Declaration

I confirm that the thesis I am submitting is wholly my own work unless otherwise stated. No part of this thesis has been accepted or is currently submitted for any degree, diploma or certificate in this University or elsewhere. The work described here was carried out during my time as a graduate student at the Ludwig Institute for Cancer Research under the supervision of Professor Xin Lu.

Investigating the role of iASPP and ASPP2 in Human Carcinoma

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The apoptotic function of p53 is specifically regulated by iASPP and ASPP2 and their additional modulation of p63, a key regulator of squamous epithelial homeostasis, has been demonstrated in mice. In this study the role of iASPP and ASPP2 in human carcinomas was explored with a particular focus on the development of squamous cell carcinomas.

The predominant expression of cytoplasmic ASPP2 is seen in the superficial, terminally differentiated cell layers of normal squamous epithelia and its loss is documented in areas of dysplasia and in squamous cell carcinomas. In addition the absence of nuclear ASPP2 in association with human papillomavirus infection suggests it could be a novel viral target.

Furthermore in a novel mouse model of lung tumourigenesis, the somatic deletion of ASPP2 is shown to promote the over expression of markers associated with specific lung progenitor cells and to be associated with the development of non small cell lung carcinomas.

Conversely nuclear iASPP expression is seen in p63 expressing, replication competent, basal and parabasal squamous epithelial cells and is increased in areas of dysplasia and in squamous cell carcinomas. Moreover the combination of nuclear iASPP, p63 and loss of ASPP2 expression is seen to be associated with cell survival and characterises the invasive edges of lingual squamous cell carcinomas.

The novel interaction of iASPP with ERα is also demonstrated together with the negative impact of nuclear iASPP on ER positive breast cancer survival, potentially identifying iASPP as a novel player in estrogen signalling and an important factor in breast tumourigenesis.

Together the data presented here provide significant corroborative evidence implicating ASPP2 and iASPP in tumourigenesis. Specifically ASPP2 is shown to promote cellular differentiation and inhibit the expansion of proliferative populations whereas nuclear iASPP promotes the survival of potentially neoplastic clones. It is likely that the balance of these proteins is a key factor in determining individual cell fate decisions.

Acknowledgments

This thesis would not have been completed without my husband Alex's support and unwavering good humour. The birth of our son, Oliver, halfway through my research, though wonderful, certainly did bring new meaning to the importance of good 'time management'. I have convinced myself that the last year and a half of full time clinical work combined with continued lab work and writing has instilled in him a good work ethic – I am just thankful his first word was 'Hiya' and not 'Goodbye'! I would also like to thank my parents, who have always prioritised my education and my work and whose emotional and practical support and great advice have always been invaluable.

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Contents

Chapter 2: Materials and Methods

Chapter 5: Results
iASPP in human carcinoma: An oncoprotein

List of Figures

Abbreviations

aa: amino acid

AAT: Acetylated alpha tubulin

APS: Ammonium persulphate

ASPP: **A**poptosis **S**timulating **P**rotein of **p**53

ATCC: American Tissue Culture Collection: The Global Bioresource Center

BADJ: Bronchioalveolar Duct Junction

BASC: Bronchioalveolar Stem Cell

Bbp: bcl-2 binding protein

BSA: Bovine Serum Albumin

CC10: Clara cell 10kDa Protein

CGRP: Calcitonin Gene-Related Peptide

CIN: Cervical Intraepithelial Neoplasia

CK: Cytokeratin

CNS: Central Nervous System

Co-IP: Co-Immunoprecipitation

CS-FBS: Charcoal stripped Foetal Bovine Serum

DAB: 3,3'-Diaminobenzidine

DCIS: Ductal carcinoma in situ

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

E2: 17β-estradiol

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal Growth Factor Receptor

ER: Estrogen Receptor

ERE: Estrogen response element

FBS: Foetal Bovine Serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HBV: Hepatitis B

HCC: Hepatocellular carcinoma

HCV: Hepatitis C

HIER: Heat induced epitope retrieval

HNSCC: Head & Neck Squamous cell carcinoma

HPV: Human Papillomavirus

HRP: Horseradish peroxidase

iASPP: inhibitory member of the ASPP family

IF: Immunofluorescence

IHC: Immunohistochemistry

LICR: Ludwig Institute for Cancer Research

LLETZ: Large Loop Excision of the Transformation Zone

Mdn: Median

MEFs: Mouse embryonic fibroblasts

MEM: Modified Eagle Medium

MEM-PR: Modified Eagle Medium without phenol red

mRNA: messenger RNA

NGS: Normal Goat Serum

NSCLC: Non-small cell lung cancer

O/N: Overnight

PAS: Periodic acid-Schiff

PBS: Phosphate Buffered Saline

PCR: Polymerase chain reaction

PD: Poorly differentiated

PIL: Personal Licence

PPL: Project Licence

PR: Progesterone Receptor

RAI: Rel-A associated inhibitor

Rb: Retinoblastoma

RGB: Red-Green-Blue

RhPV: Rhesus papillomavirus

RNA: Ribonucleic acid

RNAi: RNA interference

RT: Room temperature

RT-PCR: Reverse transcription polymerase chain reaction

SCC: Squamous cell carcinoma

SDS: Sodium dodecyl sulfate

SERMS: Selective estrogen-receptor modulators

SH3: Src homology 3 domain

SmCC: Small Cell Carcinoma

siRNA: Small interfering RNA

SNPs: Single nucleotide polymorphisms

SP-C: Surfactant Protein C

TS: Tris Buffered Saline

TEMED: Tetramethylethylenediamine

TMA: Tissue Microarray

UV: Ultraviolet

WD: Well differentiated

WB: Western Blot

wt: wild-type

Publications

ASPP2 suppresses squamous cell carcinoma via RelA/p65-mediated repression of p63.

Tordella L, Koch S, **Salter V**, Pagotto A, Doondeea JB, Feller SM, Ratnayaka I, Zhong S, Goldin RD, Lozano G, McKeon FD, Tavassoli M, Fritzsche F, Huber GF, Rössle M, Moch H, Lu X. ***Proc Natl Acad Sci U S A.*** *2013 Oct 29;110(44):17969-74.*

Restoring p53 function in human melanoma cells by inhibiting MDM2 and cyclin B1/CDK1-phosphorylated nuclear iASPP.

Lu M, Breyssens H, **Salter V**, Zhong S, Hu Y, Baer C, Ratnayaka I, Sullivan A, Brown NR, Endicott J, Knapp S, Kessler BM, Middleton MR, Siebold C, Jones EY, Sviderskaya EV, Cebon J, John T, Caballero OL, Goding CR, Lu X. ***Cancer Cell.*** *2013 May 13;23(5):618-33.*

Inhibitor of apoptosis-stimulating protein of p53 (iASPP) prevents senescence and is required for epithelial stratification.

Notari M, Hu Y, Koch S, Lu M, Ratnayaka I, Zhong S, Baer C, Pagotto A, Goldin R, **Salter V**, Candi E, Melino G, Lu X. ***Proc Natl Acad Sci U S A.*** *2011 Oct 4;108 (40):16645-50.*

Submitted

ASPP2 controls epithelial plasticity and inhibits metastasis via β-catenin-dependent regulation of ZEB1.

Yihua Wang,*, Fangfang Bu,*, Christophe Royer, Sébastien Serres, James R Larkin, Manuel Sarmiento Soto, Nicola R Sibson, **Victoria Salter**, Florian Fritzsche, Sofia Koch, Casmir Turnquist, Jaroslav Zak, Ludovico Buti, Guobin Wu, Anmin Liang, Patricia Olofsen, Holger Moch, David C Hancock, Julian Downward, Robert D Goldin, Jian Zhao, Xin Tong, Yajun Guo, and Xin Lu. *Submitted to Nature.*

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Morris EV, Cerundolo L, ten Donkelaar C, Lu M, Verrill C, Fritzsche F, **Salter V**, Thalmann G, Hamdy FC, Lu X, and Bryant RJ. *Submitted to Cell Death and Differentiation*

Chapter 1: Introduction

1.1 Classification of Malignancy

The first documented evidence of neoplastic disease can be found in an ancient Egyptian papyrus circa 3000BC, in which the treatment of breast tumours is discussed. The use of the term 'cancer' to describe tumours can be traced to Hippocrates (460-370BC) and later Celsus (28-50BC) and today this emotive term encompasses a wide variety of tumour types with huge variations in their management and ultimate prognosis (American Society of Cancer). The intervening millennia have certainly provided a greater understanding of neoplastic processes, revealing insights into the numerous molecular aberrations resulting in tumour formation and highlighting increasing numbers of potential therapeutic targets.

Clinical management depends initially on the diagnosis of and accurate categorisation of malignancy. In fact, the clinical recognition of tumours is often challenging and at times even the histopathological distinction between 'benign' and 'malignant' cannot be made for an individual tumour. It is certainly not possible, at this time, to decipher the unique molecular fingerprint of every malignancy diagnosed and as such an increasingly complex series of classifications, constantly reviewed to accommodate new research, exists primarily to guide treatment and predict outcomes.

At present malignant tumours are classified according to organ systems and then again according to the specific tissue type from which they arise (Table 1).

Tumors of epithelial origin	Benign	Malignant
Stratified squamous	Squamous cell papilloma	Squamous cell or epidermoid carcinoma
Basal cells of skin or adnexa		Basal cell carcinoma
Epithelial lining of glands or ducts	Adenoma	Adenocarcinoma
	Papilloma	Papillary carcinomas
	Cystadenoma	Cystadenocarcinoma
Respiratory passages	Bronchial adenoma	Bronchogenic carcinoma
Renal epithelium	Renal tubular adenoma	Renal cell carcinoma
Liver cells	Liver cell adenoma	Hepatocellular carcinoma
Urinary tract epithelium (transitional)	Urothelial papilloma	Urothelial carcinoma
Placental epithelium	Hydatidiform mole	Choriocarcinoma
Testicular epithelium (germ cells)		Seminoma
		Embryonal carcinoma
Tumors of melanocytes	Nevus	Malignant melanoma
Composed of One Parenchymal Cell Type		
Connective tissue and derivatives	Fibroma	Fibrosarcoma
	Lipoma	Liposarcoma
	Chondroma	Chondrosarcoma
	Osteoma	Osteogenic sarcoma
Endothelial and related tissues		
Blood vessels	Hemangioma	Angiosarcoma
Lymph vessels	Lymphangioma	Lymphangiosarcoma
Synovium		Synovial sarcoma
Mesothelium		Mesothelioma
Brain coverings	Meningioma	Invasive meningioma
Blood cells and related cells		
Hematopoietic cells		Leukemias
Lymphoid tissue		Lymphomas
Muscle		
Smooth	Leiomyoma	Leiomyosarcoma
Striated	Rhabdomyoma	Rhabdomyosarcoma

Table 1: Nomenclature of Human Tumours
Modified from http://medicinembbs.blogspot.co.uk/2011/02/neoplasia.html)

Histological examination of these tumours often identifies morphological characteristics indicating this origin, though by definition poorly differentiated tumours display few features to assist in their characterisation. In these cases the use of specific stains, such as the Periodic acid–Schiff (PAS) stain to detect mucin, and the more specialised immunohistochemical detection of retained markers, such as cytokeratins in carcinomas, can facilitate pathological classification.

Furthermore the identification of molecular markers indicating the deregulation of key pathways in specific tumour types, such as the detection of the estrogen receptor (ER) in breast carcinomas, has enabled the development of specifically tailored treatments. The identification of novel 'biomarkers', providing prognostic information, and possibly indicating potential therapeutic targets, is a rapidly expanding field of histopathological research. In the future, it is hoped that the analysis of an individual tumours' genome could allow the personalised treatment of individual tumours based on detailed assessment of their molecular make-up.

The development of cancer is considered to occur through the step wise accumulation of genetic aberrations which give neoplastic populations a survival advantage, including the ability to proliferate autonomously, evade apoptosis and replicate indefinitely. In addition these populations are eventually able to invade their surrounding tissues, establish their own blood supply and set up proliferative colonies at distant sites. Furthermore neoplastic cells are able to evade the host immune response and instigate changes within the surrounding microenvironment to facilitate their survival. This can only occur if individual cells are able to circumvent the usually robust mechanisms controlling the propagation of genetic damage, essentially fostering a background of genetic instability (Hanahan & Weinberg 2011).

Proteins considered to contribute to the survival of neoplastic cells are considered to be 'oncoproteins' in contrast to 'tumour suppressor proteins' which act, in a variety of ways, to prevent the initiation and progression of tumours. Essentially the identification of new proteins acting in either manner potentially provides a novel target through which the manipulation of tumour development can be mediated.

1.2 p53

Encoded by *TP53,* located on the short arm of chromosome 17 (17p13.1), p53 has been extensively studied over the 30 years since its initial discovery. Early experiments involved the use of mutant cDNA generated from tumour cells and it was erroneously considered to promote tumour growth for many years. In 1989 Baker *et al* studied the allelic deletions of chromosome 17 in colorectal carcinomas and suggested its frequent deletion could indicate a tumour suppressive function for the wild type p53 protein (Baker et al. 1989). The complexity of p53 activity recognised to date is such that only a very brief overview will be provided here, specifically focusing on aspects of its function pertaining to this investigation.

Nicknamed the 'guardian of the genome'; p53 has a short half-life and basal levels, within normal cells, are low. Its activation, in response to a wide variety of cellular stresses can trigger a range of cellular responses essentially designed to prevent the propagation of DNA damage and thus the development of neoplasia. The result of p53 activation is considered to be dependent on the type of stimulus received and the cellular context in which it occurs, with cells specifically directed towards cell cycle arrest and DNA damage repair, apoptosis or senescence (Levine & Oren 2009).

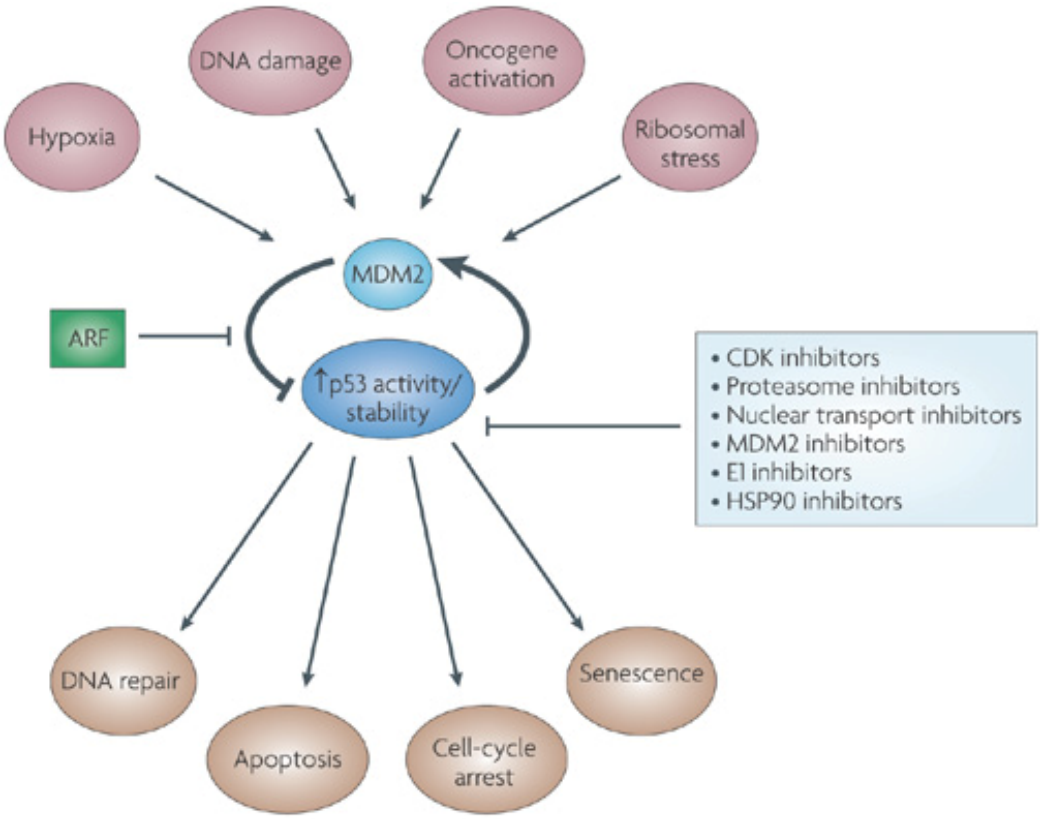

Figure 1.1: p53 activation and effect. p53 is activated by numerous cellular stresses, resulting in cell cycle arrest, apoptosis or senescence depending on stimulus and cellular context. Low basal levels are controlled by the E3 ubiquitin ligase MDM2 whose levels are controlled by p53 in an autoregulatory manner.
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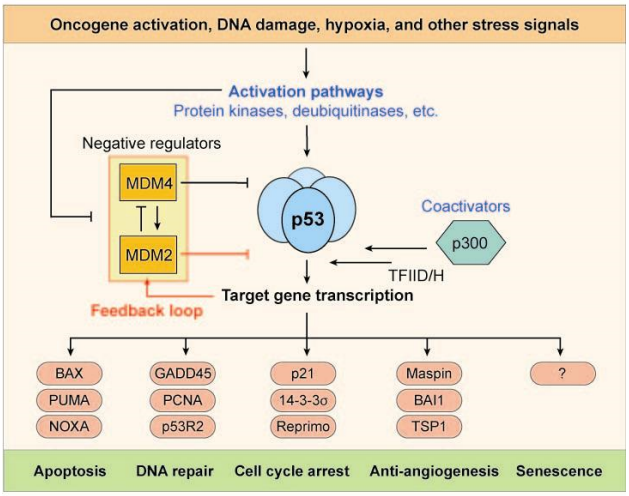
p53 is a versatile protein with a significant proportion of its tumour suppressive effects mediated through its operation as a transcription factor (Laska et al. 2010). These include the promotion of p21 transcription which is, in part, responsible for cell cycle arrest at the entry to S phase and the up regulation of pro-apoptotic proteins such as BAX and PUMA. The presence of p53 co regulators in any given cell can influence the specific outcome of p53 activation.



Joerger AC, Fersht AR. 2008.
Annu. Rev. Biochem. 77:557–82.

Figure 1.2: Core regulatory network of the p53 pathway. This simplified scheme shows the p53 response to cellular stress in more detail including a sample of proteins influenced by its activity. The influence of coregulators such as p300 is depicted together with an illustration of the tight feedback loops existing to modulate its activity.
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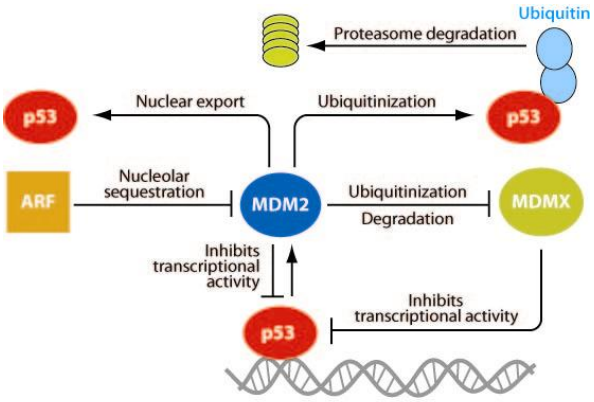
Figure 1.3 demonstrates the complex mechanisms involved in the regulation of p53 levels in more detail.



Shangary S, Wang S. 2009.
Annu. Rev. Pharmacol. Toxicol. 49:223–41

Figure 1.3: Autoregulatory feedback loop of inhibition of p53 by Mdm2. Mdm2 inhibits p53 activity and promotes its proteasomal degradation. Mdmx, a homolog of Mdm2, inhibits, but does not promote the degradation of p53.
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Mutations of p53 are seen in 50% of human tumours. These include inactivating mutations and others which can confer oncogenic activity. Clustering of mutations occur within the DNA binding region of p53 indicating that disruption of its ability to directly modulate transcription is key factor in the development of tumours (Levine & Oren 2009).

Furthermore it is widely considered that disruption to p53 pathways must also occur in those tumours exhibiting the wild type protein. Other factors regulating its function are then likely to be implicated in tumour formation (Sullivan & Lu 2007). Specifically the recognition of factors modulating p53 mediated transcription could potentially identify novel targets for therapeutic manipulation. Furthermore the elucidation of mechanisms influencing whether p53 induces cell cycle arrest or apoptosis could reveal ways in which tumour cells, expressing wild type p53, could be encouraged to trigger apoptotic pathways.

The p53 family refers to p53 together with p63 and p73, transcription factors which share structural and some functional homology with p53, but which have critical roles in mammalian development (discussed later).

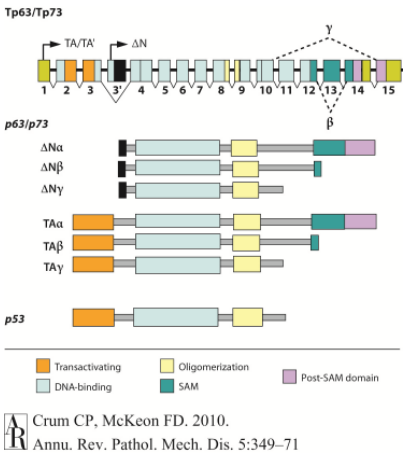

Figure 1.4: p53 family members. p63 and p73 share structural homology with p53
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1.3 The ASPP family

1.3.1 Overview

The **ASPP** (**A**poptosis **S**timulating **P**rotein of **p**53) family of proteins were originally discovered to bind and specifically regulate the apoptotic function of p53. The family consists of three proteins which share sequence similarity in their C-termini and their nomenclature also emphasises the **A**nkyrin repeats, **S**H3 domain and **P**roline-rich region characterising this region (Figure 1.5) (Samuels-Lev et al. 2001).

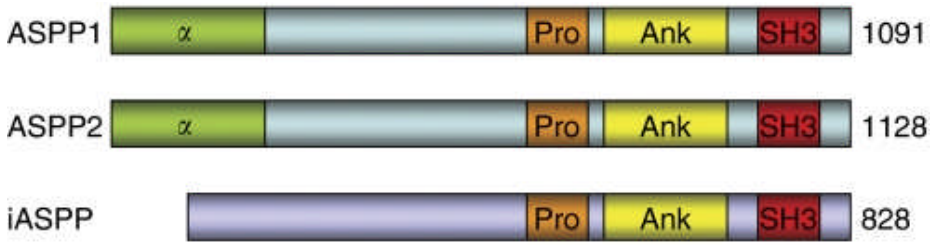

Figure 1.5: The ASPP family. These proteins share similarities in their C-termini which mediates several of their protein interactions.
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ASPP1 and ASPP2 were originally discovered to bind and specifically promote the apoptotic function of p53 whereas iASPP was shown to inhibit this function. As a result the possibility that these proteins could influence the development of tumours was postulated (Bergamaschi et al. 2003, Samuels-Lev et al. 2001).

In fact it has since been shown that individually these proteins have a significant number of other specific interactions and functions, the disruption of which could also contribute to their involvement in the formation of tumours. This investigation implicates the involvement of ASPP2 and iASPP in opposing aspects of tumourigenesis and does not expand further on the role of ASPP1.

1.3.2 ASPP2

1.3.2.1 Introduction

A truncated form of ASPP2, comprising 529 amino acids and termed p53 binding protein 2 (53BP2), was initially discovered to bind to p53 in a yeast two-hybrid assay (Iwabuchi et al. 1994). Structural analysis of the binding confirmed that 53BP2 was in fact binding to the evolutionarily conserved central DNA binding domain of p53, a common site of mutation in tumourigenesis. Furthermore six of these common p53 mutations were observed to disrupt the binding between 53BP2 and p53 (Gorina & Pavletich 1996). In a separate yeast-two hybrid assay a 1005 amino acid protein termed bcl-2-binding protein (Bbp), was discovered to bind both bcl-2 and p53, interactions which occurred via the ankyrin repeats and SH3 domain in its C-termini, which were identical to 53BP2 (Naumovski & Cleary 1996).

Early experiments suggested these truncated versions of ASPP2 could stimulate p53-mediated transcriptional activity (Iwabuchi et al. 1998). However it was not until the formal identification of the full length protein, encoded by *TP53BP2* on the long arm of chromosome 1 (1q42.1), that its specific stimulation of pro-apoptotic p53 mediated transcription was confirmed *in vitro.* In addition the first indication that this protein might function to suppress tumour formation *in vivo* was indicated in a panel of breast carcinomas which demonstrated lower levels of ASPP2, by RT-PCR, than their normal tissue counterparts (Samuels-Lev et al. 2001).

1.3.2.2 Regulation of ASPP2

The regulation of ASPP2 expression occurs via several mechanisms identified thus far. E2F has been shown to upregulate its expression, possibly sensitising those cells

progressing through the cell cycle to apoptosis and providing a link between p53 and Rb/E2F induced apoptosis (Chen et al. 2005, Fogal et al. 2005, Hershko et al. 2005).

Hypermethylation of ASPP2 has been documented in cell lines exhibiting wild type p53, silencing its action and presumably facilitating the bypass of intact p53 function in these cells (Liu et al. 2005). p53 mutations have been shown to disrupt its own binding to ASPP2 and other molecules have been seen to prevent their binding including Mdm2, Mdmx, DDA3 and Ddx42p, thereby blocking ASPP2's enhancement of p53 induced apoptosis (Bergamaschi et al. 2005; Sun et al. 2008; Uhlmann-Schiffler et al. 2009). Furthermore degradation of ASPP2 has been shown to be, at least in part, mediated by the proteasome (Zhu et al. 2005).

1.3.2.3 ASPP2: A Tumour Suppressor Protein in Mice

In this laboratory the germline inactivation of ASPP2 precipitated the development of tumours in two separate mouse models. On a mixed C57BL/6Jx129SvJ background, extreme developmental defects were associated with complete ASPP2 knockout (ASPP2 $^{\Delta exon3/\Delta exon3}$), with just 6.4% of the offspring representing this genotype, compared to the expected 25%. In addition, postnatal survival for these mice was short, with pups suffering hydrocephalus and heart defects. Consequently the tumour suppressive effects of this protein were studied in heterozygotes (ASPP2$^{\Delta exon3/+}$) compared to their wild type littermates (ASPP2$^{+/+}$). Heterozygosity of ASPP2 conferred a significant tumour susceptibility with the development of lymphomas, sarcomas and squamous cell carcinomas seen. Wild type mice developed a small number of lymphomas only. The tumours were subsequently analysed for the expression of ASPP2. The protein was demonstrated in tumours confirming ASPP2 to be acting in a haploinsufficient manner

(Vives et al. 2006a).

In vitro, ASPP2 has been shown to directly interact with p53, specifically enhancing its ability to induce apoptosis. Vives *et al*. went on to cross ASPP2$^{\Delta exon3/+}$ mice with p53 heterozygotes (p53$^{+/-}$). The resulting double heterozygotes (ASPP2$^{\Delta exon3/+}$; p53$^{+/-}$) were then intercrossed to generate compound genotypes, in order to explore this interaction in tumour development. It was discovered that the double knockout of both ASPP2 and p53 was synthetic lethal with pups dying in utero during the first half of gestation. This suggests that in mouse development at least, these two proteins have overlapping functions, corroborating the interaction seen previously. Again ASPP2$^{\Delta exon3/\Delta exon3}$ mice did not survive long enough to develop tumours and the remaining six genotypes were analysed for tumour development in this experiment. Over a period of 18 months none of the ASPP2$^{+/+}$;p53$^{+/+}$ mice developed tumours. In contrast all other combinations of gene inactivation showed some predisposition to tumour formation. The key findings included a significant decrease in tumour latency for ASPP2$^{\Delta exon3/+}$ compared to ASPP2$^{+/+}$ mice on a p53 heterozygous background, an effect lost on a p53 null background. These results suggest that in tumour development in these mice, ASPP2 and p53 co-operate with ASPP2 functioning upstream of p53.

Furthermore loss of ASPP2 appeared to alter the spectrum of tumours seen. In the previous experiment, older wild type C57BL/6Jx129SvJ mice were seen to occasionally develop lymphomas suggesting these mice are intrinsically prone to this tumour type. Moreover lymphomas were the most commonly encountered tumours in this experiment and were seen in all genetic backgrounds where tumours developed. The progressive loss of p53 on an ASPP2$^{+/+}$ background resulted in the formation of lymphomas only, with

tumour latency unaffected by ASPP2 copy number as eluded to earlier. This suggests that in lymphoma, at least, these genes have an overlapping function and as ASPP2 functions upstream its loss is less important once p53 itself has been inactivated. Conversely loss of ASPP2 resulted in a wider spectrum of tumours including sarcomas and a carcinoma, with the number of tumours seen increasing with progressive p53 loss, confirming their co-operation in tumour suppression (Vives et al. 2006a).

1.3.2.4 ASPP2: A Tumour Suppressor Protein in Humans

Evidence to support the translation of ASPP2s putative tumour suppressor role to human tumours has continued to expand with studies initially demonstrating the reduction of ASPP2 in human cancer cell lines (Trigiante & Lu 2006).

In addition, in those leukaemias, arising de novo, without complex karyotypic abnormalities, low levels of ASPP2 could, in one study, be used to predict higher risk patients who responded poorly to first line chemotherapy (Schittenhelm et al. 2013). Low mRNA levels of the protein might also be linked to a poorer clinical outcome in diffuse large B cell lymphoma (Lossos et al. 2002 , Liu et al. 2005).

Furthermore, levels of ASPP2 were seen to be significantly reduced in a diverse range of carcinomas. Higher levels of ASPP2 were seen in non-invasive pituitary adenomas compared to invasive tumours (Ma et al. 2013) with reduced levels of protein also seen in choriocarcinomas (Mak et al. 2013), gastric carcinomas (Meng et al. 2013), breast carcinomas (Patel et al. 2012), non small cell lung carcinomas (NSCLCs) (Li et al. 2012) and endometrioid endometrial carcinomas (Liu et al. 2010).

A small number of colorectal cancers exhibiting microsatellite instability were also seen to exhibit ASPP2 mutations which could contribute to the inactivation of p53 mechanisms in these tumours (Park et al. 2010). Single nucleotide polymorphisms (SNPs) at the *TP53BP2* locus have also been associated with gastric cancer susceptibility (Kampa et al. 2009).

1.3.2.5 Other mechanisms of Tumour Suppression

Mechanistically, ASPP2's modulation of p53 function has been implicated in its tumour suppressor role. Furthermore ASPP2 is able to selectively promote the apoptotic function of p63 and p73, which could represent one way in which it suppresses tumour growth in the presence of mutant p53 (Bergamaschi et al. 2004). More recently, its interaction with and modulation of oncogenic RAS has not only demonstrated a way in which RAS can influence p53's pro-apoptotic function, but has also revealed p53-independent ways by which ASPP2 can promote RAS induced senescence in non-transformed cells, which if disrupted could favour tumour formation. (X. Wang et al. 2011, Y. Wang et al. 2012, Z. Wang et al. 2013, Y. Wang et al. 2013, Godin-Heymann et al. 2013).

Furthermore the initial ASPP2 knockout mouse model demonstrated its significant role in normal development. More specifically, through its interaction with the Par complex, specifically Par-3, ASPP2 was seen to influence the maintenance of CNS polarity with ASPP2 deficient mice exhibiting severe hydrocephalus (Cong et al. 2010, Sottocornola et al. 2010). The maintenance of polarity is considered an important component of tumour suppression, particular with regards to the development of carcinomas (McCarthy 2010). This represents, yet another p53 independent way in which ASPP2 potentially protects against tumour formation.

1.3.3 iASPP

1.3.3.1 Introduction

A truncated form of iASPP, comprising 351 amino acids and termed RelA- associated inhibitor (RAI), was initially discovered to bind to p65 to in a yeast two-hybrid assay. It was recognised to share C-termini homology with the previously recognised 53BP2, and was shown to co-localise in the nucleus, binding to p65/NF-κB where it inhibited its transactivation function (J. P. Yang et al. 1999).

The full length iASPP protein, encoded by *PP1R13L* on chromosome 19 (19q13.3) in humans, was later characterised. It was found to be an evolutionarily conserved inhibitor of p53 induced apoptosis and able to co-operate with other oncogenes to transform cells *in vitro*. Furthermore overexpression of iASPP was seen to confer resistance to UV and cisplatin-induced apoptosis. In a panel of breast carcinomas, RT-PCR analysis detected the overexpression of iASPP in 7/8 tumours known to express wild type p53 and normal levels of ASPP1 and 2 (Bergamaschi et al. 2003).

iASPP has also been shown to bind the proline rich region of p53, preferentially binding to p53Pro72 rather than p53Arg72, perhaps explaining why this latter polymorphism is able to activate apoptosis more effectively than its counterpart (Bergamaschi et al. 2006).

1.3.3.2 Regulation

Published evidence of iASPP's regulation is scanty. Pin1 has been shown to reduce the interaction between p53 and iASPP, thus promoting apoptosis (Mantovani et al. 2007) and iASPP may be upregulated by GATA-2 (Xing et al. 2013).

Micro-RNA 124 (miR-124) has been seen to downregulate iASPP, promoting neuronal death after cerebral ischemia (X. Liu et al. 2013). Low levels of miR-124 have been identified in colorectal carcinoma and *in vitro* overexpression of miR-124 inhibits the expression of iASPP, inducing the upregulation of NF-κB (K. Liu et al. 2013). Furthermore in glioblastoma cell lines it has been shown that overexpression of miR-124 inhibited proliferation, G1/S transition and invasiveness and when down regulated the consequent progression was partly attributed to increased iASPP expression (Zhao et al. 2013).

1.3.3.3 iASPP: An Oncogene in Humans

Further evidence of iASPP's oncogenic effects in human tumours is a rapidly expanding field. The overexpression of iASPP mRNA was seen in three leukemic cell lines compared to normal blood levels (Liu et al. 2004) and in a separate study, its down regulation enhanced p53 dependent apoptosis in partially overlapping cell lines (Liu et al. 2009, Gillotin 2009).

Furthermore iASPP was overexpressed in the bone marrow of patients with acute leukaemia compared to healthy controls (Zhang et al. 2005). This study also indicated that iASPP's pro-tumorigenic functions may act, in part, independently of its p53 modulation as levels of iASPP did not correlate with p53 status in these samples. In a transgenic mouse model where iASPP is expressed specifically in haematopoietic cells, an increase in the number of stem cells was seen. Following irradiation, these cells were seen to have a greater degree of DNA damage, measured by γ-H2AX levels, than their wild type counterparts (Jia et al. 2014).

SNPs in iASPP have been associated with varying treatment responses in patients with advanced NSCLC (Su et al. 2007) and increased iASPP expression was seen in NSCLC cell lines, with its subsequent down regulation stimulating apoptosis (Li et al. 2012). In a second study involving human NSCLC samples, iASPP was significantly increased in tumours compared to normal lung tissue (Chen et al. 2010). iASPP was also shown to be increased in esophageal cancer compared to normal esophageal tissue (Kou et al. 2014) and in malignant ovarian tumours compared to their borderline counterparts and associated benign tissue. Furthermore iASPP overexpression was seen in ovarian tumours known to be chemoresistant and was significantly associated with a shorter overall and disease-free survival (Jiang et al. 2011).

iASPP is upregulated in prostate cancer tissue compared to paired normal controls (Zhang et al. 2011) and has been shown to be important for human bladder cancer cell proliferation (Liu et al. 2011). In human hepatocellular carcinoma (HCC) cell lines iASPP promotes proliferation and the protein is upregulated in human liver samples of cirrhosis, hepatitis B infection and HCC compared to normal liver tissue. Furthermore iASPP expression was also associated with poor survival in this cohort (Lu et al. 2010). iASPP was also increased in human endometrioid endometrial carcinoma samples and glioblastoma samples compared to their normal tissue counterparts (Liu et al. 2010, Li et al. 2011).

The data thus far implicate iASPP not only in the inhibition of apoptosis, but also the promotion of proliferation, indicating strong oncogenic potential when deregulated.

1.4 Carcinoma

1.4.1 Overview

Carcinomas are malignant tumours derived from the epithelia, a term used to describe the varied assortment of tissues covering the surfaces and tubes of the human body. The epithelia mediate interactions between diverse biological compartments (Mills 2006) and as a result are exposed to a myriad of potential carcinogens. Carcinomas are the most common tumour type, contributing to over 80% of the world's tumour burden (Cancer Research UK - http://info.cancerresearchuk.org/).

Epithelia are classified by the number of cell layers present, the morphological appearance of the constituent cells and the presence of any specialist features on the surface such as cilia (Young & Heath 2000).

Simple epithelia are composed of a single layer of flattened, cuboidal or columnar cells which are predominantly involved in absorption and secretion. Examples include the epithelia of the alveoli (flattened), kidney (cuboidal) and intestine (columnar). The lining of the large airways in the respiratory tract is composed of a special type of simple epithelium where the arrangement of the nuclei is such that it appears to be composed of several layers and is therefore referred to as 'pseudostratified'. Truly stratified epithelia are composed of two or more cell layers and can be found in the skin and other internal mucosae where resilience to physical stress is demanded. In particular, stratified squamous epithelium is especially well adapted to withstand abrasion and other external insult as its surface layers are shed without impairing its ability to self-renew. As a result it is found in close proximity to the external environment in areas including the epidermis, oral cavity, oesophagus, vagina, and cervix (Young & Heath 2000).

Cytokeratins are intermediate filaments found in epithelia, contributing to their structural integrity. The cytokeratins expressed by an individual epithelial cell is dependent upon its stage of differentiation and the tissue type in which it resides. During malignant transformation the cytokeratin make-up of a cell is often preserved and, as such, the detection of cytokeratins is a useful indicator of tumour type. For example, cytokeratin 14 (CK14) is an acidic cytokeratin which is strongly expressed in the p63 expressing basal layers of mature squamous epithelium and is lost as cells enter a programme of terminal differentiation (Velden et al. 1997)

Carcinomas are composed of malignant cells which resemble, in some way, those comprising the normal epithelium from which they arise. In well differentiated tumours morphological characteristics reveal their origin, whereas in more poorly differentiated tumours immunohistochemical detection of their cytokeratin expression is often required (Barak et al. 2004, Harnden & Southgate 1997). The initiation and development of carcinomas reflects the deregulation of normal epithelial homeostasis and generally involves the upregulation of proliferative and survival pathways with the concomitant downregulation of those involved in differentiation and apoptosis. In addition to the widely varying mechanisms governing the regulation of the different types of epithelia, tissue-specific variations are also present resulting in a myriad of different pathways, whose deregulation contributes to the development of tumours. A good understanding of these normal pathways, together with the ways in which they are commonly disrupted is of paramount importance in investigating potentially novel components in carcinoma development.

1.4.2 Squamous Cell Carcinoma

1.4.2.1 Overview

Squamous cell carcinomas originate as a consequence of the deregulation of normal squamous homeostasis. In humans step-wise progression from normal squamous epithelium to dysplastic epithelium is followed by cells eventually acquiring sufficient genetic mutations to become frankly invasive.

Self-renewal of squamous epithelia is tightly regulated. The basal layer is composed of slowly cycling stem cells, highly proliferative immature transit amplifying (TA) cells and mature TA cells which have a finite proliferative capacity (Lehrer et al. 1998, Koster 2010). Parabasal layers contain TA cells and as these are induced to differentiate they lose their capacity to proliferate, gradually moving up towards the surface where they eventually slough off (Koster 2010). An intricate network of pathways regulates this process, and dissecting these can provide insights into the genetic modifications required to initiate dysplasia and ultimately invasive carcinoma.

Epithelial stratification is in part controlled by p63. The p63 gene encodes two N-termini (TA and ΔN) and multiple C-termini (α, β, γ, δ, and ε), thus generating multiple isoforms which have made it a challenge to characterise its functions (Melino 2011).

The concept of p63 involvement in the maintenance of normal stratified squamous epithelium arose secondary to the observation of significant epidermal abnormalities in p63 null mice suggesting a lack of ectodermal commitment to the epidermal fate (Mills et al. 1999, A. Yang et al. 1999, Koster et al. 2004, Koster 2010). In addition to its function in lineage commitment, p63 also plays a critical role in the mature squamous epithelium

both maintaining the proliferative potential of basal cells and the induction of differentiation programmes. Here ΔNp63 is the predominant isoform and it is likely that in individual cells these apparently opposing directions are modulated by cofactors which ultimately determine cell fate decisions (Koster 2010).

Disruption of normal epithelial stratification together with unregulated proliferation characterises neoplastic squamous populations and here expression of p63 is somewhat controversial. Dysplasia is histologically recognisable as the disordered maturation of squamous epithelium without frank invasion of the underlying submucosa. Increasing numbers of cells demonstrate proliferative capability and express proteins, such as ΔNp63, which are normally seen only in basal and immediately parabasal cells (Y.-K. Chen et al. 2005).

Within carcinomas themselves, ΔNp63 is often seen in poorly differentiated areas, resembling more closely the proliferative layers of the normal epithelium and suggesting it might function to maintain replication competence in these areas, contributing to tumour formation (Graziano & De Laurenzi 2011). However there is evidence to suggest that the inactivation of ΔNp63 induced transcription of anti-metastatic factors such as Sharp 1 and Cyclin G2 actually promotes metastasis (Adorno et al. 2009). In fact ΔNp63 overexpression has been considered both a good and a bad prognostic marker (Graziano & De Laurenzi 2011). It is again possible that altered expression of cofactors specifically modifying different aspects of ΔNp63 function contribute to these inconsistences. Therapeutic manipulation of these cofactors could potentially promote specific anti-tumorigenic transcription and as such their identification is an important research goal.

1.4.2.2 ASPP2 and Squamous Cell Carcinoma

In order to explore the significance of ASPP2 deletion specifically in the development of carcinomas, its germline deletion was repeated on a pure Balb/c background in this laboratory. Balb/c mice are more prone to carcinoma formation on p53 deletion than C56BL6Jx129SvJ mice (Kuperwasser et al. 2000). ASPP2 deletion in Balb/c mice also conferred a significant tumour susceptibility with the development of carcinomas and specifically squamous cell carcinomas only seen in ASPP2$^{\Delta exon3/+}$ mice and in the two ASPP2 $^{\Delta exon3/\Delta exon3}$ which survived long enough to develop tumours at 19-20weeks of age.

The subsequent generation of compound mice, in this laboratory, through the intercrossing of ASPP2$^{\Delta exon3/+}$; p53$^{+/-}$ mice confirmed several of the findings described by Vives *et al*. ASPP2 and p53 were again seen to cooperate in tumour suppression with ASPP2 likely working upstream of p53. Furthermore the spectrum of tumours seen was comparable. Here a p53 null background resulted in the development of lymphomas and sarcomas only, whereas in p53$^{+/+}$ and p53$^{+/-}$ mice, progressive ASPP2 deletion resulted in an increasing proportion of carcinomas (Figure 3.1). Moreover it can be seen that heterozgosity of p53 did not significantly enhance carcinoma formation, rather the proportion of other tumour types was seen to increase (Tordella et al. 2013).

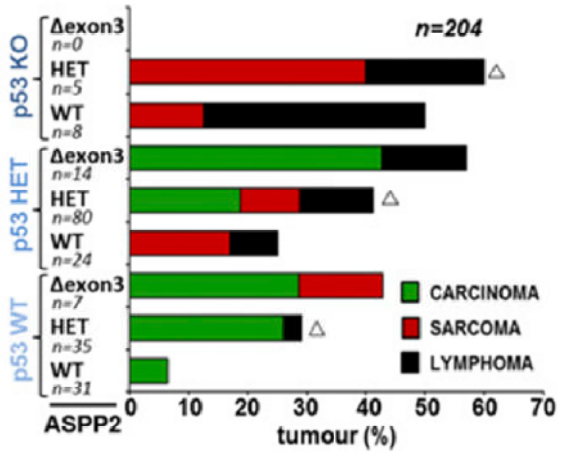

Figure 3.1: Incidence of different tumour types in indicated genotypes. On a p53 null background no carcinomas were seen. The greatest proportion of carcinomas were seen on an ASPP2 heterozygous or null background.

Modified from: Tordella et al. *PNAS,* 110(44), pp.17969-74 © 2013 National Academy of Sciences, USA

Previous *in vitro* experiments have also confirmed ASPP2's interaction with and specific promotion of apoptosis mediated via other p53 family members, namely p63 and p73 (Bergamaschi et al. 2004). Given p63's important role in squamous epithelial homeostasis, it was hypothesised that ASPP2 co-operates specifically with p63 in the development of squamous cell carcinomas. This could explain the significant increase in this particular carcinoma type seen with ASPP2 deletion on a pure Balb/c background.

Indeed, the immunohistochemical analysis of these squamous cell carcinomas demonstrated strong expression of p63. Moreover double immunofluorescence staining of those tumours developing in $ASPP2^{\Delta exon3/+}$ mice showed p63 and ASPP2 to be mutually exclusive in individual cells. Furthermore the suppression of squamous cell carcinoma formation seen in $ASPP2^{\Delta exon3/+};p63^{+/-}$ Balb/c mice compared to their $ASPP2^{\Delta exon3/+};p63^{+/+}$ counterparts confirms that heterozygosity of p63 prevents carcinoma formation (Figure 3.2).

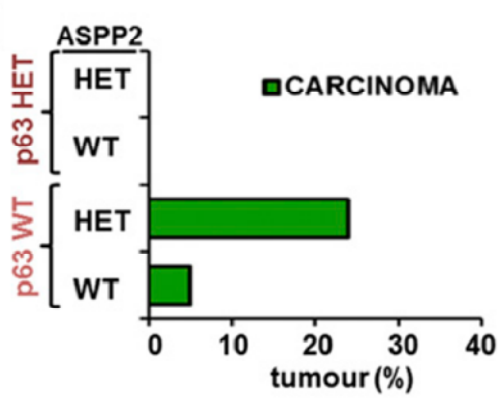

Figure 3.2: Heterozygosity of p63 prevents ASPP2Δexon3/+ BALB/c mice from developing SCC

Modified from: Tordella et al. *PNAS,* 110(44), pp.17969-74 © 2013 National Academy of Sciences, USA

Together these results suggest that repression of p63 is an important part of ASPP2's tumour suppressive function. Complementary experiments *in vitro* demonstrated that high calcium-induced differentiation of murine primary keratinocytes resulted in the induction of junctional ASPP2 and the simultaneous downregulation of ΔNp63 expression suggesting ASPP2 induced repression of ΔNp63 may also be important for normal epithelial differentiation. In fact, mechanistically ASPP2 indirectly inhibits ΔNp63 expression through its binding of IκB with the consequent nuclear accumulation of RelA/p65 which represses ΔNp63 expression (Tordella et al. 2013).

