A ROLE FOR RUNX3 IN INFLAMMATION-INDUCED EXPRESSION OF IL23A IN GASTRIC EPITHELIAL CELLS

HOR YIT TENG

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Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in this thesis.

This thesis has also not been submitted for any degree in any university previously.

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HOR YIT TENG

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Abstract

In a previous expression microarray study, *IL23A* was identified as a putative target gene of RUNX3, a transcription factor and a prominent gastric tumour suppressor. *IL23A* encodes for the unique subunit of IL-23, a heterodimeric cytokine necessary for pathogen surveillance in the gut. Using reporter gene and promoter occupancy assays, together with ectopic expression and RNAi knockdown studies, *IL23A* was demonstrated to be a genuine target gene of RUNX3 in gastric epithelial cells. Furthermore, RUNX3 is a critical requirement for the synergistic induction of IL23A by TNF-α and *Helicobacter pylori* – two key inflammatory signals strongly linked to human gastric carcinogenesis. In the presence of these stimuli, IL23A is robustly expressed and secreted by gastric epithelial cells, albeit not in its normal heterodimeric form. Lastly, stimulation of human PBMC-derived T cells with IL23A-containing supernatant revealed functions for this protein in promoting T cell proliferation and IFN-γ production. Together, these data indicate that RUNX3 functions as a tumour suppressor in part by modulating gastric inflammation and mucosal immunity.

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List of Abbreviations

AGM	aorta gonad mesonephros
AML	acute myelogenous leukaemia
AP1	adaptor-related protein complex 1
APC	antigen presenting cell
ATCC	American Type Culture Collection
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BB	Brucella's broth
BfdA	brefeldin A
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CagA	cytotoxin associated gene A
Cag-PAI	cytotoxin associated gene pathogenicity island
cAMP	cyclic adenosine monophosphate
CBFα	core binding factor alpha
CBFβ	core binding factor beta
cDNA	complementary DNA
CFSE	carboxyfluorescein diacetate succinimidyl ester
ChIP	chromatin immuno-precipitation
CNS	central nervous system
CREB	cAMP response element-binding
DAMP	damage-associated molecular pattern
DC	dendritic cell
del	deletion
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
EBI3	Epstein-Barr virus induced 3
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EMSA	electrophoretic mobility shift assay
EphB2	ephrin receptor B2
ELISA	enzyme-linked immune-sorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
F	forward
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GSEA	gene set enrichment analysis
GWAS	genome-wide association study
H. pylori/ *Hp*	*Helicobacter pylori*
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
IBD	inflammatory bowel diseases
iE-DAP	D-gamma-Glu-meso-diaminopimelic acid

IFN	interferon
IgCα	immunoglobulin constant alpha
IHC	immunohistochemistry
IκB	inhibitory protein for NF-κB
IKK	IκB kinase
IL	interleukin
IM	intestinal metaplasia
IP	immuno-precipitation
JAK	Janus kinase
LB	Luria-Bertani
LPS	lipopolysaccharide
luc	luciferase
m	mutant
MDP	muramyl dipeptide
MDR1	multi-drug resistance protein 1
MHC	major histocompatibility complex
MIR	mammalian wide interspersed repeats
MNU	*N*-methyl-*N*-nitrosurea
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-kappa B
NLR	NOD-like receptor
NOD	nucleotide oligomerisation domain
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
Pam3CSK4	porphyromonas gingivalis lipopolysaccharide and lipoteichoic acid
PAMP	pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PEBP2β	polyomavirus enhancer binding protein 2 beta
PGN	peptidoglycan
PLB	passive lysis buffer
Poly I:C	polyinosinic: polyscytidylic acid
PRR	pattern recognition receptors
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
R	reverse
RBC	red blood cell
RG	region
rhIL-23	recombinant human interleukin-23
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
RUNX	Runt-related
RNAi	RNA interference
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SEM	standard error mean
SHP2	Src homology phosphatase 2
siRNA	small interfering RNA

SMAD	small mothers against decapentaplegic
SNP	short nucleotide polymorphism
SPEM	spasmolytic polypeptide-expressing metaplasia
STAT	signal transducer and activator of transcription
SWI/SNF	switch/sucrose nonfermentable
T4SS	type IV secretion system
TCF	T-cell factor
TESS	transcription element search system
TF	transcription factor
TGF-β	transforming growth factor-beta
T_H	helper T cell
TLE	transducing-like enhancer
TLR	Toll-like receptor
TNFR	tumour necrosis factor receptor
TNF-α	tumour necrosis factor-alpha
u.d.	undetermined
UV	ultraviolet
VacA	vacuolating toxin gene A
WT	wild-type

List of Symbols/Units

bp	base pair
cm	centimeter
CO_2	carbon dioxide
C_T	threshold cycle
h	hour
H_2O	water
H_2SO_4	hydrogen sulfate
kb	kilo base
kDa	kilo dalton
L	liter
M	molar
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
$NaHCO_3$	sodium bicarbonate
NaCl	sodium chloride
ng	nanogram
nm	nanometer
nM	nanomolar
pmol	picomole
pH	potential of hydrogen
rpm	revolutions per minute
s	second
U	unit
V	volt
°C	degree Celsius
%	percentage

