the acquisition of stem cell/CSC characteristics. EBV induced upregulation of stem cell marker expression was found to have phenotypic consequences that impacted on epithelial cell proliferation and ability of cells to form tumourspheres. Studies, which have utilised this assay, have relied on the previous demonstration that isolated putative stem cell populations from tumours in nude mice. NPC derived "side population" cells, isolated by their increased Hoechst dye effluxing ability, have been shown to have increased tumour-initiating capacity (Wang et al., 2007). This side population

phenotype was later found to be induced by LMP2A expression, with a concomitant induction of an EMT, upregulation of a number of putative stem cell markers and increased tumoursphere forming capacity (Kong et al., 2010). Similarly, expression of LMP1 has previously been found to increase $CD44^{high}/CD24^{low}$ expression, a phenotype that is dependent on HH signalling in breast cancer (Tanaka et al., 2009), and induce tumour sphere formation and tumour initiation in nude mice (Kondo et al., 2011). While an increased expression of CD44 and other stem cell/CSC markers, and enhanced tumoursphere formation in response to EBV infection has been presented here, further work is required to demonstrate that this translates to increased tumour initiation in animal models and investigate if HH inhibition can reduce tumour burden. These studies may demonstrate that HH is a potential therapeutic target for the treatment of EBV associated tumours.

6.2 Targeting the HH signalling pathway in NPC treatment

The role of HH signalling in the pathogenesis of NPC appears to be highly significant owing to the fact that dysregulation of this pathway may involve multiple mechanisms, rather than centralising around a single driving mutation or the overexpression of SHH ligand. Microarray analysis highlights overexpression of a number of different HH pathway components in different NPC tumours which can lead to the activation of downstream targets. Increasingly cancers are being characterised and divided into subtypes that respond differently to therapeutic intervention. As such future work may wish to categorise this HH dysregulation in NPC into distinct subtypes. Furthermore, interrogation into the driving mutation behind different NPC tumours may find that, whilst there is interaction and co-operation between multiple signalling pathways in tumour development, tumours may

be dependent on one pathway at the expense of another. This has been the case in meduloblastoma for which transcriptome analysis have identified four distinct tumour subgroups: WNT, SHH, Group C and Group D, where the WNT and HH signalling pathways are believed to play the predominant role in pathogenesis of distinct tumour subtypes (Northcott et al., 2011). This is the age of personalised medicine, and NPC should be regarded not as a single disease but as an overriding classification of a carcinoma that can be caused by a number of distinct mutations; further interrogation will likely distinguish different subtypes of tumour that have different responses to different treatments.

This thesis identifies, for the first time, the role of EBV in the dysregulation of the HH signalling pathway not only in NPC cell lines but in epithelial carcinoma cell lines of other origin. Furthermore, activation the HH signalling pathway was seen to induce proliferation, anchorage independent growth and the induction of a cancer stem cell-like phenotype, which may contribute to the tumourigenic potential of these cells.

Further work would likely investigate the ability of HH inhibitors to impede tumour cell growth in animal models to demonstrate the dependence of NPC growth on the HH signalling pathway *in vivo*, highlighting the potential use for HH inhibitors in therapeutic intervention. The transition of this work toward a therapeutic intervention is particularly exciting as HH inhibitors have already been successfully implemented in the treatment of a number of human cancers which demonstrate deregulated HH signalling (Von Hoff et al., 2009, Rudin et al., 2009, Irvine and Copland, 2012), and Vismodegib, a SMO inhibitor, has shown positive results in phase I trials (LoRusso et al., 2011, Sekulic et al., 2012) and achieved FDA approval (Von Hoff et al., 2009).

Recently Vismodegib has progressed to phase II clinical trials for the treatment of a diverse range of cancers; including BCC, ovarian, pancreatic, and gastric cancer, and basal-cell nevus syndrome (Cirrone and Harris, 2012, Catenacci et al., 2012, Tang et al., 2012, Kaye et al., 2012, Berlin et al., 2013). As such, studies may wish to investigate the anti-tumour effects of a number of different HH inhibitors, including those with FDA approval, on pre-established NPC tumour burden by measuring tumour growth and metastasis.