So far the data suggest that ASPP2 controlled repression of ΔNp63 expression specifically results in the normal differentiation and maturation of squamous epithelia and that loss of ASPP2 results in disordered maturation with the eventual development of squamous cell carcinomas.

1.4.2.3 iASPP and Squamous Cell Carcinoma

In vitro evidence from this labratory of iASPP's interaction with ΔNp63 and antagonism of its transcriptional activity also implicates iASPP in the maintenance of stratified squamous epithelia. In fact the induced differentiation of murine keratinocytes resulted in a significant reduction in the expression of iASPP. Furthermore the conditional deletion of iASPP in the same cell type resulted in the emergence of cytokeratin markers of differentiation suggesting loss of iASPP is important in the normal differentiation of keratinocytes (Notari et al. 2011).

The expression of iASPP in murine stratified squamous epithelium confirmed its restriction to the lower layers with no expression seen in the more superficial, differentiated cells. Moreover deletion of iASPP was seen to result in a thickened epidermis with a reduction in proliferation and an expansion of differentiated layers (Notari et al. 2011).

These results suggest the persistence of iASPP expression in neoplastic squamous populations could facilitate their escape from differentiation, potentially implicating the overexpression of iASPP in the development of squamous cell carcinomas.

1.4.3 Human Squamous Cell Carcinomas

1.4.3.1 Cervix

Squamous cell carcinomas are the most common histological subtype of cervical cancer, which is the third most common cancer in women worldwide (Cancer Research UK - http://info.cancerresearchuk.org/). Persistent human papillomavirus (HPV) infection accounts for nearly 100% of cases (Gravitt 2011) and identification of the molecular environments that favour persistent infection might provide deeper insights into how HPV circumvents normal epithelial stratification to produce highly proliferative, poorly differentiated dysplastic populations.

The cervix connects the uterus with the vaginal cavity and is composed of two distinct epithelia: the simple columnar epithelium of the endocervical canal abruptly transitions into the thick stratified squamous epithelium of the ectocervix which is in contact with the vaginal cavity. Estrogen stimulation at puberty and during pregnancy causes structural changes, bringing the columnar epithelium into contact with the more hostile environment of the vaginal cavity and triggering the development of squamous metaplasia. Eventually a mature layer of stratified squamous epithelium overlies these deeper endocervical cells. The region between the original squamocolumnar junction and the new junction is termed the transformation zone (Jordan et al. 2006). The transformation zone has implications in cervical pathology as areas of squamous metaplasia are thought to be especially vulnerable to the genetic modifications necessary for neoplastic transformation (Moscicki et al. 2006).

The metaplastic process is thought to occur as a consequence of p63 induction in nests of subcolumnar basal cells (generally termed ‘reserve cells’) resulting in their hyperplasia, the undermining of the columnar lined glands and the subsequent initiation of stratification

(Figure 1.6). The origin of these reserve cells is controversial and hypotheses include: their distinction as a separate population of 'reserve' cells present in all cervices or their derivation from overlying endocervical cells or even underlying stroma (Jordan et al. 2006). Essentially how and when they arise and what triggers p63 induction is not well understood.

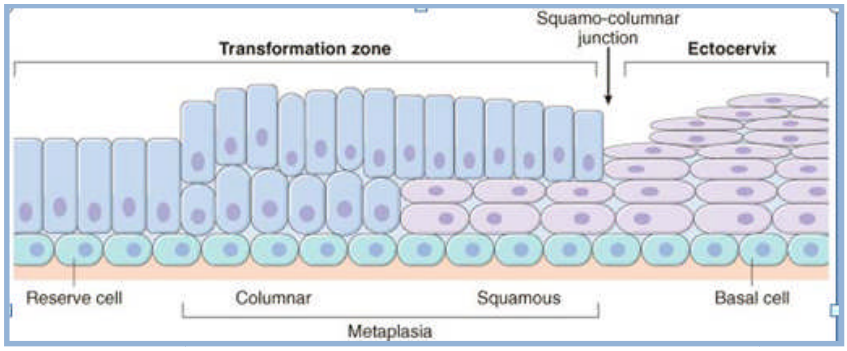

Figure 1.6: Cervical Transformation Zone. Schematic representation of the transformation zone. Modified from http://medicinembbs.blogspot.co.uk

Persistent HPV infection, arising in the transformation zone is thought to subvert the process of normal squamous maturation and promote the development of dysplasia. In the cervix, dysplasia is termed cervical intraepithelial neoplasia (CIN) and graded by severity into CIN 1 (low grade), where dysplastic cells are seen only in the lower third of the epithelium, and CIN 2&3 (high grade), where dysplastic cells are seen to extend into the more superficial layers. There is good evidence to suggest that high grade CIN is the immediate precursor to invasive squamous cell carcinoma (Dobbs et al. 2000).

1.4.3.2 Head and Neck

HPV infection also contributes to the formation of Head and Neck Squamous Cell carcinomas (HNSCCs) which make up 90% of the diverse range of cancers in this region including those of the oral cavity, pharynx and larynx. HNSCCs together feature as the sixth most common cancer in the world and overall 5-year survival rates have not improved significantly over the last two decades despite a wealth of new information regarding their molecular signatures (Sahu & Grandis 2011).

HNSCCs are a heterogeneous group of cancers in part due to the diversity of their aetiologies. Research focuses on the development of effective biomarkers to stratify tumours for alternate therapies and to predict prognosis. The two most commonly used biomarkers to date are EGFR and HPV which predict a poorer and a better outcome respectively. However even the EGFR status cannot accurately predict response to targeted therapies (Sahu & Grandis 2011). Further elucidation of the pathways involved in the development of these molecularly diverse, yet histologically similar tumours will potentially provide novel targets for more effective therapeutic manipulation and improved outcomes.

1.4.3.3 Esophagus

HPV infection may also contribute to the development of a small proportion of squamous cell carcinomas in the esophagus (Sitas et al. 2012). Worldwide, this tumour comprises the most common type seen in this organ and is often seen in its proximal aspect. However, in developed countries, the increased incidence of reflux esophagitis and consequent induction of glandular metaplasia, predominantly in the distal esophagus, has resulted in an increase in the incidence of adenocarcinomas (Brown et al. 2008).

1.4.3.4 Human Papillomavirus (HPV)

HPV infection by high risk subtypes (e.g. HPV 16 and 18) is implicated in nearly all squamous cell carcinomas of the cervix, 25-35% of HNSCCs and a small proportion of those seen in the esophagus. In the cervix, persistent high risk HPV infection is considered necessary, though not sufficient for carcinoma formation. Cofactors such as hormones and cigarette smoking have been shown to facilitate this progression (Gravitt 2011).

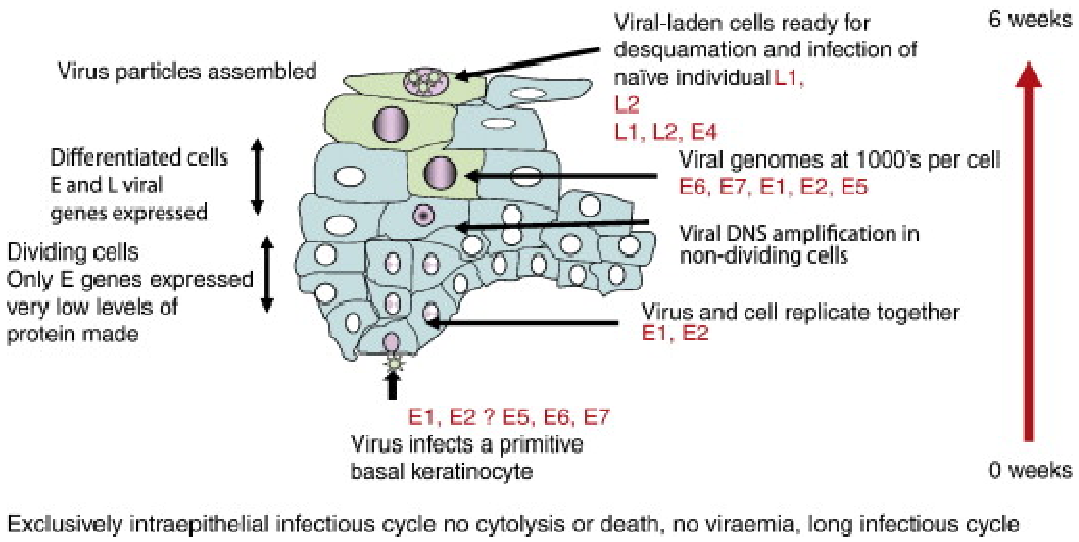

Figure 1.7: Infectious cycle of high-risk HPVs. HPV will only infect and replicate in a fully differentiated squamous epithelial cell
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As can be seen in Figure 1.7 the life cycle of HPV relies on the normal differentiation of host cells. HPV is thought to infect the basal layer of the squamous epithelium where it exists as an episome and passively replicates with the host cell. There is minimal expression of E6/E7 until the cell undergoes differentiation which significantly increases viral protein synthesis. However in order to maintain replication, viral proteins need to reactivate cellular DNA synthesis in non-cycling cells and inhibit apoptosis. This is achieved through E6's interaction with p53 and E7's interaction with Rb which prevent their normal regulation of the cell cycle and the initiation of apoptosis. The functions of

E6 and E7 are regulated in part by other viral proteins, but when, as happens in neoplastic transformation, HPV DNA integrates with cellular DNA, the inhibitory effect of these is lost and the transforming ability of E6 and E7 left unchecked (Stanley 2010, Klingelhutz & Roman 2012, Faridi et al. 2011). E6 binds to and utilises the E3 ligase activity of the ubiquitously expressed E6-AP protein to target p53 for degradation by the proteasome (Tomaić et al. 2009). E7 interacts directly with Rb and its associated pocket proteins to facilitate cell cycle progression mediated by the subsequent release of E2F transcription factors (Dyson et al. 1989; Cam & Dynlacht 2003).

It is clear that HPV has a complicated relationship with differentiation in the squamous epithelia. Ideally differentiation would occur normally in cells which have been induced to replicate. This would promote the production of complete virus particles which would slough off the surface ready to re-infect and start the cycle again. However in high risk viral infection, possibly at the point of DNA integration, the normal differentiation programs are subverted and dysplasia and eventually carcinoma develop. Identifying molecules involved in the differentiation of normal squamous epithelium, which are targeted by high risk HPV, could provide further insight into how this phenomenon occurs and perhaps identify a key point at which HPV infection initiates neoplastic transformation.

In fact ASPP2 has already been identified as a key cellular target of other oncogenic pathogens namely H.pylori (Buti et al. 2011, Nešić et al. 2014, Pacchiani et al. 2013), Hepatitis B (HBV) (Zhao et al. 2010) and Hepatitis C (HCV) (Cao et al. 2004). Furthermore high risk HPV E6 has been shown to interact with PDZ domain-containing substrates which co-ordinate cell adhesion and polarity such as Dlg and Scribble (Massimi

et al. 2004). Of note here is the critical interaction of HPV-16's close relation, Rhesus papillomavirus type 1 (RhPV-1) with Par-3 in Rhesus Macaques. Here the PDZ binding motif is found on the E7 protein rather than the E6 protein (Tomaić et al. 2009), but this disruption of the Par-3 complex is essential to RhPV-1''s ability to transform primary rodent cells (Thomas et al. 2008). It is possible, that in humans, HPV has found alternative ways to disrupt the same complex and ASPP2 is a known binding partner of Par-3 (Sottocornola et al. 2010).

Furthermore targeting cell adhesion and polarity factors would be advantageous in promoting invasion and metastasis as tumours progress. Yet even in early lesions a selective advantage would be achieved by cells able to control the symmetry of their cell division. Sottocornola et al. (2010) also demonstrated that loss of ASPP2 results in expansion of the neuroprogenitor compartment in the mouse brain. If down regulation of ASPP2 not only prevents the differentiation of replication competent cells in the squamous epithelium, but also promotes their symmetric division, it could potentially be a significant target for high risk HPV modulation.

1.4.3.5 Squamous Cell carcinomas elsewhere

Squamous cell carcinomas are also commonly seen in organs in which normal squamous epithelium is not usually described. In the cervix squamous metaplasia occurs as a physiological consequence of sexual maturity. Elsewhere, squamous metaplasia occurs in more fragile epithelia in response to chronic irritation and the continued initiation of repair mechanisms, in order to provide a more resilient surface for continued assault. However continued exposure to ongoing insult can ultimately result in the subsequent acquisition of genetic mutations resulting in the development of squamous dysplasia and carcinoma. Squamous cell carcinomas of the lung are considered to arise in this way.

1.4.3.5.1 Lung

Lung development incorporates intricate epithelial branching, resulting in the development of identifiable anatomical areas, comprising morphologically distinct epithelial cells reflecting their myriad functions (Snyder et al. 2009, Fine 2009). As mentioned previously the larger airways of the lung are lined by a pseudostratified epithelium, designed in part to facilitate airflow to the distal airways, but also incorporating important antimicrobial defences. In addition robust mechanisms for epithelial repair after exposure to the wide variety of external, potentially damaging irritants inhaled regularly exist. In contrast the most distal alveoli are lined by a simple, flattened epithelia designed to maximise the surface area in contact with the air to promote effective gas exchange. The airways connecting these two distinct compartments show gradual modifications in their epithelial lining (Figure 1.8).

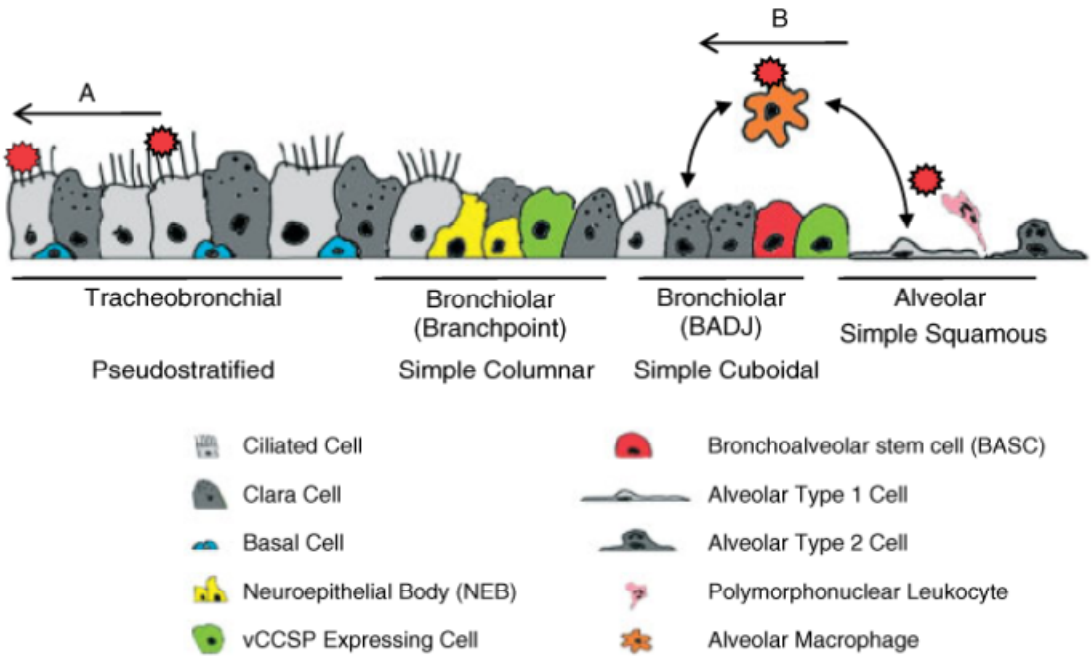

Figure 1.8: Structure and function of the airway epithelium. The schematic above demonstrates the lining of the murine airways and it can be seen that the epithelial composition, and therefore function, is markedly different from proximal (left) to distal (right) zones. The proximal, pseudostratified epithelium comprises terminally differentiated ciliated cells and basal cells with fewer secretory cells than is seen more distally. Specifically in mice, basal cells are only seen in this proximal location normally and are generally less abundant than in the bronchi of humans. The most distal alveoli are lined by type I cells and type II cells which are specialised for gaseous diffusion and surfactant production, respectively. Epithelial compartmentalization as illustrated here plays a vital role in protecting the lung from microbial, inhaled pollutants and facilitates the process of gas exchange
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Numerous mechanisms of epithelial damage repair have been described and in fact the lung appears to have several, independent niches within which cells with multipotential progenitor and stem cell like phenotypes reside. Following injury these cells have been seen to multiply and repopulate the epithelium in its entirety (Figure 1.9).

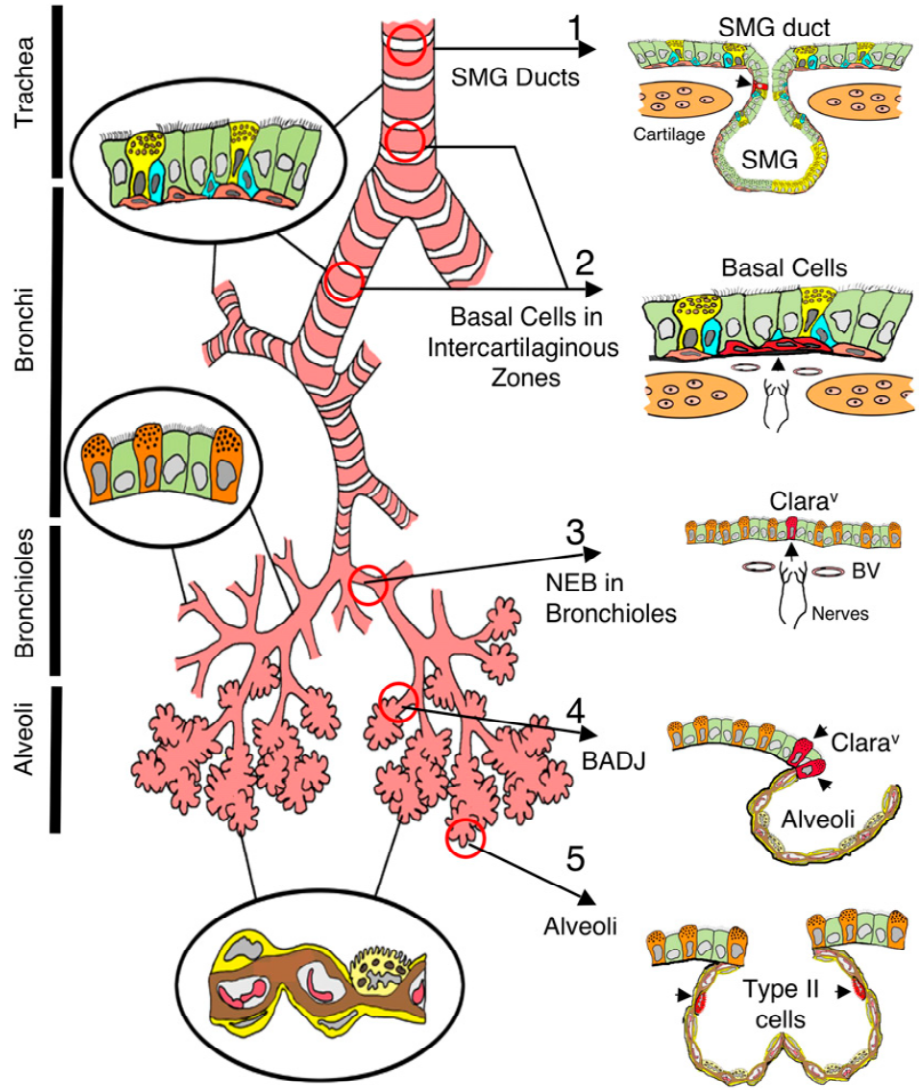


Figure 1.9: Putative Stem Cell niches in the adult mouse lung. Five potential stem cell niches are considered to exist throughout the murine airway. They include a cell type seen in association with submucosal gland ducts of the proximal trachea **1**, basal cells in the lower trachea and bronchi **2**, variant Clara cells associated with neuroendocrine bodies in bronchioles **3**, variant Clara cells associated with bronchiolar alveolar duct junctions (BADJ) **4**, and alveolar type II cells **5**
Reprinted with permission of the *American Thoracic Society:* Liu and Engelhardt. *PATS*, 5(6), pp.682-8 © 2014 *American Thoracic Society*

However it should be noted here that our knowledge of these stem cell populations has been acquired through injury models undertaken in mice. Variations in the cellular composition of murine lung epithelium must be remembered when interpreting these findings with respect to human lung physiology. Differences include the proportion and spatial orientation of submucosal glands, basal cells, clara cells and goblet cells (Snyder et al. 2009; Liu & Engelhardt 2008). Within the murine lung, as demonstrated in Figure 1.9, cells with stem cell like properties are considered to include proximally sited basal cells, cells residing in association with proximal submucosal glands and variant Clara cells and alveolar type II cells more distally (Liu & Engelhardt 2008).

Studies suggest recurrent, chronic airway inflammation and injury followed by the sustained induction of reparative mechanisms contributes to the development of cancer. It has been postulated that the stem or progenitor cells involved in the repair of airway epithelial damage might act as "tumour initiating cells". Continuous and therefore deregulated repair potentially results in the persistent presence of cells with the capacity for indefinite self-renewal and the subsequent acquisition of genetic changes favours neoplastic transformation. These tumour initiating cells could originate from stem cells, progenitor cells or differentiated cells which have reacquired the ability to self-renew (Lundin & Driscoll 2013).

Lung cancer is the most common cause of cancer mortality in both men and women and has an extremely high mortality rate which has showed relatively little improvement compared to the strides made in its molecular understanding (Yagui-Beltrán et al. 2008). In humans the histological classification of lung carcinomas is subdivided into small cell carcinomas (SmCCs) and non small cell carcinomas (NSCLCs). This division reflects key differences in the pathogenesis and behaviour of these tumours types and as such the treatment regimens which are subsequently employed. 80% of lung tumours are NSCLCs and, though lung carcinomas in general are diagnosed late, this particular group are amenable to surgical resection and to potential cure if found early. Small cell carcinomas in contrast are generally assumed to have metastasised by the time they present and, as such, are not considered amenable to surgical therapies. The mainstay of their treatment is chemotherapy, but they follow a very aggressive course and the prognosis is poor.

Thus the identification of NSCLCs and the development of effective, targeted therapy is a key research object, given that advances in this field will affect a huge number of patients. This group of tumours includes adenocarcinomas, squamous cell carcinomas and adenosquamous carcinomas.

Recurrent exposure to pollutants, particularly cigarette smoke results in the development of squamous metaplasia, particularly in the proximal airways which are particularly exposed to assault. As such the development of squamous cell carcinomas is seen commonly in the lungs of smokers, primarily in a proximal location. In contrast adenocarcinomas classically arise in a peripheral location and are the tumour type most commonly encountered in non-smokers. The differences observed in the location of these tumours has resulted in speculation that the cell of origin in their development is different

and could reflect the involvement of different progenitor cells encountered in these spatially distinct parts of the adult airway (Gomperts et al. 2011; Crystal et al. 2008).

In the last decade huge advances in our understanding of the biological basis of NSCLCs have been made, though this has not as yet translated into huge advances in its clinical management. In part this is due to the fact that though key pathways have been identified, there are a wide variety of driver mutations resulting in tumour formation. In lung tumourigenesis it appears that disruption to key signalling pathways is more important than individual mutations. This 'convergent evolution' makes therapeutic intervention difficult and increases the likelihood of subsequent drug resistance. Tumour cells able to circumvent targeted intervention points can continue activation of the same pathway. Greater understanding of the steps involved in these pathways is critical to identify intervention points with reduced potential for collateral navigation and with the least risk of significant damage to adjacent normal cells. In addition the fact that lung tumours often arise on a background of chronic tobacco exposure means numerous passenger mutations occur, which hinders research, complicating the genetic dissection of these tumours. It is important to identify those mutations important in the progression of tumours from those which are just coincidentally present. For these reasons, mouse models are particularly useful in the genetic dissection of lung tumourigenesis and have facilitated the identification of key driver mutations where lung tumours have occurred as a result of the inactivation of specific genes (Cooper et al. 2013).

Commonly occurring mutations in KRAS, EGFR and BRAF have been identified to be particularly associated with the development of adenocarcinomas and as such assessment of EGFR and KRAS mutations is undertaken routinely on histological diagnosis of this

tumour type to facilitate chemotherapeutic decisions. Specifically treatment with EGFR inhibitors represents the application of targeted therapies when this specific driver mutation has been identified. (Binder & Hegenbarth 2013; Johnson et al. 2012).

Interestingly the role of driver mutations in the development of squamous cell carcinoma, comprising 30% of NSCLCs, and the subsequent development of targeted therapies has lagged behind advances in adenocarcinoma treatment. However the identification of FGFR1 and MET amplifications, DDR2 mutations and alterations in PI3K-Akt signalling pathways has recently resulted in the development of several clinical trials for targeted drug treatment of squamous cell carcinomas (Kim 2013).

1.4.4 Breast Carcinoma

1.4.4.1 Overview

The breast is composed primarily of a simple cuboidal epithelium designed primarily for secretion. Breast cancer is one of the most common cancer types to be diagnosed in women (Lewis-Wambi & Jordan 2009), but in contrast to the organs discussed so far, squamous cell carcinomas are very rarely seen here (Hennessy et al. 2005). The predominant tumour types seen are adenocarcinomas and clinically they are further subdivided to reflect variations in origin and behaviour which influence surgical and medical management. For example, invasive ductal carcinomas, which comprise the most commonly encountered group of tumours, behave differently to lobular carcinomas which characteristically lack E-cadherin expression and have a much more discohesive pattern of growth. Lobular carcinomas can be diagnosed at a later, more advanced stage due to their infiltrative pattern of growth and classically reduced induction of an accompanying desmoplastic response. Furthermore lobular carcinoma is more likely to show bilateral involvement which can influence clinical decisions with respect to adjuvant hormone therapy and follow up (Arpino et al. 2004). Specific special types such as medullary carcinomas can be associated with inactivation of BRCA 1 and 2 and as such are seen in younger women (Iau et al. 2004). They will not be discussed further in this investigation.

The histological characterisation of breast tumours also routinely includes an assessment of tumour grade and importantly an assessment of the estrogen receptor (ER), progesterone receptor (PR) and Her2 status. These molecular results have a significant impact on prognosis and play an important role in guiding medical management. The majority of tumours retain ER expression (~70%) and the effects of estrogen signalling on breast tumour development and progression have been extensively studied.

1.4.4.2 Estrogen Signalling

Estrogens are a group of steroid hormones, primarily recognised for their critical role in the development of secondary sexual characteristics and reproductive capability in the female. In addition a wide range of cardiovascular, musculoskeletal and immune functions are mediated through estrogen signalling in both men and women. The primary compound in pre-menopausal woman is the highly potent 17β-estradiol (E2) synthesised from the ovary whereas weaker, more abundant levels of other estrogens predominate during pregnancy and in the postmenopausal period when the peripheral aromatisation of adrenal androgens is their primary source (Lewis-Wambi & Jordan 2009). Factors which increase an individual's lifetime exposure to E2, such as early menarche and late menopause are known to increase the risk of breast cancer development (La Merrill et al. 2010).

The cellular effects of estrogen signalling are predominantly mediated through gene regulation by nuclear steroid receptors, primarily ERα and ERβ, functioning as ligand dependent transcription factors. This classical model consists of ligand binding, the subsequent disassociation of the receptor from chaperones, receptor dimerization, association with nuclear coactivators/corepressors and binding to estrogen responsive elements (EREs) in the promoters of target genes to modulate transcription. However rapid effects of estrogen signalling are also described which are not dependent on the modulation of gene transcription and there is growing evidence that these are mediated via steroid receptors present at the cell membrane and also through other receptors, namely G-protein coupled receptors such as GPR30. Furthermore the activity of nuclear steroid receptors can be regulated in other, ligand independent ways including by EGFR and insulin growth factor -1 (IGF-1). Once activated, estrogen receptors can also indirectly affect gene transcription through interactions with other transcription factors including

Activator Protein 1 (AP1) and Specificity Protein 1 (SP1) adding to the complexity (Liang & Shang 2013; Edwards 2005; Prossnitz et al. 2008).

ERα and ERβ are encoded by different genes (*ESR1* and *ESR2* respectively) and show considerable homology in their C-terminus, ligand and DNA binding domains (Figure 1.10). However studies have demonstrated opposing effects on proliferation and apoptosis and suggest the effect of estrogen signalling in cells might be related to the relative proportion of the receptor subtypes. Furthermore ERα-36, a truncated form of ERα, may also inhibit its activity. As eluded to earlier, binding of steroid receptors to co activators and repressors such as p300 and E6-AP further modulates the response to estrogen signalling. The relative proportion of co regulators is therefore considered a key factor in the specific cellular response to receptor mediated estrogen signalling. All together these variables contribute to a complex and highly regulated system (Figure 1.11) (Liang & Shang 2013).

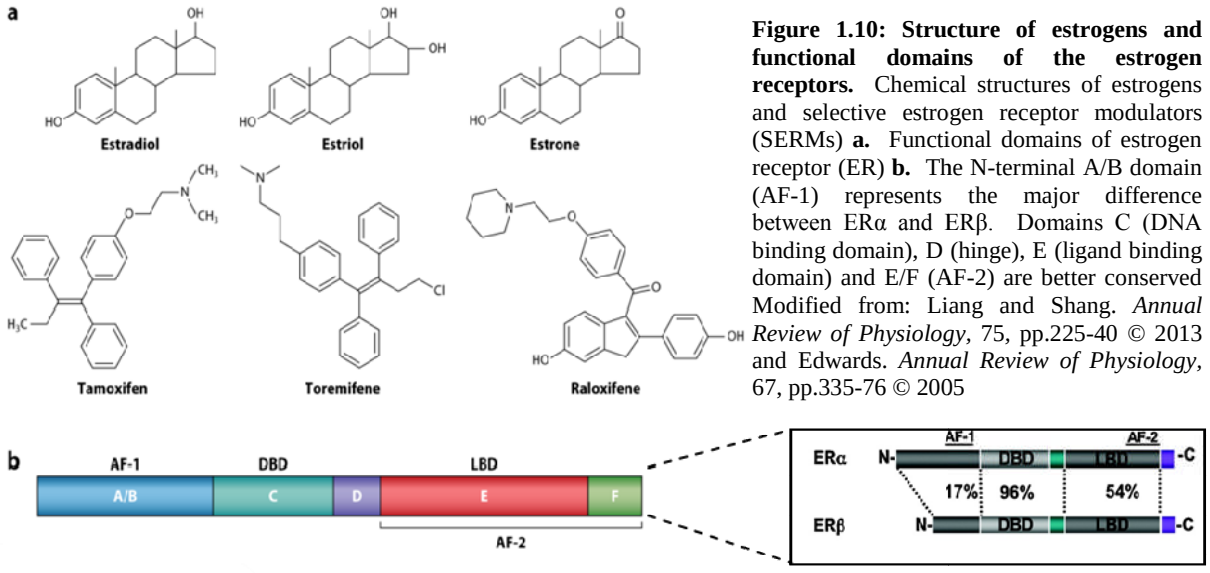
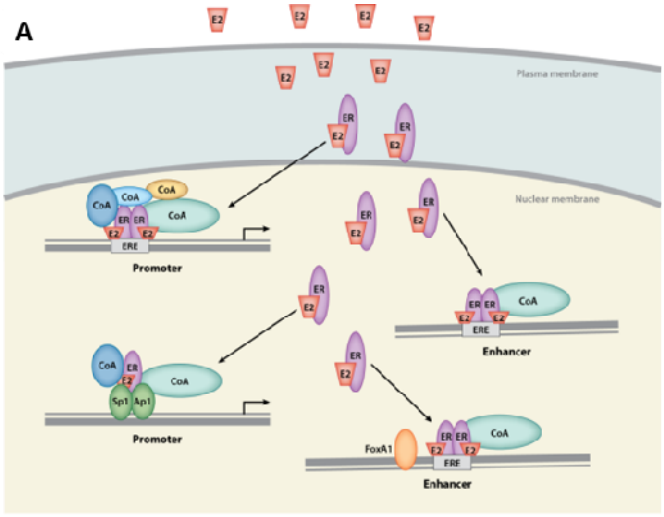
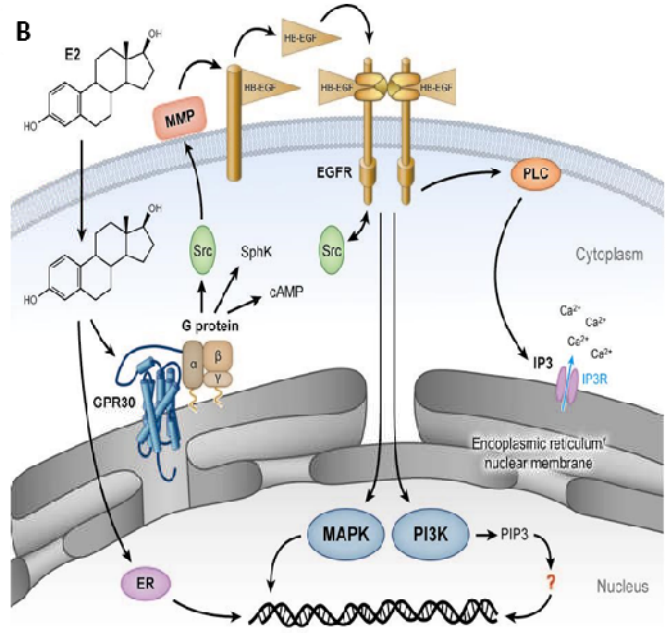


Figure 1.10: Structure of estrogens and functional domains of the estrogen receptors. Chemical structures of estrogens and selective estrogen receptor modulators (SERMs) **a.** Functional domains of estrogen receptor (ER) **b.** The N-terminal A/B domain (AF-1) represents the major difference between ERα and ERβ. Domains C (DNA binding domain), D (hinge), E (ligand binding domain) and E/F (AF-2) are better conserved Modified from: Liang and Shang. *Annual Review of Physiology*, 75, pp.225-40 © 2013 and Edwards. *Annual Review of Physiology*, 67, pp.335-76 © 2005



Liang J, Shang Y. 2013.
Annu. Rev. Physiol. 75:225–40



Prossnitz ER, et al. 2008.
Annu. Rev. Physiol. 70:165–90

Figure 1.11: Effects of estrogen receptor activation. Estrogen receptors primarily modulate gene transcription. E2 diffuses into the cell and binds to ER (ERα/ERβ). Ligand induced receptor dimerisation is followed by binding to EREs. In addition ligand bound receptors can modulate transcription by binding to with other transcription factors such as Sp1 and Ap1. The recruitment of coregulators is also shown. Recent studies show ER can bind to enhancer elements distal to target genes, occasionally requiring FoxA1. **A.** Rapid effects of estrogen signalling can occur through its interaction with GPR30. The direct transcriptional effects are also illustrated here. On activation by ligand, GPR30 activates heterotrimeric G proteins, which in turn activate multiple effectors. These can induce rapid, non genomic, effects and alternatively modulate transcription of genes without EREs **B.**
Reprinted by permission from: Liang and Shang. *Annual Review of Physiology*, 75, pp.225-40 © 2013 and Prossnitz. *Annual Review of Physiology*, 70, pp.165-90 © 2008

In breast cancer the promotion of proliferation and down regulation of apoptosis, crucial to its progression, are considered to be mediated primarily through ERα regulation of gene transcription. In fact, though ERβ is the predominant isoform in normal breast, there is evidence to suggest its down regulation in breast carcinomas (Saji et al. 2005).

Clinically, ER positivity refers to ERα positivity and determines whether hormone treatment is considered part of a patient's management. The most widely used hormonal therapy is Tamoxifen and overall its use is considered to have significantly influenced the improvements in breast cancer outcomes over the last decade (Ring & Dowsett 2004). Tamoxifen is a selective estrogen receptor modulator (SERM) which acts as an estrogen antagonist in the breast, but as an agonist elsewhere such as in the endometrium. The cellular context and relative expression of coregulators is considered to contribute to these tissue –specific differences in function (Schiff et al. 2003).

However though the majority of tumours retain ER expression, the beneficial effects of Tamoxifen treatment are by no means universal. Variations in drug metabolism have been implicated in poorer outcomes for some women, specifically for women with polymorphisms in CYP2D6, the dominant enzyme in the production of 4-hydroxy tamoxifen and endoxifen, active metabolites of tamoxifen (Higgins & Stearns 2011). Furthermore the eventual development of Tamoxifen resistance contributes to significant relapse rates for metastatic disease and the dissection of molecular mechanisms contributing to this are needed. Mechanisms implicated to date include loss of ER expression, changes in cellular coregulators, specifically loss of corepressors, and up regulation of other signalling pathways e.g. Her2.

The relative proportion of co activators and co repressors of ERα within a given cell could therefore not only influence the consequences of ERα signalling, but also tumour response to treatment. Identification of novel molecules influencing ERα activity could potentially provide attractive targets for therapeutic modulation. Of note is a particular LXXLL motif found in many of these molecules, facilitating their interaction with the receptor (Chang et al. 1999, Heery et al. 1997).

Interestingly this motif is also found in the C-terminal of iASPP. Moreover the efficient transcription of ER target genes is known to be enhanced by the recruitment of p300 and other chromatin modifiers (Catoe & Nawaz 2011). It has been reported previously that iASPP's interaction with p300 is an important part of its regulatory activity on the p53 promoter (Gillotin & Lu 2011). It is possible that iASPP, perhaps together with p300, is involved in the modulation of ER activity.

Aim of the project

The primary aim of this project is to investigate the expression of ASPP2 and iASPP, relative to p63, to corroborate their potential role in the maintenance of normal human squamous epithelium and to confirm their deregulation during neoplastic transformation. In addition ASPP2's potential regulation by HPV infection will be addressed in the human cervix and oropharynx.

The second part of the project aims to characterise the conditional deletion of ASPP2 in a novel mouse model of tumourigenesis, in order to provide new insights into the consequences of its somatic deletion on the precipitant development of carcinomas.

The third part of this investigation aims to clarify a potential interaction between iASPP and the estrogen receptor and to determine the effects of iASPP deletion on the behaviour of breast carcinoma cells. Moreover the potentially detrimental effect of iASPP expression in human breast carcinoma will be investigated.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemical Reagents

All chemicals, unless otherwise stated, were obtained from Sigma, BDH Chemicals or Fisher Scientific. All tissue culture dishes and flasks were obtained from Becton, Dickinson and Company (BD).

Ammonium Persulphate (APS)

10% (w/v) stock solution dissolved in water

Stored at -20^{o}C in aliquots

Blocking Solutions

Western Blot: 10% (w/v) fat-free milk (Marvel) dissolved in 1X TBS-T

Immunohistochemistry: 5% (v/v) Normal Goat Serum dissolved in 1X PBS

Cells – Immunofluorescence:1% Bovine Serum Albumin (BSA) dissolved in 1X PBS

β-estradiol (Sigma E2758)

1mg powder dissolved in 1ml absolute ethanol and added to 49ml media to make 20 μg/ml stock solution. Filtered and stored at -20^{o}C in aliquots.

Diluted in media to working concentration of 10^{-7}M for use

COmplete^{TM} protease inhibitor cocktail

One tablet (COmplete^{TM}, Boehringer Mannheim) dissolved in 2.0ml sterilised distilled water to make 25X stock solution. Stored at -20^{o}C (up to 3 months)

Diluted to 1X prior to use

DAPI

DAPI powder dissolved to make concentration of 1mg/ml

Stored at -20^{o}C in aliquots

Freezing Medium

50% (v/v) Media

45% (v/v) FBS

5% (v/v) DMSO

Stored at 4^{o}C

Mowiol

6ml Glycerol

2.4g Mowiol 4-88 (Calbiochem)

12ml Tris-HCL 200mM (pH 8.5)

Volume adjusted to 50ml. The solution was then heated to 60^{o}C for several hours to facilitate dissolution before filtering

Stored at -20^{o}C in aliquots

DABCO added prior to use

NETN buffer

50mM Tris pH 8.0

150mM NaCl

1mM EDTA

1% (v/v) NP40

Stored at Room Temperature (RT) and at 4^{o}C prior to Co-IP experiments

Paraformaldehyde Solution (4%)

100µl 10M NaOH

4g Paraformaldehyde

98ml PBS

Powder dissolved in PBS using heat and then filtered

Stored at -20^{o}C in aliquots

Phosphate Buffered Saline (PBS)

10X PBS (Gibco) diluted in distilled water to 1X

Ponceau S (10X)

5% (v/v) Acetic acid

2% (v/v) Ponceau S (sodium salt)

30% (w/v) Trichloroacetic acid CCl_3.COOH

30% (w/v) 5-sulfosalicyclic acid $C_7H_6O_6S.2H_2O$

The solution was dissolved in water to 1X

Protein G Sepharose

Protein G Sepharose beads (Pharmacia Biotech) stored in 20% ethanol at 4^{o}C

Washed with PBS and suspended as slurry (1:1) prior to use.

SDS Solution

10% (w/v) SDS (Severn Biotech)

Dissolved in water and stored at RT

2 x SDS-PAGE loading dye

100mM Tris-HCL (pH 6.8)

4% (w/v) SDS

20% (v/v) Glycerol

0.2% (w/v) Bromophenol Blue

Volume adjusted to 50ml with distilled water

5% (v/v) β-mercaptoethanol was added prior to use

4 x SDS-PAGE loading dye

Purchased from Invitrogen

5% (v/v) β-mercaptoethanol was added prior to use

10x SDS-PAGE Running Buffer

720g Glycine

150g Tris

50g SDS

Volume adjusted to 5L with distilled water and diluted to 1X prior to use

10x SDS-PAGE Transfer Buffer

725g Glycine

145g Tris

Volume adjusted to 5L with distilled water

Diluted to 1X with distilled water and 20% (v/v) ethanol prior to use

Stripping Buffer

62.mM 15.5ml of 1m Tris-HCL, pH6.7

100mM 1.75ml β-mercaptoethanol

2% 5g SDS

Volume adjusted to 250ml with distilled water

This was used at 50^{o}C for 20-30mins until signal obscured

TO-PRO

Purchased from Invitrogen and stored at -20^{o}C

Tris Solutions

Tris base solutions were dissolved in water to provide a range of different strength solutions with pH adjusted with concentrated HCL for individual experiments

10X Tris Buffered Saline Tween (TBS-T)

24.2g Tris

7.3g NaCl

50ml Tween-20

pH adjusted to 7.6 with HCL, adjusted to volume of 1L and diluted to 1X prior to use

Triton

0.1% (v/v) Triton dissolved in PBS

Stored at 4^{o}C

Urea Buffer

8M Urea

1M Thiourea

0.5% CHAPS

50mM DTT

24mM Spermine

Stored at -20^{o}C in aliquots

Water

Nanopure water (Type 1) generated from MilliQ water system was used for all procedures

2.1.2 SDS-polyacrylamide gels

	Resolving (ml)				Stacking (ml)
	6%	8%	10%	12%	4%
H_2O	5.3	4.6	4.0	3.3	6.8
Acrylamide	2	2.7	3.3	4.0	1.7
1.5M Tris-HCL pH8.8	2.5	2.5	2.5	2.5	_
1.0M Tris-HCL pH6.8	_	_	_	_	1.25
10% SDS	0.1	0.1	0.1	0.1	0.1
10% APS	0.1	0.1	0.1	0.1	0.1
TEMED	0.008	0.006	0.004	0.004	0.01
Total	10	10	10	10	10

Abbreviations: Ammonium Persulphate (APS), N,N,N',N',-tetramethyl-ethylenediamine (TEMED), Tris(hydroxymethylaminomethane) (Tris),Sodium dodecyl sulphate (SDS), Water (H_2O)

2.1.3 Antibodies

2.1.3.1 Primary antibodies

Antigen	Name/Clone	Host	Type	Source	Application & Concentration
ASPP2	DX54.10	Mouse	M	LICR	IHC 1:400, IF 1:100, WB 1:500
iASPP	LX049.3	Mouse	M	LICR	IHC 1:400, IF 1:100, WB 1:1000
Actin	C-2	Mouse	M	Santa Cruz	WB 1:200
Acetylated alpha tubulin (AAT)	611B-1	Mouse	M	Sigma	IF 1:200
Clara Cell Protein (CC10)	T-18	Goat	P	Santa Cruz	IF 1:100
CGRP	-	Rabbit	P	Calbiochem	IF 1:100
Cytokeratin 5 (CK5)	-	Rabbit	P	Covance	IF 1:500
Cytokeratin 14 (CK14)	-	Rabbit	P	Covance	1F 1:750
Cleaved caspase 3	Asp 175	Rabbit	P	Cell Signaling	IHC 1:200, IF 1:100
E6-AP	-	Rabbit	P	Bethyl Laboratories	WB 1:10000
Estrogen Receptor (ER)	SP-1	Rabbit	M	Thermo Scientific	IF 1:100, WB 1:1000
Estrogen Receptor α (ERα)	D8H8	Rabbit	M	Cell Signaling	WB 1:1000
Estrogen Receptor β (ERβ)	-	Rabbit	P	Santa Cruz	WB 1:1000
GAPDH	-	Mouse	M	Abcam	WB 1:2000
Involucrin	-	Rabbit	P	Covance	IF 1:100
Ki67	-	Rabbit	M	Vector Labs	IHC 1:200
Ku80	-	Rabbit	M	Abcam	WB 1:200
Mucin	Muc5AC	Mouse	M	Abcam	IF 1:100
p53	DO-1	Mouse	P	LICR	WB 1:2000
p63	4A4	Mouse	M	Santa Cruz	IHC 1:400, IF 1:100
Prosurfactant Protein (SP-C)	-	Rabbit	P	Millipore	IF 1:100
Synaptophysin	-	Rabbit	P	Abcam	IF 1:500
V5-tag	-	Mouse	M	AbD Serotec	WB 1:500

Abbreviations: Calcitonin Gene Related Peptide (CGRP), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Immunohistochemistry (IHC), Immunofluorescence (IF), Ludwig Institute for Cancer Research (LICR), Monoclonal (M), Polyclonal (P), Western Blot (WB)

Validation of ASPP Primary Antibodies

The specificity of the ASPP antibodies was validated using tissue from ASPP2 and iASPP knockout mice. The following tissues were provided by Sofia Koch (Oesophagus $ASPP2^{+/+}$ vs $ASPP2^{\Delta 3/\Delta 3}$) and Kathryn Chung (Skin $iASPP^{+/+}$ vs $iASPP^{\Delta 8/\Delta 8}$) (Figure 2.1).