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Chapter 1:

Introduction

1.1 The RUNX family members

Runt-related (RUNX) proteins are key developmental transcriptional regulators whose dysregulation are often associated with human cancers (Ito, 1999). The founding member of the RUNX gene family is *runt*, a *Drosophila "*pair-rule" gene that controls segmentation in embryos and is required for neurogenesis and sex determination (Duffy and Gergen, 1991; Duffy et al., 1991; Ingham and Gergen, 1988; Kania et al., 1990). Subsequently, *RUNX* genes are found in phylogenetically diverse organisms including nematode worm *Caenorhabditis elegans*, zebrafish *Danio rerio* and mammals (Sullivan et al., 2008). Despite their evolutionary divergence, the DNA-binding domain for these proteins remains highly conserved even in the most basic metazoan, indicating that RUNX proteins play fundamental role in cell biology (Sebe-Pedros et al., 2011; Sullivan et al., 2008).

Mammalian RUNX was originally discovered by independent groups to be a nuclear factor that binds to the transcriptional enhancers in polyomaviruses and murine leukemia viruses (Kamachi et al., 1990; Speck et al., 1990). Shortly after, *RUNX* gene was found to be frequently targeted by t(18;21) chromosome translocation in human acute myelogenous leukemia (Miyoshi et al., 1991; Miyoshi et al., 1997). Due to the concurrent discovery of RUNX genes by various groups, RUNX genes were given a variety of names including polyomavirus enhancer-binding-protein-2 alpha (PEBP2α), core binding-factor alpha (CBFα) and acute myelogenous leukemia (AML). Eventually, the term 'RUNX' was adopted to refer to genes encoding the runt-related proteins (van Wijnen et al., 2004).

To date, three mammalian *runt*-related genes have been characterised; *RUNX1 (AML1/CBFA2/PEBP2αB), RUNX2 (AML3/CBFA1/PEBP2αA),* and *RUNX3*

(AML2/CBFA3/PEBP2αC) (Ito, 2004). A high level of sequence and structural homology is shared among all three members which include an enormously conserved 128 amino acid Runt domain and a five amino-acid VWRPY domain (Coffman, 2003; Ito, 1999; Levanon et al., 2003; Rennert et al., 2003). The high degree of sequence similarity suggests the crucial importance of these domains to the function of RUNX proteins. The amino-terminal Runt domain is critical for RUNX proteins to function as transcription factors as it confers sequence specific DNA-binding and dimerisation with their non DNA-binding subunit, core-binding factor beta (CBFβ) (Kamachi et al., 1990). Although the Runt domain alone is able to bind DNA, its binding affinity and hence transcriptional activity are fully activated only when dimerised with CBFβ (Ogawa et al., 1993a; Ogawa et al., 1993b). The carboxyl-terminal VWRPY motif is responsible for repressing the transcriptional activity of RUNX proteins by acting as a platform to recruit co-repressors including transducing-like enhancer (TLE)/Groucho (Aronson et al., 1997; Imai et al., 1998; Levanon et al., 1994). In spite of these similarities, each RUNX gene product has seemingly distinct biological functions, which is best reflected in their respective murine knockout phenotypes (Levanon and Groner, 2004).

1.2 The pleiotropic roles of RUNX genes in development and diseases

1.2.1 RUNX1 in haematopoiesis and leukaemia

Amongst the *RUNX* genes, *Runx1* is the most studied gene in both mouse and human. RUNX1 is a master regulator of haematopoiesis and its functional dysregulation often leads to leukaemia. Homozygous deletion of *Runx1* in mice obliterated the fetal liver haematopoiesis and impaired the formation of vascular capillaries, demonstrating the importance of *Runx1* in definitive haematopoiesis (Okada et al., 1998; Okuda et al., 1996; Takakura et al., 2000; Wang et al., 1996). Subsequently, it was discovered that Runx1 is essential for the emergence of hematopoietic stem cells (HSCs) from the hematogenic endothelial clusters in the embryonic aorta-gonad-mesonephros (AGM) region (North et al., 1999; Yokomizo et al., 2001). These discoveries highlight the critical role of Runx1 in the initiation of the hematopoietic system. In adult mice, Runx1 is essential for terminally differentiation of the multi-lineage hematopoietic cells. Conditional ablation of *Runx1* in adult mice led to defective megakaryocytes, T- and B-lymphocyte development (Growney et al., 2005; Taniuchi et al., 2002). During T cell development, Runx1 is involved in the active repression of *CD4* in $CD4^{-}CD8^{-}$ double negative immature thymocytes via direct binding to two *Runx* binding motifs in the *CD4* silencer (Taniuchi et al., 2002).