Whilst EBV has been demonstrated to have a causative role in the pathogenesis of NPC our understanding of how the virus alters cell signalling and associated characteristics leading to oncogenic progression, and the stage during NPC pathogenesis that this dysregulation occurs, is still incomplete. Whilst inhibitors of the HH signalling pathway may prove a useful tool in the treatment of NPC, the induction of latently EBV infected epithelial cells into lytic cycle may provide a viable therapeutic strategy. In NPC, the latent EBV viral protein expression pattern is restricted to gene products (both RNA, miRNAs and proteins) that are necessary for tumour growth but, in addition, may allow evasion of the host immune system (Middeldorp et al., 2003). The induction of lytic cycle activates the expression of immediate early, early and late viral antigens that are known to be immunogenic (Hislop et al., 2007) and allows tumour cells to be targeted by the host immune system and antiviral therapy (Wildeman et al., 2012). Indeed, Wildeman et al., (2012) demonstrated that lytic induction by gemcitabine (GCB) and valproic acid (VPA), as measured by the increased expression of ZEBRA (BZFL1), as well as other EBV lytic genes, increased the cytotoxicity of valganciclovir (GCV) treatment in the C666.1 cell line. They went on to demonstrate that a combined therapy of GCB, VPA and GCV;

cytolytic virus activation therapy, demonstrated a biological response in three patients with end-stage NPC and even reduced tumour size. Additionally, an increase in viral DNA load in the blood during recovery periods between therapies was postulated to represent a recovering immune system with increased clearance of lytic reactivated cells; however, no increase in anti-EBV immune reactivity was observed (Wildeman et al., 2012). This work demonstrates that HH may have a role in maintaining viral latency and as such inhibition of the pathway may have a dual effect to target both tumour cell proliferation and assist in the immune detection of EBV infected tumour cells.

Finally, we cannot overlook the case for a prophylactic EBV vaccine in the prevention of this disease, a top down approach to NPC treatment which would bypass the need for more the more complete comprehension of how cell signalling pathways are dysregulated in human cancer; an area of research which over the next few decades is likely to shed light on the mechanisms of oncogenic transformation.

6.3 Implications of this work on our understanding of the origin of NPC

Finding presented in this thesis, along with recent developments by other research groups, impact on our understanding on the aetiology of NPC by supporting the existence of CSC populations within NPC tumours. A number of groups have identified sub-populations of cells in NPC that express stem cell/CSC markers or have CSC-like characteristics (Wang et al., 2007, Kong et al., 2010, Zhang et al., 2010, Lun et al., 2012); however, the origin of these cells is currently under debate. While work presented in this thesis suggest that EBV is involved in the expansion of this population, through aberrant activation on HH and/or other stem cell

maintenance pathways, a number of possibilities exist. Firstly, EBV may preferentially infect and subsequently expand an undifferentiated early progenitor cell, such as a stem cell, which already expresses stem cell markers. Alternatively, EBV engagement of stem cell maintenance pathways may promote the dedifferentiation of a more committed cell inducing the expression of stem cell/CSC-markers and the acquisition of CSC-like characteristics. Whilst findings from *in vitro* studies suggest that EBV may induce epithelial cell de-differentiation, it cannot rule out the possibility that stable latently infected epithelial cells are generated from CSC-like populations resident in the cell lines used in this study. Future studies should aim to purify these resident populations, based on stem cell or SP-like properties, and examine the efficiency with which stable EBV infected cell lines can be generated from sorted and unsorted populations.