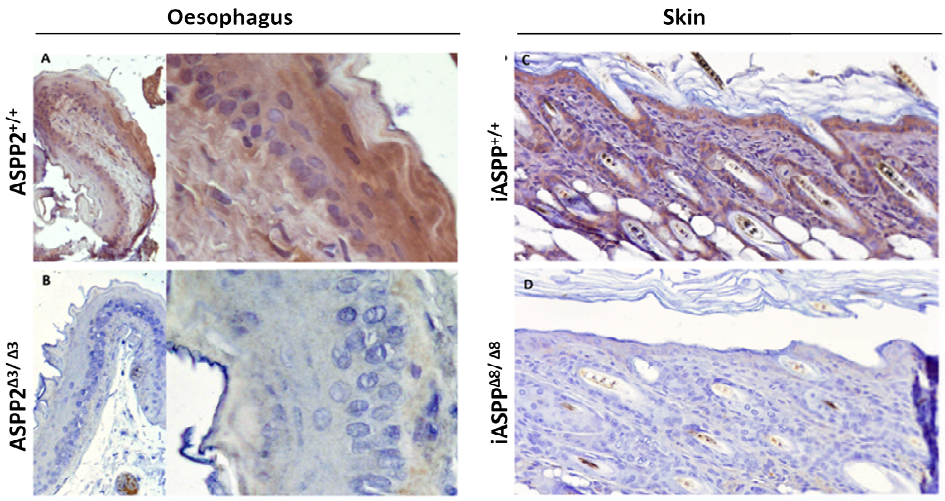


Figure 2.1: iASPP and ASPP2 primary antibody specificity controls. Antibodies for iASPP (LX049.3) **C&D** and ASPP2 (DX54.10) **A&B** show significantly reduced staining in the transgenic mice where their substrates have been deleted.

Figures 2.2 and 2.3 demonstrate the methods used in the generation of the knockout mice used in this experiment (taken from the literature).

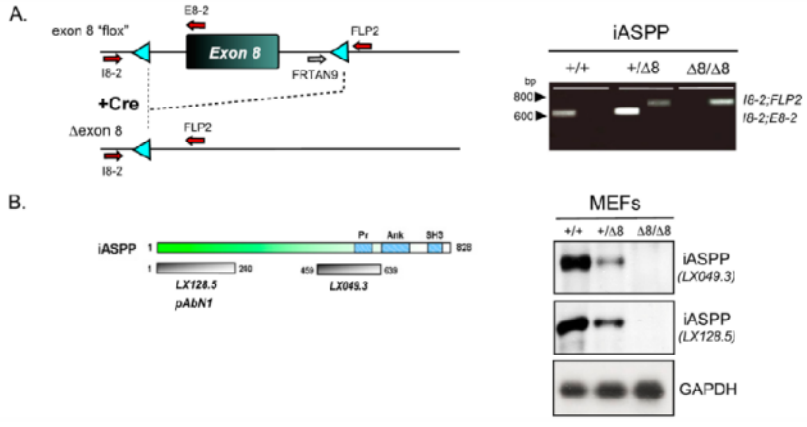


Figure 2.2: Generation of the transgenic mouse in which iASPP expression is under the control of the Cre/LoxP system. Schematic representation of part of the conditional iASPP KO allele with details of the primers used to confirm deletion of exon 8 (red arrows). The blue triangles represent loxP sites *(left panel)*. Deletion of exon 8 was confirmed by PCR **A.** Schematic representation of the antigen used to generate the iASPP antibodies relative to the iASPP protein with absence of iASPP protein confirmed by immunoblot analysis using two different antibodies spanning **B**
Modified from: Notari et al. *PNAS,* 108(40), pp.16645-50 © 2011 National Academy of Sciences, USA

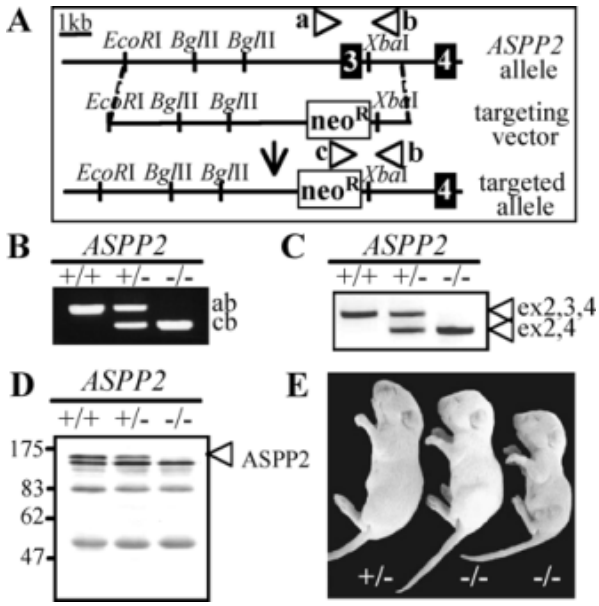


Figure 2.3: Generation of the transgenic mice with a targeted disruption of the ASPP2 gene. Schematic representation of part of wild-type *ASPP2* (*top*), targeting vector (*middle*), and targeted *ASPP2* allele (*bottom*) with primer locations **A.** PCR confirmation of DNA deletion **B&C.** Western blot confirmation of protein down regulation **D.** Phenotype of P9 pups with indicated *ASPP2* genotypes **E**
Modified from:Vives et al. *Genes and Development* 20(10), pp.1262-7 © 2006**.**

2.1.3.2 Secondary antibodies

Antibody	Host	Source	Application & Concentration
Alexa Fluor® 488 Anti-Mouse IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 488 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 546 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 647 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF 1:400
Biotinylated Anti-Mouse IgG	Goat	Vector Labs	IHC 1:250
Biotinylated Anti-Rabbit IgG	Goat	Vector Labs	IHC 1:250
Anti-Mouse immunoglobulins/HRP	Rabbit	DAKO	WB 1:3000
Anti-Rabbit immunoglobulins/HRP	Swine	DAKO	WB 1:3000

Abbreviations:Horseradish Peroxidase (HRP), Immunohistochemistry (IHC), Immunofluorescence (IF), Ludwig Institute for Cancer Research (LICR), Monoclonal (M), Polyclonal (P), Western Blot (WB)

2.1.4 Cell Lines

Cell Line	Organ	Source	Notes
Caski	Cervix	ATCC	Epidermoid carcinoma. Cell Line derived from small intestinal metastatic deposit
MCF-7	Breast	LICR	Invasive breast ductal carcinoma. Cell line derived from pleural effusion. Wild type p53, ER positive.
MDA-MB-231	Breast	LICR	Invasive breast ductal carcinoma. Cell line derived from pleural effusion. Mutant p53, ER negative.

Abbreviations: Ludwig Institute for Cancer Research (LICR), *American Tissue Culture Collection*: The Global Bioresource Center (ATCC)

The generation of MCF-7 cells stably transfected with truncated versions of the iASPP N-terminus (1-478) and C-terminus (479 – 828) tagged with V5 has been described previously (Slee et al. 2004).

2.1.5 Small interfering RNAs

siRNA	Source	Sequences
PP1R13L iASPP	Thermo Scientific 03815-00-0002	AGUAAAGUCUAGCAGGAUA
		GCACGGGUGUUGGCGGAAA
		GCAGACGUCGAGCAGAGUA
		UCGAGAAGUGCGACCCUUA
UBE3A E6-AP	Thermo Scientific M-005137-00	GUACAGAGCUUCCGGAAAG
		CCAGAUUGCUCUCUAAUGA
		GAAAGGCGCUAGAAUUGAU
		GCAGUUGAAUCCAUAUUUG
RISC-free Control	Thermo Scientific D-001220-01-05	Sequence is property of Dharmacon

2.2 Methods

2.2.1 Human Tissue Analysis

Ethical approval for the analysis of human tissue samples in this study is incorporated in "**The role of signalling pathways in human disease". Ethics Ref: 09/H0606/78.**

2.2.1.1 Sample Details

A combination of full tumour and tissue microarray sections were used to analyse the expression of the ASPP proteins in a variety of human tissues in this investigation.

2.2.1.1.1 Full Tumour sections

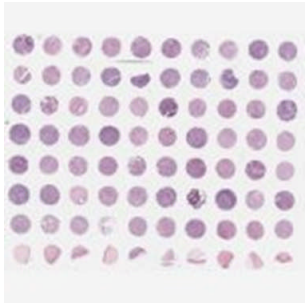
Organ	Number & Details	Source Contact	Institution
Buccal	3 SCC + adjacent normal squamous epithelium	Professor Mahvash Tavassoli	King's College Hospital, London
Cervix	29 samples - CIN + adjacent normal	Professor Robert Goldin	St Mary's Hospital, London
Esophagus	25 biopsies - 15 with normal squamous epithelium	Dr Richard Gillies	University of Oxford
Larynx	3 SCC + adjacent normal squamous epithelium	Professor Mahvash Tavassoli	King's College Hospital, London
Tongue	6 SCC + adjacent normal squamous epithelium	Professor Mahvash Tavassoli	King's College Hospital, London
Tonsil	6 SCC + adjacent normal squamous epithelium (3xHPV+ve, 3xHPV-ve)	Professor Mahvash Tavassoli	King's College Hospital, London

Abbreviations: Cervical Intraepitheial Neoplasia (CIN), Human Papillomavirus (HPV), Squamous Cell Carcinoma (SCC)

2.2.1.1.2 Tissue Microarray sections

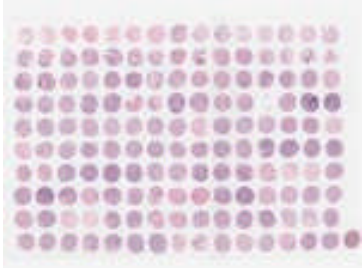
2.2.1.1.2.1 Source: US Biomax, Ltd

Cervical Cancer CR803



Cores:	Total (80), Normal Tissue(10), Tumours(70)
Median Age:	46 years
Histology:	Squamous Cell Carcinoma(70)
H_score iASPPn:	Median 0, Mean 15.893
H_score ASPP2c:	Median 0, Mean 21.893
p63:	Median 0, Mean 30.21

Breast Cancer BR1503b

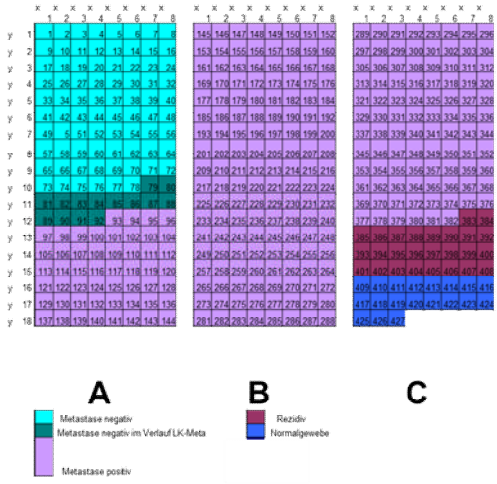


Cores:	Total (134), DCIS (14), Tumours(120)
Median Age:	50 years
Histology:	Ductal adenocarcinoma(120)
Grade:	1(8), 2(91), 3(15)
ER:	Negative(81), Positive(39)
Ki67:	Low<10(94), High>10(26)
H_score iASPPn:	Median 40 (Low<40, High>40)

2.2.1.1.2.2 Source: University Hospital Zurich, Professor Holger Moch

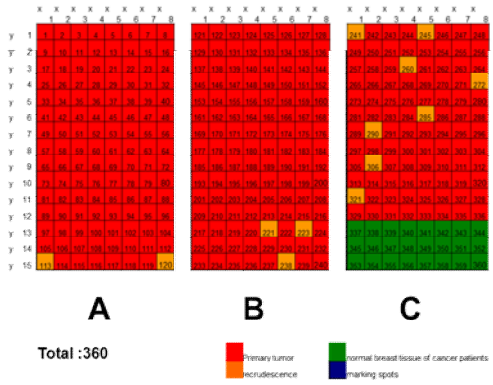
Oral/Oropharyngeal Cancer ZTMA 42

Cores:	Total (427), Normal Tissue(19), Tumours(408)
Histology:	Squamous Cell Carcinoma(408)
Clinical:	Non-metastatic(92), Metastatic(160), LN Deposits(130), Recurrences(26)
H_score ASPP2c:	Median 0
H_score iASPPn:	Median 30



Breast Cancer ZTMA 26

Cores:	Total (360), Normal Tissue(24) Tumours(336)
Median Age:	62 years
Median Followup:	86 months
Histology:	Ductal adenocarcinoma(281) Lobular adenocarcinoma(36) Others(9)
Grade:	1(34), 2(146), 3(132)
pT:	1(129), 2(143), 3(20), 4(31)
pN:	0(130), 1(105), 2(32), 3(14)
ER:	Negative(53), Positive(271)
PR:	Negative(90), Positive(234)
Intensity iASPPn:	Negative vs Positive
ASPP2N&C:	Median 160, Upper Quartile 200



Tissue sections were derived from paraffin embedded samples in all instances. The commercially bought Cervical TMA slides (CR803) were further preserved in a thick layer of wax which was melted at 60^{O}C prior to processing as described below.

2.2.1.2 Haematoxylin and Eosin Staining

Sections were dewaxed, rehydrated, immersed in Harris Haematoxylin (3mins), differentiated with acid alcohol (1 dip), blued in Scotts Water (30s) and then immersed in Eosin (5mins) with washes in between steps. Sections were then dehydrated and permanently mounted (Vectamount – Vector Labs).

2.2.1.3 Immunostaining

2.2.1.3.1 Conventional Immunohistochemistry: iASPP, ASPP2

Sections were dewaxed, rehydrated and incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity (10mins). Sections were then blocked with 5% normal goat serum (NGS), incubated overnight (O/N) at 4^{o}C with the primary antibody against iASPP (LX049.3) or ASPP2 (DX54.10) diluted in NGS to 1:400, followed by the biotinylated secondary antibody for 40 min at room temperature (RT). The Avidin–Biotin immunoperoxidase-Complex (ABC) Vectasatin Elite Reagent (Vector Labs) was used to amplify the signal and catalyse the hydrogen peroxide oxidation of 3,3'-Diaminobenzidine (DAB - Vector Labs) to allow substrate detection. The sections were then counterstained with haematoxylin, dehydrated and permanently mounted.

Sections were then analysed and in the case of TMA's scored appropriately. iASPP and ASPP2 expression were scored based on the intensity of staining (0-none, 1-weak, 2-moderate, 3-strong) and the proportion of cells exhibiting staining (0-100% in 5-10% increments). The overall expression is a product of these two scores (Expression = Intensity x Proportion (0-300)). Statistical analysis was undertaken in SPSS.

Furthermore the objective analysis of DAB staining could be undertaken. The original images were inverted (Photoshop), unmixed (ZEN) and signal intensity could be measured (ImageJ).

2.2.1.3.2 Conventional Immunohistochemistry: Other antibodies

Sections were processed exactly as described above with an additional heat induced epitope retrieval (HIER) step undertaken prior to quenching of the endogenous peroxidase activity. Sections were immersed in 10mM Sodium Citrate pH 6.0 and microwaved for 10 mins. Sections were incubated O/N at 4^{o}C with the primary antibody and the subsequent steps were as detailed above.

TMA Scoring: p63 scores reflected the proportion of positive cells (0-100% in 5-10% increments).

2.2.1.3.3 Double Immunohistochemistry: iASPP or ASPP2 with p63

The endogenous peroxidase activity was quenched in dewaxed and rehydrated sections prior to blocking with NGS and then incubation with iASPP (LXO49.3) or ASPP2 (DX54.10) overnight (O/N) at 4^{o}C followed by the biotinylated secondary antibody for 40 mins at RT. ABC complexes were added for 15 mins before visualising the substrate using DAB. Instead of washing in H_2O here prior to haematoxylin counterstaining, the sections were washed in PBS and then immersed in Sodium Citrate at pH 6.0 in the microwave for 10 minutes. Lan et al 1995 demonstrated than in addition to facilitating the antigen retrieval for the second substrate, this step also successfully blocks any overlap which could be expected from the use of two mouse monoclonals and two peroxidase based systems. An additional immersion in 3% hydrogen peroxide in methanol was undertaken here to ensure no background peroxidise activity remained. The sections could then be blocked with NGS, incubated O/N with p63 (4A4) primary antibody and the steps repeated the next day up until visualisation with Vector VIP (Vector Labs) rather than

DAB. The sections were then dehydrated and permanently mounted with no haematoxylin counterstain.

ASPP2 and p63 were thought to be mutually exclusive and this staining pattern was a good control to demonstrate the lack of non-specific staining when two mouse monoclonal primaries were used together. Singly stained ASPP2 and p63 were also visualised with DAB and VIP respectively to allow pure profiles to be extracted in the Image J to facilitate the design of a matrix to enable clean spectral unmixing. These single controls were unmixed with the same matrix as the double stained sections and demonstrated clean separation as seen in the Results.

2.2.1.3.4 Conventional Immunofluorescence

Rehydrated sections underwent HIER with 10mM Sodium Citrate at pH 6.0 microwaved for 10mins, or with Tris EDTA buffer at pH9.0 microwaved for 20mins (p63), prior to overnight incubation with one or two primary antibodies. Sections were then incubated for 40mins at RT with AlexaFluor-conjugated mouse secondary and/or AlexaFluor-conjugated rabbit secondary antibodies (at appropriate wavelengths) with DAPI (1:2000) or TO-PRO (1:1000). Sections were mounted in a Mowiol/glycerol based solution containing anti-fading agent DABCO.

2.2.1.3.5 Double Immunohistochemistry and Immunofluorescence

The endogenous peroxidase activity was quenched in dewaxed and rehydrated sections prior to blocking with NGS and then incubation with iASPP (LXO49.3) or ASPP2 (DX54.10) O/N at 4^{o}C followed by the biotinylated secondary antibody for 40 mins at RT. ABC complexes were added for 15 mins before visualising the substrate using DAB.

Instead of washing in H_2O here prior to haematoxylin counterstaining, the sections were washed in PBS and then immersed in Sodium Citrate at pH 6.0 in the microwave for 10 minutes. The sections were then briefly washed in PBS, blocked with NGS and incubated O/N with the second and occasionally third primary antibodies (these antibodies were raised in different hosts). The next day sections were incubated for 40mins at RT with appropriate AlexaFluor-conjugated secondary antibodies with TO-PRO (1:1000). Sections were mounted as in conventional immunofluorescence protocols. Again appropriate controls were undertaken prior to experimental procedures to ensure the accuracy of the technique.

Figure 2.4 demonstrates the mutual exclusive staining of Cytokeratin 14 and nuclear p63 in the ectocervix using this technique and confirming the lack of overlap. However appropriate controls were undertaken (if not always shown) in every experiment to confirm the spatial localisation of the proteins seen corresponded to that seen in the singly stained controls.

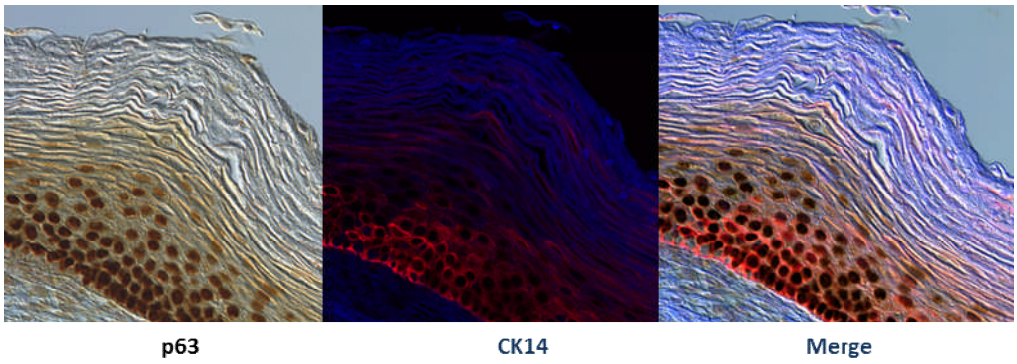


Figure 2.4: Double immunohistochemistry and immunofluorescence staining control. The figure demonstrates double staining for p63 and CK14 which are mutually exclusive. For further objective analysis the brightfield image can be inverted, unmixed and merged with the fluorescent image as is demonstrated in Chapter 4.

2.2.2 Mouse Tissue Analysis

2.2.2.1 Mouse Colonies

Animals were housed in the Wellcome Building and all conditions and procedures were carried out as determined in the Home Office Animals (Scientific Procedures) Act 1986, under Project Licence (PPL):**70/6380** and Personal Licence (PIL):**30/8797**.

ASPP2$^{flox/+}$ mice were generated by InGenious Targeting Laboratory in a mixed C57BL/6Jx129vJ background. ASPP$^{flox/+}$ conditional mice were crossed with R26Cre^{+}-ER^{T}mice (Schwenk et al. 1995), enabling the induction of exon 3 deletion on treatment with 4-OHT (Sigma). Genotyping of offspring was undertaken by PCR using primers detailed in Figure 2.5.

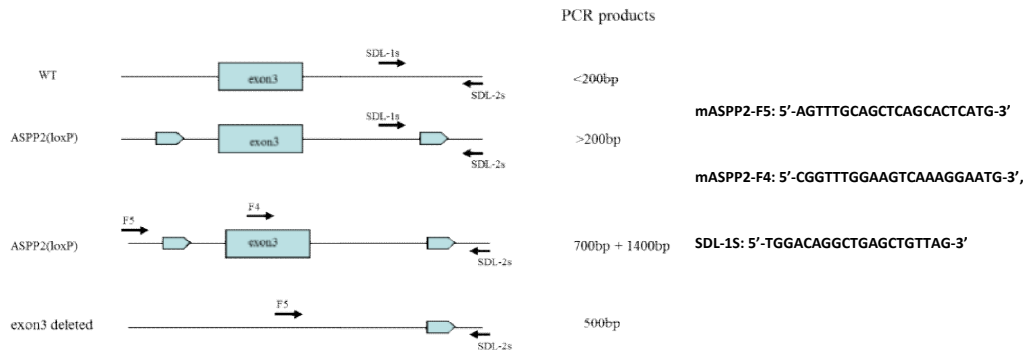


Figure 2.5: Generation of conditional ASPP2 knockout mice. Schematic representation of part of the targeting strategy resulting in the deletion of ASPP2 exon 3 with list of primers used to genotype offspring. With thanks to S.Zhong, LICR for image.

Previously genotyped ASPP2$^{flox/flox}$;Cre^{+}ER^{T} mice were obtained for the tumour study. Intraperitoneal injections of 4-OHT (100µl (10mg/ml stock in sunflower oil)) or sunflower oil alone (control) were administered to the mice for 5 consecutive days to induce gene deletion. Mice were then observed until any of the end points governing our tumour studies was reached. Mice were culled and underwent post mortem examination with a particular emphasis on the identification of tumours.

2.2.2.2 Tissue Processing

After dissection, mouse tissue was fixed in 10% buffered formalin for at least 24 hours. Tissues were dehydrated in an ethanol series and cleared in xylene in an automated machine. Tissues were then embedded in paraffin wax. Sectioning of the tissue was undertaken at a thickness of approximately 4µm and sections dried thoroughly prior to further analysis.

2.2.2.3 Analysis

H&E and immunostaining was undertaken as detailed in 2.2.1.2 for human tissue sections.

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2.2.3 Tissue Culture

2.2.3.1 Media and Supplements

Dulbecco's Modified Eagle Medium (DMEM) and Modified Eagle Medium without phenol red (MEM-PR) were purchased from Gibco. OptiMEM was purchased from Invitrogen. All media was stored at 4^{o}C. Fetal bovine serum (FBS) was purchased from PAA laboratories and stored at -20^{o}C in 50ml aliquots. Charcoal stripped fetal bovine serum (CS-FBS) was purchased from Gibco and stored at -20^{o}C in 50ml aliquots. L-Glutamine (200mM) and Penicillin/Streptomycin (10000 units/ml) were purchased from Gibco and stored at -20^{o}C in 5ml aliquots.

2.2.3.2 Routine maintenance of Cell Lines

Cell lines were grown in DMEM supplemented with 10% (v/v) FBS, 2mM L-Glutamine and 200units/ml streptomycin/penicillin at 37^{O}C with 5% CO_2. Routine maintenance involved media changes every 2-3 days depending on cell line. After reaching confluence, cells were washed with 1X PBS, incubated with 0.05% Trypsin-EDTA (Gibco) at 37^{o}C until detachment of cells was seen. Fresh media (Volume: 4X trypsin) was added to inhibit trypsin and cells were reseeded at an appropriate density in fresh flasks/dishes.

After reaching ~80%-90% confluency, cells were trypsinised and centrifuged at 1000rpm for 5 minutes. The resulting pellet was resuspended in 1.8ml of freezing medium and stored in cryovials (Corning). Vials were cooled at 1^{o}C/min in a Nalgen Cryo 1^{o}C freezing container placed at -80^{o}C for at least 24 hours. Long term storage was in liquid nitrogen. Thawing cells involved the rapid warming of vials in a 37^{o}C water bath for 2-3 minutes followed by the resuspension of cells in pre-warmed media and seeding in small flasks for overnight incubation. The media was changed the following day to aid recovery.

2.2.4 Protein Analysis

2.2.4.1 Protein Extraction

Cells were washed and trypsinised as described above. They were then collected and centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and the resulting cell pellet resuspended in urea buffer. The solution was left for 30 minutes at room temperature and then centrifuged at 13,200rpm for 20 minutes. The supernatant was then collected and stored on ice for subsequent protein quantification.

2.2.4.2 Protein quantification

The BioRad protein assay reagent system was used to determine the concentration of protein within the supernatant. 1µl of supernatant was mixed with 200µl of 1X BioRad reagent. Duplicate measurements were taken at 595nm in a spectrophotometer (Anthos Labtech instrument) and compared against a standard curve created at the same time using known concentrations of BSA (Invitrogen) measured in the same way.

2.2.4.3 Western Blotting

Polyacrylamide gels with varying concentrations of acrylamide (6-12%), depending on the size of the protein of interest, were made in casting units (BioRad) with isopropanol solution on the surface. Once polymerisation had taken place, this was rinsed away with water and replaced with stacking gel (4%) with wells delineated by an appropriate insert.

Known concentrations of protein were mixed with 4X loading buffer and boiled for 5 minutes. Samples of equal concentration were then loaded into the polyacrylamide gel adjacent to a broad range protein marker (New England Biolabs). The gels were

submerged in 1X running buffer and run at a voltage of 110-150V for 1-2 hours depending on gel concentration (Biorad system). Gels were transferred onto a nitrocellulose membrane (Whatmann) at 70-80V over 2.5 hours at 4^{o}C in a wet cassette with 1X transfer buffer (containing 20% ethanol). Transfer success was determined using Ponceau S solution and membranes were then washed (1X TBS-T), blocked with 5% milk solution for 1 hour and incubated O/N at 4^{o}C or for 3 hours at RT with the primary antibody diluted in 5% milk solution. Membranes were then washed thoroughly with 1X TBS-T and incubated for 40mins at RT with the appropriate secondary HRP-conjugated antibody. After further washing, the results were visualised by enhanced chemiluminescent detection (Amersham Pharmacia Biotech) using X-ray film (Fujifilm). Any necessary further probing was undertaken immediately or after treating the membrane with stripping buffer followed by reblocking prior to incubation with the next primary antibody.

2.2.4.3 Co-Immunoprecipitation

Cells were grown in flat dishes rather than flasks to facilitate cell harvesting. Cells were washed 3X with PBS and lysed with NETN buffer containing protease inhibitors (500µl per dish). Cells were then scraped using a sterile cell scraper and collected in eppendorfs. These were left on ice for 30 minutes, centrifuged at 13,200rpm for 20 minutes at 4^{o}C and the supernatant collected for assessment of protein concentration (BioRad assay). 50µl of the lysate was saved for use as the input control.

Protein G sepharose beads were washed 3X with cold PBS. 30µl of beads (50% slurry with PBS) were used to preclear 1000µl of lysate for 1 hour at 4^{o}C on a rotating wheel. Lysates were then centrifuged at 4000rpm for 2 minutes at 4^{o}C with collection of the supernatant.

Experimental tubes contained the pre cleared lysate, 30µl pre washed protein G sepharose beads and ~ 2µg of purified antibody. Tubes were left O/N at 4^{o}C on a rotating wheel. The next day, the tubes were centrifuged at 4000rpm for 2 minutes with collection of the beads and disposal of the supernatant. The beads were washed thoroughly 3 times with cold NETN buffer, mixed with 30µl 2X sample buffer and boiled for 5 minutes. Samples were then centrifuged at 13,200rpm for 30 seconds with the supernatant then carefully loaded into polyacrylamide gels and processed as described above for subsequent immunoblotting. Each experiment included the appropriate controls and was repeated at least twice.

2.2.5 Cell Assays

2.2.5.1 Immunofluorescence

Cells were grown on 13mm coverslips to ~80% confluency. Media was extracted and the cells washed with PBS prior to being fixed in 4% paraformaldehyde for 15 mins at RT. Cell permeabilisation was achieved using 0.1% Triton in PBS for 4 mins on ice. Cells were blocked using 0.1% bovine serum albumin (BSA) prior to incubation with one or two primary antibodies for 30 minutes (RT). After washing, cells were incubated for 20mins at RT with the appropriate AlexaFluor-conjugated secondary antibodies and Topro. Sections were washed again and mounted in a Mowiol/glycerol based solution containing anti-fading agent DABCO.

2.2.5.2 Small interfering RNA (siRNA) Transfection

Cells were plated at pre-determined optimum densities in 6cm dishes (for initial experiments). 4 different siRNA oligonucleotides targeting the protein of interest (Dharmacon) were used at final concentrations of 10nM (E6-AP) and 40nM (iASPP) and transfected using 5µl of Dharmafect 1 reagent in 195µl OptiMEM. The appropriate concentration of siRNA was added to make a total of 200µl in OptiMEM. The reagent and siRNAs were mixed and after 20mins at RT were added to 1600µl OptiMEM in wells. Cells were harvested 96 hours later with replacement of media with DMEM when necessary.

2.2.5.3 Hormone Treatments

To maximise the effects of E2 addition to estrogen sensitive cell lines, the cells were grown in phenol red free MEM (MEM-PR) supplemented with charcoal stripped FBS (CS-FBS) for 3 days prior to the addition of 10^{-7}M E2 (Sigma) for 24 hours. In experiments

combining siRNA and hormone treatments, OptiMEM was replaced with MEM after 24 hours for 3 days before the addition of E2 for 24 hours. Control experiments confirmed the maintenance of iASPP knockdown over this period.

2.2.5.4 MTT Assay

After optimisation studies, 16,000 MCF-7 and 8,000 MDA-MB-231 cells were seeded in individual wells of a 96 well plate. Cells were either incubated with normal culture media, MEM-PR or having undergone siRNA transfection, all with or without additional hormone treatment. Assessment of proliferation was then undertaken using the MTT assay (ATCC). The experiments were repeated twice in triplicate. 10µl of (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) was added to each well and incubated for 4 hours at 37^{o}C. 100µl of detergent was then added and the plate was left in the dark at RT for at least a further 4 hours. The absorbance was measured for each well at a wavelength of 570nm (with a reference taken at 750nm). The results were then analysed in SPSS and the differences analysed using a students t-test.

2.2.5.5 Cell Motility Assay

MCF-7 control and siRNA (iASPP) treated cells were grown in 24 well plates for 5 days until 90-100% confluent. Cells were washed and a scratch made with a sterile 200µl pipette tip before new media was added. The cells were incubated at 37^{o}C on a timelapse microscope which imaged the scratched area in each well every 15 minutes over a period of 30 hours. The time taken for the scratched area to close was calculated using ImageJ and graphs created in Excel.

Chapter 3: Results

ASPP2 in human carcinoma: A tumour suppressor protein

ASPP2 binds to and specifically promotes the apoptotic function of p53 and p63 (Samuels-Lev et al. 2001). In this laboratory the germline inactivation of ASPP2 precipitated the development of tumours in two separate mouse models. Furthermore the generation of compound mice suggestsASPP2 co-operates with both p53 and p63 in the development of specific tumour types.

In particular the data from these experiments suggest that ASPP2 promotes the normal maturation of stratified squamous epithelium in part due to its control of p63 repression. The loss of ASPP2 is implicated in the deregulation of normal squamous homeostasis and therefore also the development of squamous cell carcinomas (Tordella et al. 2013).

The first part of this project investigates the expression of ASPP2 in normal human stratified squamous epithelia and its deregulation in emerging dysplastic populations and squamous cell carcinomas.

3.1 Normal Squamous Epithelium

If ASPP2 is involved in the promotion and maintenance of human squamous maturation, its expression would be expected in the normal mature squamous epithelium. Moreover it would be expected in the superficial, more differentiated layers of normal stratified squamous epithelium, which lack p63 expression.

3.1.1 ASPP2 is expressed in the superficial layers of mature squamous epithelia

To investigate the expression of ASPP2 in human squamous epithelia, conventional immunohistochemical staining for ASPP2 was undertaken on sections from the morphologically normal squamous mucosae lining the ectocervix and oropharynx. Staining of24 samples from the ectocervix had been started prior to this project by Anna Pagotto, Emily Ruban and Beyza Vurusaner and its continuation and analysis is reported here. In addition a further 6 samples of normal ectocervix were stained and examined. Figure 3.3 demonstrates that the strongest expression of ASPP2 is in the cytoplasm of cells in the more superficial, differentiated layers of the epithelia examined.

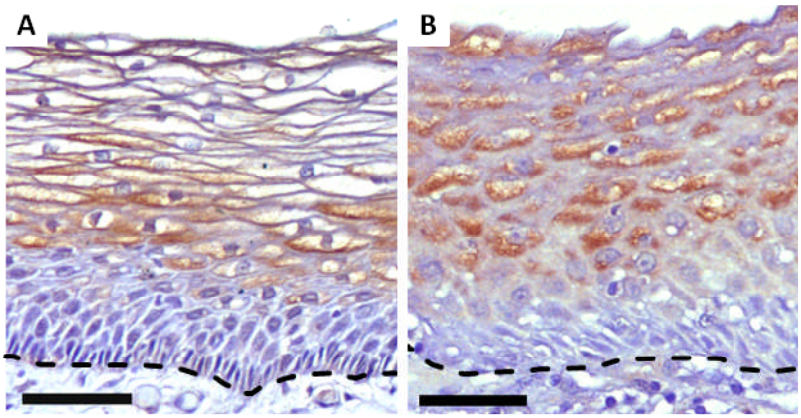

Figure 3.3: ASPP2 is expressed in the superficial layers of mature squamous epithelia. Conventional immunohistochemical staining for ASPP2 in the ectocervix **A** and tonsil **B.** ASPP2 expression spares the basal and parabasal layers in all samples analysed. Dashed lines delineate the basement membrane. Scale bar - 50µm.

Conventional labelling in immunohistochemistry utilises the horseradish peroxidase (HRP) catalysed hydrogen peroxide oxidation of 3,3'-Diaminobenzidine (DAB) to give a yellow-brown stain, providing good contrast against a purple haematoxylin counterstain and an excellent result for subjective, visual analysis. However automated methods of measuring staining intensity, for example in immunofluorescence, usually rely on the signal of interest occupying only one of the Red-Green-Blue (RGB) channels. Brown and purple stains would each generate signal in more than one channel rendering any objective analysis inaccurate. However linear unmixing can be used to interpret this type of conventional bright field image, after first inverting the image to ensure low concentrations of protein correspond to low pixel values. This allows the image to be reconfigured in such a way that the haematoxylin and DAB now each occupy just one of the RGB channels (Newberg & Murphy 2008). Objective quantification of the DAB signal is then possible providing robust and statistically analysable evidence of differences in protein expression levels.

In Figure 3.4 the original images demonstrate the expression of cytoplasmic ASPP2 in the upper two-thirds of the ectocervix. Furthermore profiling the red channel, now representing the DAB signal, clearly demonstrates an increase in ASPP2 in the more superficial, differentiated cells. This differential pattern of expression suggests that the induction of ASPP2 expression either results from or contributes to the normal differentiation of squamous epithelium.

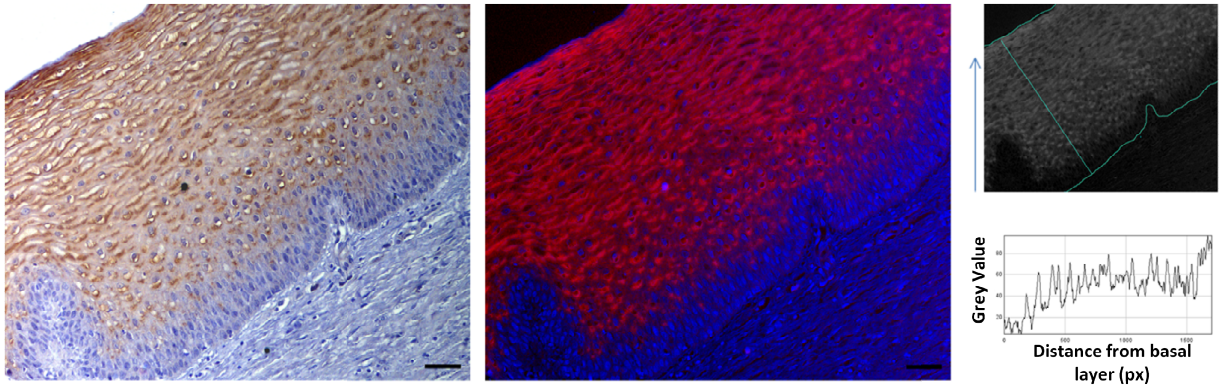

Figure 3.4: ASPP2 expression spares the basal and parabasal layers in the morphologically normal ectocervix. Conventional immunohistochemical staining of the ectocervix with the corresponding unmixed image. Detailed analysis of the DAB, now red, signal was undertaken using ImageJ and clearly demonstrates the increase in ASPP2 expression as the superficial, more differentiated, layers are reached. Scale bar – 50µm

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3.1.2 CK14 highlights sparing of the basal and parabasal layers by ASPP2

More detailed analysis of ASPP2 expression in the squamous epithelium was undertaken in the ectocervix. Conventional double immunofluorescence staining for ASPP2 and CK14 confirms sparing of the basal and parabasal layers of the ectocervix by ASPP2 (Figure 3.5).

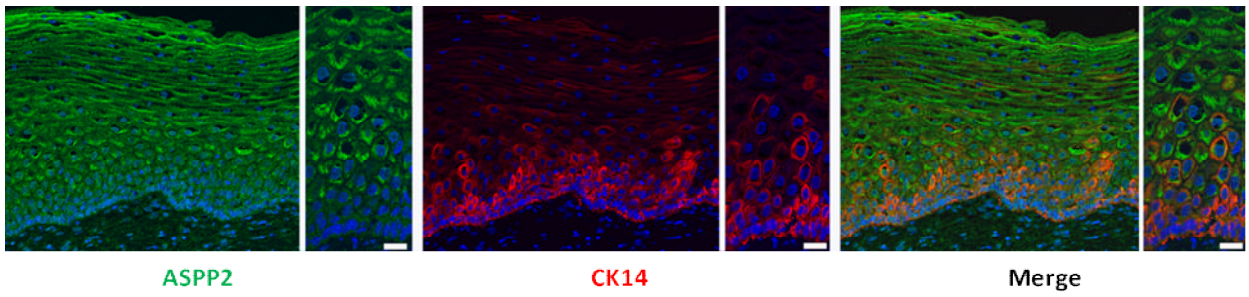

Figure 3.5: CK14 expressing basal and parabasal layers are spared by ASPP2. ASPP2 and CK14 double immunofluorescence staining. ASPP2 (green) spares the basal and lower parabasal layers where CK14 (red) expression is highest . Scale bar – 10µm

In vitro, the induction of ASPP2 expression coincides with the upregulation of differentiation associated keratins and the downregulation of p63. In addition the overexpression of ASPP2 indirectly inhibits p63 expression through its binding to IκB, inducing the subsequent nuclear accumulation of p65/NF-κB (Tordella et al. 2013). The relationship between p63 and ASPP2 expression in human squamous epithelia was therefore investigated.

3.1.3 p63 and ASPP2 are mutually exclusive in the ectocervix

The immunofluorescent staining protocols used in Figure 3.4 involved the use of heat induced epitope retrieval (HIER) with slides immersed in sodium citrate buffer at pH6.0. However technical difficulties were encountered in double staining human tissue for the ASPP family when more intensive HIER with Tris-EDTA at pH9.0 was used to effectively label nuclear p63. Here ASPP2 expression was markedly then impaired. Efforts were made to enhance the signal by incorporating additional amplification steps, as is inherent in the conventional immunohistochemical staining protocol, which unfortunately resulted in significant non-specific background fluorescence. It was therefore important to devise a different way to label the antigens concurrently to enable investigation of their spatial relationship in squamous epithelia. The use of immunohistochemistry to co-stain tissue for multiple markers using enzyme based detection systems rather than fluorochromes has been described (van der Loos 2008). Furthermore it is possible to utilise HIER midway through the procedure to enhance the detection of just one antigen (Lan et al. 1995). Figure 3.6 demonstrates the successful application of these techniques for p63 and ASPP2 costaining. In this case, the use of spectral separation in ImageJ allowed profiles for DAB and Vector VIP to be created from the singly stained controls, thus allowing accurate unmixing of the inverted images (Figure 3.6A).

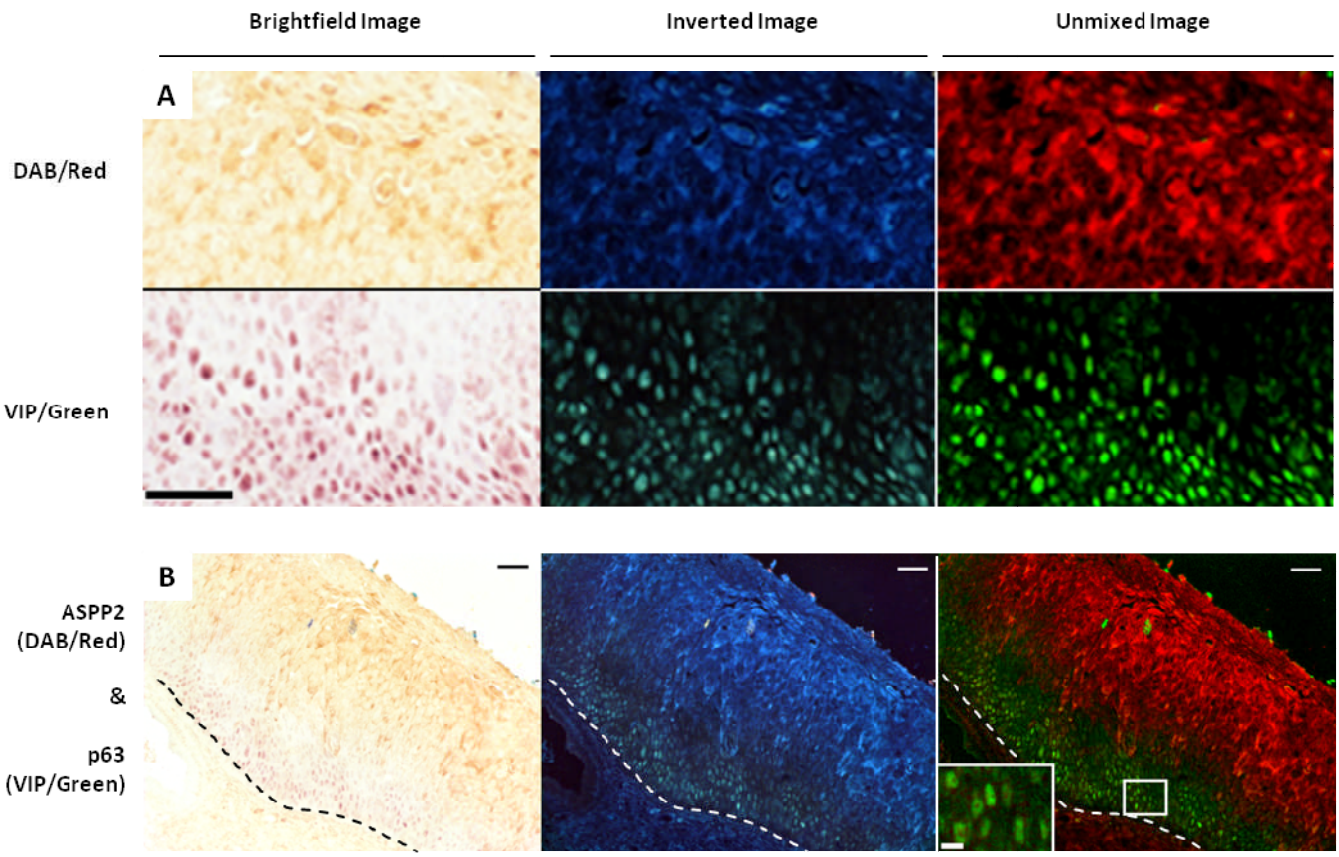

Figure 3.6: p63 and ASPP2 are mutually exclusive in the ectocervix. ASPP2 and p63 double immunohistochemical staining in the ectocervix demonstrates the successful use of two mouse primary antibodies and two peroxidase based enzyme detection systems (DAB and Vector VIP, Vector Labs) with the detection of just one antigen enhanced by HIER midway through the staining process. The expression of ASPP2 (red) and p63 (green) is mutually exclusive **B.** The inverted images were unmixed in ImageJ and the controls, which comprised sections singly stained for ASPP2 and p63 alone using conventional methods, but without a counterstain, show no significant overlap **A.** Scale bar – 50μm

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Figure 3.6B demonstrates the mutually exclusive expression of ASPP2 and p63 in the morphologically normal ectocervix. This pattern of expression confirms the lack of ASPP2 in the replication competent compartment of this type of epithelium. This data supports the *in vitro* observations that ASPP2 expression is implicated in terminal differentiation.

3.2 Squamous Dysplasia

Cervical intraepithelial neoplasia (CIN) is characterised by lack of differentiation, unregulated proliferation and p63 expression; features which characterise the replication competent basal and parabasal layers (Quade et al. 2001). ASPP2 is expressed in differentiated cells and is known to promote apoptosis. Loss of ASPP2 is predicted to occur in areas of CIN.