In human, *RUNX1* is one of the most frequently disrupted genes in acute myelogenous leukaemia (AML) (Look, 1997; Osato, 2004; Speck and Gilliland, 2002). Classical chromosomal translocations that inhibit RUNX1's function through the generation of chimeric proteins are prevalent in various types of leukaemia including acute myelogenous leukaemia (AML1-ETO), chronic myelogenous leukaemia (AML1-Evi1) and childhood acute lymphoblastic leukaemia (TEL/AML1)

(Golub et al., 1995; Mitani et al., 1994; Nucifora et al., 1993). Moreover, loss-of-function *RUNX1* mutations were also found in both sporadic and familial cases which predispose the patients to the development of acute myeloid leukaemia (Osato et al., 1999; Song et al., 1999). Interestingly, gain-of-function mutations caused by amplification of the *RUNX1* locus and increased in its promoter activity are also leukemogenic (Niini et al., 2000; Wotton et al., 2002). Thus, it appears that RUNX1 expression has to be precisely regulated to prevent leukaemia. Taken together, RUNX1 is a global regulator of embryonic and adult haematopoiesis whose dysregulation is strongly link to leukemogenesis.

1.2.2 RUNX2 in bone development

RUNX2 is a central regulator of bone development in mammals and vertebrates. Genetic ablation of *Runx2* in mice resulted in impaired osteoblasts maturation and osteogenesis, thereby leading to complete lack of bone tissue formation (Komori et al., 1997; Otto et al., 1997). Consistent with this phenotype, Runx2 regulates the expression of a major set of osteoblast-related genes including *osteocalcin*, *osteopontin*, *collagenase 3* and *MMP9* that define the osteoblast *lineage* from mesenchymal stem cells (Ducy et al., 1999; Ducy et al., 1997; Geoffroy et al., 1995; Jimenez et al., 1999; Pratap et al., 2005). While *Runx2*$^{-/-}$ mice died from asphyxiation due to systematic lack of ossification, including the absence of ribs, *Runx2*$^{+/-}$ mice displayed skeletal abnormalities that are characteristic of human heritable skeletal morphorgenesis disorder, cleidocranial dysplasia (Lee et al., 1997; Mundlos et al., 1997). Indeed, loss-of-function mutations of *RUNX2* are found in patients suffering from this disease (Otto et al., 2002; Tessa et al., 2003; Xuan et al., 2008).

Similar to the other two Runx transcription factors, Runx2 displays anti-proliferative function in normal osteoprogenitors and pre-osteoblasts by affecting cell cycle progression at G1 phase, implicating a role in stringent cell growth control in this cell types (Galindo et al., 2005; Pratap et al., 2003; Teplyuk et al., 2008; Young et al., 2007b; Zaidi et al., 2007). Paradoxically, unscheduled expression of RUNX2 has also been reported in a subset of osteosarcomas (Blyth et al., 2005; Pratap et al., 2006). The robust expression of RUNX2 in osteosarcomas correlates with metastasis and poor chemotherapy response by controlling genes linked to cell motility and adhesion (Lucero et al., 2013; Pereira et al., 2009; Sadikovic et al., 2010; San Martin et al., 2009; van der Deen et al., 2012). Furthermore, overexpression of RUNX2 is not restricted to osseous cancer but also observed in nonosseous cancers including prostate and breast cancers indicating that RUNX2 may have an oncogenic function in tumourigenesis (Akech et al., 2010; Das et al., 2009; Leong et al., 2010; Pratap et al., 2009; Pratap et al., 2011; Pratap et al., 2008; van der Deen et al., 2010).

1.2.3 RUNX3 in neuron and lymphocyte development

RUNX3 is often considered the most primitive forms among all mammalian RUNX family members, as it retains the highest level of mammalian wide interspersed repeats (MIR), which are elements enriched in most ancient genes (Bangsow et al., 2001). It is also the smallest member that possesses all the structural hallmarks common to the *RUNX* family members (Bangsow et al., 2001). Unlike RUNX1 and RUNX2 which play fundamental roles in a restricted number of organs, RUNX3's roles appear more pleiotropic. In mouse, *Runx3* is expressed in specific cell types in the peripheral nervous system, haematopoietic cells as well as epithelial cells in gastrointestinal tract (Inoue et al., 2002; Levanon et al., 2002; Li et al., 2002;

Taniuchi et al., 2002). In line with this, *Runx3*-null mice displayed numerous anomalies including motor dis-coordination, reduction in peripheral cytotoxic T cells, and hyperplastic gastrointestinal epithelium (Inoue et al., 2002; Levanon et al., 2002; Li et al., 2002).

In the nervous system, Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons and thus, the ablation of *Runx3* leads to severe ataxia in mice due to less neurite outgrowth (Inoue et al., 2002). In the hematopoietic system, RUNX3 is necessary for establishing the epigenetic silencing of *CD4* in mature $CD4^-$ $CD8^+$ cytotoxic T lymphocytes (Taniuchi et al., 2002; Woolf et al., 2003). *Runx3*-deficient $CD4^-CD8^+$ cytotoxic T cells, but not $CD4^+CD8^-$ helper T cells, failed to proliferate and displayed defective cytotoxic activity, suggesting that Runx3 has critical function in lineage specification and homeostasis of CD8-lineage T lymohocytes (Collins et al., 2009; Taniuchi et al., 2002).