The fact that NPC is a relatively rare tumour which displays distinct geographical and ethnic prevalence is interesting given that EBV is a ubiquitous virus that infects greater than 95% of the population worldwide. Although still speculative, it suggests that EBV infection must occur in cells which harbour some genetic mutations, which promote the establishment stable latent infection in an epithelial background. Data in support of this idea come from the recent finding that hTert-immortalised nasopharyngeal epithelial cells, engineered to overexpress cyclin D1, are permissive for stable latent infection with EBV (Tsang et al., 2012), highlighting the importance of specific genetic and/or environmental factors in the initiation of this disease.

In summary, findings presented in this thesis demonstrate that dysregulation of the HH signalling pathway is important for stable latent infection of epithelial cells and,

moreover, contributes to the proliferation and anchorage independent growth of EBV infected epithelial cells *in vitro*. As such, these findings may lead to a novel target for the treatment of NPC and other EBV-associated diseases.

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Review

The role of the EBV-encoded latent membrane proteins LMP1 and LMP2 in the pathogenesis of nasopharyngeal carcinoma (NPC)

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ABSTRACT

Although frequently expressed in EBV-positive malignancies, the contribution of the oncogenic latent membrane proteins, LMP1 and LMP2, to the pathogenesis of nasopharyngeal carcinoma (NPC) is not fully defined. As a key effector in EBV-driven B cell transformation and an established "transforming" gene, LMP1 displays oncogenic properties in rodent fibroblasts and induces profound morphological and phenotypic effects in epithelial cells. LMP1 functions as a viral mimic of the TNFR family member, CD40, engaging a number of signalling pathways that induce morphological and phenotypic alterations in epithelial cells. Although LMP2A plays an essential role in maintaining viral latency in EBV infected B cells, its role in epithelial cells is less clear. Unlike LMP1, LMP2A does not display "classical" transforming functions in rodent fibroblasts but its ability to engage a number of potentially oncogenic cell signalling pathways suggests that LMP2A can also participate in EBV-induced epithelial cell growth transformation. Here we review the effects of LMP1 and LMP2 on various aspects of epithelial cell behaviour highlighting key aspects that may contribute to the pathogenesis of NPC.

1. Introduction

Epstein-Barr virus (EBV) is a human gammaherpesvirus found as a widespread and largely asymptomatic infection throughout the world. The virus exploits the physiology of normal B cell differentiation to persist within the memory B cell pool of the immunocompetent host as a life-long latent infection. EBV replication occurs in both B cells and in mucosal epithelium lining the nasopharynx. It is the aberrant establishment of latent EBV infection at these sites that results in the development of both lymphoid and epithelial tumours [1]. The oncogenic potential of EBV was demonstrated through its association with multiple human malignancies including Burkitt's lymphoma (BL), NPC, Hodgkin's lymphoma (HL), post-transplant lymphoproliferative disease (PTLD), some NK/T-cell lymphomas, and a proportion of gastric carcinomas [1]. Different forms of EBV latent gene expression are observed in these tumours. In NPC EBV latent gene expression is restricted to EBNA1, the LMP2A/B proteins, the EBER and BamHIA transcripts with variable expression of the LMP1 protein [2]. The presence of monoclonal EBV episomes in NPC indicates that virus infection preceded the clonal expansion of the malignant cell population [2]. Limited analysis of premalignant lesions in the nasopharynx also found monoclonal EBV episomes along with LMP1 expression suggesting a role for this viral oncogene in the early stages of NPC pathogenesis [3].

2. Latent membrane protein 1 (LMP1)

LMP1 is one of five key EBV-encoded viral proteins required for B cell immortalisation [1]. While early experiments performed in rodent fibroblasts identified LMP1 as a viral "transforming" gene, the advent of recombinant EBV technology established the requirement for LMP1 in EBV-mediated B cell immortalisation [1]. While expression of LMP1 in human epithelial cells is not normally associated with growth transformation, LMP1 does exert a variety of growth-promoting effects in this cell type [4]. Whereas targeted expression to the epidermis of transgenic mice is associated with epidermal hyperplasia [5], expression in non-transformed epithelial cell lines induces a cellular phenotype similar to "wounded" or psoriatic keratinocytes [6]. When expressed in tumourigenic epithelial cells, LMP1 potentiates anchorage-independent growth and greatly enhances cell motility and invasion [4,7–9]. Taken as a whole, these findings demonstrate that LMP1 can induce profound effects in epithelial cells, many of which may account for its oncogenic properties.