3.2.1 Cervical Intraepithelial neoplasia (CIN) lacks ASPP2 expression

The expression of ASPP2 was investigated by conventional immunohistochemistry in areas of CIN identified in 23 of the 24 cervical samples available. Figure 3.7 demonstrates reduced ASPP2 in areas of high grade CIN, compared to the normal ectocervix from the same patient. The same pattern was seen in low grade CIN, suggesting loss of ASPP2 occurs early in the neoplastic transformation of cervical squamous epithelium.

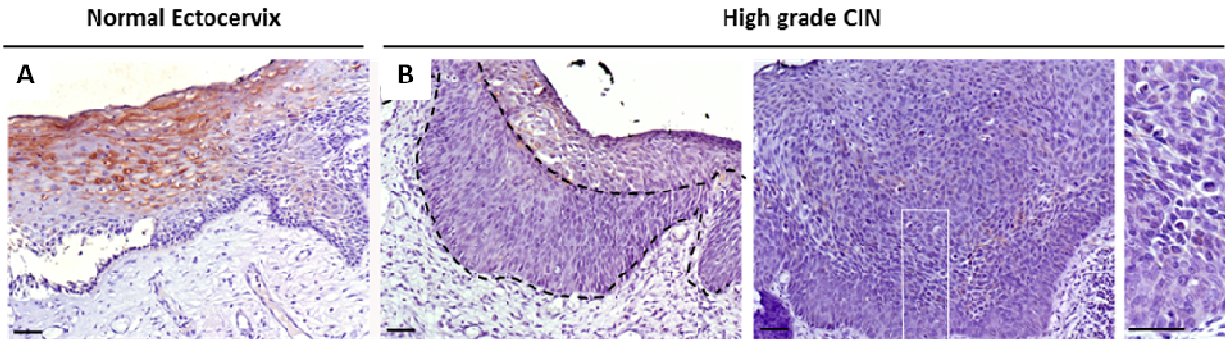


Figure 3.7: Loss of ASPP2 expression is seen in CIN. ASPP2 immunohistochemical staining in normal ectocervix **A** and corresponding high grade Cervical Intraepithelial Neoplasia (CIN) **B** from the same sample. CIN lacks ASPP2 expression in all samples analysed. Scale bar – 50µm

These data provide evidence that loss of ASPP2 expression is implicated in the development of pre-invasive neoplastic transformation in human squamous epithelium. This phenotype is predicted to persist in invasive cervical squamous cell carcinoma.

3.3 Squamous Cell Carcinoma

CIN is characterised by lack of differentiation, but squamous cell carcinomas often show a heterogeneous pattern of differentiation, reflecting the emergence of different clones during tumour progression. It is hypothesised that though ASPP2 levels will be reduced in carcinomas, well differentiated areas may show some re-expression of this protein.

3.3.1 Reduced levels of ASPP2 are seen in cervical squamous cell carcinomas

Immunohistochemical staining of Tissue Microarray CR803 (TMA CR803), comprising 70 cervical squamous cell carcinoma cores and 10 normal cervical biopsies, was used to investigate the expression of ASPP2 in squamous cell carcinomas of the cervix.

51.4% (36/70) of the tumour cores showed no ASPP2 expression at all and in the remaining tumours cytoplasmic expression was reduced compared to the normal ectocervix and seen primarily in well differentiated (WD) regions (Figure 3.8A-C).

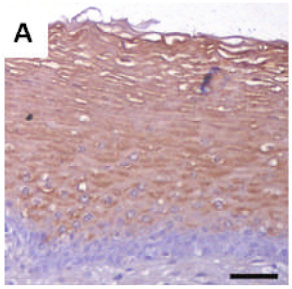
Tumour cores expressing p63 tended to show reduced ASPP2 expression, though statistically this did not reach significance (Figure 3.8D). More importantly in individual tumours it was observed that well differentiated areas expressing ASPP2 were negative for p63 whereas the converse was true in adjacent less well differentiated areas (Figure 3.8C).

The data thus far in CIN and squamous cell carcinomas suggest that loss of ASPP2 expression is a key feature of early neoplastic transformation and of the poorly differentiated, potentially more aggressive areas of invasive carcinomas.

Figure 3.8: TMA CR803 – Cervix. Two slides were stained for ASPP2 to validate the reproducibility of the staining procedure. Staining of these slides were analysed by two independent scorers and agreement between them also validated (kappa = 0.79). One slide was stained for p63.

ASPP2 expression was scored based on the intensity of staining (0-none, 1-weak, 2-moderate, 3-strong) and the proportion of cells exhibiting staining (0-100% in 5-10% increments). The overall expression is a product of these two scores (Expression = Intensity x Proportion (0-300)). The proportion of cells positive for nuclear p63 staining was documented (0-100% in 5-10% increments). Statistical analysis was undertaken in SPSS.

Normal squamous epithelium

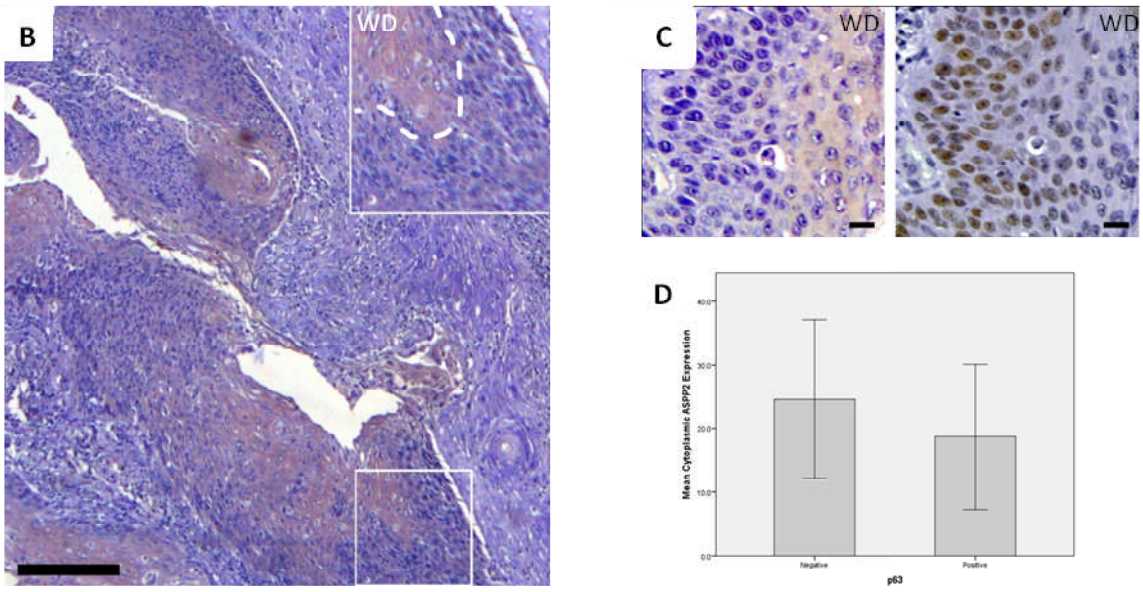


ASPP2 expression is greater in the normal ectocervix **A** compared to squamous cell carcinomas **B&C.** In those ASPP2 positive carcinomas showing heterogeneity of differentiation, ASPP2 expression is seen in the more well differentiated areas **B&C.** These areas are negative for p63 **C.**

Overall there is no significant difference in the ASPP2 expression in p63 positive and negative tumours. However the trend suggests less ASPP2 is seen in p63 positive tumours **D**.

Scale bar 50µm **A** , 100µm **B**, 10µm **C** Error bars = 95% CID

Squamous Cell Carcinoma of the Cervix



3.3.2 ASPP2 is lost in squamous cell carcinomas of the oropharynx

To confirm that loss of ASPP2 is seen in squamous cell carcinomas originating elsewhere, ASPP2 expression was analysed in full cross-sections of six tonsillar and three lingual tumours using conventional immunohistochemical methods.

Figure 3.9 demonstrates that, in comparison to the adjacent normal squamous epithelium, there is a reduction in ASPP2 expression in areas of invasive carcinoma. This pattern is seen in both the classical and basaloid squamous cell carcinomas represented in this sample. In addition, as was seen in TMA CR803, though the majority of the tumour cross sections examined showed loss of ASPP2 expression, areas which demonstrated better differentiation did express the protein.

As has been eluded to earlier, the objective quantification of immunohistochemical methods of staining is difficult to do without adopting one of a variety of different scoring techniques. In particular, scoring antigen expression in the normal squamous epithelium is difficult as few of these scoring systems will take into account the spatial differences in protein expression. An attempt was made to quantify the difference in ASPP2 expression in a tonsillar squamous cell carcinoma comparing an area of normal and an area of adjacent tumour in which some ASPP2 expression was retained. The images were inverted and unmixed as described previously in figure 3.4. Twelve areas were measured, six of which incorporated the strongly positive superficial layers of the normal epithelium and six the weakly staining basal layers. Though not ideal, this enabled a quantitative significant difference in the expression of ASPP2 to be recorded (Figure 3.9).

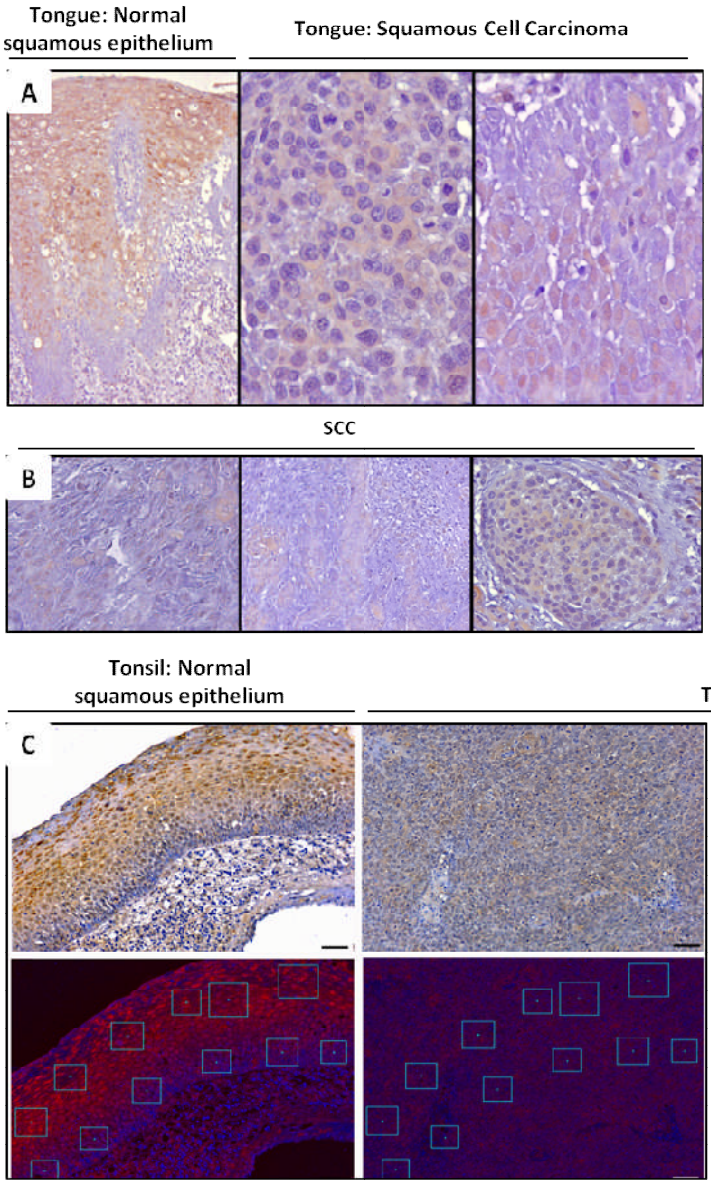
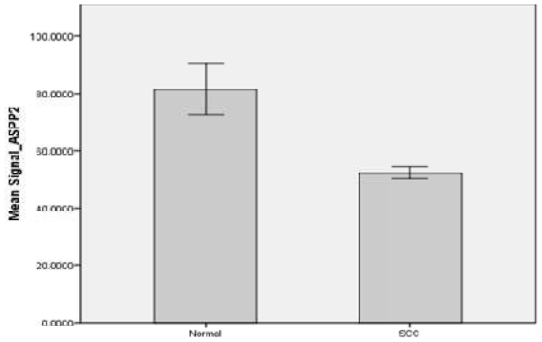


Figure 3.9: Oropharynx. Loss of ASPP2 expression is seen in an SCC compared to adjacent normal lingual squamous epithelium **A.** Two further lingual SCCs demonstrate little ASPP2 expression **B.** ASPP2 immunostaining in normal squamous epithelium and adjacent SCCs was examined in six tonsils. The corresponding unmixed images for one is seen in **C.** Here the intensity of the red signal (DAB) was measured in 12 different areas in both the normal tissue and the carcinoma using Image J. There is a significant reduction in the intensity of red signal in the carcinoma compared to the normal epithelium ($U=0.000$, $r=-0.85$, $p<0.001$) **C.** Scale bar - 50 µm. Error bars – 95% CI



3.3.3 Reduced ASPP2 is seen specifically at invasive margins in HNSCCs

Further analysis, specifically of the lingual carcinoma cross-sections which retained the best orientation, enabled an appreciation of ASPP2 expression patterns. Figure 3.10 shows cells with the lowest expression of ASPP2 were predominantly located at the invasive tumour edges. To characterise these areas further serial sections were probed for p63 and caspase-3.

p63 expression was most intense in the areas of lowest ASPP2 expression. In addition these areas showed the most apoptosis, as illustrated by caspase-3 expression, indicating that loss of ASPP2 is associated with cell survival at the invasive edges.

p63's role in tumorigenesis is rigorously debated. In 81-100% HNSCCs ΔNp63 overexpression is seen. ΔNp63 is known to maintain the replication competence of the normal epithelium (Truong et al. 2006, Senoo et al. 2007) and thus its presence in the invasive margin could be advantageous. However in head and neck tumours p63 overexpression has been linked with both a better and poorer prognosis (Graziano & De Laurenzi 2011). It has also been shown that loss or inactivation of p63 transcriptional activity by mutant p53 can promote metastasis (Adorno et al. 2009). It is possible that mutant p53 has other functions which promote aggressive behaviour which mask this inactivation of p63, but this remains speculation at present.

In addition the differential expression of ASPP2 seen in the centre and at the invasive tumour edges, potentially implicates hypoxia and/or nutrient deprivation in its regulation. However further investigation into this observation is beyond the scope of this investigation.

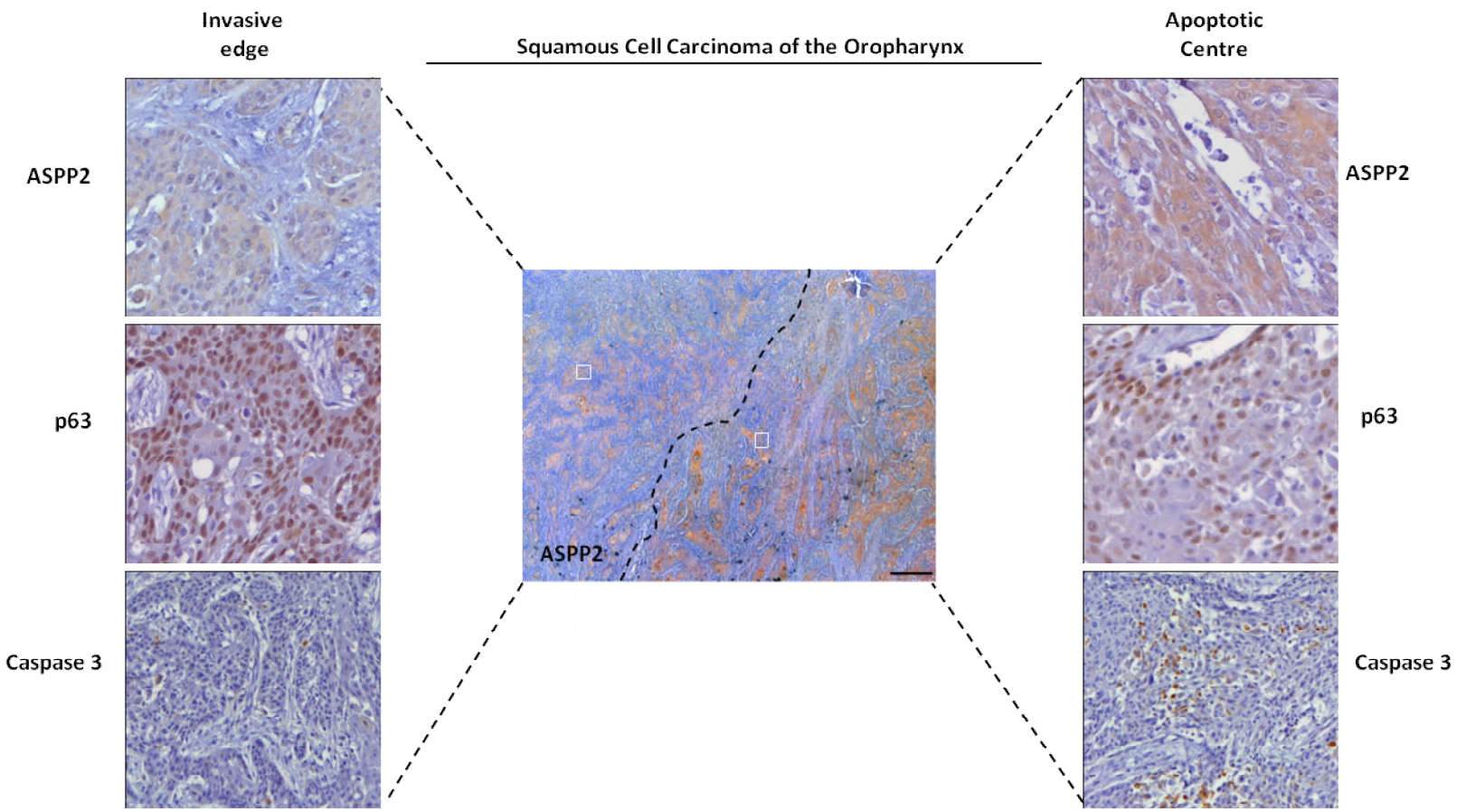


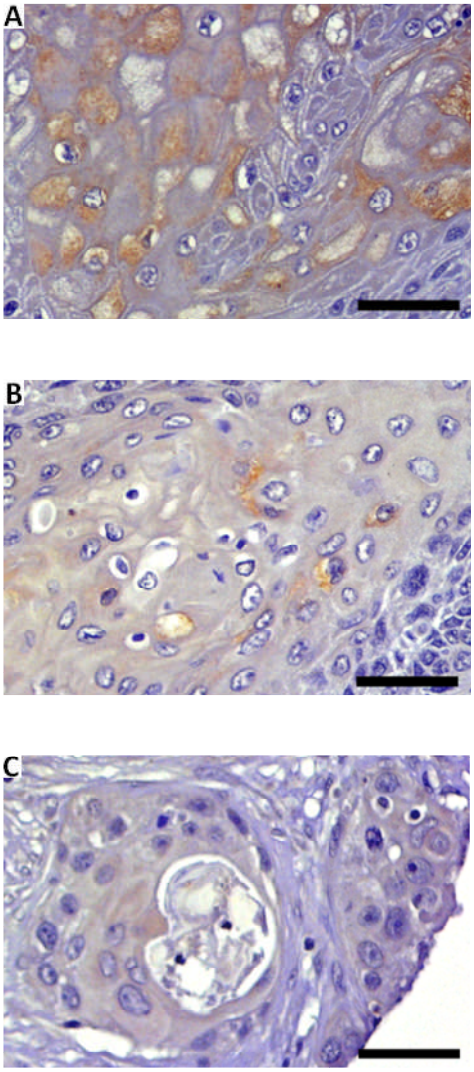
Figure 3.10: Reduced ASPP2 is found at the invasive edge of lingual squamous cell carcinomas. ASPP2 expression in a full SCC cross-section with corresponding p63 and caspase 3 immunohistochemistry. The invasive edge shows a lack of ASPP2 expression with prominent p63 positivity and lower levels of apoptosis, illustrated by caspase-3, compared to the centre of the tumour. Scale bars - 500 µm

The data thus far, in CIN and squamous cell carcinomas of the cervix and oropharynx, suggest that loss of ASPP2 expression is a key feature of early neoplastic transformation and its loss is maintained in areas of tumours which remain poorly differentiated, specifically at invading tumour edges. Given that well differentiated tumours appear to re-express ASPP2 it can be hypothesised that lack of ASPP2 expression might promote the progression of tumours and the formation of distant metastases.

3.3.4 Metastatic HNSCCs show further loss of ASPP2

To determine if loss of ASPP2 is associated with tumour progression it was necessary to examine its expression in a tissue microarray incorporating squamous cell carcinomas with and without evidence of metastases, using conventional immunohistochemistry. ZTMA 42.2 is composed of over 400 primary oral/oropharyngeal squamous cell carcinomas with and without evidence of distant metastases and also separate lymph node metastatic deposits.

Figure 3.11 shows that cytoplasmic ASPP2 expression was significantly lower in primary tumours with evidence of metastases than those with no evidence of metastases. Furthermore, the expression of ASPP2 in metastatic lymph node deposits was significantly lower than in all primary tumours supporting the hypothesis that loss of ASPP2 is associated with tumour progression. In addition ASPP2 expression in p63 positive tumour cores is reduced compared to those with no p63 expression (Figure 3.11E).



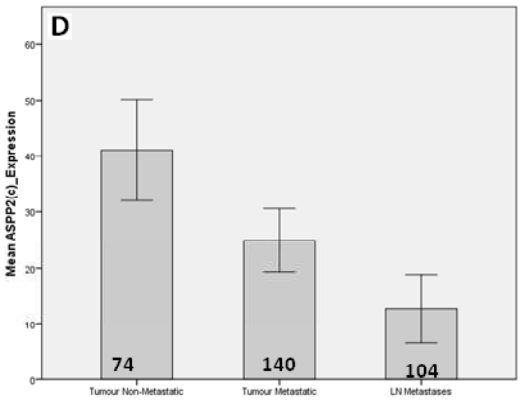


Figure 3.11: ZTMA 42.2 – Oropharynx. ASPP2 expression was scored as for TMA CR803 (Fig 3.8). Results were analysed by the Kruskal-Wallis Test (H) with Mann-Whitney tests (U) used for *post hoc* procedures. The effect size is denoted by $r=z/\sqrt{n}$ *(>0.3 = medium effect, >0.5 = large effect)*. A Bonferroni correction was applied so all effects are reported at a 0.0167 (0.05/3) level of significance.

The overall expression of cytoplasmic ASPP2 is significantly different across the categories of tumour type ($H(2) = 43.962$, $p<0.01$) in 318 carcinoma cores analysed **D**.

The expression of cytoplasmic ASPP2 is significantly lower in primary tumours with evidence of metastases ***(B** Mdn 0.0)* compared to those with no evidence of metastases *(**A** Mdn 40.0)* $U=3781.500, r=-0.23, p<0.0167$.

Lymph node metastases *(**C** Mdn 0.0)* show significantly lower ASPP2 expression than primary tumours with or without evidence of metasases. $U=5330.000$, $r=-0.27$, $p<0.0167$ and $U=1836.500$, $r=-0.49$, $p<0.0167$ respectively. Scale bar - 50μm Error bars = 95% CI

The data described provides strong evidence to support an association between ASPP2 expression and squamous differentiation in human stratified epithelium. Loss of ASPP2 is associated with early pre-invasive neoplastic change, poorly differentiated areas of carcinoma and progression to metastasis in human squamous cell carcinomas of the head and neck.

3.4 ASPP2 and HPV infection

The data thus far, suggest that the sustained loss of ASPP2 contributes to the progression of squamous cell carcinomas of the cervix and oropharynx. Data from dysplastic lesions of the cervix suggests that ASPP2 is already down regulated at an early stage of tumourigenesis. It is therefore critical to understand the mechanisms contributing to its initial downregulation.

The pathogenesis of cervical squamous cell carcinoma is unique in that is almost 100% dependent on infection by HPV. It should be emphasised here that all the morphologically normal ectocervical epithelia analysed in this study were from patients with evidence of high grade CIN elsewhere. HPV infection is seen in 99% cases of high grade CIN and is also known to produce a field effect of genomic change. Therefore if a protein's expression is targeted by HPV, it should be considered that in neighbouring morphologically normal tissue, molecular modifications might already have occurred (Pinto et al. 2004, Kalantari et al. 2009). HPV induced neoplastic transformation is characterised by the disruption of normal terminal differentiation and maintenance of replication competence (Stanley 2012, Moscicki et al. 2006). Given that ASPP2 is known to modulate squamous differentiation and is seen to be disrupted in CIN, it could be postulated that HPV targeting of ASPP2 could provide HPV with a way to control squamous homeostasis.

If so, it can be predicted that ASPP2 expression would be greater in the epithelium adjacent to HPV negative squamous cell carcinomas.

3.4.1 Nuclear ASPP2 is detected in HPV negative squamous epithelium

ASPP2 expression in the morphologically normal ectocervical squamous epithelium of patients presumed to have HPV was therefore compared to morphologically normal squamous epithelium from the tonsils of three patients with squamous cell carcinomas known to be HPV negative using conventional immunohistochemistry.

Nuclear ASPP2 is not seen in ectocervical squamous epithelium from patients presumed to be HPV positive. Figure 3.12 demonstrates that in HPV negative tonsil samples, intermittent nuclear ASPP2 is seen in the basal and suprabasal layers in the morphologically normal squamous epithelium adjacent to tumours. In addition islands of the three HPV negative invasive tumours showed nuclear ASPP2, a pattern not seen in any of the cervical squamous carcinomas in this investigation so far.

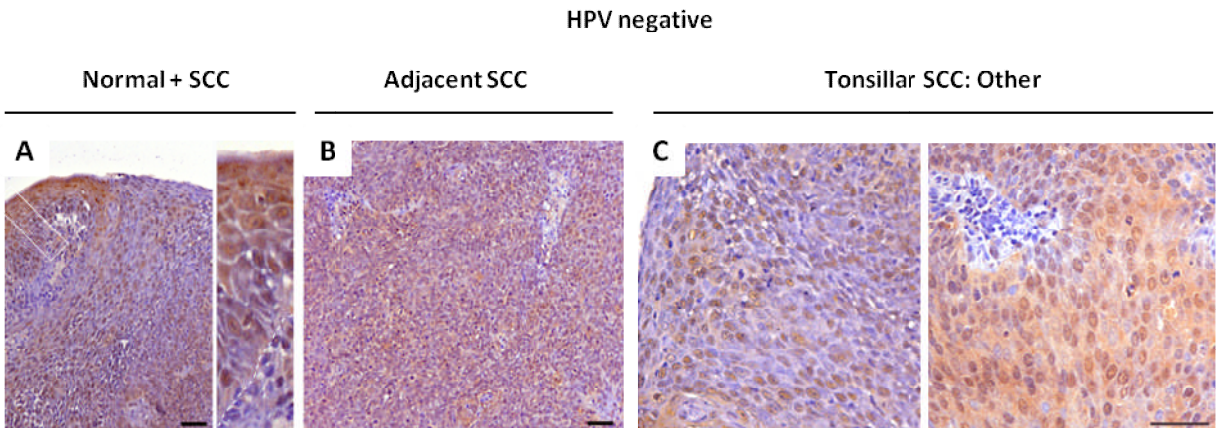


Figure 3.12: Nuclear ASPP2 expression is seen in normal squamous epithelium and adjacent SCCs in HPV negative tonsillar samples. ASPP2 immunohistochemical staining in HPV negative tonsillar normal squamous epithelium **A,** adjacent SCC **B** and two further SCC samples **C.** Scale bar – 50μm

Furthermore, although an association between high risk HPV infection and esophageal squamous cell carcinoma exists, no such association with esophageal adenocarcinoma has been identified (Syrjanen 2002, Chen et al. 2014).

In 19 esophageal biopsies analysed, 15 contained areas of morphologically normal squamous epithelium and all 15 (100%) showed nuclear ASPP2 in suprabasal layers (Figure 3.13). Of these 15 biopsies, 6 (40%) had adjacent areas of adenocarcinoma, 1 (7%) had an area of squamous cell carcinoma (as confirmed by p63 staining) and 8 (53%) had no evidence of concurrent tumour. These data do not contradict the hypothesis thus far.

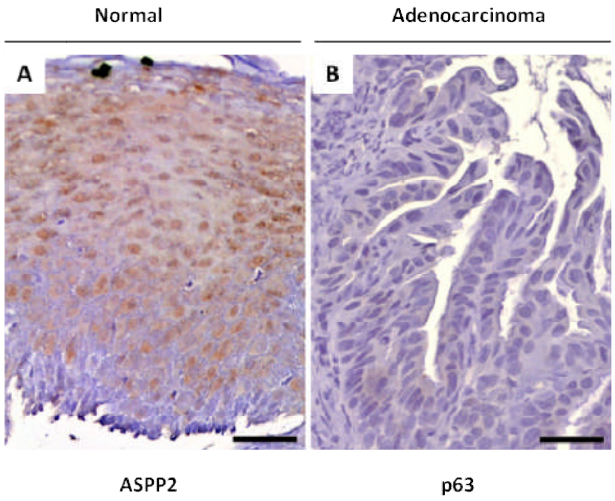


Figure 3.13: Nuclear ASPP2 is seen in normal esophageal squamous epithelium. ASPP2 immunohistochemical staining demonstrates the presence of nuclear ASPP2 in the suprabasal layers of esophageal squamous epithelium **A** adjacent to adenocarcinoma (as confirmed by p63) **B**. Scale bar – 50µm

3.4.2 Expression of nuclear ASPP2 is lost in HPV positive sections

In order to determine if loss of nuclear ASPP2 is also associated with HPV infection in the tonsil and that this phenomenon is not just seen in the ectocervix, a further three tonsillar sections from squamous cell carcinomas known to be HPV positive were stained for ASPP2 using conventional immunohistochemistry.

Figure 3.14 shows that the pronounced nuclear ASPP2 expression seen in the HPV negative squamous epithelium is not present in HPV positive samples and none of these three invasive carcinomas showed any evidence of nuclear ASPP2 expression.

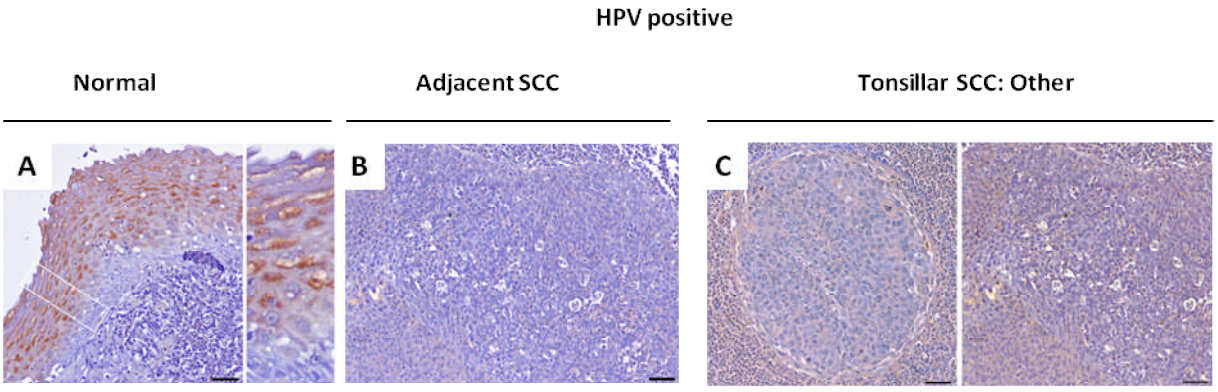


Figure 3.14: Nuclear ASPP2 is not seen in squamous epithelium and adjacent SCCs in HPV positive tonsillar samples. ASPP2 immunohistochemical staining in HPV positive tonsillar normal squamous epithelium **A,** adjacent SCC **B** and two further SCC samples **C.** Scale bar – 50μm

The data appear to suggest that infection by HPV correlates with loss of nuclear ASPP2, suggesting a novel mechanism by which HPV could deregulate normal squamous maturation. Perhaps, loss of ASPP2 is indicative of HPV infection prior to overt koilocytosis and dysplastic change.

In addition it can be hypothesised that in the normal cervix and tonsil, free from HPV infection, the maintenance of normal squamous epithelial maturation is controlled, at least in part, by nuclear ASPP2's modulation of p63 mediated transcription. ASPP2 then translocates to the cytoplasm where it interacts with IκB, promoting nuclear accumulation of p65/NF-κB and the consequent repression of p63, resulting in a commitment to differentiation. ASPP2 is then free to translocate to the cell junctions in differentiated cells, reinforcing their stability.

However, as indicated, few samples were available for analysis and further evaluation of the morphologically normal squamous epithelia for other indications of definite HPV infection was not possible. Furthermore it is not clear from these results whether the difference in ASPP2 expression seen between the tonsil and cervix reflects fundamental differences in these epithelia. The risk of persistent HPV infection and subsequent development of tumourigenesis in the cervix may be higher because the ectocervix already lacks nuclear ASPP2. Another explanation could be that, in those HPV negative tumours of the tonsil other well documented causative agents such as alcohol and/or tobacco have induced the expression of nuclear ASPP2.

It is well known that the E6 protein of HPV binds to and utilises the E3 ligase activity of the ubiquitously expressed protein E6-AP to target p53 for premature degradation by the proteasome (Talis et al. 1998 Huibregtse et al. 1994). The proteasome has also been implicated in the regulation of ASPP2 (Zhu et al. 2005). It is feasible then, that E6 also targets ASPP2 via proteasomal degradation mediated through E6-AP.

3.4.3 ASPP2 appears to be down regulated when E6-AP is silenced

To understand whether E6-AP might also have a regulatory effect on ASPP2, small interfering RNAs were used to silence the expression of E6-AP in the HPV positive Caski cell line. Figure 3.15 shows its effective silencing, as determined by the elevation of p53. However, very low levels of ASPP2 expression made detecting any consequent changes very difficult. These results even suggest that in Caski cells ASPP2 may be down regulated on silencing of E6-AP, possibly contradicting the hypothesis, but this would need to be examined further in larger scale experiments where greater quantities of protein could be harvested.

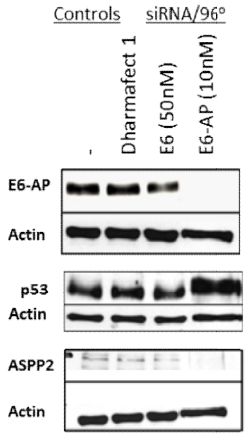


Figure 3.15: ASPP2 levels are reduced after successful knockdown of E6-AP in Caski cells. Caski cells were treated with E6-AP RNAi for 96 hours. Controls included no treatment, treatment with the transfection reagent alone and treatment with HPV 16 E6 RNAi. Immunoblots demonstrate up regulation of p53 as expected and downregulation of ASPP2.

Furthermore it should be noted that Caski cells were originally derived from a metastatic deposit (small intestine) of cervical carcinoma and our hypothesis implicates an ASPP2:E6-AP interaction in the initial stages of HPV infection, prior to established dysplastic change. Molecular interactions may not therefore be comparable. The data in this project has demonstrated some reexpression of ASPP2 in invasive carcinomas which may mask any initiating mechanisms. It is likely that the specific mechanisms involved in HPV targeting of ASPP2 would be better investigated in a laboratory with a specialist interest in HPV and though this might promote future collaborations, it will not be pursued further in this project.

3.5 ASPP2 and the cervical transformation zone

The data so far suggest that HPV infection is associated with the down regulation of nuclear ASPP2 in the replication competent compartment of ectocervical squamous epithelia. In those areas where infection becomes established and koilocytic and dysplastic change is seen, further down regulation of cytoplasmic ASPP2 is seen. If this is the case it might be possible to see evidence of nuclear ASPP2 expression in the cervix, at the site where HPV infection occurs, in cells which may not have acquired molecular evidence of such infection.

The cervical transformation zone, as described in the introduction, represents an area including foci of squamous metaplasia demonstrating the transformation from glandular to squamous epithelia in areas newly exposed to the harsher environment within the vaginal cavity. Here the precise regulation of squamous differentiation is essential in ensuring the normal development of the new squamous lining. Furthermore it is here, where the epithelia is particularly vulnerable to genetic modification, that HPV induced neoplastic transformation is thought to occur (Moscicki et al. 2006). This physiological squamous metaplasia takes place under the influence of estrogen. The metaplastic process is thought to be initiated by p63 induction in subcolumnar basal cells though the precise origin of these subcolumnar ‘reserve’ cells, the molecular trigger for p63 induction and relationship to estrogen signalling are unclear.

ASPP2 interacts with p63, appears to play a key role in epithelial stratification and its nuclear expression is postulated to be down regulated early in neoplastic transformation, possibly due to HPV infection. This makes its focal nuclear expression in the transformation zone probable.

3.5.1 ASPP2 is not expressed in the endocervical epithelium

In order to explore this further the transformation zone was identified in cervical LLETZ specimens and conventional immunohistochemical staining for ASPP2 was undertaken.

Figure 3.16 demonstrates that ASPP2 is not expressed in the mature endocervical epithelium.

3.5.2 Nuclear and Cytoplasmic ASPP2 are seen in subcolumnar basal cells and areas of immature squamous metaplasia

However, in the transformation zone it is apparent that both nuclear and cytoplasmic ASPP2 are expressed in the subcolumnar basal ‘reserve’’ cells, thought to be site of the p63 induction which initiates and facilitates squamous metaplasia (Figure 3.16). As the section progresses caudally towards the ectocervix the number of ASPP2 expressing cell layers underlying the endocervical cells can be seen to increase as the area of squamous metaplasia matures.

Double immunohistochemical staining of ASPP2 with p63, as described before, was undertaken on the transformation zone. Here it can be seen that it is the ASPP2 expressing population of cells which intermittently also co-express p63 (Figure 3.16). Subcolumnar basal cells, as highlighted by ASPP2 expression, which do not express p63 are also seen here.

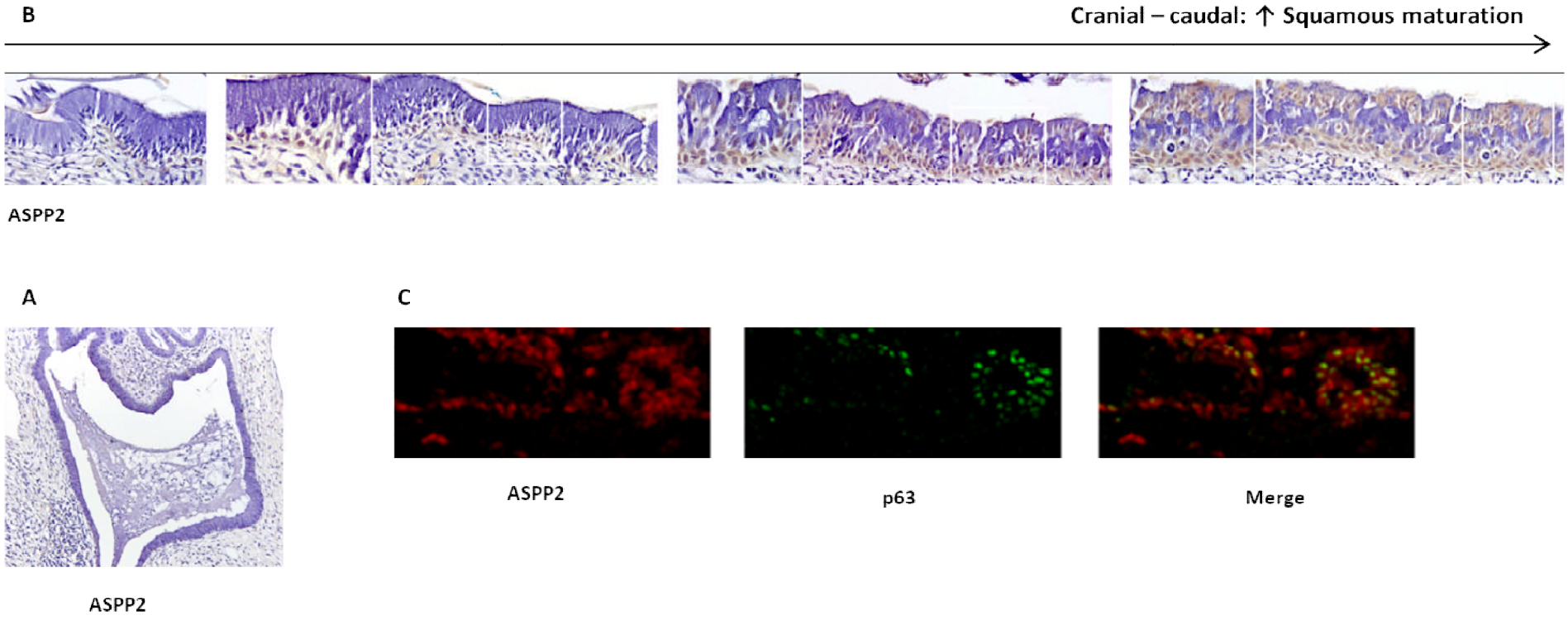


Figure 3.16: Nuclear and cytoplasmic ASPP2 is seen in the transformation zone. The transformation zone was examined in three cervical samples. ASPP2 is not expressed in mature endocervical cells **A.** Nuclear and cytoplasmic ASPP2 is seen in a subcolumnar cell population in areas of immature squamous metaplasia. Cranially the ASPP2 expressing cells appear in a single layer underlying the endocervical cells. As the section progresses caudally the cells are seen in an increasing number of layers as the squamous metaplasia matures. This pattern was seen in all 3 of the 24 samples in which adequate areas of squamous metaplasia were available for assessment **B**. p63 expression occurs in a population of these ASPP2 expressing cells **C.**

These results suggest nuclear ASPP2 potentially plays an important role in facilitating the induction of squamous metaplasia and, if deregulated at this early stage, also neoplasia.

The expression of nuclear ASPP2 in this part of the cervix fits with our knowledge of its interactions with nuclear p63. Whether the presence of nuclear ASPP2 is essential for maintaining the reserve cell population, for effective p63 regulation in the transformation zone or simply to modify the p63 transcription programme once metaplasia begins is as yet unclear.

However the data in this first chapter implicate ASPP2 in the maintenance of normal human squamous epithelia and provide evidence to corroborate observations in the ASPP2 knockout mouse model. Furthermore they raise the possibility of important roles for ASPP2 in the progression and metastases of human squamous cell carcinomas and implicate the loss of nuclear ASPP2 as a possible marker of high risk HPV infection. Further investigation is therefore warranted.

Chapter 4: Results

The somatic deletion of ASPP2 results in a novel mouse model of lung tumourigenesis

As discussed in Chapter 3, the germline deletion of ASPP2 on mixed and pure Balb/c backgrounds demonstrates its ability to co-operate with both p53 and p63 in the development of tumours. Mechanistically ASPP2 has been shown to specifically inhibit the apoptotic function of p53 and repress the expression of p63 (Vives et al. 2006; Tordella et al. 2013; Samuels-Lev et al. 2001).

Specifically, on a pure Balb/c background the germline inactivation of ASPP2 results in an increased susceptibility to the development of squamous cell carcinomas, in part due to the consequent impaired repression of p63 expression. Subsequent data from human tumours, presented in Chapter 3, corroborates these findings and indicates that loss of ASPP2 expression is seen in early dysplastic lesions, and that particularly low levels of ASPP2 are associated with more aggressive, metastatic tumours.

At present there is little data on the effect of germline deletion of ASPP2 on the normal development of squamous epithelia. However given ASPP2's likely role in the normal development of this epithelial type it is likely that the development of squamous cell carcinomas in ASPP2 knockout mice arises on a predicted background of abnormal squamous development. Consequently this will not accurately reflect the natural history of sporadic squamous cell carcinoma development seen much more commonly in humans.

In order to better characterise the sporadic loss of ASPP2 the timed somatic deletion of the protein was undertaken in mice aged 15 and 18 months to determine the effect of ASPP2 loss on a background of presumed normal squamous development.

Intraperitoneal injections of 4-OHT were administered to $ASPP2^{flox/flox}$;$Cre^{+}ER^{T}$ mice over a period of 5 days, to induce ASPP2 deletion, after which mice were observed until any of the end points governing our tumour studies was reached. Control mice were injected with sunflower oil alone. The mice were then culled and a post mortem examination was undertaken specifically to identify tumours which were subsequently analysed. From this point onwards mice injected with 4-OHT will be referred to as ASPP2Δexon3 mice and control mice will be $ASPP2^{+/+}$.

Given the association between germline ASPP2 inactivation and squamous cell carcinomas already documented and the striking loss of ASPP2 seen in human squamous neoplastic populations, it was hypothesised that in this experimental model, the precipitation of squamous cell carcinomas would be seen. It was also predicted that these tumours would take longer to develop than those arising on a background of abnormal squamous development.

4.1 The somatic deletion of ASPP2 precipitates the development of tumours

Significantly more tumours developed in those mice injected with 4-OHT than in control mice. The two age groups were analysed together due to the small number of mice available for eventual assessment in each group. 64.0% (16/25) of the experimental mice developed tumours compared to 31.3% (5/16) injected with sunflower oil only (Figure 4.1).

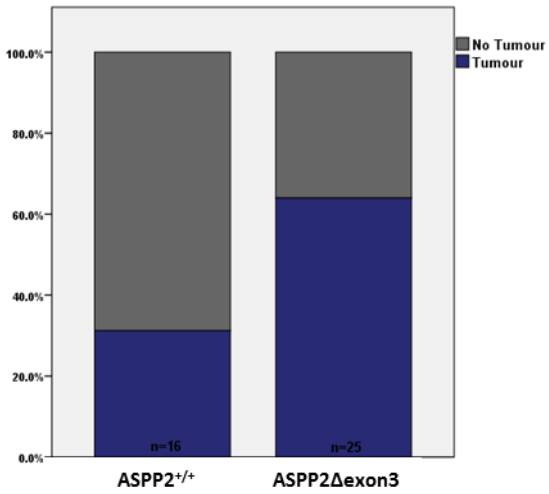


Figure 4.1: The sporadic deletion of ASPP2 precipitates the development of tumours. There was a significant association between the development of tumours and the injection given $\chi^2(1) = 4.188$, $p<0.05$. This reflects the fact that, based on the odds ratio, the odds of the mice developing tumours were 3.9 times higher if they were injected with 4-OHT, to induce the sporadic deletion of ASPP2, than if they were injected with sunflower oil only. A medium effect can therefore be documented for 4-OHT injection (Cramer's V = 0.320,$p<0.05$).