1.2.4 RUNX3, a tumour suppressor for gastrointestinal cancers

A role of RUNX3 in the gastrointestinal epithelium was first implicated by the pronounced hyperplastic gastric epithelia of the *Runx3* knockout mice (Li et al., 2002). Further analysis revealed that the fundic and pyloric region of the stomach displayed excessive proliferation. This was attributed to the suppression of apoptosis and reduced sensitivity to the growth inhibitory effect of transforming growth factor-beta (TGF-β) (Ito, 2008; Li et al., 2002). The dysregulated proliferation and apoptosis experienced by gastric epithelial cells following the loss of Runx3 are consistent with its role as a tumour suppressor in this tissue type. However, the neonatal death of C57/BL6 *Runx3*$^{-/-}$ mice has hampered detailed examination of the phenotype at the later stage of life. To overcome this problem, immortalised mouse gastric epithelial

cell lines, termed GIF lines were established from *Runx3*$^{+/+}$ and *Runx3*$^{-/-}$ E16.5 embryos in a *p53*$^{-/-}$ background (Li et al., 2002). Concordant with the tumour suppressive properties of Runx3, allografting of *Runx3*$^{-/-}$.*p53*$^{-/-}$ GIF cell lines but not their *Runx3*$^{+/+}$.*p53*$^{-/-}$ counterparts formed tumours in immuno-compromised nude mice (Li et al., 2002).

More recently, *Runx3*-null mice in Balb/c genetic background that survived up to one year were generated for the examination of preneoplastic lesions in adult mice (Ito et al., 2011). Analysis of these mice revealed a phenotype that is remarkably similar to spasmolytic polypeptide-expressing metaplasia (SPEM), a preneoplastic lesion found in human gastric cancer (Goldenring and Nomura, 2006; Ito et al., 2011). As Runx3 is prominently expressed in pepsinogen-positive chief cells and Muc5AC-positive surface mucous cells, it may be involved in the differentiation of these lineages. Indeed, loss of chief cells and antralisation of the stomach were observed in adult *Runx3*$^{-/-}$ mice (Ito et al., 2011). This was likely consequent to the blockage in chief cells differentiation or trans-differentiation of chief cells into SPEM cells (Ito et al., 2011). Remarkably, the induction of an intestinal phenotype characterised by the upregulation of intestinal markers, Cdx2 and Muc2, was observed in the gastric mucosa of *Runx3*$^{-/-}$ adult mice (Ito et al., 2011). These mixed characteristics of gastric and intestinal phenotypes in *Runx3*$^{-/-}$ gastric epithelium indicates the occurrence of intestinal metaplasia (IM) (Ito et al., 2011). The above evidence suggests that loss of Runx3 in the gastric epithelium disrupts the identity of the epithelial cells causing the differentiation pathways to be easily altered by extracellular morphogenetic cues. More importantly, SPEM observed in *Runx3*-null mice was readily transformed into gastric adenocarcinoma when induced with a carcinogen, *N*-methyl-*N*-nitrosurea (MNU), indicating that the loss of Runx3 induces a pre-malignant state in the stomach

(Ito et al., 2011). Moreover, genetic ablation of *Runx3* induced hyperplasia of gastric epithelia in the absence of *Helicobacter pylori* (*H. pylori*) infection or inflammation indicating that this is an epithelial cell autonomous phenomenon (Ito et al., 2011; Ito, 2008).

The tumour suppressive function of Runx3 in mice is firmly supported by human clinical data. In human, loss of RUNX3 expression is strongly correlated to the genesis and progression of gastrointestinal cancer. Inactivation of *RUNX3* was found in more than 80% of primary gastric tumours and gastric cancer cell lines due to hemizygous deletions, epigenetic silencing and protein mislocalisation (Ito et al., 2005; Li et al., 2002). Furthermore, silencing of *RUNX3* was prevalent in human colorectal carcinomas in which *RUNX3* was inactivated in 40% of primary colorectal tumours and 60% of colorectal cancer cell lines (Ito et al., 2008). In addition, downregulation of RUNX3 is frequently observed in intestinal metaplasia (IM) which is often regarded as a precancerous state in gastric cancer (Li et al., 2002). Similarly, inactivation of RUNX3 induced intestinal adenomas in both human and mice, which provide favourable conditions for the progression of these adenomas to malignant adenocarcinomas (Ito et al., 2008).

Chromosome *1p36*, where *RUNX3* resides, harbours a cluster of tumour suppressor genes, and is a deletion hotspot in diverse cancers of epithelial, hematopoietic, and neural origins (Bagchi and Mills, 2008). Examination of gastric cancer cell lines SNU1, NUGC3 and AGS together with clinical specimens revealed hemizygous deletions of *RUNX3* occurred at frequencies which increase with tumour grade (Li et al., 2002). Moreover, epigenetic silencing of *RUNX3* by promoter hypermethylation was found to be the most common aberrant methylation events in gastric and colorectal cancers (Ahlquist et al., 2008; Li et al., 2002; Oshimo et al.,

2004; Soong et al., 2009; Subramaniam et al., 2009). The CpG dinucleotide sequence in the *RUNX3* exon 1 region were completely methylated in *RUNX3*-negative cell lines whereas those that expressed *RUNX3* were entirely methylation free (Li et al., 2002). Subsequently, this phenomenon is also observed in bladder, breast, lung, pancreatic, brain cancers and hepatocellular carcinoma (Jiang et al., 2008; Kim et al., 2004; Kim et al., 2005; Lau et al., 2006; Mueller et al., 2007; Sato et al., 2006; Woolf et al., 2003).