2.1. LMP1 structure and function

Recent reviews have described in detail the structural and functional aspects of LMP1 signalling in epithelial cells [4,7–9].

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ORIGINAL PAPER

Epstein–Barr virus induction of the Hedgehog signalling pathway imposes a stem cell phenotype on human epithelial cells

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Abstract

Nasopharyngeal carcinoma (NPC) is a cancer common in southern China and South East Asia that is causally linked to Epstein–Barr virus (EBV) infection. Here, we demonstrate that NPC displays frequent dysregulation of the Hedgehog (HH) pathway, a pathway implicated in the maintenance of stem cells, but whose aberrant activation in adult tissues can lead to cancer. Using authentic EBV-positive carcinoma-derived cell lines and nasopharyngeal epithelial cell lines latently infected with EBV as models for NPC *in vitro*, we show that EBV activates the HH signalling pathway through autocrine induction of SHH ligand. Moreover, we find that constitutive engagement of the HH pathway induces the expression of a number of stemness-associated genes and imposes stem-like characteristics on EBV-infected epithelial cells *in vitro*. Using epithelial cells expressing individual EBV latent genes detected in NPC, we show that EBNA1, LMP1, and LMP2A are all capable of inducing SHH ligand and activating the HH pathway, but only LMP1 and LMP2A are able to induce expression of stemness-associated marker genes. Our findings not only identify a role for dysregulated HH signalling in NPC oncogenesis, but also provide a novel rationale for therapeutic intervention.

Keywords: EBV; nasopharyngeal carcinoma; cancer stem cell; Hedgehog signalling

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Introduction

Nasopharyngeal carcinoma (NPC) is a highly metastatic cancer that is relatively rare in western countries but is particularly prevalent in South East Asia and southern China [1]. Unlike other squamous carcinomas of the head and neck, the poorly differentiated and undifferentiated forms of NPC are consistently associated with Epstein–Barr virus (EBV) infection. Although the contribution of EBV to NPC development is poorly understood, it is recognized that EBV can alter a variety of cellular processes that contribute to tumour progression [2]. EBV latent gene expression in NPC is restricted to EBNA1, EBER1/2, BARF1, the BamHIA transcripts (BARTs), and variable expression of the oncogenic membrane proteins LMP1 and LMP2A [2]. These proteins and RNAs alter the behaviour of epithelial cell lines in culture, influencing cell growth, differentiation, apoptosis, angiogenesis, motility, and invasion [3,4].

Although the cellular origin of NPC is unknown, an emerging theory proposes that like other carcinomas, NPC may originate from populations of cancer stem cells (CSCs), genetically altered stem cells or early progenitor cells which possess the capacity for unlimited self-renewal [5,6]. Although controversial, evidence in support of this concept comes from studies showing that NPC biopsies contain small numbers of cells expressing embryonic stem cell markers [7], whilst NPC-derived cell lines contain minor subpopulations which express stemness-related genes and possess stem-like characteristics [8–10]. The notion that EBV plays a direct role in the induction and/or maintenance of CSC populations is supported by the demonstration that the EBV-encoded membrane proteins LMP1 and LMP2A can induce expression of stemness-related genes and impose stem-like characteristics on epithelial cell lines *in vitro* [11,12].

The Hedgehog (HH) signalling pathway maintains normal stem cell populations in a number of adult tissues [13]. While HH signalling functions during embryogenesis to regulate tissue morphogenesis and patterning, sustained or aberrant activation in adult tissues can facilitate cancer progression [14]. Dysregulation of the HH pathway has been reported in a range of malignancies, including those of the skin, breast, lung, brain, prostate, and digestive tract [15]. In many cases, this dysregulation is linked to the maintenance of CSC populations [5].