In a comparable experiment, examining the sporadic deletion of p53, Hinkal *et al* (2009) demonstrated a significant difference in the time taken for mice of different ages to develop tumours. Older mice developed tumours significantly more quickly and this was considered to reflect the increasing number of mutations already accumulated over their lifetime, indicating loss of p53 to be a critical final step in promoting the development of tumours. The difference in time taken for mice injected with 4-OHT to develop tumours was analysed in the two different age groups.

4.1.2 No difference in time taken to tumour formation was seen in ASPP2Δexon3 mice

In contrast to the somatic deletion of p53, the inactivation of ASPP2 at 15 and 18 months of age resulted in no significant difference in the time taken to develop tumours (Figure 4.2). This could corroborate the hypothesis that loss of ASPP2 is an important initiating factor in tumour development and that the tumours occurring do not depend on prior genetic mutations. However it should be considered that 15 months is older than the oldest mice in the p53 study (12 months) and it could reflect the fact that the necessary initiating mutations have already occurred. The small number of mice analysed is also a consideration.

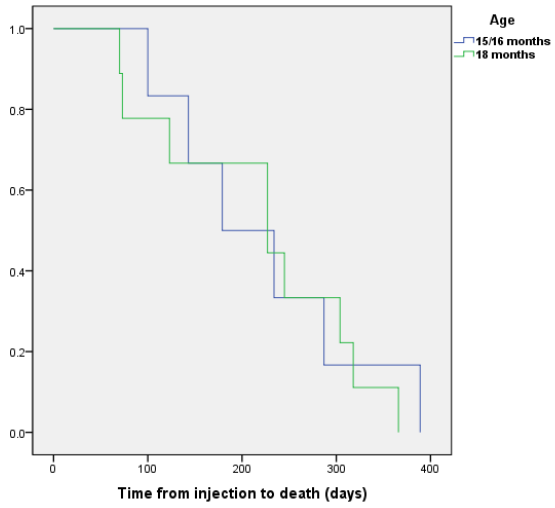


Figure 4.2: No significant difference in the ime taken to tumour formation was seen in the two age groups examined. The curve demonstrates the time taken to tumour development in the two groups (15/16mths n=6, 18mths n=9). No significant difference was seen $p>0.05$.

Interestingly in mice with a germline deletion in ASPP2, tumours were seen to develop in just 19-20 weeks (133-140 days) in those few homozygous knockout mice which survived the immediate post natal period. In this experiment tumours were seen after a median of 191 days. It is impossible to determine if this is constitutes a significant difference, but it could suggest that, as expected, the development of tumours takes longer in mice which have undergone normal development prior to the deletion of ASPP2.

4.1.3 The somatic deletion of ASPP2 primarily results in the development of lung tumours

Tumours were predominantly seen in the lungs of ASPP2Δexon3 mice. In those mice, available for post mortem examination, 69% (11/16) of ASPP2Δexon3 had lung tumours, specifically distal lung tumours, compared to 40% (2/5) of $ASPP2^{+/+}$ mice (Figure 4.3)

This does represent a significant difference in location of tumours compared to other mouse models of ASPP2 deletion (Tordella et al. 2013; Vives et al. 2006).

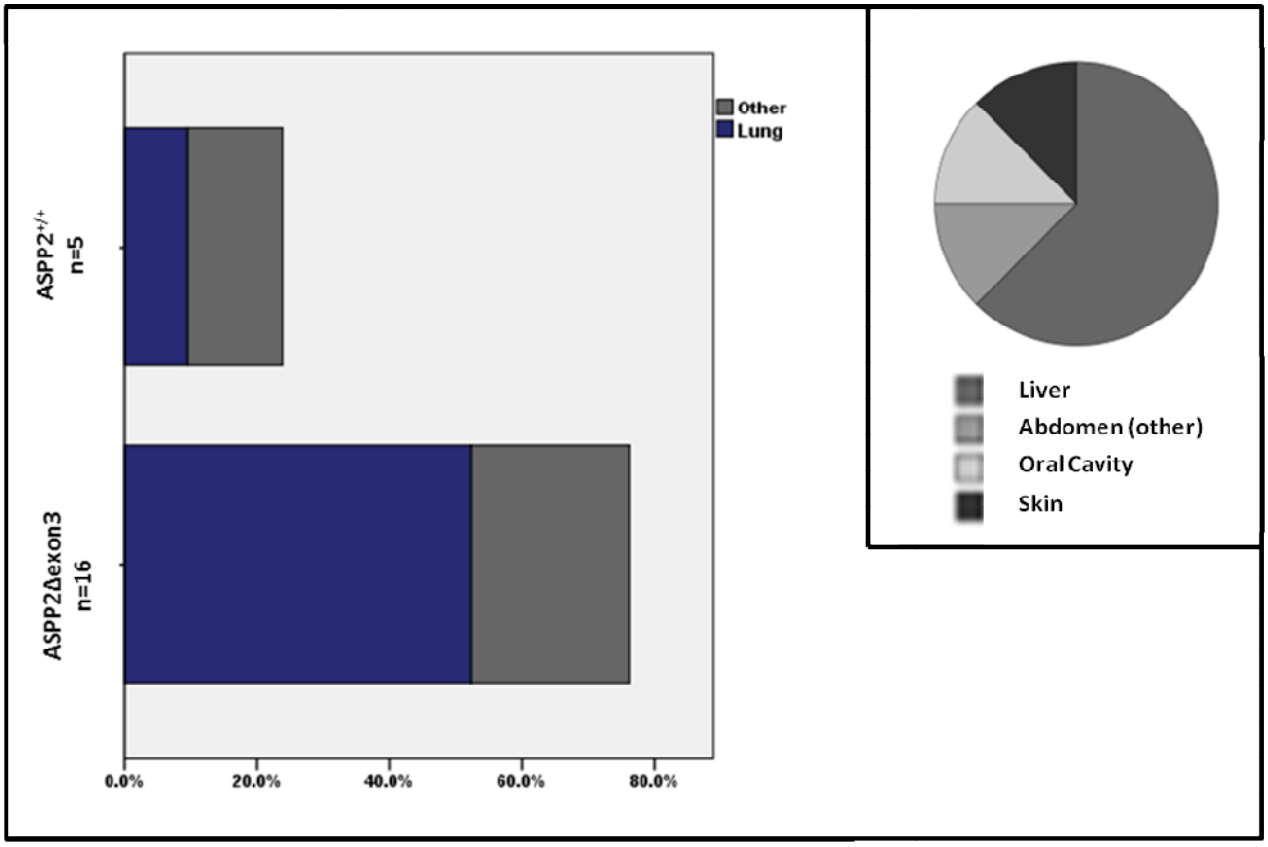


Figure 4.3: The somatic deletion of ASPP2 primarily results in the development of lung tumours. 52% (11/21) of the tumours seen in this experiment were in the lungs of mice injected with 4-OHT. The insert shows the spectrum of other tumours seen in both groups.

4.1.4 ASPP2 expression was significantly reduced in ASPP2Δexon3 lungs

Conventional immunofluorescent labelling of ASPP2 confirms the loss of this protein in the lungs of ASPP2Δexon3 mice. This confirms the experimental conditions were successful in this organ and suggests loss of ASPP2 could be associated with the development of tumours.

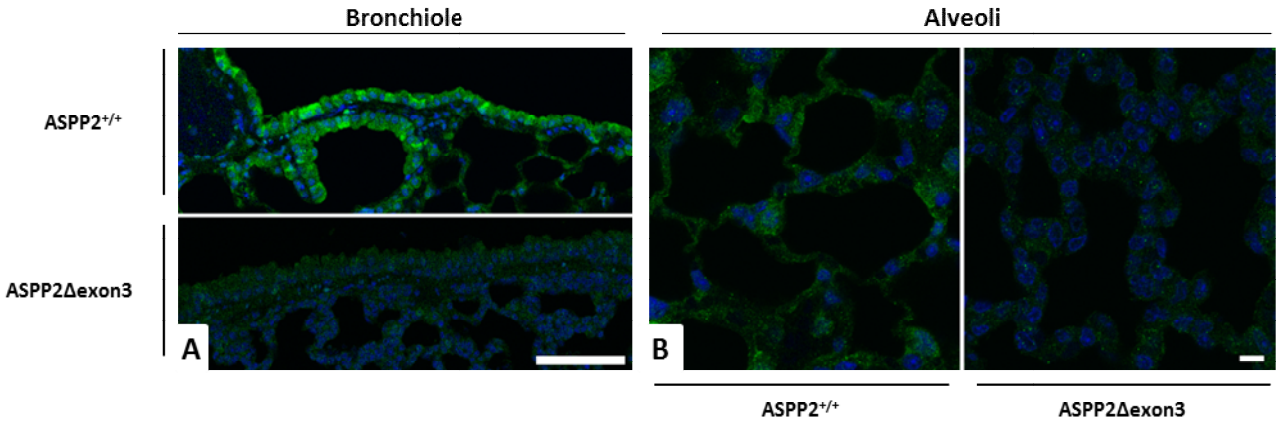


Figure 4.4: ASPP2 expression is lost in experimental mice lungs. ASPP2 expression is seen, particularly in the distal bronchiole **A** compared to the cells lining the alveolar spaces **B.** Furthermore ASPP2 expression is lost in both regions under experimental conditions. Scale bar – 50µm **A,** 10µm **B.**

Furthermore, as can be seen in Figure 4.4, expression of ASPP2 was particularly strong in the bronchiolar epithelial cells compared to cells lining the alveoli. The bronchiole here is lined by a pseudostratified epithelium comprising Clara cells, expressing Clara Cell 10 (CC10) protein and a specific subset of Clara cells, expressing CC10 and Surfactant Protein C (SP-C), which probably act as stem cells. In addition ciliated cells are present which are identified by the expression of acetylated alpha-tubulin (AAT) in the cilia. As can be seen above, ASPP2 expression in the bronchiole is not homogenous and it will be important to determine exactly which cells express the protein, before the mechanisms influencing tumour development on its deletion can be further elucidated.

ASPP2 is known to promote a differentiated phenotype in squamous epithelium and if it acts in a similar manner in the pseudo stratified epithelium of the lung, its expression would be expected in differentiated cells.

4.2 ASPP2 is expressed in terminally differentiated cells of the normal murine lung

To explore the expression of ASPP2 in murine lungs a different imaging protocol was developed. In Chapter 3, ASPP2 expression is well preserved using conventional immunohistochemical techniques without HIER. In order to examine ASPP2 here a combination of immunohistochemical and immunofluorescent staining was used. This two step procedure, undertaken over three days, comprised conventional ASPP2 immunohistochemical staining, without a haematoxylin counterstain, followed by HIER and subsequent fluorescent labelling. Fluorescent (green and blue) and bright field images were obtained concurrently via confocal microscopy. Using spectral separation techniques the bright field images were then inverted, unmixed and the DAB signal converted into red. Imaging software was used to merge both images to enable co-localisation studies.

4.2.1 ASPP2 is not seen in CC10 or SP-C expressing Clara cells

As demonstrated in Figure 4.4, ASPP2 expression is greater in the bronchiolar epithelium compared to alveoli in the distal lung. Unfortunately the trachea and proximal airways were not retrieved in these mice for further analysis of their epithelial linings. Figures 4.5 & 4.6 show ASPP2 and CC10 and ASPP2 and SP-C to be mutually exclusive.

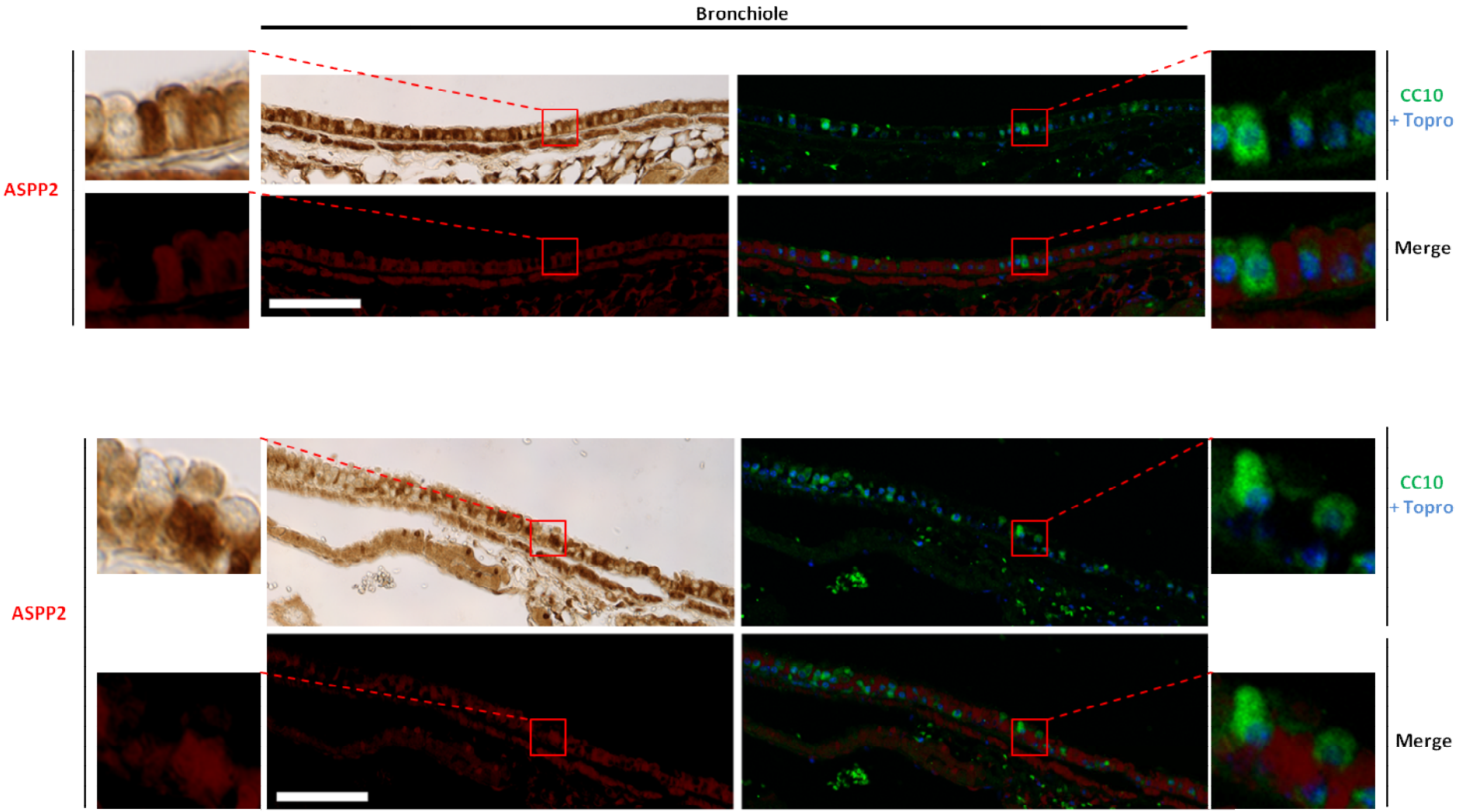


Figure 4.5: ASPP2 and CC10 expression are mutually exclusive in the bronchiolar epithelium. The strongest expression of ASPP2 is in the cells which had surface cilia. Cells without cilia do not express ASPP2 (Dab/Red). These cells express CC10 (green). ASPP2 and CC10 are mutually exclusive.
Scale Bar - 50µm

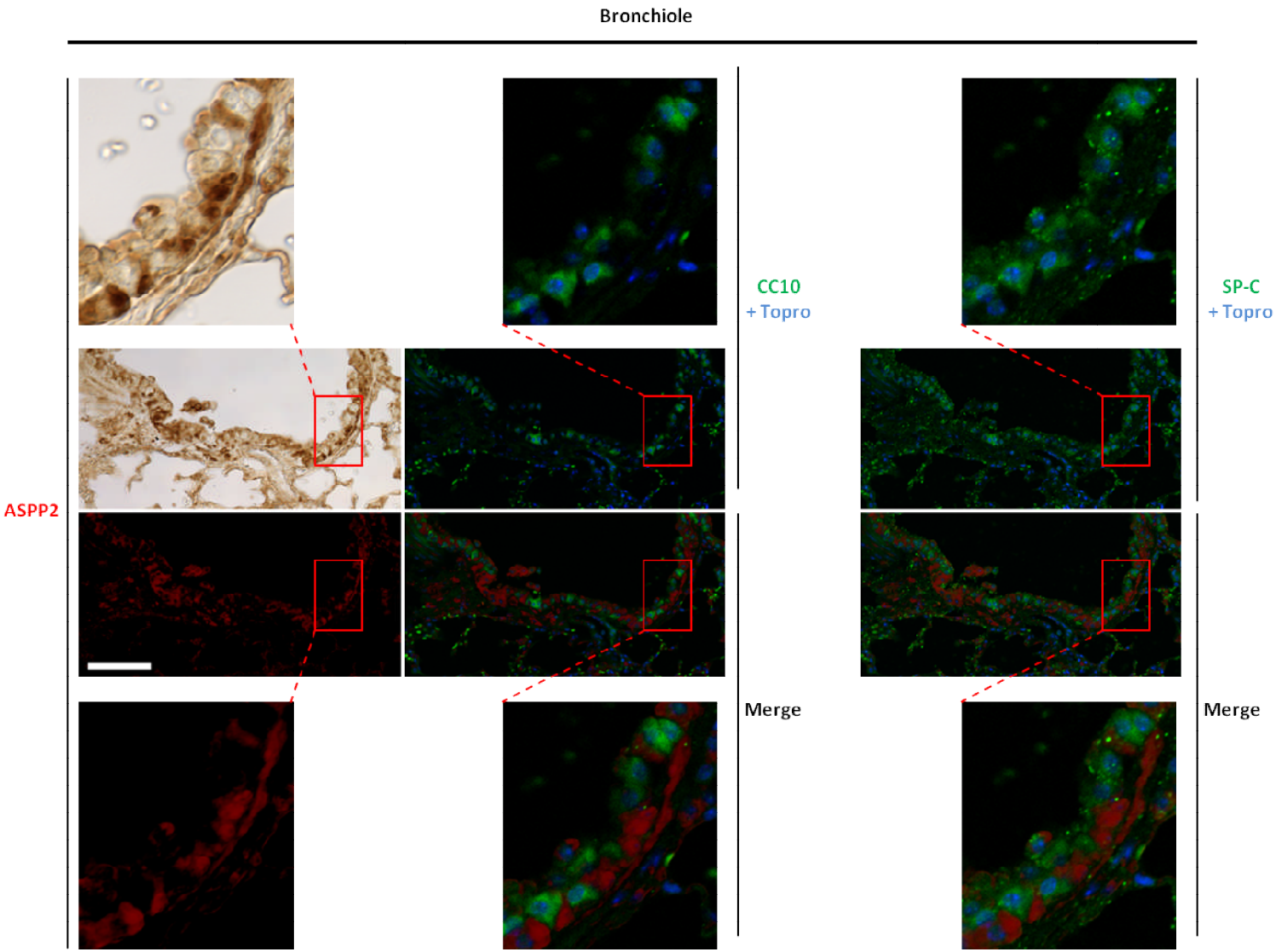


Figure 4.6: ASPP2 is not expressed with CC10^{+} or SP-C^{+}. Cells which express CC10 and/or SP-C (Green) do not express ASPP2 (Dab/Red). Scale Bar - 50µm

4.2.2 ASPP2 is strongly expressed in ciliated cells

Figure 4.7 shows the strongest expression of ASPP2 to be in cells with surface cilia. Cilia express acetylated alpha-tubulin (AAT) and to confirm this double immunohistochemical, immunofluorescent staining was undertaken for ASPP2 and AAT.

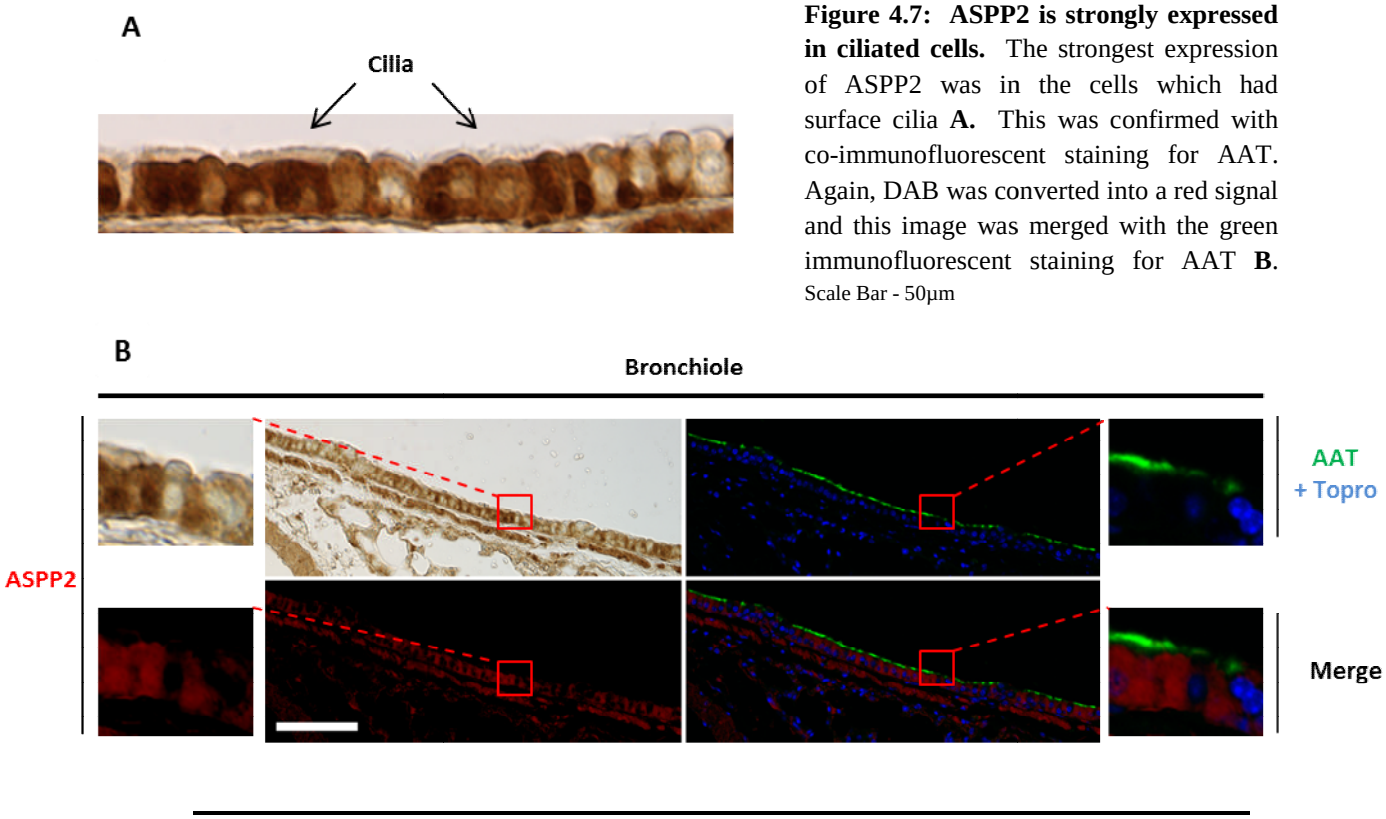


Figure 4.7: ASPP2 is strongly expressed in ciliated cells. The strongest expression of ASPP2 was in the cells which had surface cilia **A.** This was confirmed with co-immunofluorescent staining for AAT. Again, DAB was converted into a red signal and this image was merged with the green immunofluorescent staining for AAT **B.** Scale Bar - 50µm

These results confirm the expression of ASPP2 in ciliated columnar cells and in adult control mice with no apparent lung injury, these likely represent terminally differentiated ciliated cells, corroborating a possible role for ASPP2 in the promotion of differentiation in lung epithelium. Follow up studies could explore the distribution of this specific cell type in lungs no longer expressing ASPP2 to establish if this protein is important in the maintenance of the differentiated phenotype.

4.3 Characterisation of lung epithelium in ASPP2Δexon3 mice

The genetic heterogeneity of human lung carcinomas primarily results from prolonged exposure to mutagenic tobacco smoke. Consequently numerous passenger mutations successfully mask the more significant driver mutations, making their identification difficult. As a result mouse models are extremely important for the study of this tumour type and the development of lung tumours, and in particular malignant lung tumours, secondary to single gene deletions or mutations potentially identifies novel therapeutic targets (Kwon & Berns 2013).

The majority of tumours occurring spontaneously and in genetically modified mouse models to date are non small cell lung carcinomas (NSCLCs) and of these, adenomas and adenocarcinomas predominate. Squamous cell carcinomas are seen rarely in experimental models, none of which involve a single gene, and have not been reported to occur spontaneously (Nikitin et al. 2004).

Squamous cell carcinomas develop through a well characterised sequence of events reflecting repetitive injury to the pseudo stratified mucosa. The first histologically identifiable consequence is basal cell hyperplasia, followed by squamous metaplasia which, on continued exposure to the insult, is rendered dysplastic and eventually frankly invasive. Basal cells are therefore considered to be a candidate cell of origin of squamous cell carcinoma. In contrast to human airways, basal cells are only found in the mouse trachea and not in the more distal airways or alveoli. This relative paucity of basal cells in the mouse airway might explain their comparative resistance to the development of this tumour type (Snyder et al. 2009).

Recent elegant experiments have provided further, compelling evidence that basal cells may be the initiating cell of interest in this tumour type. Squamous cell carcinomas are most commonly seen in a central location due to their association with smoking. It has been postulated that the stem or progenitor cells involved in the continued repair of this repeated proximal epithelial damage might act as "tumour initiating cells". Basal cells are characterised by their expression of p63 and CK5. A subset, which is rapidly inducted following injury, is characterised by CK14 expression, producing a phenotypically heterogeneous progeny demonstrating self-renewal, proliferation and multipotency, after which levels quickly returned to normal (Kratz et al. 2010). This suggests the expression of CK14 is tightly regulated and the cells associated with repair either originate from CK14 positive cells or from CK5 positive basal cells which have acquired the ability to express CK14 under those conditions. Furthermore in human samples taken from patients with chronic obstructive airways disease, significant CK14 expression was seen in areas of reserve cell hyperplasia and squamous metaplasia, areas associated with chronic inflammation. Regions of squamous dysplasia also showed CK14 expression and its expression was then examined in NSCLCs. As expected, CK14 expression was higher in squamous cell carcinomas, but there was expression in other histological subtypes where it correlated with a poorer prognosis, but only in smokers i.e. where the lungs are undergoing chronic injury and repair and its expression was not seen to be associated with proliferation (Ooi et al. 2010). Hong *et al* demonstrated that on naphthalene induced depletion of Clara cells, basal cell hyperplasia occurs, resulting in the complete repopulation of the epithelium including the appearance of Clara cells and ciliated cells which do not characteristically express p63 or CK14 (Hong et al. 2004a; Hong et al. 2004b).

ASPP2 is known to repress p63 expression, reduce CK14 transcription and promote a differentiated squamous phenotype. The epithelium in the distal mouse airways was examined using H&E staining with the expression of CK14 analysed by conventional immunofluorescence.

4.3.1 Alveolar wall thickening is seen in ASPP2Δexon3 mice

Figure 4.8 demonstrates the morphological changes in the background lung of ASPP2Δexon3 mice. H&E demonstrates thickening of the alveolar walls with rounded cells replacing the normally flattened epithelium seen here. Immunofluorescence shows these cells to express CK14.

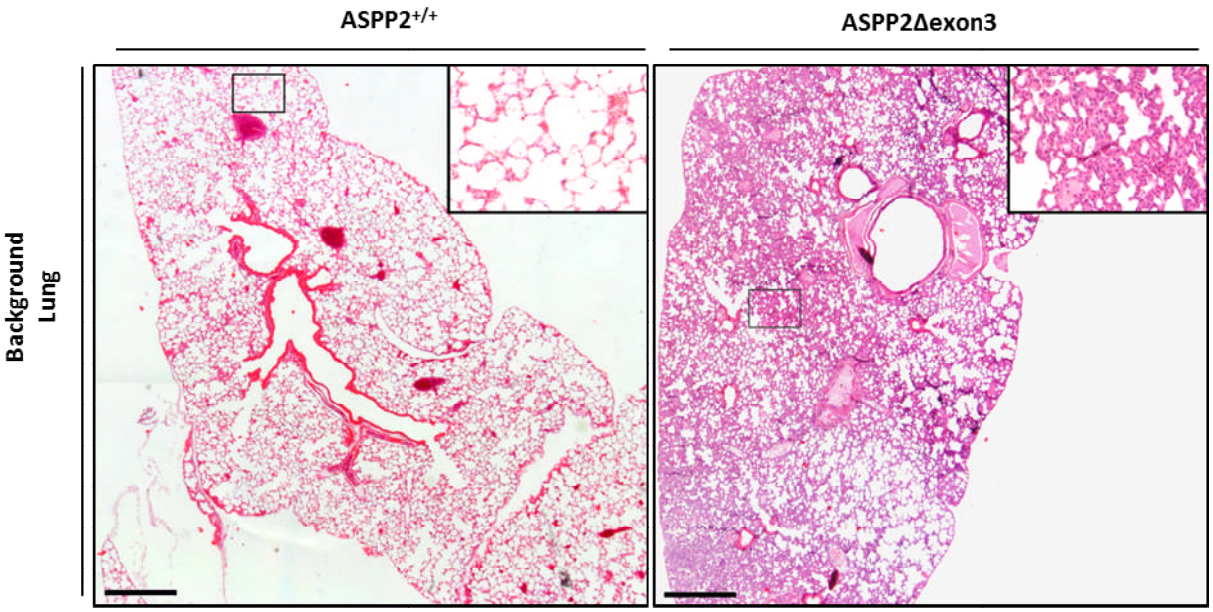


Figure 4.8: The somatic deletion of ASPP2 results in alveolar morphological changes. Rounded cells are seen to replace the normal, flattened alveolar epithelium giving the appearance of wall thickening. Scale bar – 500µm.

4.3.2 CK14 expression is increased in the distal lung in ASPP2Δexon3 mice

Figure 4.9 demonstrates the morphological changes in the background lung of ASPP2Δexon3 mice. Conventional immunofluorescence staining demonstrates the rounded cells replacing the normal, flattened alveolar epithelium express CK14.

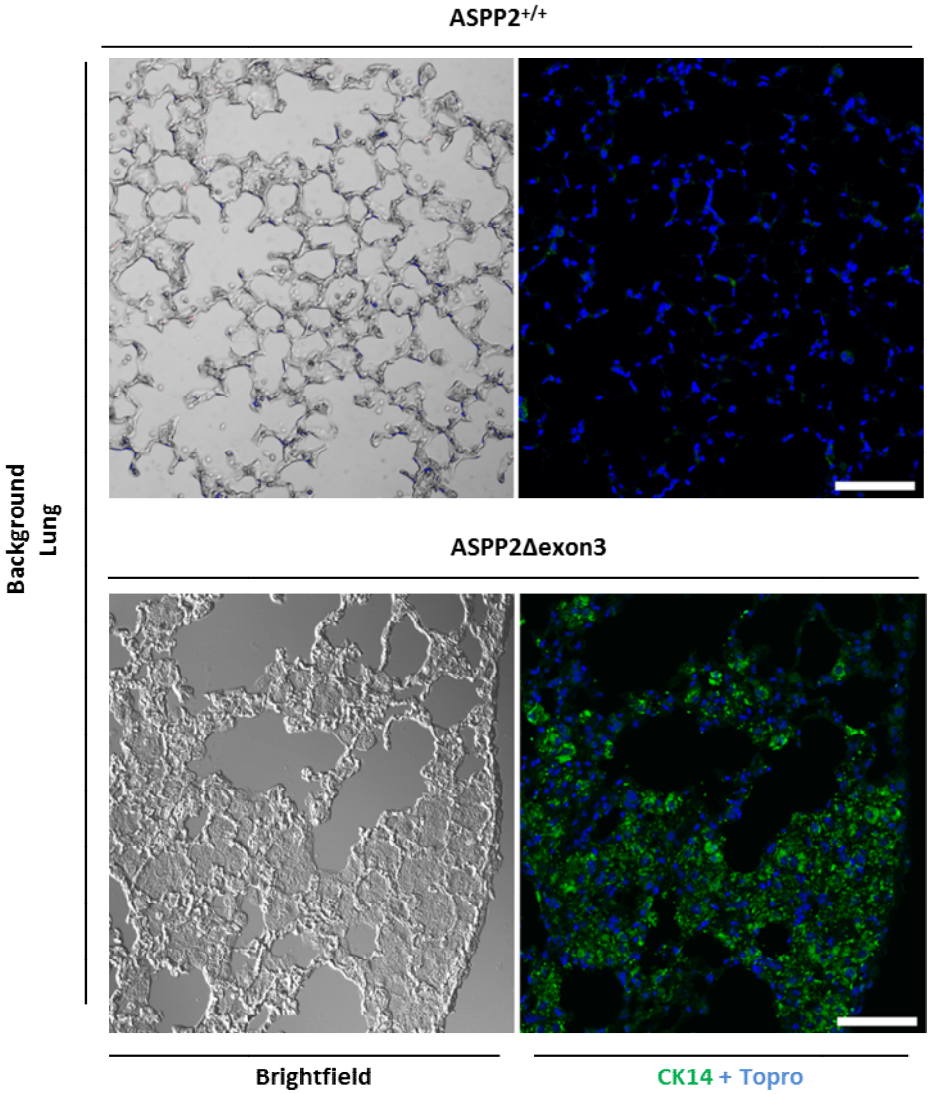


Figure 4.9: The somatic deletion of ASPP2 results in overexpression of CK14 in the distal lung. The rounded cells lining the alveoli in ASPP2Δexon3 mice alveoli are seen to express CK14 Scale bar – 50µm.

Dakir *et al* generated a mouse model in which the human keratin 14 gene hK14 was constitutively expressed in mouse airway progenitor cells using a Clara cell specific 10kDa protein (CC10) promoter. The study was undertaken to specifically investigate the development of pulmonary squamous cell carcinomas. Experimental animals were seen to develop multifocal airway hyperplasia, squamous metaplasia and an increasing number of lung tumours. Interestingly these lung tumours were a mix of adenomas and poorly differentiated carcinomas and did not show histological evidence of overt squamous maturation. Immunohistochemical labelling suggested multi-directional differentiation suggesting CK14 modulates divergent pathways in the lung (Dakir et al. 2008).

4.3.3 Squamous metaplasia is identified in a single ASPP2Δexon3 mouse

Experimental mouse lungs were analysed specifically for areas of squamous metaplasia and a possible focus was identified in a single ASPP2Δexon3 mouse (Figure 4.10). This was confirmed with conventional immunohistochemistry for p63. Of note p63 expression was not seen in the lungs of other ASPP2Δexon3 mice.

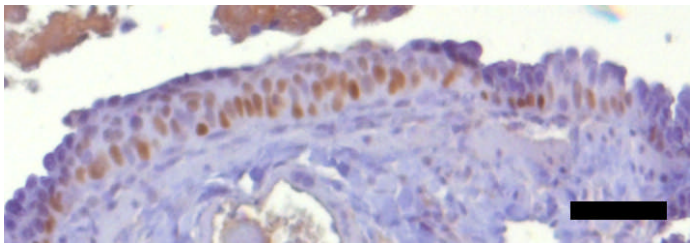


Figure 4.10: Squamous metaplasia is identified in a single ASPP2Δexon3 mouse. A single focus of squamous metaplasia, confirmed here by positive p63 expression is seen in one ASPP2Δexon3 mouse lung. Scale bar – 50μm.

4.4 Characterisation of lung tumours

To summarise, the somatic deletion of ASPP2 results in the overexpression of CK14 in the distal lungs, resulting in hyperplastic change and foci of squamous metaplasia. Dakir *et al* saw similar appearances and the consequent tumours showed multidirectional differentiation with no overt squamous differentiation. Further classification of the lung tumours seen in experimental mice here is therefore important.

4.4.1 ASPP2 expression was also lost in lung tumours arising in ASPP2$^{+/+}$ mice

Previous experiments have confirmed ASPP2 to act as a haploinsufficient tumour suppressor with the wild type protein identified in tumours arising in heterozygotes (Vives et al. 2006).

Figure 4.11 shows loss of ASPP2 expression in the rare lung tumours arising in ASPP2$^{+/+}$ mice. This raises the interesting possibility that the development of these lung tumours might be dependent on ASPP2 loss of heterozygosity. In order to investigate this further the expression of ASPP2 RNA could be measured using RT-PCR or in situ hybridisation techniques.

The other possibility is that these tumours have arisen from cells which do not normally express ASPP2. Given that the cell of origin for tumours seen in this model could be the CK14 expressing basal cell which would not be expected to express ASPP2, this is more likely. It should be emphasised that only two lung tumours developed in ASPP2$^{+/+}$ mice and these results may therefore not be reproducible.

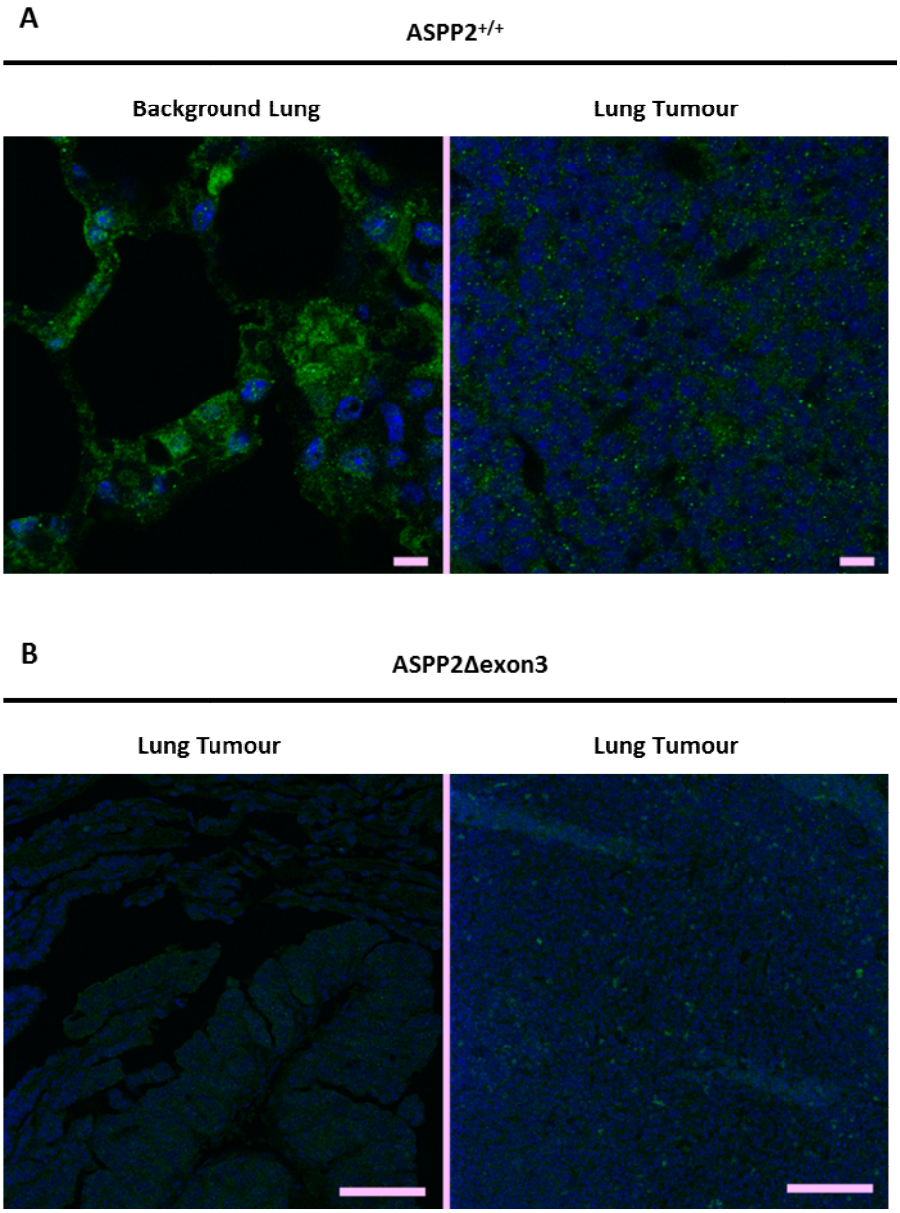


Figure 4.11: ASPP2 expression is lost in lung tumours. Compared to the background alveoli, ASPP2 expression is lost in the lung tumour from the same ASPP2$^{+/+}$ mouse **A.** ASPP2 expression is not seen in tumours from ASPP2Δexon3 mice **B.** Scale bar – 10μm **A,** 50μm **B.**

4.4.2 Tumours in ASPP2Δexon3 mice show glandular differentiation

Sections from lung tumours were stained with H&E and their morphological characteristics examined with the light microscope. Tumours were poorly circumscribed focally and in some cases numerous lobules were connected by areas demonstrating a lepidic pattern of growth. Within the nodular areas, papillary structures with well defined fibrovascular cores were seen adjacent to solid, more poorly differentiated areas. The recognisable features were more in keeping with a glandular differentiation and no areas of overt squamous differentiation were identified (Figure 4.12).

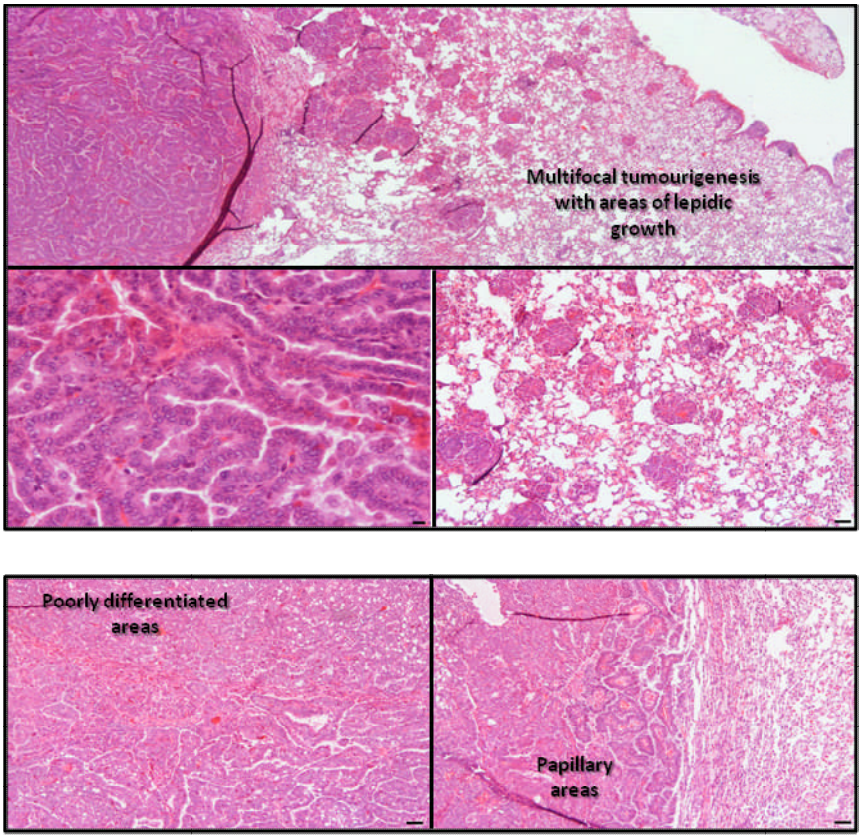


Figure 4.12: Lung tumours arising in ASPP2Δexon3 mice show features consistent with glandular differentiation. Morphological features identified include areas of lepidic growth, glandular structures and papillary areas. No overt squamous differentiation was seen. Poorly differentiated areas were also apparent. Scale bar – 50µm.

Furthermore, occasional foci of mucin expression were detected, consistent with areas of glandular differentiation (Figure 4.13).

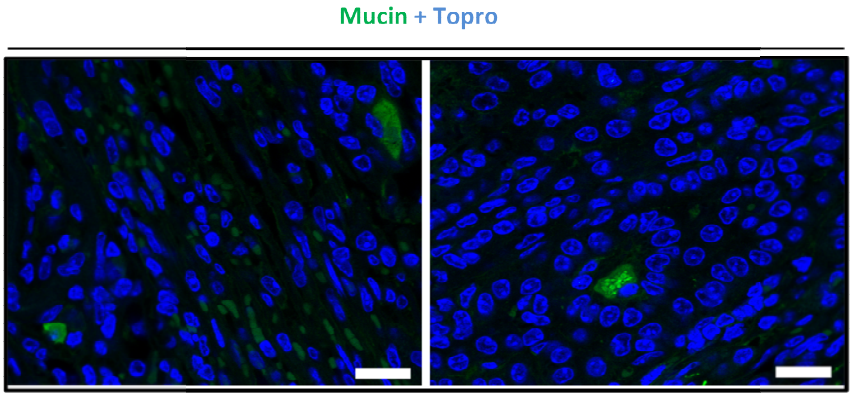


Figure 4.13: Lung tumours arising in ASPP2Δexon3 mice show foci of mucin expression. Conventional immunofluorescence staining show small foci of mucin (Muc5C) production. Scale bar – 50μm.

4.4.3 Immunohistochemical analysis is not consistent with squamous differentiation.

The histological identification of squamous cell differentiation is aided in poorly differentiated tumours by the positive expression of p63 and CK5. These markers, together with involucrin, were used to stain the tumours retrieved from experimental mice and the results do not support evidence of squamous differentiation in any of the tumours available for assessment (Figure 4.14)

4.4.4 Small foci of CK14 expression are seen

However given the increase in CK14 expression in the background lung parenchyma in the ASPP2Δexon3 mice, the tumours themselves were also probed for CK14. Small foci expressing CK14 were identified, though the expression is considerably reduced when compared to the levels seen in the background lung (Figure 4.14). These could represent small foci of early squamous differentiation, though the focal expression of CK14, in the absence of p63, has been reported in adenocarcinomas (Ooi et al. 2010).