Cytoplasmic sequestration of nuclear factor RUNX3 was also found in 38% of gastric cancer cases and in multiple cancer cell lines (Ito et al., 2005; Ito, 2008). Remarkably, cytoplasmic retention of RUNX3 in SNU16 cells resulted in larger tumour formation in nude mice (Ito et al., 2005). Localisation of transcription factor to the cytoplasm is thought to keep them in an inactive state. Therefore, the observation in SNU16 cells highlights the importance of the transcriptional activity of RUNX3 for its tumour suppressive function. Lastly, although the mutation of *RUNX3* turned out to be rare, a mutation R122C bears an amino acid substitution of arginine to cysteine was isolated from a gastric cancer patient (Li et al., 2002). This R122C mutation occurs within the evolutionarily conserved Runt domain and is capable of abolishing the tumour suppressive effects of RUNX3 (Li et al., 2002). The relevance of this mutation is underscored by the discovery of a mutation (R169Q) at the equivalent position in *RUNX2* from a patient with the skeletal disorder cleidocranial dysplasia. Taken together, the above evidence strongly suggests that RUNX3 is a tumour suppressor causally related to the genesis and progression of gastric cancer.

1.3 Gastric carcinogenesis

Gastric cancer is the fourth most prevalent malignancy accounting for approximately one million new cases annually in the world (Ferlay et al., 2010). The incidence of gastric cancer is particularly high in Asia (especially in Korea, Japan and China), Eastern Europe, and parts of Central and South America, and it is about twice as high among men than among women (Figure 1.1) (Garcia et al., 2011). Although the mortality rates have been substantially declining for several decades, the absolute number of new cases per year is increasing due to an aging population (Aaltonen et al., 2000; Ferlay et al., 2010; Garcia et al., 2011).

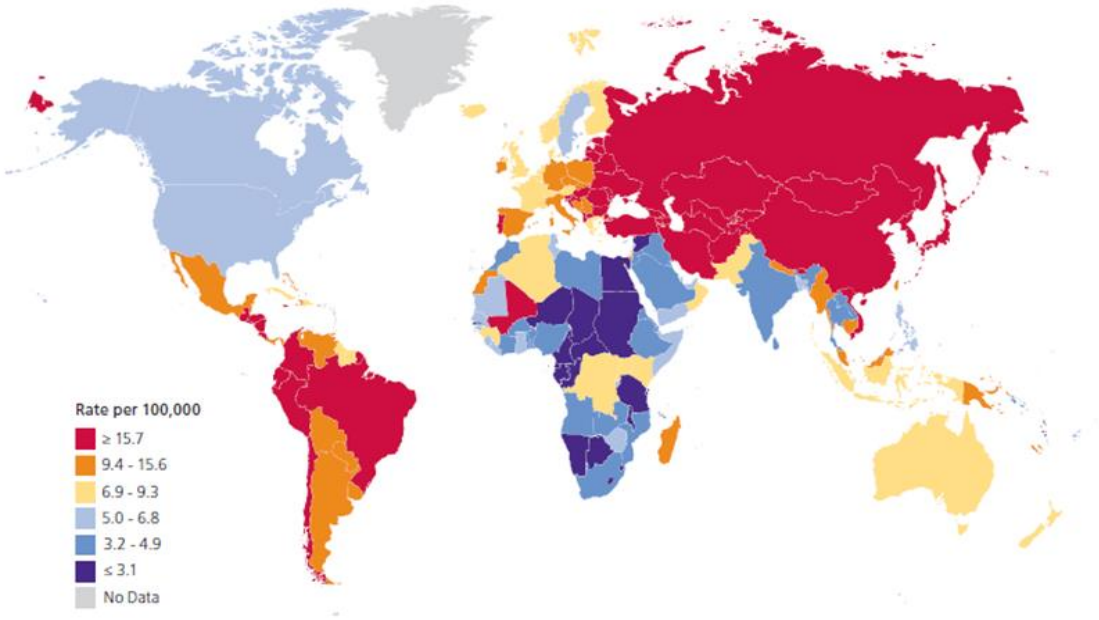


Figure 1.1. Global variation in age-standardised stomach cancer incidence rates. Worldwide incidences of stomach cancer per 100,000 males (all ages). The incidence of gastric cancer is particularly high in Asia, Eastern Europe, and parts of Central and South America (Adapted from epidemiology study of Global cancer facts & figures, 2008).

More importantly, it remains the third leading cause of cancer death worldwide, exhibiting a persistently high mortality rate (9.7% of all cancer deaths per year) irrespective of gender or geographical region (Ferlay et al., 2010). The prognosis for stomach cancer is generally rather poor, with 5-year relative survival below 30% in most countries (Brenner et al., 2009). The exceedingly poor prognosis of gastric cancer is attributed to its typical late diagnosis, high recurrence rate post-surgical resection and limited range of treatment options (Carl-McGrath et al., 2007; Macdonald et al., 2001). Therefore, understanding the pathogenesis of gastric cancer at the molecular level is of great importance for providing better diagnosis of gastric cancer at early stages and wider range of therapeutic alternatives.

1.3.1 Aetiology of gastric cancer

Human gastric carcinogenesis is a multi-factorial and multi-step process. It involves a temporal sequence of chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, primary carcinoma, invasive carcinoma and metastatic tumour (Carl-McGrath et al., 2007; Correa, 1992; Yasui et al., 2005). This precancerous cascade reflects the accumulation of genetic and epigenetic alterations that acquire the cells with hallmark capabilities of cancer. Based on Laurén classification, histologically, gastric adenocarcinoma is generally subdivided into two main categories: intestinal-type carcinomas and diffuse-type carcinomas (Lauren, 1965). For many decades, the aetiology of gastric cancer was totally obscure. Considerable efforts were made to explore and identify the complex aetiology of environmental and genetic risk factors which influence the initiation, promotion and progression of gastric cancer (Chan et al., 2001; Correa, 2002; Kelley and Duggan, 2003; Stadtlander and Waterbor, 1999). In the early days, dietary and lifestyles factors were thought to

be the two major risk factors for gastric cancer (Correa et al., 1975). Indeed, more recently, wide-ranging prospective study has confirmed that high intake of salted, smoked and nitrated foods and low intake of fresh fruits and vegetables increase the risk of developing stomach cancer (Gonzalez, 2006; Howson et al., 1986; Kramer and Johnson, 1995; Odenbreit et al., 2000). Such diet causes stomach irritation by damaging the mucosa and disturbing the homeostatic balance between the gut flora and the epithelium, leading to atrophic gastritis. Injuries in the stomach epithelium cause excessive cell replication and increase the mutagenicity of carcinogens, thereby leading to cancer (Stadtlander and Waterbor, 1999; Takahashi et al., 1994; Tatematsu et al., 1975). On the other hand, the contribution of lifestyle factors such as alcohol consumption and cigarette smoking to stomach cancer had been intensively studied but the results are inconclusive (Hansson et al., 1994; Kabat et al., 1993; Nomura et al., 1990; Vaughan et al., 1995).

The landmark discovery of *Helicobacter pylori* (*H. pylori*) in 1983 and its causal role in gastritis and peptic ulcers effected a re-examination of this classical view of gastric carcinogenesis. *H. pylori* are spiral-shaped, microaerophilic, Gram-negative bacteria that are able to survive in acidic environment and colonise human gastric epithelium. The gastroenterologist Barry Marshall and the pathologist Robin Warren, in the 1980's, first postulated for the association between *H. pylori* infection and human gastritis and gastric cancer (Kidd and Modlin, 1998; Marshall et al., 1985; Marshall and Warren, 1984; Warren and Marshall, 1983). This strong association was subsequently demonstrated in numerous studies in which independent cohorts of gastric cancer patients were retrospectively examined (Ekstrom et al., 2001; Forman et al., 1991; Fox and Wang, 2001; Nomura et al., 1991; Parsonnet et al., 1991; Uemura et al., 2001). *H. pylori* induces pre-neoplastic lesions that are similar to

spasmolytic polypeptide-expressing metaplasia (SPEM) in the stomachs of human (Craanen et al., 1992; Rugge et al., 1996; Schmidt et al., 1999) and experimental animals (Hirayama et al., 1996; Honda et al., 1998; Sugiyama et al., 1998; Watanabe et al., 1998). The disappearance of the inflammatory reaction post-eradication of the bacteria in human using antibiotics further supports that *H. pylori* is a causative agent (Blaser, 1992). During the past 20 years of research, the initially tentative association between persistent *H. pylori* infection and the development of gastric cancer has been well established, prompting the International Agency for Research on Cancer to classify *H. pylori* as type I carcinogen (WHO, 1994).

H. pylori infects more than half of the world's population from infancy, making it one of the most successful human pathogens (Suerbaum and Michetti, 2002). *H. pylori* infection is highly prevalence in Asia, Africa and South America (Figure 1.2) (Bauer and Meyer, 2011). More than 50% of new gastric cancer cases and a 2.7- to 12-fold increase in the risk of developing gastric cancer can be attributed to *H. pylori* infection (Cover and Blaser, 1995; O'Connor et al., 1996; Parkin, 2006). This includes both intestinal-type and diffuse-type gastric cancers (Forman et al., 1991; Nomura et al., 1991; Parsonnet et al., 1991; Talley et al., 1991; Uemura et al., 2001). Critically, the prevalence of *H. pylori* infection mirrors the gastric cancer incidence in Asia, implying a strong association between them in this region (Figure 1.2) (Bauer and Meyer, 2011). Among East Asian countries, the overall prevalence rate was 59.6% in South Korea, 58.07% in China, 54.5% in Taiwan and 39.3% in Japan (Fujisawa et al., 1999; Hoang et al., 2005; Teh et al., 1994; Wang and Wang, 2003). Among Southeast Asian countries, the reported prevalence rate was 57% in Thailand, 35.9% in Malaysia and 31% in Singapore (Deankanob et al., 2006; Fock, 1997; Goh and Parasakthi, 2001).