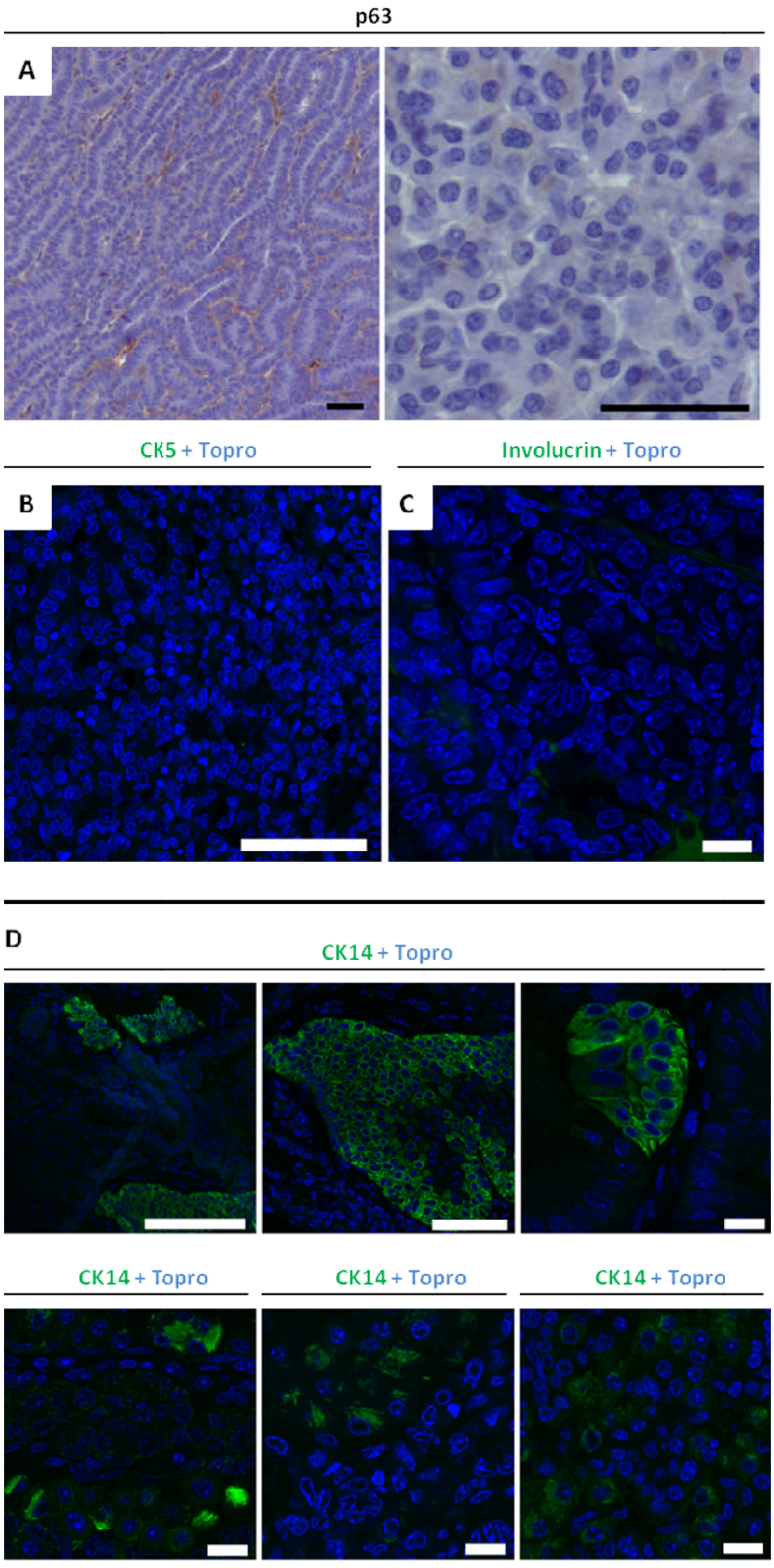


Figure 4.14: Lung tumours show no overt squamous differentiation. Conventional immunohistochemical and immunofluorescent staining shows no expression of p63, CK5 and Involucrin seen **A,B&C.** However small foci of CK14 expression are apparent **D.** Scale bar – 50µm **A&B,** 10µm C**&D.**

4.4.5 Small foci of neuroendocrine differentiation are seen

Lung tumours were also probed for the neuroendocrine markers synaptophysin and calcitonin gene related peptide (CGRP) with small foci of positivity identified (Figure 4.15). There were however no morphological characteristics to suggest these tumours should be classified as neuroendocrine and foci of positive staining were small. Interestingly, given the results discussed so far, it has been suggested that the particularly aggressive nature of adenosquamous carcinomas might be attributable to promotion of neuroendocrine pathways (Bastide et al. 2010). It would be extremely interesting to examine a larger cohort of tumours induced in the same way to determine if ASPP2 deletion could facilitate the development of this tumour type. In this experiment none of the tumours could be diagnosed as adenosquamous tumours on morphological or immunohistochemical grounds as criteria generally require each component to comprise at least 10% of the tumour (Shimoji et al. 2011). Overt squamous differentiation was not seen in the small sample of tumours examined here.

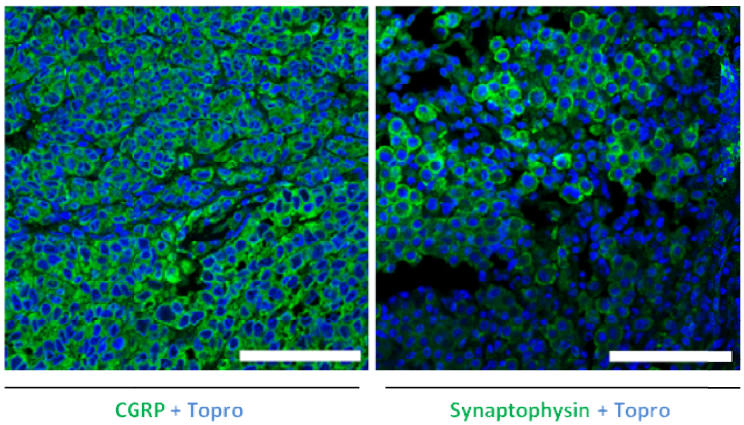


Figure 4.15: Lung tumours foci of neuroendocrine differentiation. Markers of neuroendocrine differentiation show focal positivity in lung tumours from ASPP2Δexon3 mice. Scale bar – 50μm

Hyperplasias and adenomas arise much more frequently in mice than their malignant counterparts. Furthermore the definite identification of invasive adenocarcinoma as opposed to the much more commonly occurring adenoma in the mouse lung is controversial. Hard criteria for malignancy include fewer tumours with a tumour size >5cm, definite invasion into the underlying airways or blood vessels and identification of distant metastases with the ability to grow on transplantation. Other softer criteria include nuclear atypia, increased number of mitoses and architectural irregularities such as the presence of well defined papillary structures with fibrovascular cores (Nikitin et al. 2004). The development of true carcinomas has been seen in those mouse models genetically engineered to express important, well-recognised driver mutations in lung cancer including K-RAS and EGFR. In other models, the development of these aggressive tumours is only seen with a combination of genetic modifications (Nikitin et al. 2004).

The specific morphological characteristics delineating an aggressive phenotype were then examined in our experimental system. The hypothesis was that loss of ASPP2 alone results in marked basal cell hyperplasia and is therefore potentially a key driver mutation in lung carcinogenesis. We would therefore expect the tumours to show an aggressive phenotype.

4.4.6 Lung tumours in ASPP2Δexon3 mice show features of invasive carcinomas

Table 2 and figure 4.16 indicate the features which could be analysed in the tumours available for assessment. As can be seen the tumours seen in ASPP2Δexon3 mice were often single, significantly larger than 5cm with marked nuclear pleomorphism, numerous mitoses and features suggestive of stromal invasion. Distant metastases were not specifically looked for and the brains in these mice could not be examined at the time of post mortem. However even in the small number of tumours analysed there is evidence to corroborate the hypothesis that the tumours seen in mice with the somatic deletion of ASPP2 show features consistent with invasive carcinoma.

Case	Age/mths	Number of Tumours	Size/mm	Ayptia	Mitoses/10hpf	Invasion	Preferred diagnosis
172	15	1	2	0	1	0	Adenoma
208	18	2	9	1	18	1	Carcinoma
173	15	3	7.5	1	14	2	Carcinoma
184	15	>10	2.7	1	1	2	Adenoma
291	15	1	18	1	19	2	Carcinoma
212	18	1	6	1	22	2	Carcinoma
223	18	1	17	1	10	2	Carcinoma
245	18	1	10	1	9	2	Carcinoma
277	18	1	1.9	1	2	0	Adenoma
278	18	1	10	1	18	1	Carcinoma

Table 2: Analysis of lung tumour parameters to facilitate accurate classification. Cases highlighted in blue represent control mice compared to experimental mice highlighted in pink. Atypia was classified as 0=Minimal and 1= Marked. Invasion was difficult to assess particularly in samples with little adjacent lung and was scored 0 = No, 1= Possible, 2 = Probable and 3 = Definite.

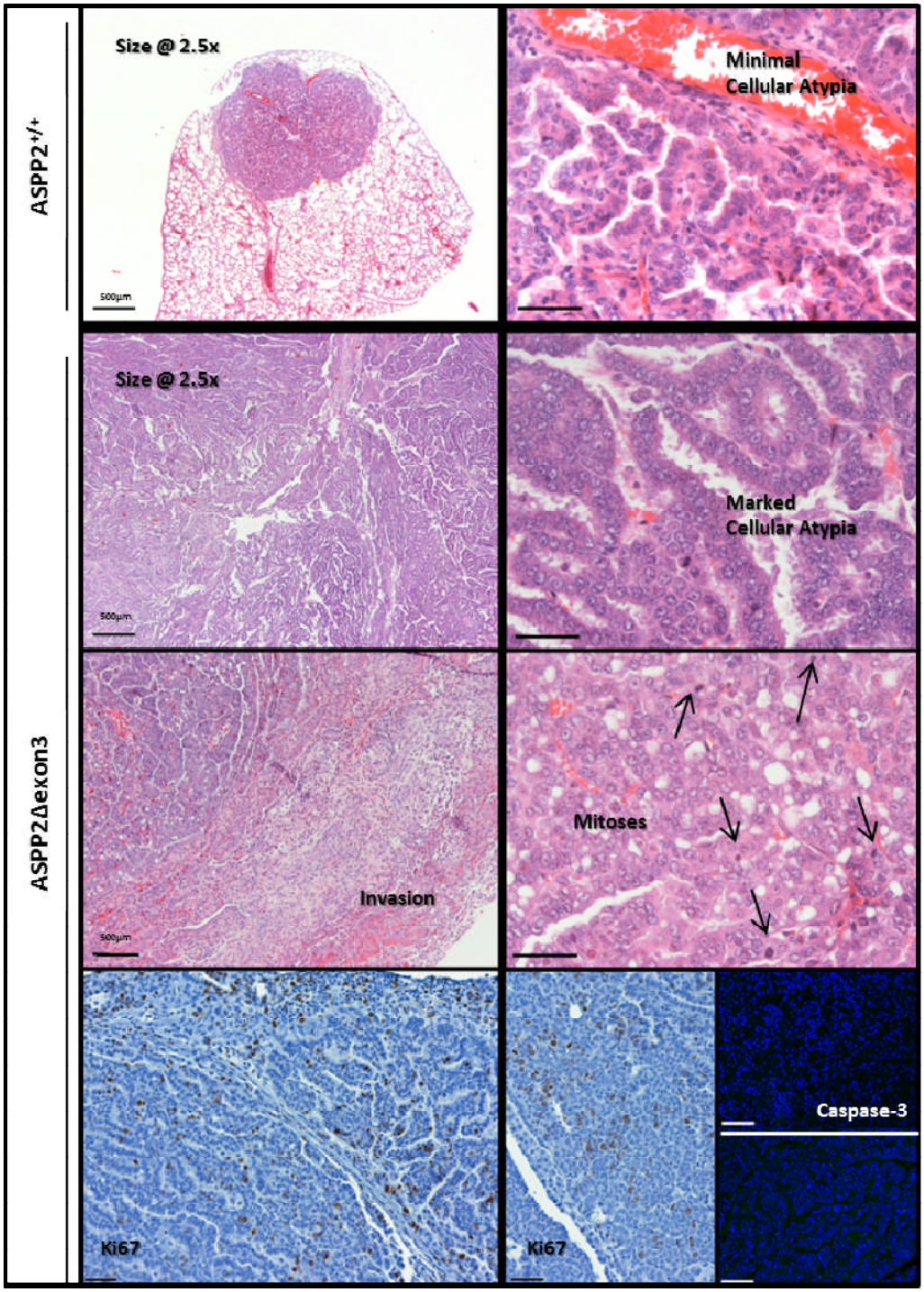


Figure 4.16: Analysis of lung tumour parameters to facilitate accurate classification. Lung tumours developing in ASPP$^{+/+}$ mice were small and showed minimal cellular atypia. In contrast, tumours developing in ASPP2Δexon3 mice were much larger, showed marked cellular atypia, foci of stromal invasion and numerous mitoses. Furthermore the Ki67 index was >20% and there was no evidence of apoptosis as illustrated by negative immunofluorescence staining for cleaved caspase -3 (green). Scale bar - 50μm (unless otherwise indicated).

It can therefore be concluded from this part of the investigation that the somatic deletion of ASPP2 on a mixed background results in basal cell hyperplasia, foci of squamous metaplasia and the development of aggressive NSCLCs which may show multidirectional differentiation and warrant further investigation in a larger study. These results are very similar to those reported by Dakir *et al* (2008) and suggest that ASPP2's repression of CK14 expression contributes to this phenotype. This model represents a novel mouse model of tumourigenesis resulting from the deletion of single gene, suggesting loss of ASPP2 could be a key initiating factor in the development of lung cancer.

Furthermore the results corroborate ASPP2's promotion of the differentiated phenotype in normal epithelium and implicate ASPP2 as a key modulator of epithelial homeostasis in both squamous epithelia and the pseudostratified epithelium of the lung.

Chapter 5: Results

iASPP in Human Carcinoma: An Oncoprotein

iASPP binds to and specifically inhibits the apoptotic function of p53 and its family members p63 and p73 (Bergamaschi et al. 2004). Furthermore the generation of transgenic mice in which the expression of iASPP was controlled by the Cre/LoxP recombination system identified a role for iASPP in the maintenance of normal stratified squamous epithelium via its negative regulation of alternate p63-dependent transcription. In the nucleus, iASPP binds to and inhibits p63's transcription of keratins associated with differentiation. In mice this translates as the epidermal expansion of differentiated layers and a reduction in proliferative capacity on iASPP deletion (Notari et al. 2011).

As discussed, in humans the development of squamous cell carcinoma occurs with the step-wise progression from normal squamous epithelium to dysplastic epithelium with cells eventually acquiring sufficient genetic mutations to become frankly invasive. Dysplasia is histologically recognisable as the disordered maturation of squamous epithelium with increasing numbers of cells demonstrating proliferative capability and expressing proteins normally seen only in basal and parabasal cells. This would appear to be in direct contrast to the appearance of the epidermis described above, in the conditional iASPP knockout mouse.

Together these data suggest that the overexpression of iASPP in human squamous epithelia would inhibit differentiation and promote proliferation, thereby potentially contributing to subsequent neoplastic transformation. The first part of this chapter explores the role of iASPP in human squamous cell carcinoma formation.

5.1 Normal Squamous epithelium

If iASPP does indeed maintain the proliferative potential of human squamous epithelium, its expression would be expected in normal mature tissue. Moreover it would not be expected in the superficial, differentiated layers, but in the basal and parabasal layers of the normal stratified squamous epithelium where p63 is usually expressed.

5.1.1 iASPP is expressed in the lower two thirds of mature squamous epithelia

To investigate the expression of iASPP in human squamous epithelia, conventional immunohistochemical staining for iASPP was undertaken on sections from the morphologically normal squamous mucosae lining the ectocervix and oropharynx. As for ASPP2, staining of the ectocervix in 24 samples had been started prior to this project by Anna Pagotto, Emily Ruban and Beyza Vurusaner and its continuation and analysis is reported here. Again a further 6 samples were also stained and analysed.

In figure 5.1, nuclear and cytoplasmic iASPP is seen to be expressed in the lower two thirds of the epithelia examined. The superficial layers are spared.

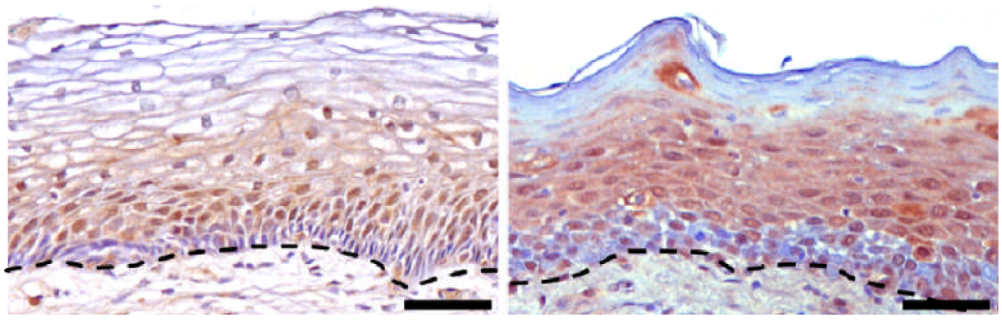


Figure 5.1: iASPP expression predominates in the lower two thirds of mature squamous epithelia. Conventional immunohistochemical staining for iASPP in the ectocervix **A** and tongue **B.** iASPP expression spares the superficial, differentiated layers In all samples analysed. Scale bar - 50µm.

Profiling of the red channel, now representing the DAB signal, clearly shows a decrease in iASPP in the superficial differentiated cells of the ectocervix. Furthermore figure 5.2 clearly demonstrates the predominance of nuclear iASPP in the basal and parabasal layers. This differential pattern of expression suggests that the persistence of nuclear iASPP, in particular, is either a consequence of, or contributes to the immaturity of cells in these lowest layers.

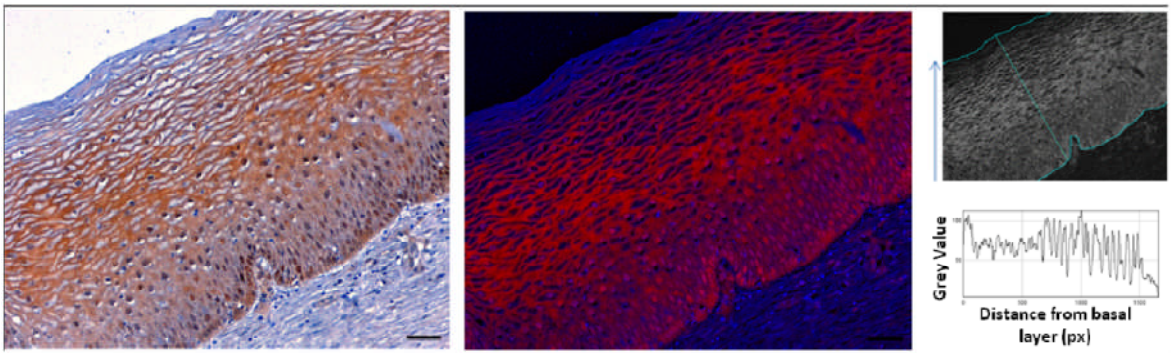


Figure 5.2: iASPP expression predominates in the lower two thirds of the morphologically normal ectocervix. Conventional immunohistochemical staining of the ectocervix with the corresponding unmixed image. As in Chapter 1, the original image was inverted, unmixed and the detailed analysis of the DAB, now red, signal clearly demonstrates the decrease in iASPP expression as the superficial, more differentiated, layers are reached. Scale bar – 50µm

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5.1.2 CK14 highlights the iASPP expressing basal and parabasal layers

Again the use of CK14 was used to confirm the expression of iASPP in the lower two thirds of the ectocervix. Conventional double immunofluorescence staining confirms that iASPP localises in the same cells as CK14 in these lowest layers (Figure 5.3).

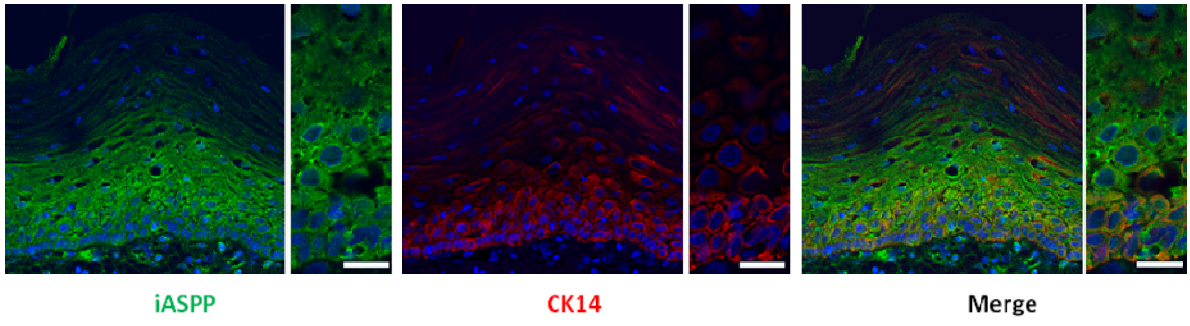


Figure 5.3: CK14 highlights the iASPP expressing lower layers. iASPP and CK14 double immunofluorescence. iASPP (green) localises in the same cells as CK14 (red) in these lowest layers. Scale bar – 10µm

These observations support a role for iASPP in maintaining the replication competent compartment of the squamous epithelium. Moreover, the prominent expression of nuclear iASPP, as seen in figure 5.2 could facilitate its modulation of nuclear p63 here.

5.1.3 p63 co-localises with iASPP in the ectocervix

To investigate the relationship between iASPP and p63 expression in human squamous epithelium, double immunohistochemical staining of the ectocervix was undertaken. The technique described in Chapter 3 was used again as figure 5.3 demonstrates the loss of nuclear iASPP after HIER in conventional immunofluorescence protocols. Figure 5.4 demonstrates the nuclear colocalisation of iASPP and p63 in the basal and parabasal layers of ectocervical squamous epithelium. This result suggests iASPP could also regulate human epithelial stratification via its interactions with p63.

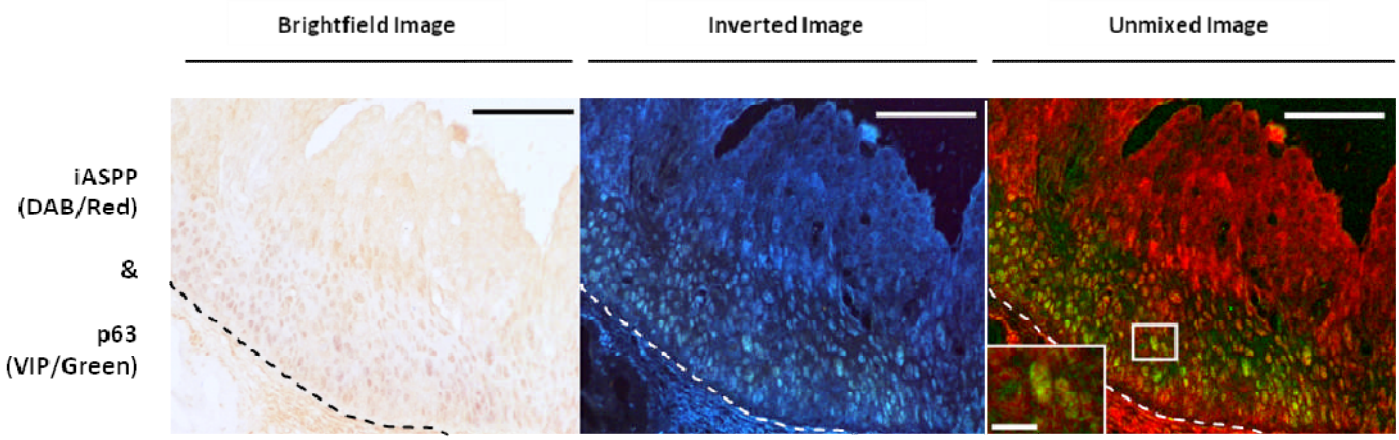


Figure 5.4: p63 colocalises with iASPP. iASPP and p63 double immunohistochemical staining in the ectocervix demonstrating the colocalisation of nuclear iASPP (red) and p63 (green). Scale bar – 50μm.

5.2 Squamous Dysplasia

As described previously CIN is characterised by lack of differentiation, unregulated proliferation and p63 expression; features which characterise the replication competent basal and parabasal layers (Quade et al. 2001). It is hypothesised that nuclear iASPP's ability to maintain these layers is manipulated in the development of CIN and its overexpression will be seen in these areas.

5.2.1 Cervical Intraepithelial Neoplasia (CIN) expresses predominantly nuclear iASPP

To determine whether there is evidence of nuclear iASPP expression in CIN, these areas were examined, using conventional immunohistochemistry, in 23 of the 24 cervical samples available. Figure 5.5 demonstrates the increased expression of nuclear iASPP in high grade CIN compared to the normal ectocervix of the same patient.

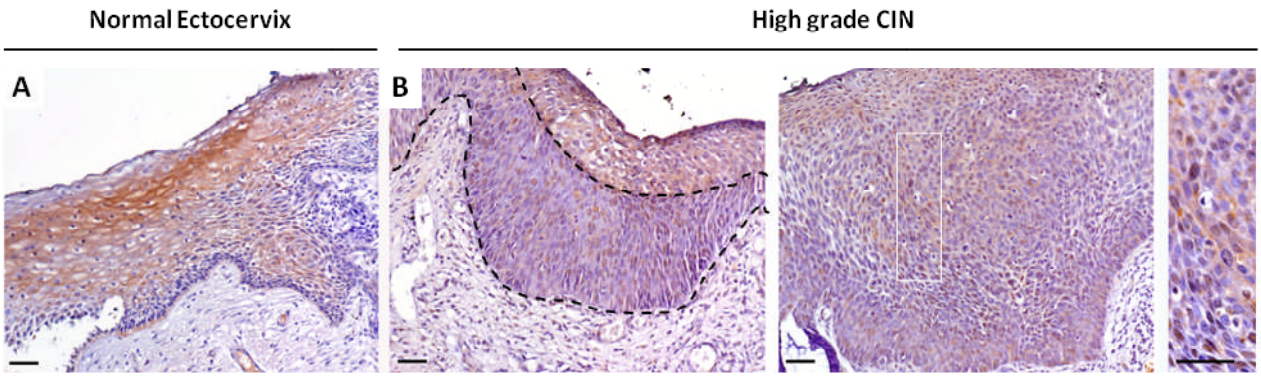


Figure 5.5: Increased nuclear iASPP expression is seen in CIN. iASPP immunohistochemical staining in normal ectocervix **A** and corresponding high grade CIN **B** from the same sample. Areas of CIN shows predominantly nuclear iASPP in all the samples analysed. Scale bar – 50μm

These results support a role for nuclear iASPP in the development of pre-invasive dysplastic lesions, a phenotype expected to persist in invasive cervical squamous cell carcinoma.

5.3 Squamous Cell Carcinoma

The heterogeneous pattern of differentiation seen in squamous cell carcinomas is expected to be reflected by a heterogeneous expression of iASPP.

5.3.1 Cervical squamous cell carcinomas show a heterogeneous expression of iASPP

TMA CR803 was used to analyse the expression of iASPP in cervical squamous cell carcinomas.

iASPP expression was observed to be markedly heterogeneous with cytoplasmic and/or nuclear staining seen in 74% (52/70) of tumours. Figure 5.6A&B demonstrate that in iASPP positive tumours, poorly differentiated (PD) areas expressed predominantly nuclear iASPP.

5.3.2 Nuclear iASPP is expressed with p63 in cervical squamous cell carcinomas

Further analysis of nuclear iASPP in individual tumour cores showed that its expression correlated with the expression of p63 in these less well differentiated areas (Figure 5.6B). In fact overall, there is a significant increase in nuclear iASPP expression in cores which were p63 positive (Figure 5.6C).

The data in CIN and squamous cell carcinomas of the cervix suggest that nuclear iASPP expression is a key feature of early neoplastic transformation and of poorly differentiated tumours. Though tumour grade is a controversial prognostic factor in cervical carcinoma (Royal College of Pathologists: DataSet for Cervical Carcinoma,2011) this observation suggests nuclear iASPP is present in more aggressive parts of invasive carcinomas.

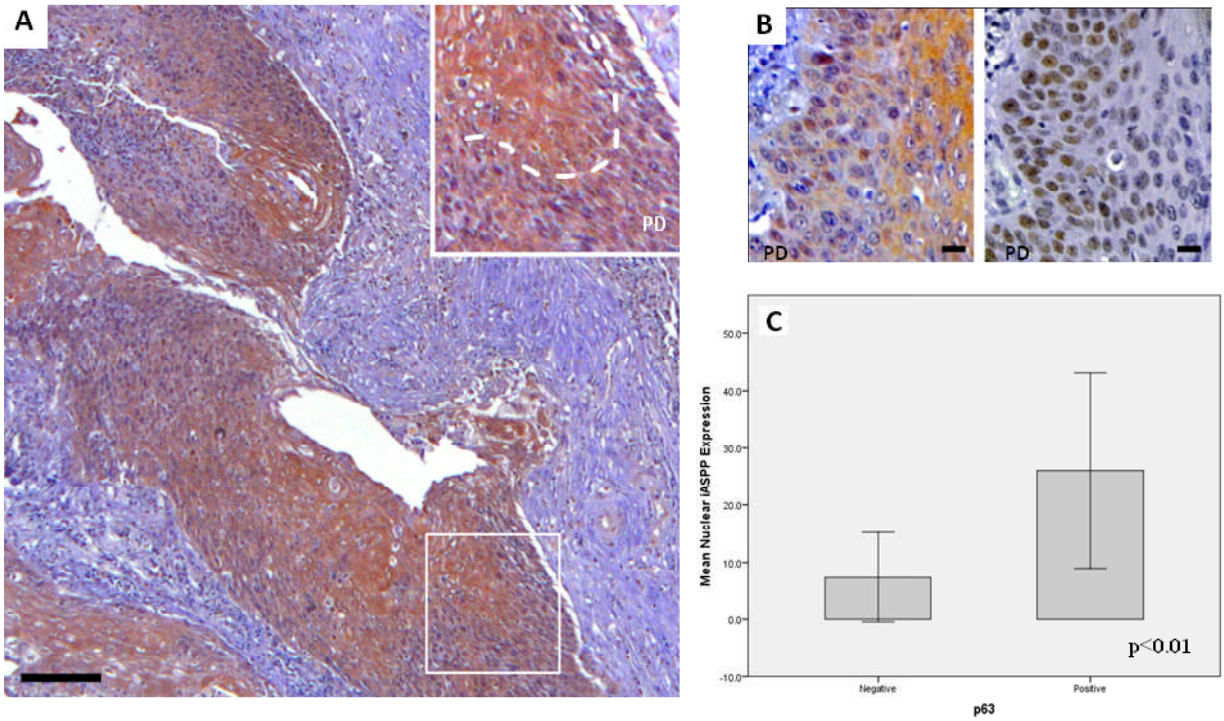


Figure 5.6: TMA CR803 – Cervix: Two slides were stained for iASPP to validate the reproducibility of the staining procedure in this context. Staining of these slides were analysed by two independent scorers and agreement between them also validated (kappa = 0.82). One slide was stained for p63. iASPP expression was scored in the same way as for ASPP2 and the results analysed using the same methods (Chapter 3, Fig 3.8). Statistical analysis was undertaken in SPSS. Results were analysed by the Mann Whitney test and the effect size is denoted by $r=z/\sqrt{n}$ (*>0.3 = medium effect, >0.5 = large effect).*

iASPP expression in squamous cell carcinomas is markedly heterogeneous with areas of nuclear, cytoplasmic and mixed staining seen **A**. In those iASPP positive carcinomas showing heterogeneity of differentiation, predominantly nuclear iASPP is seen in the less well differentiated areas (PD) **A**. These areas also co-express p63 **B**. Overall there is a significant increase in nuclear iASPP expression in cores which were p63 positive ($U=381.500$, $r=-0.37$, $p<0.01$) **C**. Scale bars - 100µm **A**, 10µm **B**, Error bars 95% CI **C.**

5.3.3 Oropharyngeal squamous cell carcinomas show heterogeneity of iASPP expression

To further investigate iASPP heterogeneity in squamous cell carcinomas, its expression was analysed in full cross-sections of six tonsillar, six lingual, three laryngeal and three buccal tumours using conventional immunohistochemical methods. Figure 5.7 demonstrates the key findings.

The most striking feature was the variation in iASPP expression in different areas of the same tumour. In those tumours showing morphologically identifiable changes in differentiation, nuclear iASPP was, as in cervical squamous cell carcinomas, seen in the poorly differentiated areas, in contrast to those better differentiated areas which showed predominantly cytoplasmic iASPP expression (Fig 5.7A). However it was also noted that in other tumours with a more homogenous morphological appearance, stark differences in the expression of iASPP were detected in adjacent, otherwise similar, islands of tumour. As can be seen in Fig 5.7B, although the underlying morphology of these tumour islands is preserved, there is a clear change of iASPP from predominantly cytoplasmic to nuclear expression.

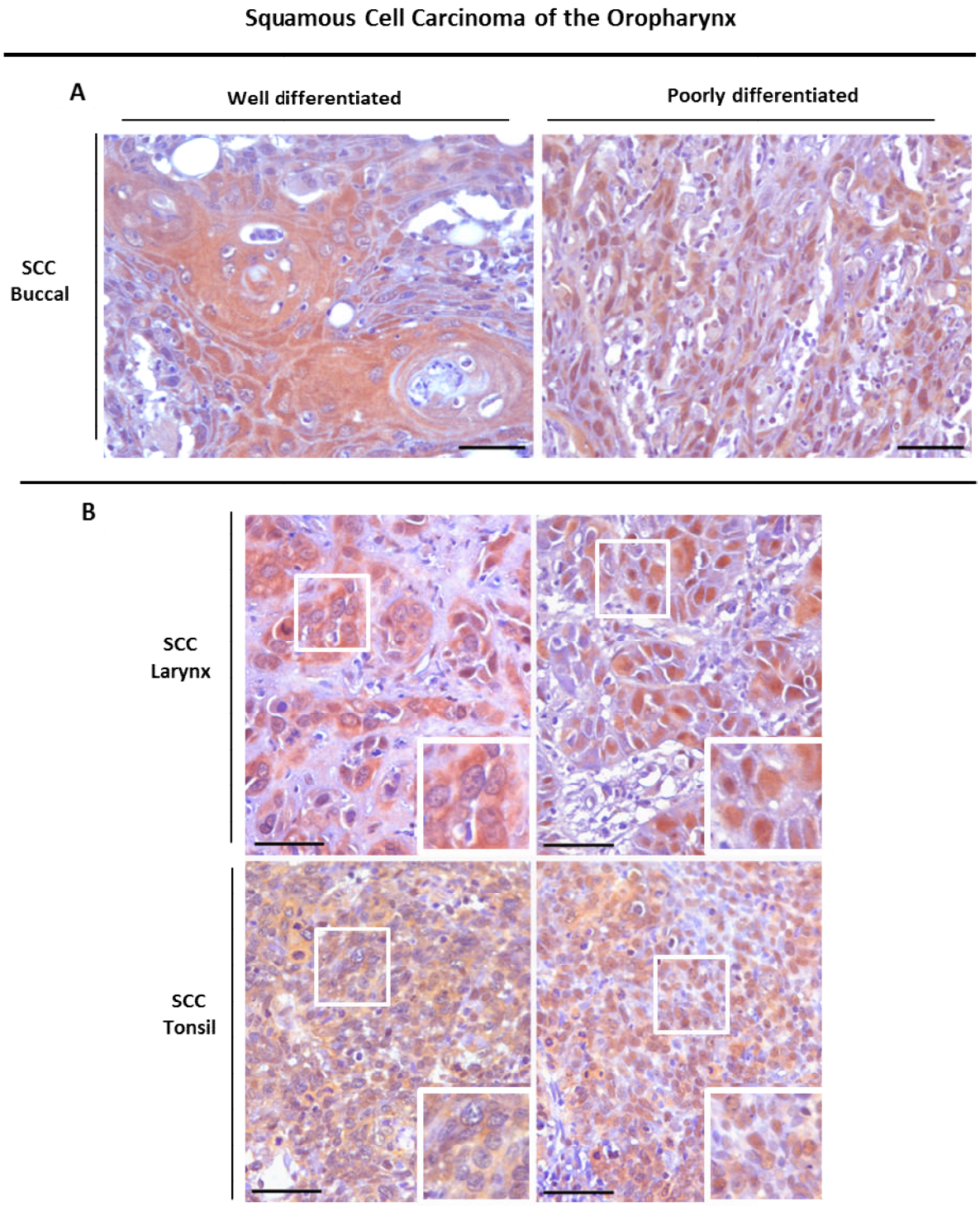


Figure 5.7: A heterogeneous pattern of iASPP expression is seen in oropharyngeal squamous cell carcinomas. In carcinomas demonstrating variable differentiation in morphologically distinct areas, nuclear iASPP is seen primarily in more poorly differentiated areas **A**. In carcinomas with a more homogenous morphological appearance, islands with predominantly cytoplasmic iASPP expression are seen adjacent to islands of malignant cells expressing nuclear iASPP in the same section **B.** Scale bar – 50μm

5.3.4 Predominantly nuclear iASPP is seen specifically at invasive margins in HNSCCs

The three lingual carcinoma sections showed the best orientation and these were therefore further analysed to determine if a pattern to the areas of similar iASPP expression could be discerned.

Figures 5.8 and 5.9 show that islands of malignant cells, with predominantly nuclear iASPP expression, were located at the peripheral, invasive edges of carcinomas. These were the same areas which had, in Chapter 3, demonstrated reduced expression of ASPP2 and caspase-3, but the highest levels of p63. In fact figure 5.9 demonstrates the co-localisation of iASPP and p63 in these peripheral tumour islands. The two antigens were detected using the dual immunohistochemical, immunofluorescence staining method described in Chapter 4.

These results appear to demonstrate that the expression of nuclear iASPP is associated with cell survival at the invasive edges and not with the potentially hypoxic, nutrient scarce, apoptotic centre of the tumours.

The data thus far, in CIN and squamous cell carcinomas of the cervix and oropharynx, suggest that nuclear iASPP is a key feature of early neoplastic transformation and its expression is maintained in areas of tumours which remain poorly differentiated carcinomas, specifically at the invading tumour edges. It can therefore be hypothesised that the nuclear expression of iASPP might promote tumour progression and the formation of distant metastases.

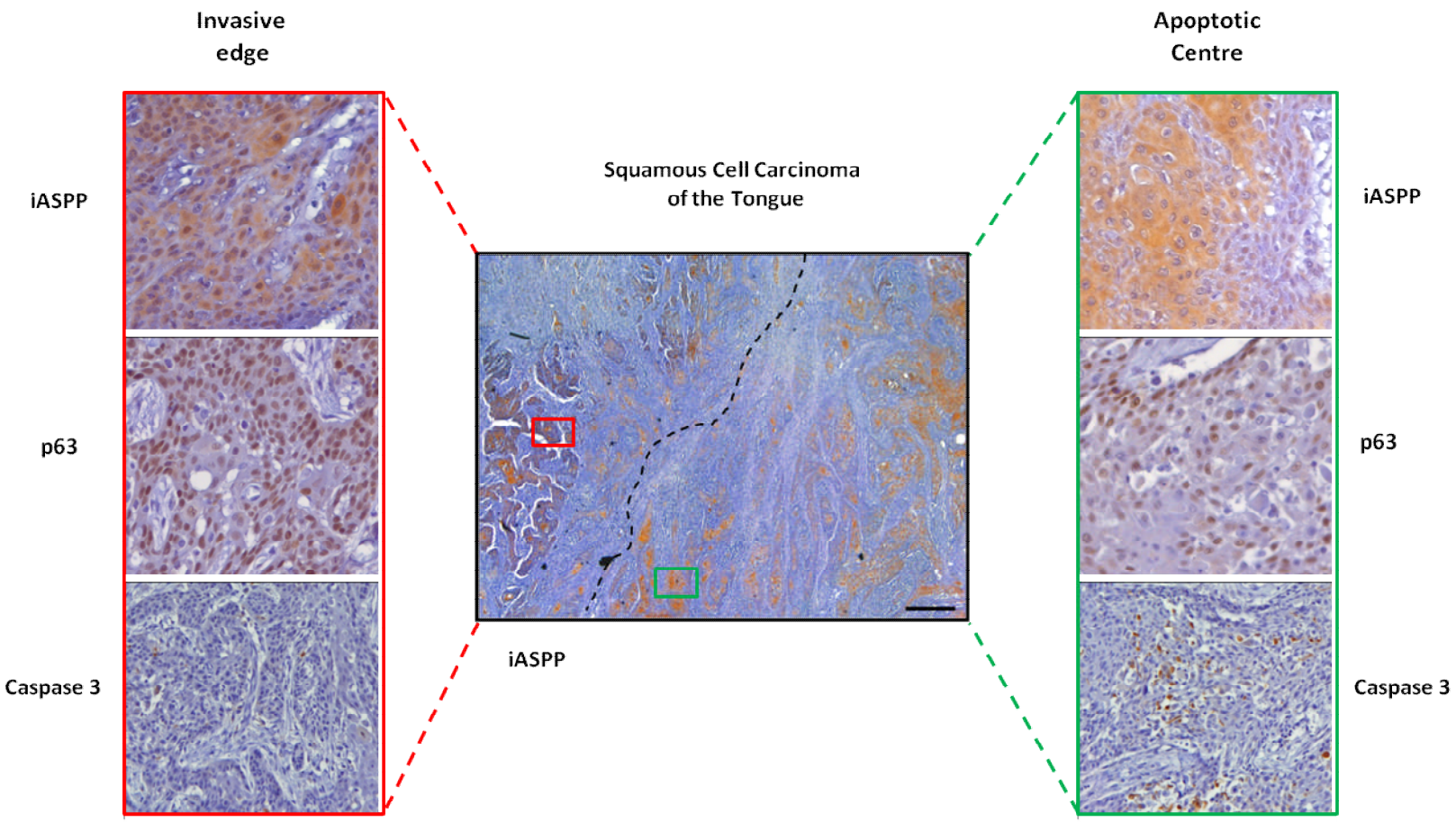


Figure 5.8: Predominantly nuclear iASPP expression is found at the invasive edge of squamous cell carcinomas. iASPP expression in a full SCC cross-section with corresponding p63 and caspase 3 immunohistochemistry. The invasive edge shows nuclear iASPP, p63 and lower levels of apoptosis as illustrated by caspase-3. Scale bars - 500 µm

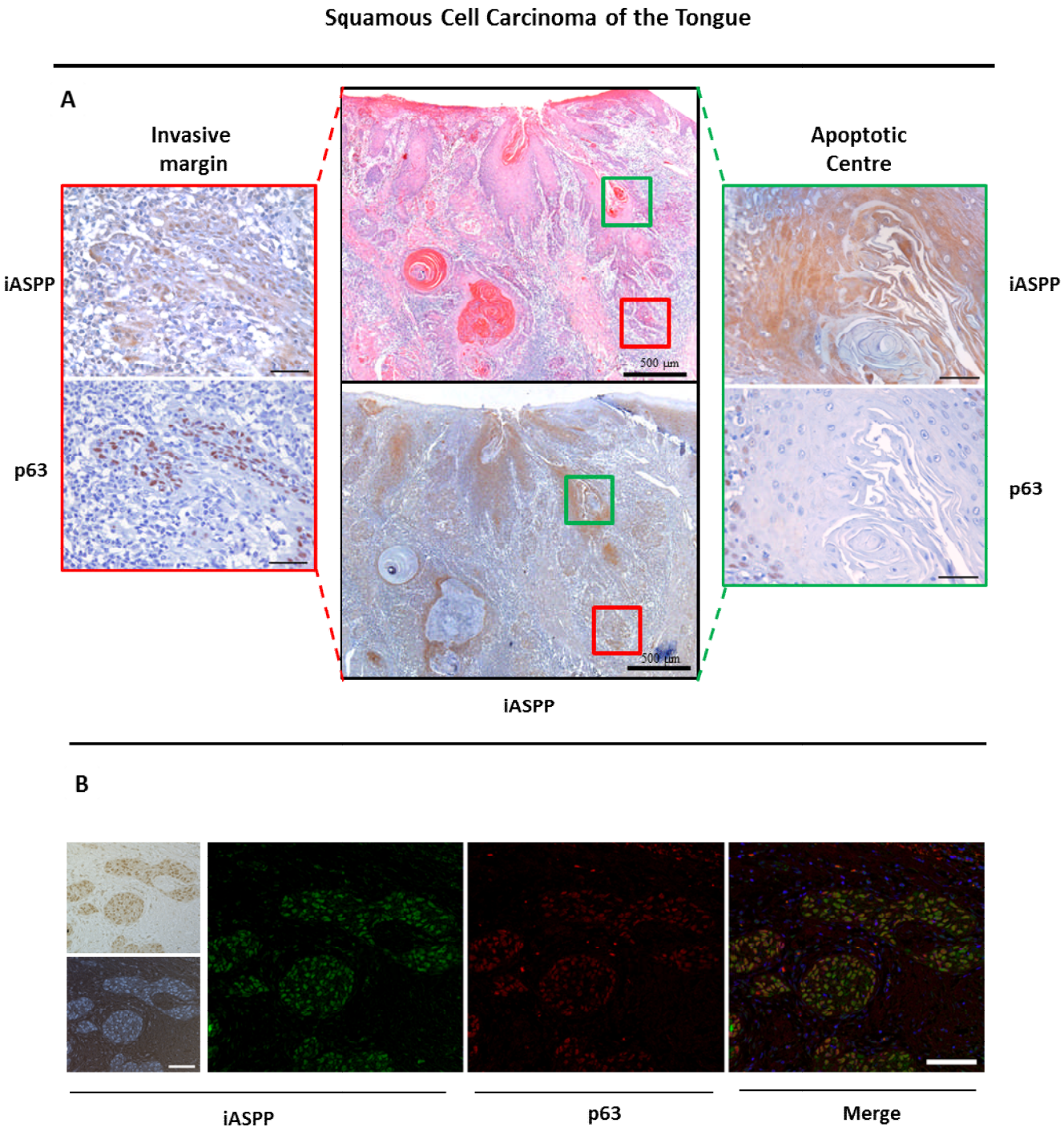
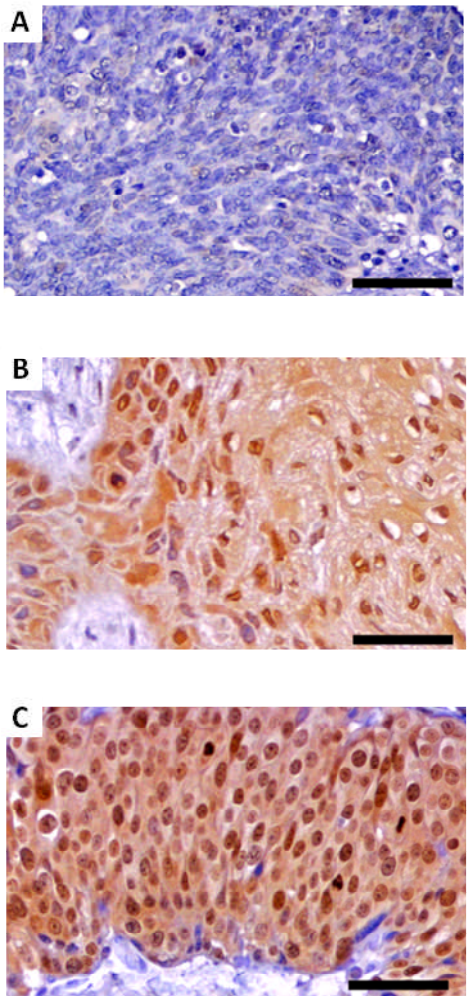


Figure 5.9: Nuclear iASPP co-localises with p63 at the invasive margin of squamous cell carcinomas. Nuclear iASPP expression is seen to localise with p63 at the invasive edges of a second lingual SCC **A.** Dual immunohistochemistry and immunofluorescence staining demonstrates that nuclear iASPP and p63 are expressed in the same cells in a third lingual SCC **B.** Scale bars - 50 µm (unless otherwise specified)

5.3.5 Metastatic HNSCCs show an increase in nuclear iASPP

ZTMA 42.2 was again used to determine if nuclear iASPP is associated with tumour progression in the oropharynx.