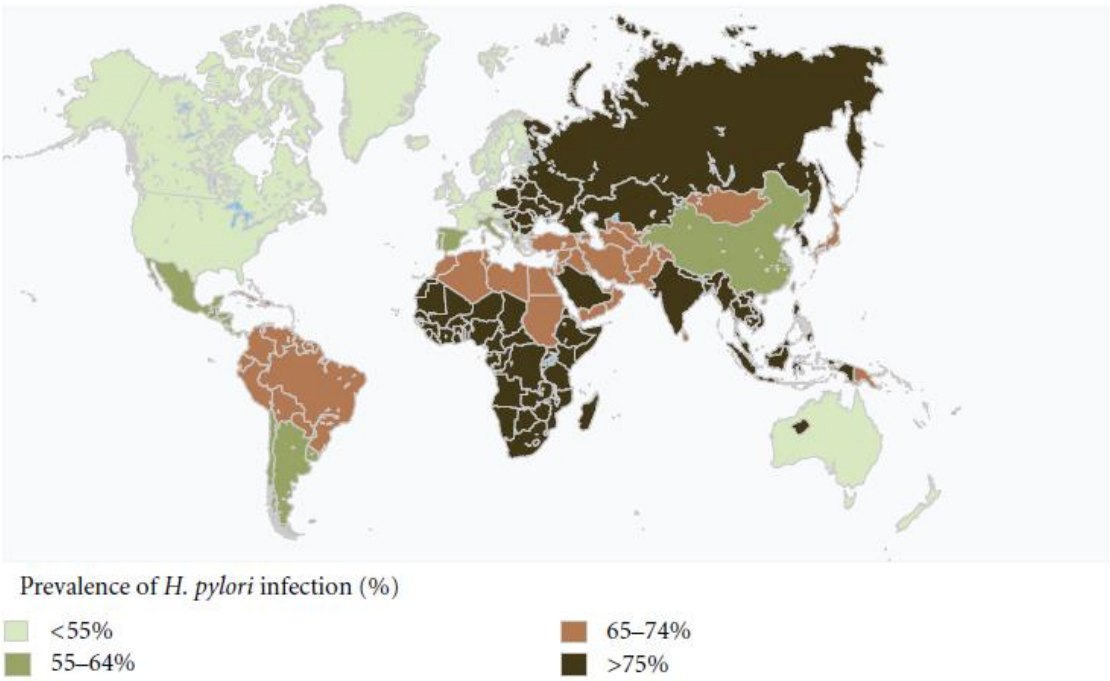


Figure 1.2. Worldwide prevalence of *H. pylori* infection. Bacterial infection rates were presented in percentage (%). *H. pylori* infection is highly prevalence in Asia, Africa and South America. (Bauer and Meyer, 2011).

Interestingly, there is considerable genetic heterogeneity among the strains of *H. pylori* circulating in different geographical regions. Studies show that certain genotypes are more prevalent in gastric cancer patients than in control population, and are therefore regarded to be of high virulence or oncogenic potential (Atherton et al., 1995; Tomasini et al., 2003). One of these genetic factors is the "cag pathogenicity island" (cag-PAI), which is a 40 kilobase fragment of the *H. pylori* genome that encodes 31 genes. This fragment contains the coding sequences for the oncoprotein CagA and a type IV secretion system for the injection of CagA and other bacterial materials into gastric epithelial cells (Odenbreit et al., 2000). Translocation of CagA into host cells is well-known to cause dramatic morphological changes in the cells as reflected by strong actin polymerisation and cellular elongation, term the

'hummingbird' phenotype (Segal et al., 1999). The status of CagA as a marker of pathogenic disease resulted from the observation that patients infected with CagA-positive strain of *H. pylori* showed higher incidences of both gastric inflammation and gastric adenocarcinoma compared to CagA-negative strains (Blaser et al., 1995a; Huang et al., 2003; Nomura et al., 2002; Queiroz et al., 1998; Wu et al., 2003). Another virulence factor carried by 50% of *H. pylori* strains is vacuolating toxin A (VacA) which is responsible for epithelial cell damage and is associated with gastric cancer development (de Figueiredo Soares et al., 1998; Peek and Blaser, 2002). Given the profound influences that *H. pylori* exerts on host cell biology, understanding the mechanisms of how it 'hijacks' the host cells is critical for gaining novel strategies for gastric cancer treatment.

Besides bacterial infection, infection with human herpes virus 4, or Epstein-Barr virus (EBV) was also detected in approximately 10% of gastric carcinoma cases throughout the world (Takada, 2000). It has been reported that the antibody titres against EBV were significantly higher in subjects who later developed EBV-associated gastric cancer than those subsequently developed non-EBV-associated gastric cancer or control subjects (Levine et al., 1995). EBV was found to be associated with both intestinal- and diffuse-type gastric cancers (Shibata and Weiss, 1992), and appeared to be more prevalent in the male than in female (Tokunaga et al., 1993). The mechanism of EBV-mediated gastric carcinogenesis remains largely unclear. However, chronic inflammation due to virus infection is a likely cause for the disease.