Figure 5.10 shows that expression of nuclear iASPP is significantly greater in lymph node metastatic deposits and in primary tumours known to have metastasised compared to those primary tumours with no evidence of metastases.



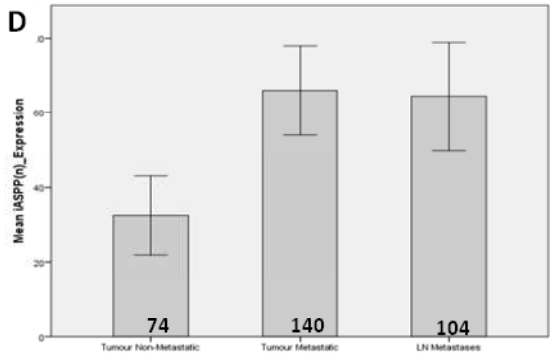


Figure 5.10: ZTMA 42.2 - Oropharynx. iASPP expression was scored as for TMA CR803. Results were analysed by the Kruskal-Wallis Test (H) with Mann-Whitney tests (U) used for *post hoc* procedures and the effect size is denoted by $r=z/\sqrt{n}$ *(>0.3 = medium effect, >0.5 = large effect).* A Bonferroni correction was applied so all effects are reported at a 0.0167 (0.05/3) level of significance

The overall expression of nuclear iASPP is significantly different across the categories of tumour type ($H(2) = 13.145$, $p<0.01$) in 318 carcinoma cores analysed **D.**

The expression of nuclear iASPP is significantly greater in lymph node metastatic deposits (**C** *Mdn 35.0)* and in those primary tumours known to have metastasised (**B** *Mdn 40.00,)* compared to those primary tumours with no evidence of metastases (**A** *Mdn 10.0)* $U=3698.500$, $r=-0.24$, $p<0.0167$ and $U= 2848.000$, $r=-0.22$, $p<0.0167$ respectively. Scale bar - 50µm Error bars = 95% CI

Thus far, it has been possible to demonstrate that iASPP's role in the maintenance of murine squamous epithelium (Notari et al. 2011) can potentially translate to human squamous epithelia. Furthermore the persistence of nuclear iASPP, in co-operation with p63, is likely to promote the development and progression of dysplastic, malignant and metastatic squamous lesions, specifically of the cervix and oropharynx.

5.4 iASPP and the cervical transformation zone

The persistence of nuclear iASPP is associated with the progression of squamous cell carcinomas of the cervix and oropharynx. As described in detail in Chapter 3 the pathogenesis of this tumour type in the cervix and not uncommonly in the oropharynx is dependent on prior infection by HPV. As discussed, HPV infection in the cervix is thought to be specifically tropic to those areas of physiological squamous metaplasia occurring in the cervical transformation zone. The reserve cells here are known to express p63 and it is therefore hypothesised that they will also express nuclear iASPP.

5.4.1 iASPP is not expressed in the endocervical epithelium

To explore this further the transformation zone was identified in cervical LLETZ specimens and conventional immunohistochemical staining for iASPP was undertaken.

Figure 5.11 demonstrates that iASPP is not expressed in the mature endocervical glandular epithelium.

5.4.2 Nuclear and Cytoplasmic iASPP are seen in subcolumnar basal cells and areas of immature squamous metaplasia

However figure 5.11 demonstrates the nuclear and cytoplasmic expression of iASPP in the subcolumnar “reserve” cells and double immunohistochemical staining with p63 shows that the iASPP expressing population of cells intermittently also co-express p63 (Figure 5.11C). Subcolumnar basal cells, as highlighted by iASPP expression, which do not express p63 are also seen here.

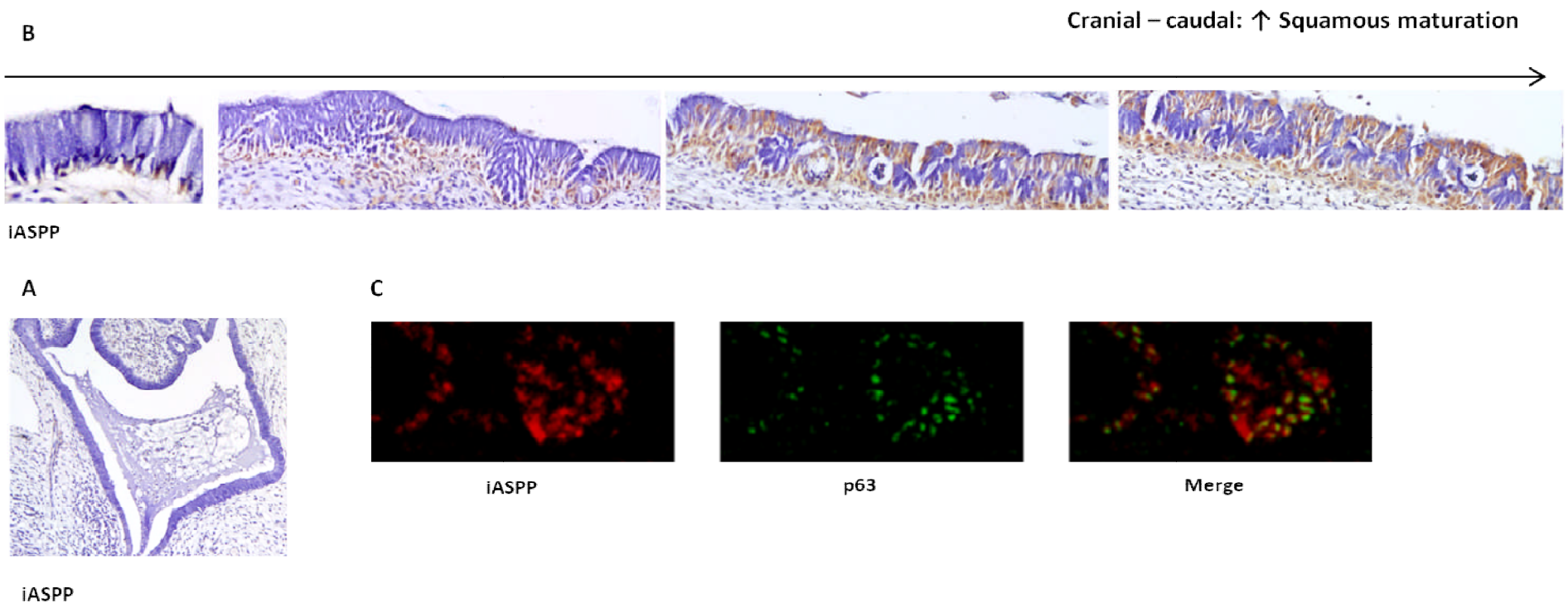


Figure 5.11: Nuclear and cytoplasmic iASPP is seen in the transformation zone. iASPP is not seen in the mature endocervical cells **A.** Nuclear and cytoplasmic iASPP is seen in a subcolumnar cellular population in areas of immature squamous metaplasia. Cranially the iASPP expressing cells appear in a single layer underlying the endocervical cells. As the section progresses caudally the cells are seen in an increasing number of layers as the squamous metaplasia matures. This pattern was seen in all 3 of the 24 samples where adequate areas of squamous metaplasia were available for assessment **B**. p63 expression occurs in a population of these iASPP expressing cells **C**.

These results demonstrate the presence of nuclear iASPP in a population of cells thought to initiate squamous metaplasia in the cervical transformation zone, providing possible evidence that iASPP is involved in this differential switch. The expression of nuclear iASPP here fits with our knowledge of its interactions with nuclear p63. Whether the presence of nuclear iASPP is essential for maintaining the reserve cell population, for effective p63 regulation in the transformation zone or simply to modify the p63 transcription programme once metaplasia begins is unclear. It can also be deduced then, that HPV infection of these cells either promotes, or at least does not preclude, the persistence of nuclear iASPP expression.

However there is an element of cervical physiology which has, until this point of the investigation, been overlooked. The cervical epithelium, as part of the female reproductive system, is exquisitely sensitive to the effects of circulating estrogen. In fact the squamous epithelium of the ectocervix in pre and postmenopausal women is atrophic and composed of basal and parabasal cells only. Estrogen signalling, translated predominantly through ERα, directs the complex cell fate decisions and epithelial stromal remodelling which characterises the initiation of squamous metaplasia and formation of the transformation zone. Specifically, it promotes both the proliferation of parabasal layers and the maturation and terminal differentiation of the midzone resulting in the development of the superficial layers and the consequent thickening of the ectocervix.

Furthermore the role of estrogen signalling in cervical carcinogenesis is controversial. It is considered to play an important role, at least in the initial development of cervical neoplasia. The expression of ERα is much higher in the transformation zone, compared to mature ecto or endocervical epithelium (Remoue et al 2003). Mouse models of cervical

squamous carcinogenesis, initiated by HPV show that estrogen signalling is essential for the initiation of squamous dysplasia at the transformation zone (Chung et al. 2010). Clinically, the prolonged exposure to exogenous estrogen in the form of the oral contraceptive pill is a known risk factor for human cervical carcinogenesis.

However the progression from early dysplastic lesions through high grade in situ lesions to frankly invasive SCC has been shown in many studies to be dependent on the loss of *ESR1* and the down regulation of ERα as detected by conventional immunohistochemical methods. In fact the persistence of ER has been reported to predict those cases of high grade CIN which are less likely progress to invasive disease.

In the mature ectocervix, ER has been reported to be expressed predominantly in the basal and parabasal layers and in reserve cells of the transformation zone (Jordan et al 2006). This described distribution would appear to reflect that of nuclear iASPP. Thus iASPP and ER are apparently both expressed in the same tissue location and both implicated, to some extent, in the promotion of squamous proliferation and initiation of early squamous dysplasia. Conversely ER, but not iASPP has been shown to promote squamous differentiation and iASPP, but not ER is shown to persist in more aggressive malignant lesions. It can therefore be hypothesised that the presence of iASPP and ER together in an individual cell could have different implications for its particular fate than when these proteins are expressed in isolation.

The genomic effects of estrogen signalling, as mediated by ERα, are considered to be dependent on tissue and cell type and more particularly on the proportions of other factors present which act either to promote or repress different signals. It is possible that in the

normal ectocervix, iASPP acts to promote the mitogenic effects of ER in the cells in which it is present and where it is not present, the cells are instead directed towards differentiation. Conversely the presence of ER could act to control iASPP mediated activity, curbing the over expression seen in neoplasia. Thus in the later stages of tumourigenesis the presence of ER would dampen the effects of nuclear iASPP expression, reducing the potential for progression to invasive disease. In any case, further investigation of the possible interaction between estrogen signalling and iASPP would appear to be warranted.

In order to explore the relationship between iASPP and ER further double staining was undertaken in the human cervical tissue used previously and in normal human breast tissue. The breast was chosen as the second tissue type in which to examine this relationship as it is also exquisitely sensitive to the effects of estrogen signalling. Furthermore estrogen signalling, again mediated predominantly through ERα, plays a key role in the development and progression of breast carcinomas. In fact routine histological diagnosis of breast carcinoma is accompanied by an assessment of ER expression which is very important in determining treatment regimens. As such, the roles of estrogen and specifically ERα have been extensively studied in the breast where they are better understood than elsewhere.

5.5.1 Nuclear iASPP and ER co-localise in the morphologically normal ectocervix

Unfortunately lack of adequate cervical tissue precluded analysis of the relationship between iASPP and ER in the transformation zone itself. However figure 5.12 demonstrates that there is extensive co-localisation of ER, nuclear iASPP and p63 in the parabasal layers of the mature ectocervix. Furthermore there are cells, particularly in the more superficial, differentiated layers, in which there is strong ER expression, but no accompanying expression of either nuclear iASPP or p63 indicating that these proteins are lost as cells are directed to differentiate.

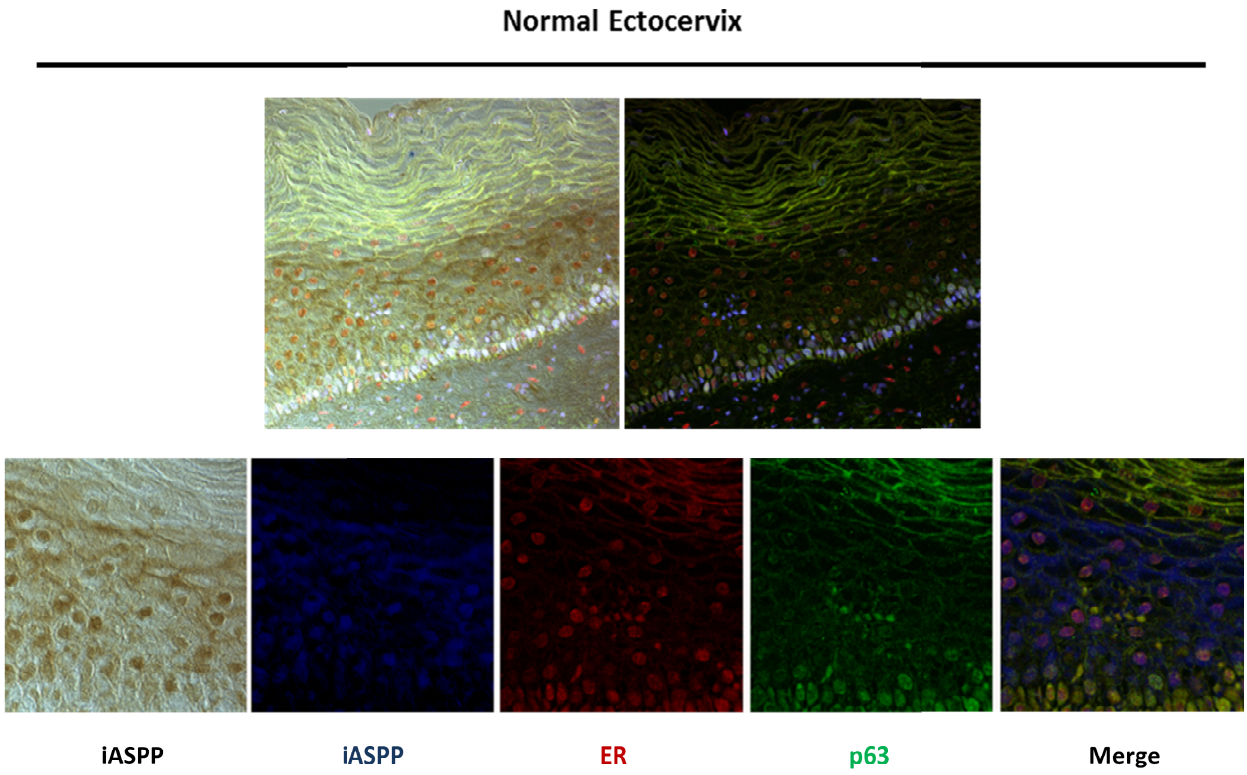


Figure 5.12: Expression of iASPP in the morphologically normal ectocevix. Dual immunohistochemistry and immunofluorescence staining demonstrates the co-localisation of iASPP, ER and p63, particularly in the parabasal layers in this section. ER expression is seen to extend more superficially than either iASPP or p63. Scale bars – 50µm

5.5.2. iASPP is predominantly expressed in luminal cells of the normal breast

Breast epithelium is glandular and the spatial arrangement of p63 and ER is different to that seen in the cervix thereby presenting a good opportunity to identify if any relationship between ER and iASPP exists, independently of p63 expression. p63 is expressed in the basal/myoepithelial layer of cells with patchy ER expression seen in the luminal cells of the normal breast glands.

Figure 5.13 identifies iASPP in the luminal cells of normal breast glands with sparing of the myoepithelial, classically p63 expressing, layer.

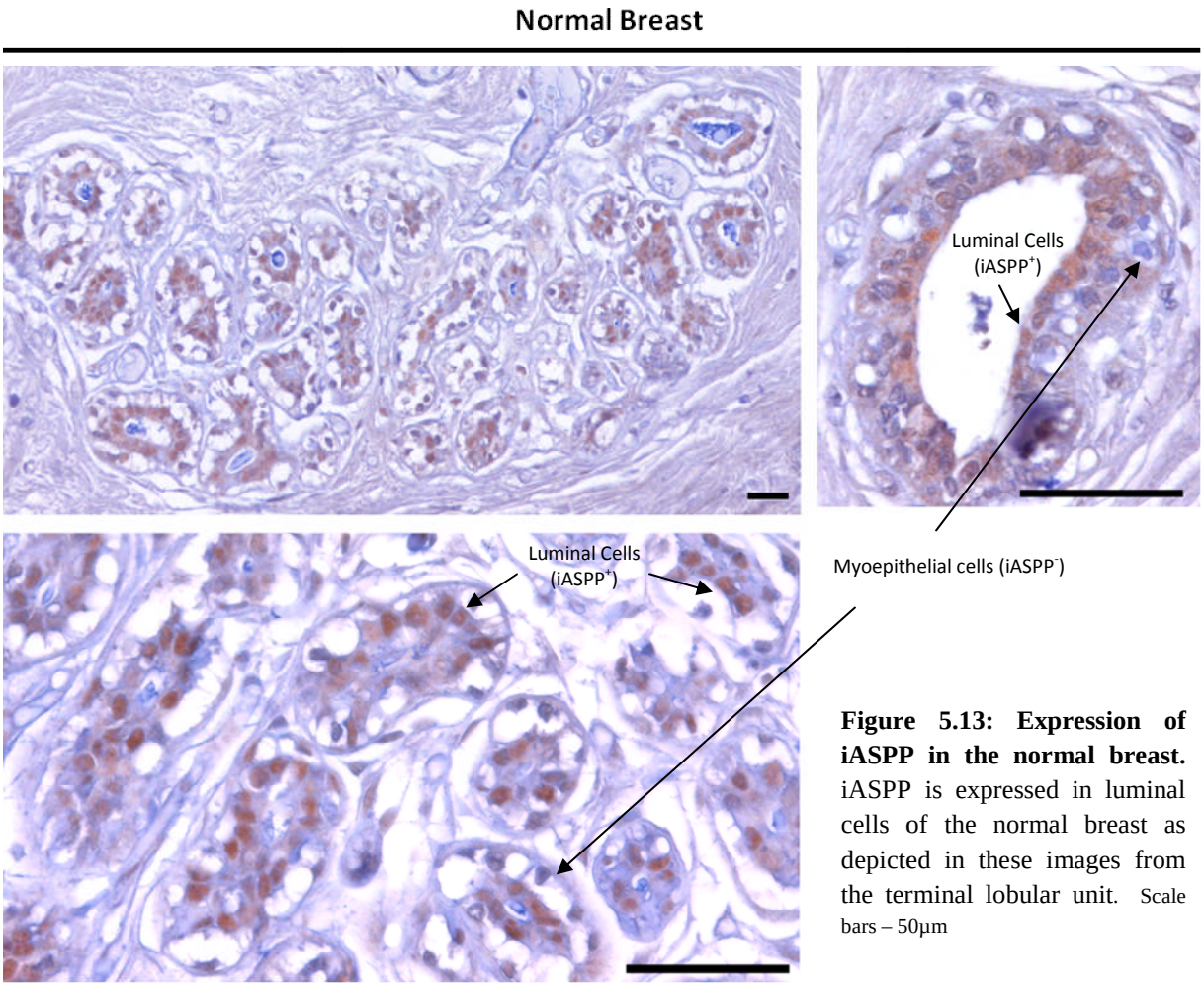


Figure 5.13: Expression of iASPP in the normal breast. iASPP is expressed in luminal cells of the normal breast as depicted in these images from the terminal lobular unit. Scale bars – 50µm

The data thus far suggests fundamental differences between the interactions of iASPP and p63 in ectocervical squamous epithelium and breast glandular epithelium. It also suggests a variation in iASPP function itself in the cervix and the breast, given that iASPP is not expressed in endocervical glandular epithelium, but rather in the subcolumnar reserve cell population in the transformation zone which also expresses p63. Conversely in the breast iASPP spares the basal, p63 expressing layers and is seen in mature glands.

Interestingly the effect of estrogen signalling in these two tissue types is also different, given that squamous metaplasia is not normally promoted in the breast. Moreover the effects of SERMS such as Tamoxifen exert different effects in these two tissue types. As described in the introduction Tamoxifen is used in the clinical management of patients with hormone responsive breast cancer due to its estrogen antagonistic effects in this tissue. Conversely studies have suggested it to have, at least, a partial estrogen agonist effect in the ectocervix. Here it has been observed that Tamoxifen treatment results in the down regulation of proliferation (estrogen antagonistic effect) and promotion of differentiation (estrogen agonist effect). Of note, these same effects have also been reported in the epidermis on down regulation of iASPP (Notari et al. 2011). The literature suggests that the tissue-specific effects of tamoxifen might be due to the presence of different ER co activators/co repressors in cells. This would seem to raise some intriguing possibilities as to the interaction between ER and iASPP, particularly given the presence of the LXXLL motif in the C-terminal of iASPP.

Given their co-localisation in the breast and ectocervix it is feasible that iASPP and ER directly interact.

5.5.3 iASPP and ER co-localise in the nucleus of MCF-7 cells

In order to investigate a potential interaction between these two proteins, their expression and potential binding was investigated using the MCF-7 breast cancer cell line. This cell line was selected due its known expression of ER, wild-type p53 and iASPP (Liu et al. 2008).

Figure 5.14B demonstrates the co localisation of iASPP and ER in the nucleus of MCF-7 cells. Furthermore this experiment suggests that iASPP is located primarily in the cytoplasm in cells which are ER negative and is actually only expressed in the nucleus of those cells which co express ER.

5.5.4 iASPP binds to ER in MCF-7 cells

In order to investigate binding between these proteins, levels of ER were maximised by growing cells in phenol red free media (MEM-PR) supplemented with 5% charcoal stripped foetal bovine serum (CS-FBS). Previous studies have shown that this increases the number of estrogen receptors and pilot studies with this cell line confirmed this to be the case in this experimental system (Figure 5.14A).

Co-immunoprecipitation (Co-IP), repeated several times, indicates strong, reciprocal binding between ER and iASPP when antibodies to both ER and iASPP were used to pull down (Figure 5.14C)

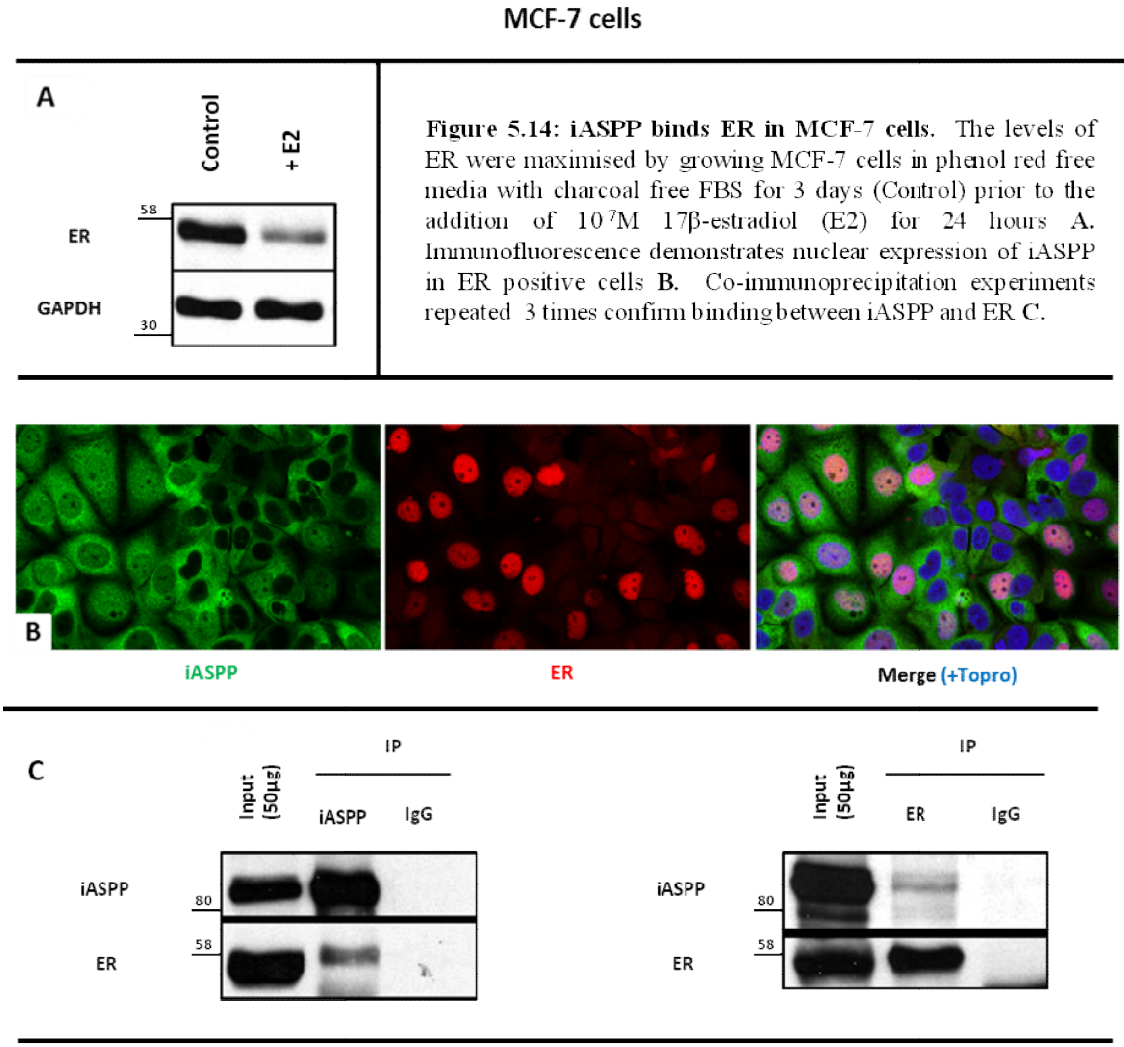


Figure 5.14: iASPP binds ER in MCF-7 cells. The levels of ER were maximised by growing MCF-7 cells in phenol red free media with charcoal free FBS for 3 days (Control) prior to the addition of 10^{-7}M 17β-estradiol (E2) for 24 hours **A.** Immunofluorescence demonstrates nuclear expression of iASPP in ER positive cells **B.** Co-immunoprecipitation experiments repeated 3 times confirm binding between iASPP and ER **C.**

5.5.5 iASPP binds specifically to ERα

Furthermore, the antibody used in the experiments outlined above has been shown to bind both ERα and ERβ. Therefore specific antibodies to ERα and ERβ were used instead to assess binding to iASPP and Figure 5.15 demonstrates that iASPP specifically binds to ERα. There is no demonstrable interaction with ERβ in these cells, though the protein itself is expressed.

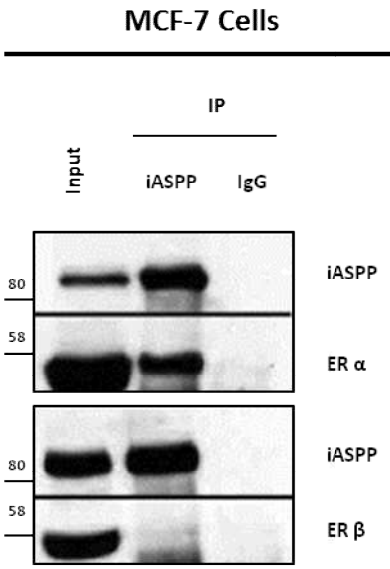


Figure 5.15: iASPP binds specifically to ERα in MCF-7 cells. Strong binding is reproduced between ERα and iASPP. No binding is seen between ERβ and iASPP, though ERβ is expressed in MCF-7 cells.

5.5.6 The C-terminus of iASPP binds to ERα

The interaction between iASPP and ERα was investigated further in MCF-7 cells which had previously been stably transfected with N- and C-termini of iASPP tagged with V5 (Slee et al. 2004). The C-terminus contains the LXXLL motif and has also been shown to be the predominantly nuclear component of iASPP. We would therefore predict that this would be the part of iASPP responsible for the interaction with ER. Figure 5.16 shows the C-terminus of iASPP (iASPP-RAI) colocalises with ER and Co-IP experiments confirm their interaction.

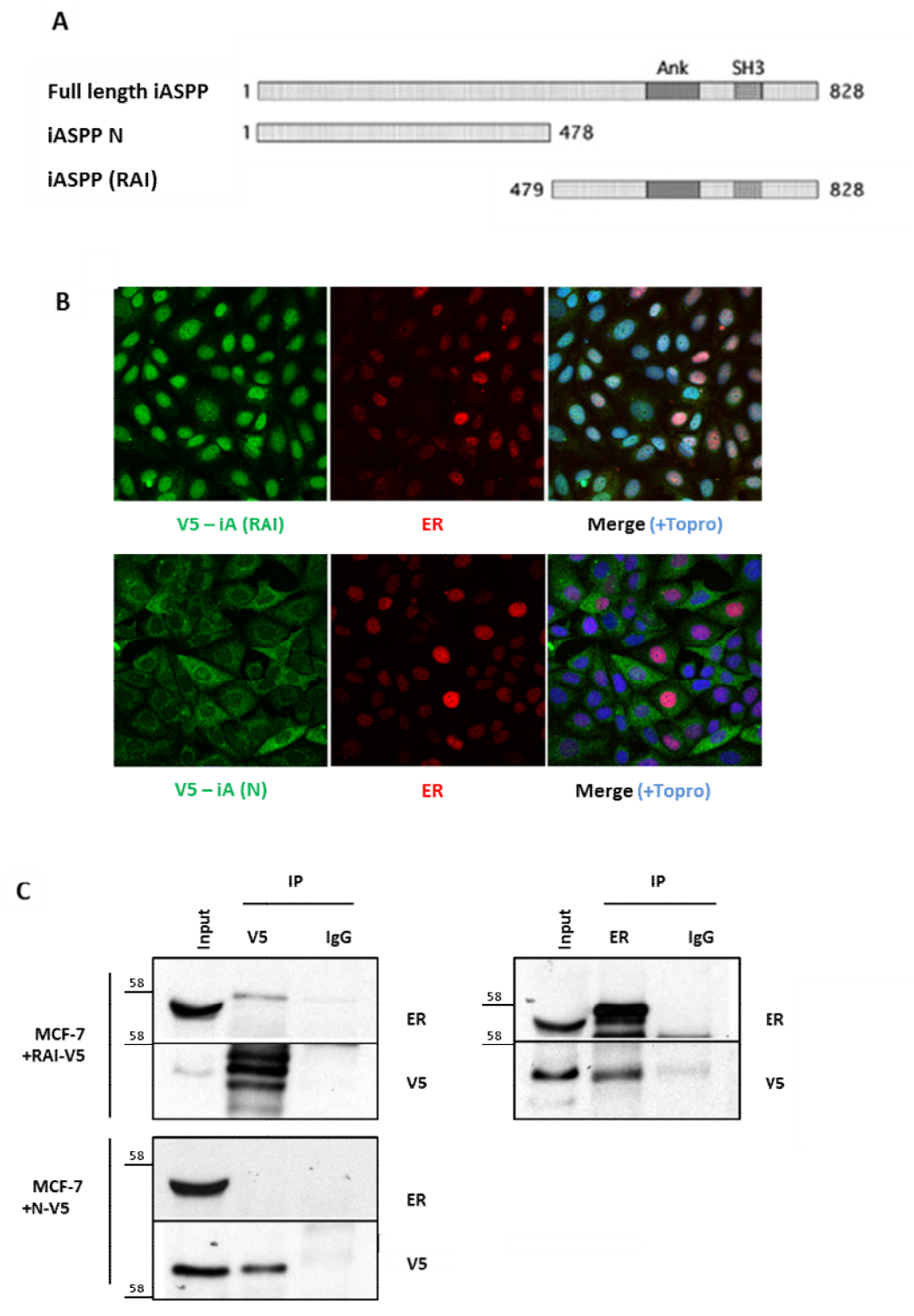


Figure 5.16: The C-terminus of iASPP binds to ER in MCF-7 cells. Cells were grown in phenol red free media with charcoal stripped FBS for 3 days prior to experiments. Immunofluorescence shows colocalisation of ER and V5-iASPP-RAI (C-terminus) in MCF-7 cells **A&B.** Co-IP experiments, using whole cell extracts, confirm their interaction with no binding seen between ER and the N-terminus of iASPP as detected using antibodies for V5 and ER (SP-1) **C.** Input = 5%
Part A modified from: Slee et al. *Oncogene* 23(56), pp9007-16 © 2004

These experiments have established that iASPP binds to ERα and this interaction is likely mediated via its C-terminus. Further experiments will be needed to dissect this binding in greater detail and to determine if mutations which disrupt it can alter cellular characteristics. This is however beyond the scope of this investigation. Instead, the potential effects of this interaction in breast cancer were investigated.

5.6 Estrogen Signalling

5.6.1. Estrogen signalling promotes iASPP nuclear recruitment

Figure 5.17 demonstrates that after short term estrogen depletion, with cells grown in MEM-PR for 3 days, the addition of 17β–estradiol (E2) promotes iASPP recruitment to ER positive nuclei.

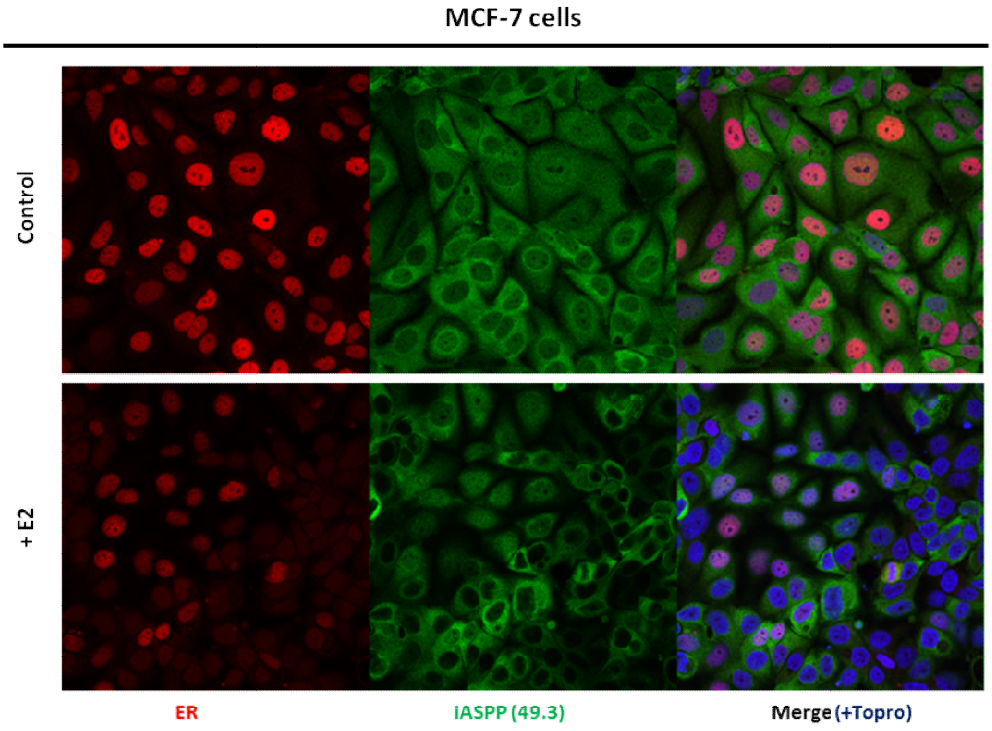


Figure 5.17: Estrogen signalling promotes iASPP nuclear recruitment. Double immunofluorescence staining demonstrates that on the addition of 10^{-7}M 17β-estradiol (E2) for 24 hours to cells previously grown in phenol red free media with charcoal stripped FBS for 3 days (Control), levels of ER are reduced and in the ER positive cells, iASPP expression is now predominantly nuclear.

iASPP directly interacts with ER and it is possible that this is interaction is preserved in breast cancer, specifically ER-positive breast cancers, which comprise 70% of tumours diagnosed. Given our knowledge of the pro-proliferative, anti-apoptotic actions of iASPP investigated to date, it would seem possible that the presence of iASPP might also promote the progression of this tumour type.

To date our understanding of iASPP's tumourigenic effects include its interaction with p63 in the development and progression of squamous neoplasia and its ability, in vitro, to interact with and to modify the apoptotic response of wild type p53. We now know that iASPP also interacts with ER in one breast cancer cell line and that it is recruited to ER positive nuclei on estrogenic stimulation. In breast carcinoma the upregulation of ER is thought to promote disease through its ability to stimulate proliferation and inhibit apoptosis.

In clinical practice the identification of p63 in breast specimens is usually indicative of in-situ, rather than invasive disease and very few breast tumours have been shown to demonstrate significant p63 expression. Interestingly these tumours have a very poor prognosis, but as a special sub-type their analysis is beyond the scope of this investigation.

In addition p53 mutations have been described in only ~20% of breast carcinomas. ER positive tumours however, appear to be resistant to wild type p53 induced apoptosis (Bailey et al). Given that iASPP has been shown to inhibit the apoptotic ability of wild type p53, it is possible that the upregulation of iASPP in ER positive tumours could contribute to their progression by enhancing the inhibition of p53 induced apoptosis.

In fact Liu *et al* (2008) have already demonstrated that in MCF-7 cells the down regulation of iASPP results in an increase in levels of apoptosis. It is possible that in the presence of nuclear iASPP, ER is able to inhibit p53 induced apoptosis to promote tumourigenesis and that this effect is reduced when iASPP is down regulated.ER recruitment of nuclear iASPP may be one mechanism in the crosstalk between p53 and ER in the regulation of apoptosis.

5.6.2 Down regulation of iASPP reduces proliferation

In squamous epithelia, iASPP is considered an important factor in the maintenance of proliferative potential. ER is also known to promote the proliferation of breast cancer cells. In order to investigate the effect of iASPP on breast cancer cell proliferation an MTT assay, using MCF-7 cells and the ER negative cell line MDA-MB-231, was undertaken.

Figure 5.18 demonstrates that down regulation of iASPP does inhibit proliferation in MCF-7 cells. However this effect was only significant in estrogen stimulated cells, that is, cells grown either in ordinary DMEM or in MEM-PR (+CS-FBS) for 3 days, then supplemented with E2 for 24 hours. In cells which remained estrogen depleted at the time of analysis, a further reduction in proliferation was seen with the down regulation of iASPP, but this did not reach significance in a student's t-test. This result suggests a level of interaction between estrogen signalling and iASPP in promoting proliferation in MCF-7 cells. Conversely MDA-MB-231 cells, which do not express ER, only demonstrated a significant reduction in proliferation on iASPP down regulation in cells which remained estrogen depleted at the time of analysis. The significance of this is unclear. However it does demonstrate the loss of the interaction, observed in MCF-7 cells, between ER signalling and iASPP in the promotion of proliferation.

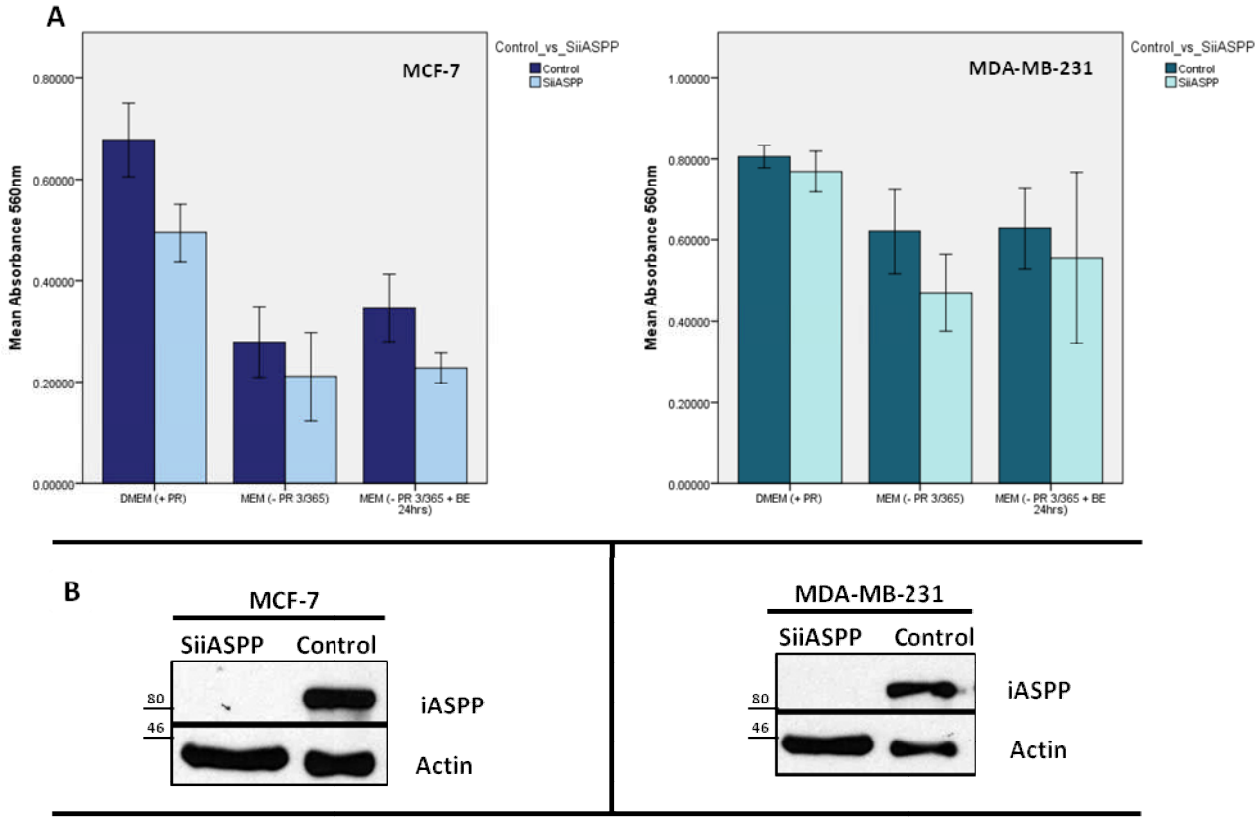


Figure 5.18: Down regulation of iASPP reduces proliferation. Down regulation of iASPP using iASPP RNAi inhibits proliferation in estrogen stimulated MCF-7 cells compared to cells treated with Control RISC-free RNAi $p<0.05$ (student's t-test). Cells were treated with iASPP or Control RISC-free RNAi for 72 hours before the addition of 17β-estradiol (10^{-7}M) for a further 24 hours. Experiments were repeated twice. The resultssuggest a level of interaction between estrogen signalling and iASPP in promoting proliferation in these cells. This interaction was lost in the ER negative cell line MDA-MB-231 **A** Immunoblots confirm the successful down regulation of iASPP in both cell lines after 96 hours treatment with iASPP RNAi **B.**
Error bars = 95% CI.

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These data suggest that iASPP may function to inhibit apoptosis and promote proliferation specifically in ER positive breast cancer cells. Together these actions would be expected to enhance the aggressiveness of these tumours.

5.6.3. Down regulation of iASPP inhibits cell motility

In order to determine if these effects facilitate tumour progression a cell motility assay was undertaken. Figure 5.19 demonstrates that loss of iASPP significantly reduces the ability of MCF-7 cells to fill the gap created in wells in the time period for analysis (30 hours). This data confirms that the inhibition of apoptosis and promotion of proliferation enhanced by iASPP is critical in maintaining the aggressive nature of these cells.

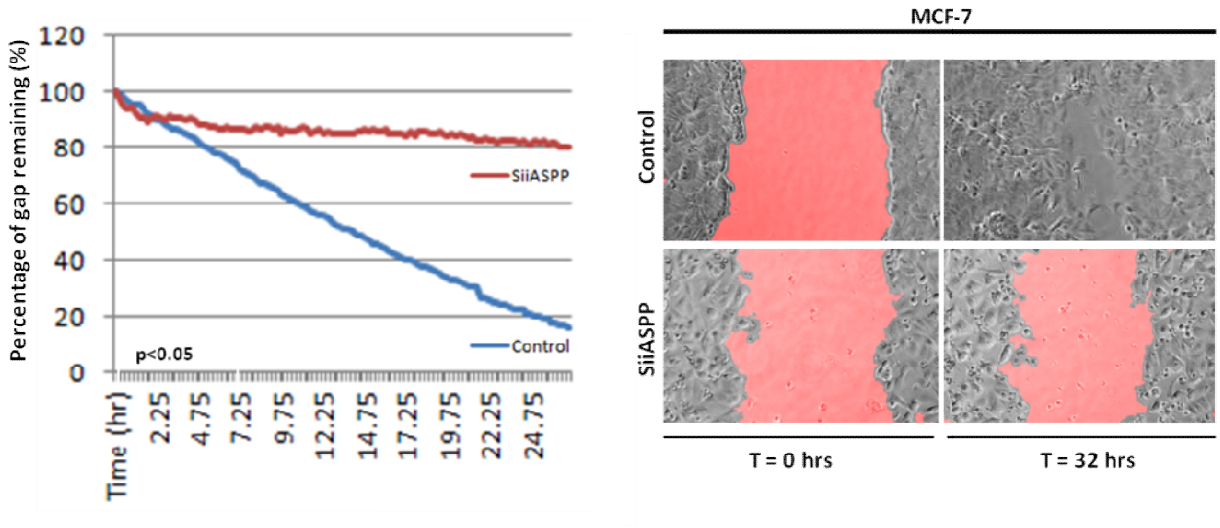


Figure 5.19: Down regulation of iASPP reduces cell motility. MCF-7 cells were grown in phenol red free media with charcoal stripped FBS for 3 days prior to treatment with iASPP RNAi or control RISC-free RNAi for 48 hours. A scratch was then made in each well and the cells observed for a further 32 hours. Cells were plated in triplicate twice for each experiment. Experiments were repeated twice. Down regulation of iASPP inhibits cell motility in these cells suggesting iASPP expression is critical in maintaining their aggressive nature, potentially both through the inhibition of apoptosis and promotion of proliferation $p<0.05$ (student's t-test).

In summary iASPP appears to be recruited to the nucleus of ER positive cells in a breast cancer cell line and is involved in the inhibition of apoptosis and promotion of proliferation which enhances the invasive nature of these cells in a cell motility assay. It could be predicted then that in human breast carcinomas we would see the retention of nuclear iASPP, possibly a correlation between the expression of iASPP and ER and a detrimental impact of nuclear iASPP expression on survival.

5.7.1 Nuclear iASPP expression is significantly higher in Grade 1 carcinomas

The Breast Cancer Tissue Microarray (TMA BR1503b) consisted of breast tissue cores comprising 14 ductal carcinoma in situ (DCIS) and 120 human invasive ductal adenocarcinoma samples. The expression of iASPP was explored using conventional immunohistochemistry. Data regarding ER and Ki67 expression was made available with the array.

Strong expression of nuclear iASPP is seen in high grade DCIS. Furthermore the overall expression of nuclear iASPP is significantly different across different grades of breast carcinoma with the highest levels seen in lower grade carcinomas which also expressed high levels of ER (Figure 5.20). Interestingly this is opposite to the role of nuclear iASPP in the most aggressive squamous cell carcinomas and suggests a strong association between nuclear iASPP and ER expression in this tumour type.

These initial results corroborate our data suggesting ER and nuclear iASPP are co expressed in breast carcinomas.

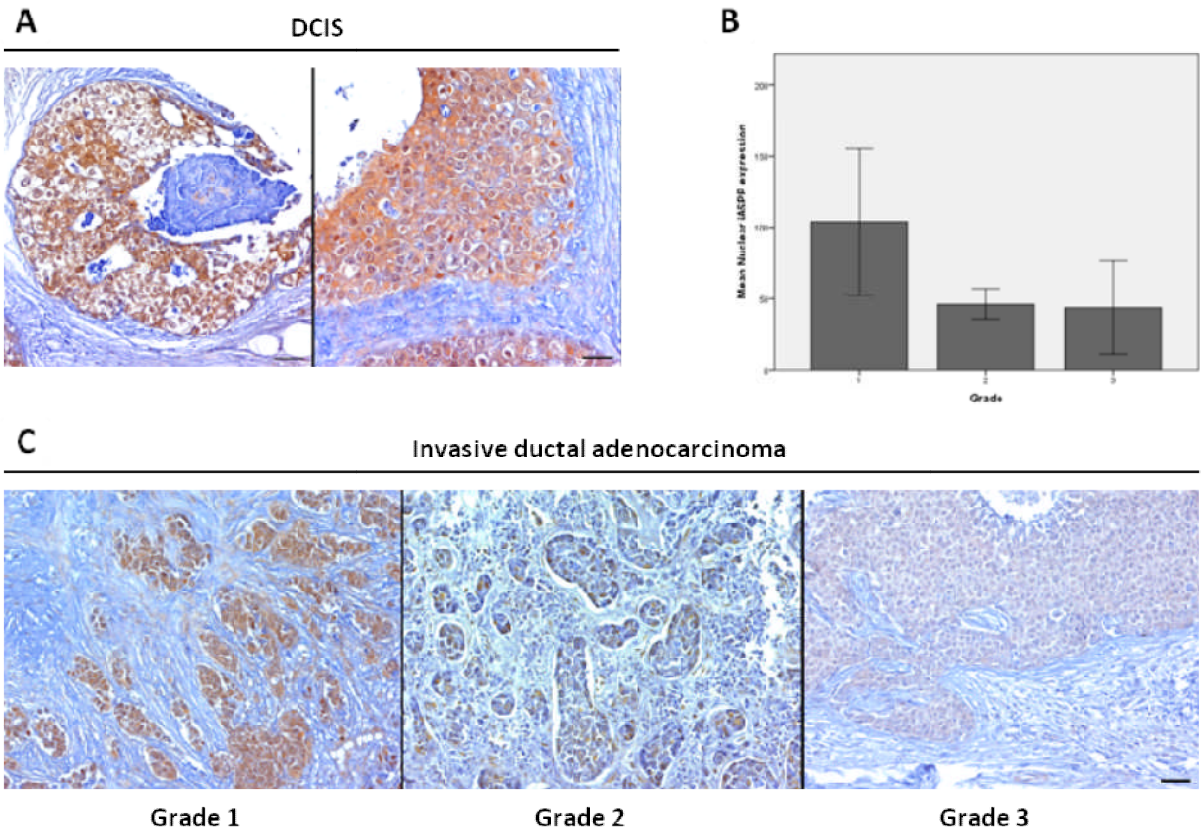
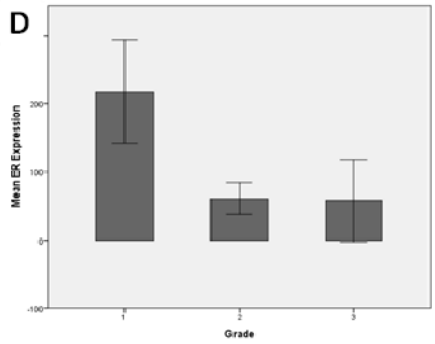


Figure 5.20: High levels of ER and nuclear iASPP are associated with low grade breast carcinomas TMA BR1503b. iASPP expression was scored as for TMA CR803. Results were analysed by the Kruskal-Wallis test (H) with Mann-Whitney tests (U) used for *post hoc* procedures and the effect size is denoted by r=z/√n*(>0.3 = medium effect, >0.5 = large effect).* A Bonferroni correction was applied so all effects are reported at a 0.0167 (0.05/3) level of significance.

Strong expression of nuclear iASPP is seen in high grade DCIS **A.** The overall expression of nuclear iASPP is significantly different across different grades of breast carcinoma ($H(2) = 6.489$, $p<0.05$) in 120 carcinoma cores **B&C.** The expression of nuclear iASPP is significantly higher in Grade 1 compared to Grade 2 $U=152.500$, $r=-0.26$, $p<0.0167$ and Grade 3 carcinomas $U=23.000$, $r=-0.47$, $p<0.0167$ (1-tailed) **B.**



Furthermore in the same cohort the overall expression of ER is significantly different across different grades of breast carcinoma ($H(2) = 16.649$, $p<0.05$) in 120 carcinoma cores. The expression of ER is also significantly higher in Grade 1 compared to Grade 2 $U=99.000$, $r=-0.40$, $p<0.0167$ and Grade 3 carcinomas $U=17.000$, $r=-0.62$, $p<0.0167$ **D.**
Error bars = 95% CI

5.7.2 There is a significant association between nuclear iASPP and ER expression

To quantify the relationship between nuclear iASPP and ER in these carcinomas further analysis of their expression was undertaken in the invasive cores. A significant association between their expression was seen with significantly more nuclear iASPP identified in ER positive tumours (Figure 5.21).

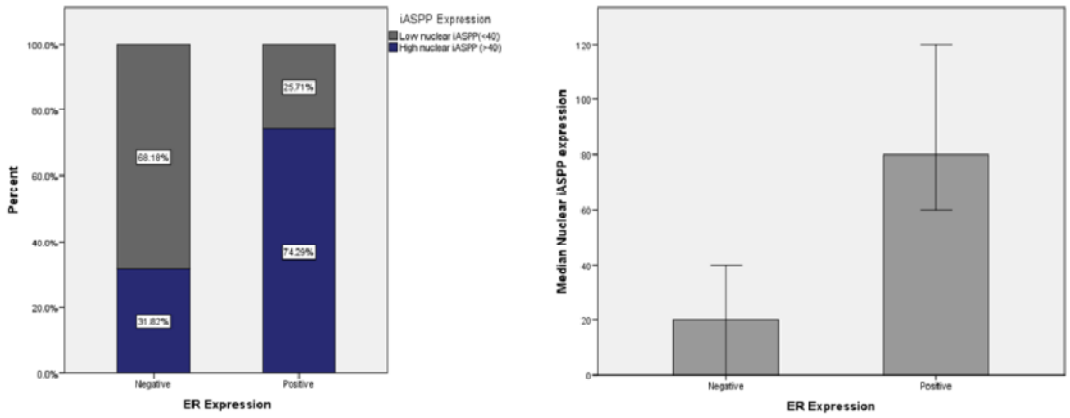


Figure 5.21: There is a significant association between nuclear iASPP and ER expression. In fact a significant association was seen between nuclear iASPP and ER expression $\chi^2(1) = 16.579$, $p<0.001$. This reflects the fact that, based on the odds ratio, the odds of the tumour being ER positive are 6.2 times higher if they express nuclear iASPP than if they do not. A medium effect can therefore be documented for this association (Cramer's $V = 0.405$,$p<0.001$). Levels of nuclear iASPP were significantly increased in ER positive carcinomas $U=504.500$, $r=-0.47$, $p<0.001$. Error bars = 95% CI

5.7.3 There is a significant association between nuclear iASPP and Ki67 expression

To determine if there is an association between the Ki67 index and the level of nuclear iASPP expression, the levels of these two proteins were analysed (Figure 5.22)

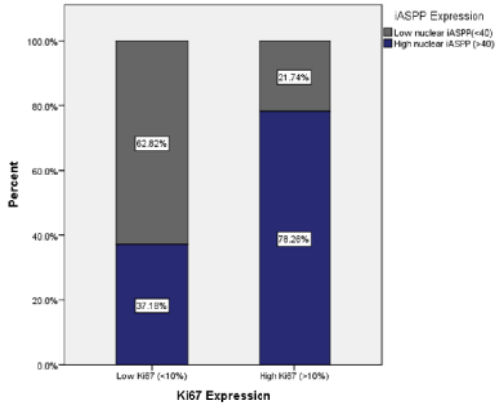


Figure 5.22: There is a significant association between nuclear iASPP and Ki67 expression. In fact a significant association was seen between nuclear iASPP and higher Ki67 expression $\chi^2(1) = 12.049$, $p<0.01$. This reflects the fact that, based on the odds ratio, the odds of the tumour having a higher Ki67 are 6.1 times higher if they express nuclear iASPP than if they do not. A medium effect can therefore be documented for this association (Cramer's $V = 0.345$,$p<0.01$).

5.7.4 Nuclear iASPP influences survival in Breast Cancer

A second Breast Cancer Tissue Microarray ZTMA 26, with comprehensive clinical follow up, was used in this part of the investigation, specifically to assess the effect of nuclear iASPP on patient survival. This TMA was originally obtained by Dr Florian Fritzsche from the University of Zurich and was stained and scored by him during his time in the laboratory. The subsequent detailed analysis of the results was undertaken as part of this project and the results are discussed here.

Firstly the selection of tumours was confirmed to be a random sample with characteristics reflecting the usual clinical situation. A significant survival advantage was seen in tumours with a lower pT score and in tumours which showed no evidence of nodal spread (pN0), as would be expected (Figure 5.23).

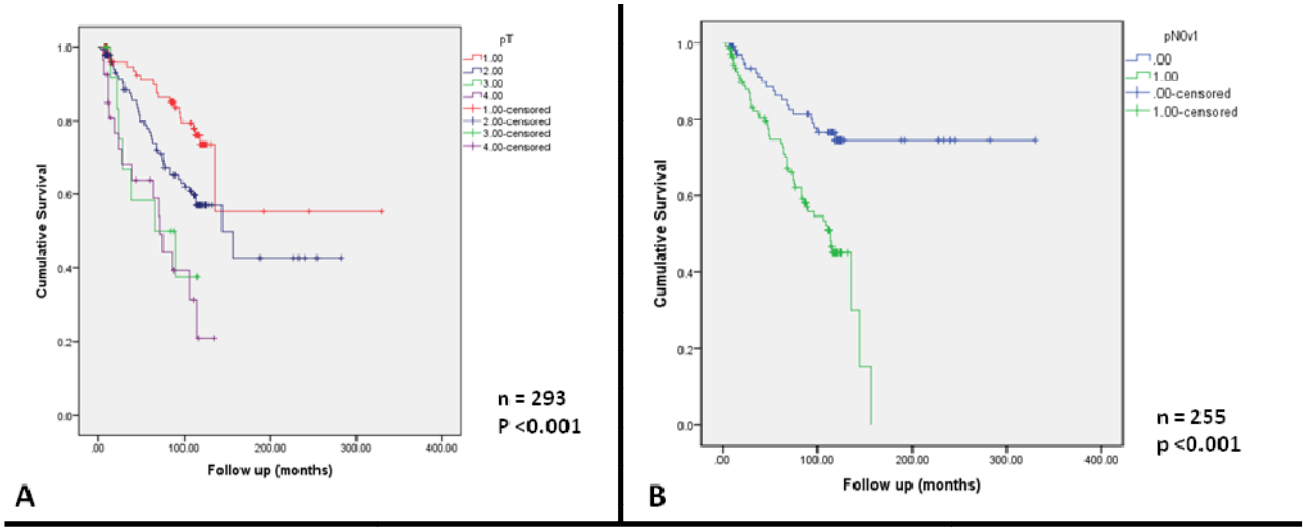


Figure 5.23: Breast cancer staging influences breast cancer survival in this cohort. As would be expected a significant survival advantage is associated with lower pT **A** and pN **B** scores $p<0.001$.

Furthermore the histopathological diagnosis of breast tumours is routinely accompanied by the analysis of hormone receptors, namely estrogen receptor (ER) and progesterone receptor (PR). The expression of the hormone receptors ER and PR is associated with a better prognosis and in this study their expression confirmed this significant survival advantage(Figure 5.24). ER positive tumours comprised 84% of the tumours which is similar to the proportion of tumours known to express ER (70%) on initial diagnosis.

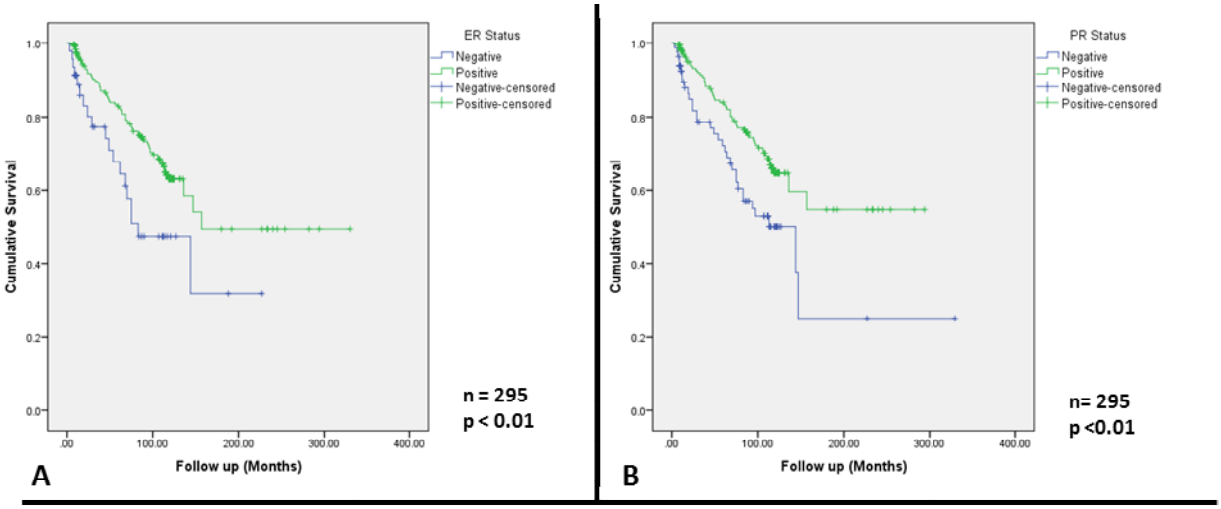


Figure 5.24: Hormonal status influences breast cancer survival in this cohort. As would be expected a significant survival advantage is associated with ER **A** and PR **B** positivity p<0.01.

Analysis of iASPP expression again demonstrates a significant association between the expression of ER and nuclear iASPP in this tumour cohort with 71% of ER positive tumours demonstrating positivity for nuclear iASPP (Figure 5.25A). The association here is not as powerful as in TMA BR1503b and this may reflect differences in the analysis of nuclear iASPP expression.

A straightforward analysis of the effect of nuclear iASPP expression showed no significant survival disadvantage (Figure 5.25B). The tumours were then separated by ER status and again, the presence of nuclear iASPP did not alter survival significantly in either ER positive or ER negative carcinomas (Figure 5.25C).

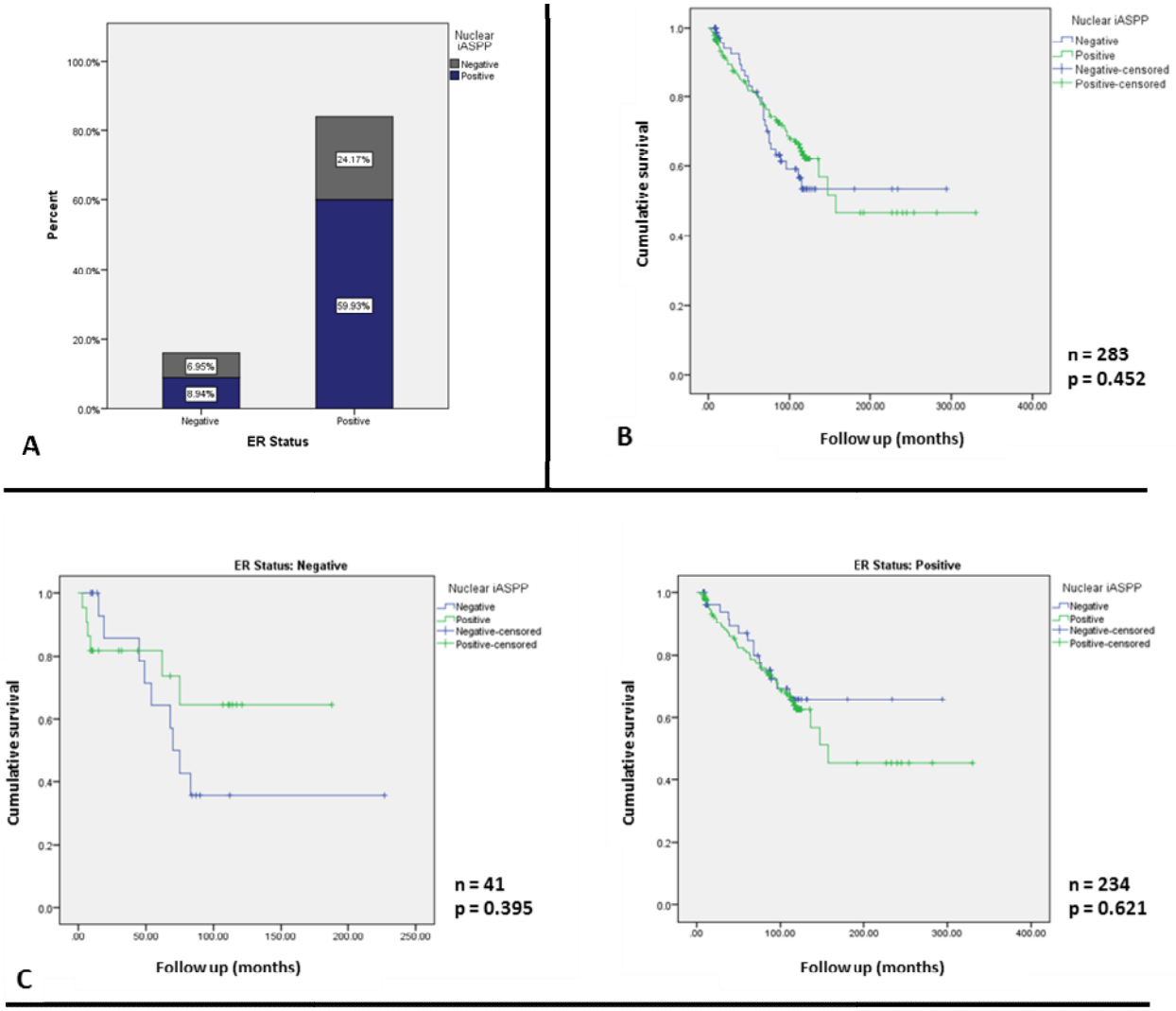


Figure 5.25: There is significant association between ER and iASPP expression in this cohort. A significant association was seen between nuclear iASPP and ER expression $\chi^2(1) = 4.342$, $p<0.05$ with 59.9% of tumours expressing both ER and nuclear iASPP. Based on the odds ratio, the odds of the tumour being ER positive are 1.9 times higher if they express nuclear iASPP than if they do not. A small effect can therefore be documented for this association (Cramer's V = 0.119,$p<0.05$) **A.** The expression of iASPP does not affect survival in the initial analysis **B&C** $p>0.05$.

However, when the tumours were separated by iASPP status, the survival advantage obtained with ER expression was only retained in the small number of tumours showing no nuclear expression of iASPP (Figure 5.26).

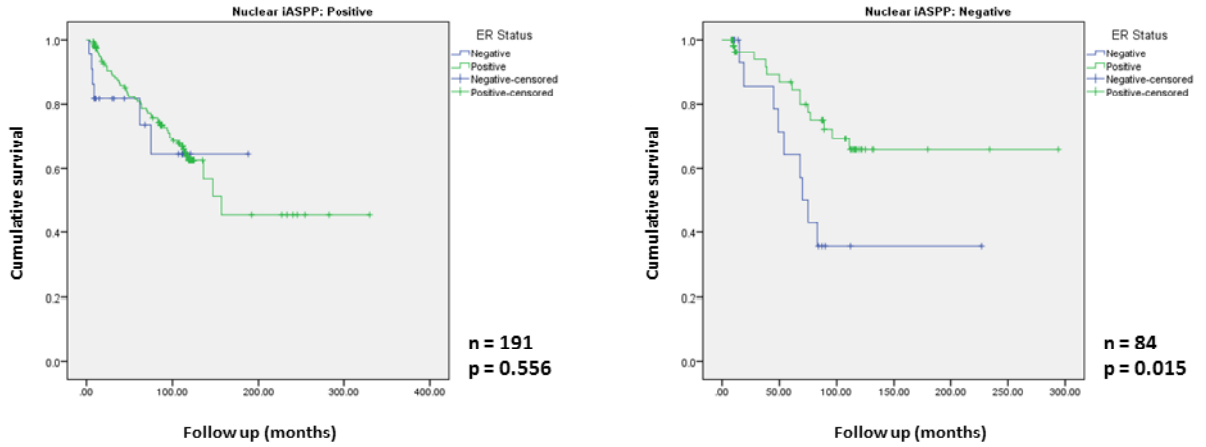


Figure 5.26: The survival advantage obtained with ER expression is only retained in iASPP negative tumours. In tumours expressing nuclear iASPP, the survival advantage gained by ER expression lost. Furthermore this represents a much larger cohort of patients than those not expressing nuclear iASPP.

Importantly these comprised only 29% of ER positive tumours. In the remaining 71% of ER positive tumours, their ER status was no longer seen to confer a survival advantage. It would appear that in this sample of tumours, the survival advantage conferred by ER positivity is only applicable in the small number of tumours which do not express nuclear iASPP. Moreover this effect is so pronounced that even though it is seen in less than a third of the ER positive tumours it is enough to skew the survival statistics for the whole group.

If this effect were to be translated into the general population it could represent a large number of patients with ER positive breast tumours, who are considered to have a better prognosis, when in fact this effect has been nullified by the fact that their tumours also express nuclear iASPP. It will be extremely important to verify this seemingly detrimental impact of nuclear iASPP on survival further and to establish the precise reasons for it as this could represent an exciting new target for therapeutic intervention.

The data presented in this part of the investigation demonstrate an interaction between iASPP and ERα in one breast cancer cell line and further evidence to suggest their strong association in human breast carcinomas. In addition down regulation of iASPP *in vitro* modulates the effects of normal estrogen stimulation, possibly identifying iASPP as a novel player in estrogen signalling. Moreover the presence of nuclear iASPP in ER positive breast tumours could indicate a significantly poorer prognosis for these patients and when verified, clinically could be used to either stratify ER positive tumours requiring more aggressive treatment or to predict tumours more likely to relapse. Further work should analyse the effect nuclear iASPP on the response to hormonal therapies. If iASPP is, as predicted, a novel coregulator of ER, it is possible that its presence influences the effectiveness of SERMS in breast cancer treatment. The role of nuclear iASPP in breast carcinoma is worthy of more intensive investigation.

Chapter 6: Discussion

ASPP2 and iASPP were initially implicated in tumourigenesis through their interaction and modulation of p53 mediated pro-apoptotic transcription. Subsequent studies have revealed the increasingly complex role these two members of the ASPP family play in normal cell signalling and several alternative ways they potentially influence tumourigenesis. The aim of this study was to identify if recent *in vitro* work in our laboratory, specifically implicating iASPP and ASPP2 in the maintenance of normal squamous epithelia could be translated to human tissues. Moreover the suggestion that the deregulation of ASPP2 and iASPP is associated with the development of squamous cell carcinomas was also investigated in human tumours, specifically to determine their potential role in prognostication and as potential novel therapeutic targets.

As a putative tumour suppressor, ASPP2 has been shown, in our laboratory, to directly influence p63 mediated transcription and to indirectly repress p63, suggesting a role inducing squamous epithelial maturation. This was reflected in a mouse model of tumourigenesis in which the germline deletion of ASPP2 on a Balb/c background resulted in the development of squamous cell carcinomas. In Chapter 3 the predominant expression of cytoplasmic ASPP2 in the superficial, differentiated layers of normal human squamous epithelia was confirmed in the ectocervix and oropharynx. Moreover ASPP2 expression was seen to be lost early in the process of cervical neoplastic transformation and to persist in poorly differentiated areas of cervical squamous cell carcinomas. These findings were confirmed in the oropharynx where the additional identification of ASPP2 loss, specifically at the invasive tumour edges, suggested a role in progression and metastasis. This was borne out when tumour samples from established lymph node metastases were probed for ASPP2 expression.

One common feature of squamous cell carcinogenesis in the two regions examined thus far is the association with prior infection with high risk HPV. The identification of nuclear ASPP2 in known HPV negative only samples from the head and neck implicated it as a potential target of HPV down regulation. Conversely the possibility that nuclear ASPP2 expression could be a direct consequence of other tumour initiating events in HPV negative tumours, such as smoking and alcohol exposure could not be ruled out. However given that ASPP2 is known to translocate to the nucleus, after phosphorylation induced by oncogenic RAS, to facilitate its interaction with p53 and presumably p63, it is likely that an element of nuclear ASPP2 expression can be expected in normal tissues. Furthermore the experiments detailed here identify nuclear ASPP2 in the reserve cell population of the cervical transformation zone caudally located to areas of CIN. It can thus be postulated that HPV infection targets nuclear ASPP2 initially for degradation, followed by cytoplasmic ASPP2 in established, persistent infections. The consequences of this would include the prevention of p63 induced pro-differentiation transcription, apoptosis and the normal repression of p63 expression in more differentiated layers resulting in the morphological appearances of dysplasia.

The mechanisms involved in this targeted down regulation will need to be elucidated. The possibility of its proteasomal degradation should be explored further as this is a well-recognised way in which HPV targets tumour suppressors such as p53 and moreover ASPP2 is known to be susceptible to this method of regulation (Zhu et al. 2005).

In addition to its potential targeting by HPV, alternative methods of ASPP2 regulation have been described. In lingual squamous cell carcinomas, the sustained down regulation of ASPP2 at the invasive tumour front is associated with p63 expression and lower levels of apoptosis than in the centre of the tumour. The nature of the invasive front of human HNSCCs is commented upon in routine histological reporting. This is because the presence of an infiltrative margin as opposed to a more cohesive pattern of growth has been associated with a higher risk of tumour recurrence. Furthermore an infiltrative margin might indicate the presence of hypoxia triggering the emergence of more aggressive clones (Eustace et al. 2013; Harris 2002). The lingual tumours examined in this study all exhibited an infiltrative, discohesive invasive front.

Interestingly the presence of hypoxia has been associated with the down regulation of ASPP2 in two separate *in vitro* studies. In the first ASPP2 degradation was shown to be promoted by the ubiquitin ligase Siah2. An increase in Siah2 levels were seen in hypoxic conditions, triggering ASPP2 down regulation and the consequent weakening of epithelial tight junctions through disruption of its binding to Par-3 (Kim et al. 2013). ASPP2 can also be hydroxylated at N986 in an FIH-1 dependent manner. Though this was not seen to impact the levels of ASPP2 present, it was shown to facilitate its interaction with Par-3. In hypoxic conditions FIH-1 is inhibited, predicting a weakening of this interaction and corroborating the theory that hypoxia results in more aggressive clones, at least in part through modulation of ASPP2 function (Janke et al. 2013). However in this investigation the presence of vascular networks or hypoxia or was not established in the lingual tumours examined and this would perhaps be useful in future studies.

The novel mouse model described in Chapter 4 provides further corroborative evidence for ASPP2's tumour suppressive role with its somatic deletion resulting in the development of a significant number of lung tumours. A key observation in the normal lungs was the expression of ASPP2 in the terminally differentiated ciliated cells, reflecting ASPP2s importance in the maintenance of a differentiated cellular phenotype. Furthermore the diffuse up regulation of CK14 expression throughout the distal lung fields, after ASPP2 deletion, demonstrates ASPP2s role in suppressing the expression of this cellular marker, which usually characterises a multipotential progenitor cell population, identified in small groups in the mouse trachea. It is not known whether this reflects a huge increase in the number of "basal cells", which characteristically express CK14, or if it represents the acquired expression of this cytokeratin in other cell types. Interestingly the morphological appearances of the concurrent tumours reflect those seen in a previous mouse model where CK14 was deliberately over expressed in the distal lung (Dakir et al. 2008). These results suggest that loss of ASPP2 results in up regulation of CK14 and the subsequent development of NSCLCs which exhibit multidirectional differentiation, reflecting CK14's origin in multipotential progenitor cells.

There is however significant room for improvement in this study. Due to unforeseen circumstances the full range of age groups in the planned experiment (6,12,15 and 18months) could not be included. Furthermore a significant delay between the presentation of tumours and their subsequent analysis is likely to have had a detrimental impact on the quality of the tissue examined and the ease with which antigens could therefore be accurately illustrated. Prolonged fixation in formalin prior to histological examination can provoke artefactual results, in particular decreasing the immunoreactivity of antigens (Wester & Wahlund 2000). Therefore the negative results reported in this chapter, specifically pertaining to the lack of p63 expression compared to CK14

expression, will need to be verified before firm conclusions can be drawn. In addition due to these technical difficulties, proportionally small numbers of tumours were available for assessment. Moreover in future studies the analysis of tracheal and proximal airway epithelium should be undertaken in more detail as this represents the normal location of CK14 expressing basal cells in the murine lung.

However the results have been reported here as they represent a potentially fascinating facet of ASPP2 function. It appears that ASPP2 expression in the lung is essential for directing the proper differentiation of cells after basal cell hyperplasia, seen characteristically following proximal injury and implicated as an early step in the development of squamous cell carcinomas. Interestingly this is a function which has in the past been attributed to intact Notch signalling and in our laboratory ASPP2's cooperation with Notch in squamous epithelial maintenance has been demonstrated (Luca Tordella - unpublished results). These results certainly indicate that ASPP2's role in lung tumourigenesis is worthy of further investigation.

As a putative oncoprotein, iASPP has been shown, in the laboratory, to directly repress p63 mediated transcription, promoting epithelial proliferative capacity and maintaining normal epithelial stratification.

In Chapter 5 it was possible to confirm the expression of predominantly nuclear iASPP in the basal and parabasal layers of normal human squamous epithelium as exemplified in the ectocervix and oropharynx. Moreover nuclear iASPP was seen in early areas of CIN and persisting in poorly differentiated areas of invasive carcinomas from this region. These findings were confirmed in the oropharynx where the additional identification of nuclear iASPP at invasive tumour margins suggested a role in invasion and metastasis. This was borne out when tumours samples from established lymph node metastases were probed for iASPP expression.

During the course of this investigation, Cao *et al* confirmed the correlation of nuclear iASPP over expression in cervical squamous cell carcinomas with their poor prognosis (Cao et al., 2013). Furthermore Liu *et al* published evidence of the up regulation of both cytoplasmic and nuclear iASPP in HNSCCs. In addition this study showed a significant correlation between iASPP expression and a poorer prognosis in the 109 laryngeal and hypopharyngeal carcinomas studied (Liu et al. 2012). The work described in Chapter 5 complements these results by implicating nuclear iASPP in the progression of oral carcinomas as well, thereby confirming its oncogenic role in HNSCCs.

One of the most striking aspects of this investigation is how the write-up of Chapters 3 and 5 reflects the diametrically opposing roles of ASPP2 and iASPP in normal squamous epithelial maintenance and thus also in the development of dysplastic and frankly invasive squamous lesions. It can, in fact, be postulated that the relative contributions of these two proteins in any given nucleus determines the consequent effect of p63-mediated transcription. Their presumed co-expression in the reserve cell population of the cervical transformation zone would appear to corroborate this. Persistent, nuclear ASPP2 in HPV negative parabasal layers could be expected to facilitate the exquisite, at least partially p63-mediated, regulation of normal proliferation and differentiation occurring here. Furthermore as cells commit to terminal differentiation, a key molecular event would appear to be the translocation of ASPP2 into the cytoplasm where it functions to indirectly repress p63 expression in the nucleus.

Further investigation into the regulation of these ASPP proteins in normal epithelial homeostasis could provide important information as to how their balance is altered in the promotion of tumourigenesis. The down regulation of ASPP2, perhaps, as suggested, by HPV, would appear to be an excellent way in which nuclei could not only be induced to overexpress p63, but where p63 would then be exposed to unopposed iASPP modulation. Furthermore, the overexpression of nuclear iASPP, by as yet undetermined mechanisms, in combination with the HPV induced down regulation of ASPP2, could promote persistent HPV associated dysplastic transformation. Potentially the absence of nuclear iASPP overexpression could facilitate the clearance of high risk HPV infection in the 98% of women who do not go on to develop neoplastic disease as a consequence. It would also be important in the future to determine if these proteins have direct or indirect effects on each other's regulation as this would add yet another level of complexity.

This interaction could also perhaps explain the controversy surrounding the role of p63 in carcinoma progression. As seen in Chapters 3 and 5, the emergence of different clones in tumour progression, results in spatial variation in differentiation and also in the relative expression of ASPP2 and iASPP. It is perhaps unsurprising that the aggressive, invasive edges of the lingual tumours examined show a reduction in ASPP2 expression in combination with nuclear iASPP, p63 and reduced apoptosis. Perhaps this could be extrapolated to suggest that the relative amounts of iASPP and ASPP2 in a particular tumour will, at least in part, determine whether the effect of p63 expression is to promote or prevent tumour progression. For example, in a tumour with ASPP2 expression, but inactivation of iASPP, p63 could be expected to promote the emergence of well differentiated areas showing less proliferation in a tumour with a better prognosis than one in which there is over expression of nuclear iASPP.

Again, further investigation into the mechanisms controlling the expression of ASPP proteins will be key to understanding what other factors promote tumourigenesis.

Interestingly since this investigation began, further work in the laboratory has identified one mechanism by which nuclear iASPP is regulated. In malignant melanoma cells the phosphorylation of iASPP, by cyclin B1/CDK1, results in its translocation into the nucleus with consequent increased inhibition of wild type p53. In fact, alongside the investigations detailed here, I was able to demonstrate the translation of these *in vitro* results to human melanoma samples. In a TMA containing 142 primary and metastatic melanoma cores, nuclear iASPP was enriched in metastatic deposits. Moreover increased nuclear iASPP expression was significantly associated with a poorer survival (Figure 6.1A-C). Furthermore strong cyclin B1 staining was significantly associated with high nuclear iASPP expression (Fig 6.2 C&D) with weak cyclin B1 and low nuclear iASPP expression observed in ~ 81% of high primary melanomas (26/32) and ~ 83% of lymph node metastases expressing strong cyclin B1 nuclear iASPP expression (5/6). A larger cohort of patients with full clinical follow up will be needed to confirm the trend seen between high cyclin B1 expression and poor survival to determine if overall those tumours expressing strong cyclin B1 and nuclear iASPP are associated with the worst survival outcome, as predicted here (Lu et al. 2013)

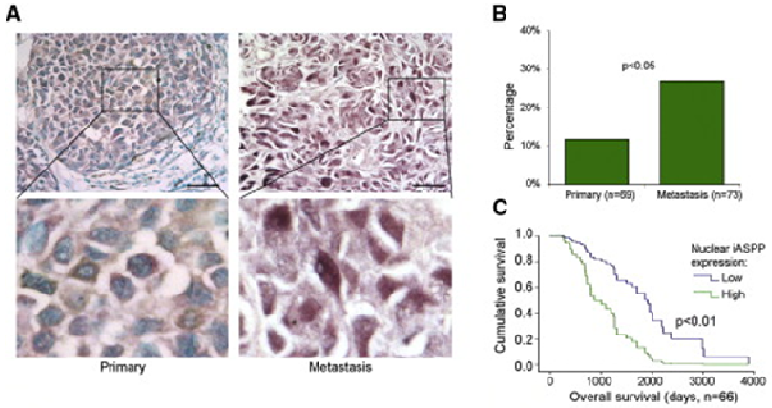


Figure 6.1: Taken from Figure 1 (Lu et al. 2013)**.** Example of iASPP staining pattern **A** and the percentage of samples expressing high nuclear iASPP **B** in 142 human primary and metastatic melanoma tissue array cores. Scale bar -50 μm. $p<0.05$. Survival curves of patients with low or high nuclear iASPP expression in 66/142 samples which had full clinical follow up information $p<0.01$.
Modified from: Lu et al *Cancer Cell*, 23(5), pp618-33, © 2013

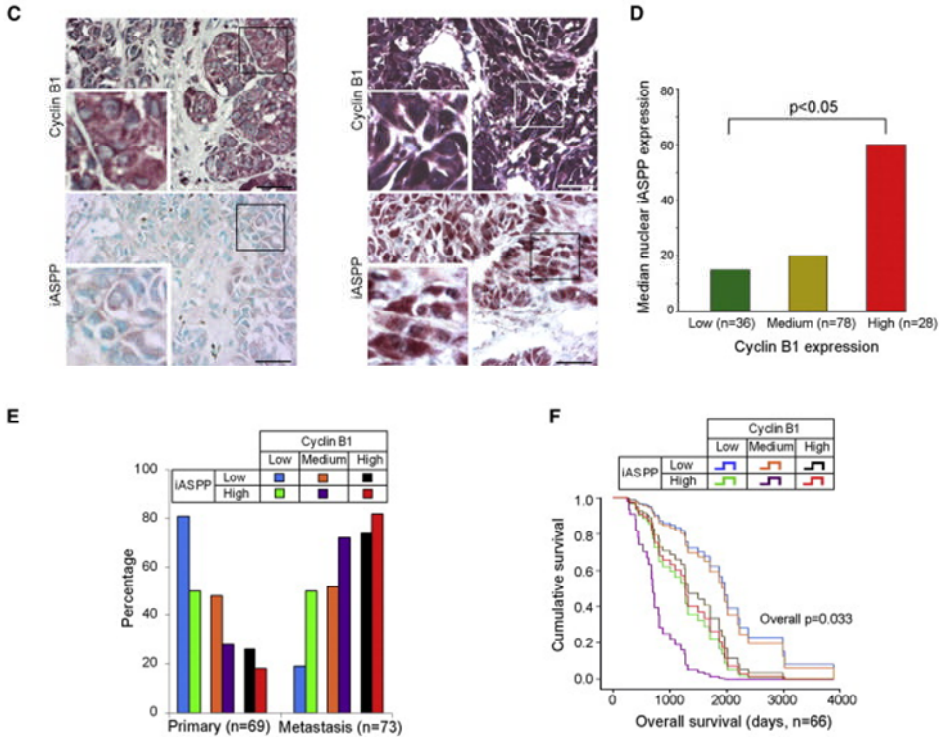


Figure 6.2: Taken from Figure 6 (Lu et al. 2013)**.** Representative iASPP and cyclin B1 staining **C.** Scale bar - 50 μm. The median nuclear iASPP expression levels in samples that expressing low, medium, or high cyclin B1 ($U = 340.500$, $r = -0.28$, $p < 0.05$) **D**. Percentage of samples expressing various cyclin B1 levels and nuclear iASPP levels from lymph node metastases and primary tumors, respectively **E**. Survival curve of patients expressing various cyclin B1 levels and nuclear iASPP levels **F**.
Modified from: Lu et al *Cancer Cell,* 23(5), pp618-33, © 2013

Importantly this study as a whole demonstrates one further way in which iASPP regulation can occur in a specific cellular context. It will be interesting to follow further developments in iASPP regulation as they pertain to squamous epithelium or indeed to breast carcinoma development.

In fact in Chapter 5 iASPP is also shown to interact, via its C-terminus, with ERα and its presence is seen to promote cell survival, implicating iASPP as a novel player in estrogen signalling. Furthermore the expression of nuclear iASPP has a negative impact on the survival of patients with ER positive breast carcinomas. As such, its identification could have a significant impact on the clinical management of patients, 70% of whom present with this tumour type. The expression of nuclear iASPP could represent one way in which these patients can be further subdivided to facilitate prognostic predictions and even to guide their medical management. This possibly is one of the most exciting outcomes of this investigation.

Further work will obviously include dissecting the binding between these proteins more specifically, determining if mutations affect the binding and identifying mechanisms which promote and disrupt the interaction. In addition, determining the molecular pathways influenced by this binding are all things which are likely to impact on the feasibility of targeting nuclear iASPP in breast carcinoma treatment.

The relationship between iASPP and ASPP2 modulation is fascinating and it raises interesting ideas as to the possibility of an interaction between ASPP2 and ERα, given the conservation of C-termini between ASPP family members. It could be postulated that in the ectocervix, in those cells definitely co-expressing iASPP and ERα and possibly ASPP2, proliferation is promoted, whereas in those more differentiated cells which still express ERα and ASPP2 and have lost iASPP expression pro-differentiation signals predominate. Furthermore it is possible to postulate a role for ASPP2 in breast carcinoma, especially given that one of the first indications of ASPP2's tumour suppressive function in humans was the identification of its down regulation in a panel of breast carcinomas (Samuels-Lev et al. 2001). In fact in order to explore this, ZTMA 26 was probed for ASPP2 expression and figure 6.3 demonstrates that there is a significant association

between ASPP2 expression and overall survival in breast cancer with tumours known to have spread to lymph nodes exhibiting less ASPP2 than their counterparts. However on analysis with ER expression no significant associations were identified and if an interaction between the two proteins was to be identified in the laboratory this would need to be explored further in a larger sample.

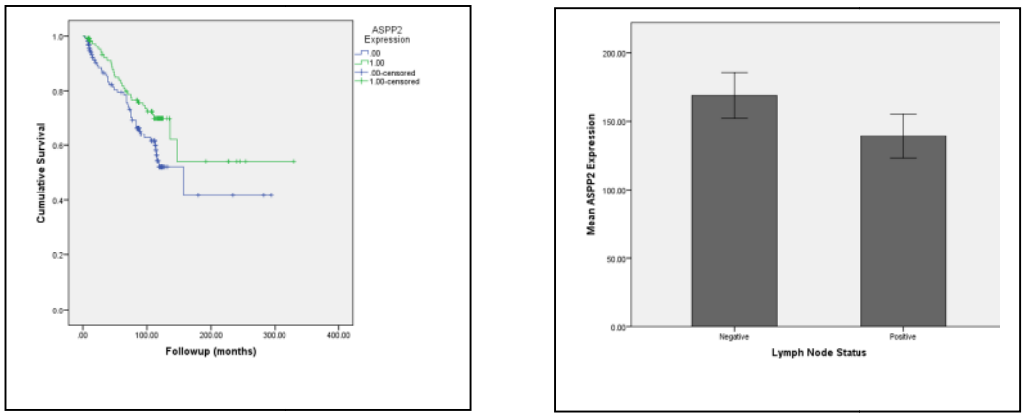


Figure 6.3: ASPP2 influences survival in breast cancer. Loss of ASPP2 expression is associated with poorer survival in this cohort $p<0.05$, n=275. Furthermore the expression of ASPP2 in tumours known to have metastasised is significantly lower than in those which have not. ($U = 6397.000$, $r=-0.15$, $p<0.05$) Negative (n=16), positive (n=134).

In addition, the development of several alternative methods to conventional immunohistochemistry was undertaken throughout his project. The spatial association of proteins within a tissue type is an important first step in determining any probable relationship between them. In Chapters 4 - 6 the use of double immunohistochemical and double immunohistochemical and immunoflourescence staining procedures are demonstrated together with the use of linear and spatial unmixing techniques to enable the construction of composite images for subsequent analysis. The establishment of these techniques within the laboratory will continue to ensure the accurate assessment of ASPP2 and iASPP protein localisation in paraffin embedded human tissue samples.

In conclusion, together the data presented here provides significant corroborative evidence implicating ASPP2 and iASPP in tumourigenesis. Specifically ASPP2 is shown to promote cellular differentiation and inhibit the expansion of proliferative populations whereas nuclear iASPP promotes the survival of potentially neoplastic clones. It is likely that the balance of these proteins is a key factor in influencing individual cell fate decisions and their specific regulation would appear to be an important determinant of cellular outcome.

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