1.3.2 Inflammation and gastric cancer

Inflammation is part of the complex biological response of cells to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani et al., 2007). It is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation can be classified into acute or chronic. Acute inflammation is the body's defence system to eliminate assaulting agents and prevent the dissemination of these agents in the body. However, if the body fails to end the inflammatory cycle, it may disturb the homeostatic balance of the immune system and result in chronic inflammation that exacerbates the wounds. Continual repairing of the wounding tissue by enhancing cell proliferation in a microenvironment enriched with inflammatory cells, growth factors, activated stroma and mutagenic agents potentiates or promotes cancer formation (Coussens and Werb, 2002). Indeed, the similarities between conditions conducive to healing wound and tumorigenesis have prompted some researchers to consider tumours as wounds that never heal (Dvorak, 1986).

The link between inflammation and cancer was first proposed by Rudolph Virchow in 1863 when he observed leukocyte infiltration in neoplastic tissue. The original hypothesis has been revisited numerous times in the subsequent years and a formidable body of evidence has been generated that corroborate inflammation-mediated oncogenesis (Coussens and Werb, 2002). As a result of these researches, the causal relationship between inflammation and cancer is now well-accepted and tumour-promoting inflammation has been listed as one of the emerging hallmarks of cancer (Hanahan and Weinberg, 2011). The intimate link between chronic inflammation and cancer is particularly notable in the gastrointestinal tract where microbial contacts are frequent. Classical examples include hepatocellular carcinoma, inflammatory bowel disease (IBD)-associated colorectal cancer and *H. pylori*-

associated gastric cancer. The aetiological agents of inflammation can be either infective, such as bacteria or viruses; or non-infective, such as physical or chemical irritants from the environment and diet.

In recent years, the long-suspected influence of genetic susceptibility has come to the forefront. Current understanding points to the role of gene polymorphisms as yet another major determinant of aetiological forces (Correa and Schneider, 2005). The most prominent host polymorphisms associated with an elevated risk for gastric cancer were found in the Interleukin-1 (*IL1*) gene clusters, including *IL1B* (Interleukin-1 beta gene) and *IL1RN* (IL1 receptor antagonist gene) (El-Omar et al., 2000; El-Omar et al., 2003; Machado et al., 2001). Such polymorphisms were shown to affect the production of the proinflammatory cytokine IL-1β in response to *H. pylori* infection (Hwang et al., 2002). Subsequent study in mice demonstrated that ectopic production of IL-1β in the stomach leads to gastric inflammation and cancer (Tu et al., 2008). In addition to *IL1* gene cluster polymorphisms, pro-inflammatory genotypes of *TNFA* (tumour necrosis factor alpha gene) and *IL10* (Interleukin-10 gene) have also been identified as risk factors for gastric cancer (El-Omar et al., 2003). Not surprising that carriage of multiple proinflammatory polymorphisms of *IL1B*, *IL1RN*, *TNFA* and *IL10* conferred greater risk for the disease (El-Omar et al., 2003). More recently, genetic variations in the IL-12 family of cytokines and receptors were also found to be associated with inflammatory disease in the gastrointestinal tract. In particular, the polymorphisms in the Interleukin-12 (IL-12) cytokine family genes including, *IL12A* and *IL12B* are associated with increased risk of gastric cancer in *H. pylori* infected individuals (Navaglia et al., 2005). Interestingly, similar associations have been observed for IL-12 receptor family. For example, a non-synonymous short nucleotide polymorphism

(SNP) of *IL23R* (Interleukin-23 receptor gene) was found to be associated with elevated risk of gastric cancer (Chen et al., 2011; Chen et al., 2010).

In general, the genes that encode cytokines involved in the regulation of inflammatory responses are genetically polymorphic and different genotypes may determine the disease outcome and response to drug treatment. These genetic variations add to the complex interplay between the host system and environmental risk factors, which together often lead to a state of chronic inflammation in the gastric mucosa (Carl-McGrath et al., 2007). The above clinic-pathological evidence points to a strong link between inflammation and gastric cancer.

1.4 RUNX proteins function as transcription factors

RUNX proteins are context-dependent transcriptional regulators. Studies in *Drosophila*, mammalian and other systems revealed that RUNX proteins are able to either increase or actively inhibit gene expression by cooperating with different subsets of transcription factors or cofactors in specific cells or tissue types (Wheeler et al., 2002). The transcriptional role of RUNX factors are dictated by the Runt homology domain that confers sequence specific binding and interaction with their universal binding partner CBFβ (Ito, 2004; Kamachi et al., 1990). Although the Runt domain has intrinsic DNA-binding capabilities, its binding affinity increases markedly when complexed with CBFβ (Ogawa et al., 1993a; Ogawa et al., 1993b). The interaction between RUNX and CBFβ is sufficiently strong that they were originally co-purified as a heterodimer in H-ras transformed NIH 3T3 cells (Kamachi et al., 1990). The consensus RUNX target DNA sequence was further defined by Kamachi and colleagues as PuACCPuCA, whereby 'Pu' denotes a purine base. Subsequently, the physical binding between Runt domain, CBFβ and DNA was also resolved using X-ray crystallography (Kamachi et al., 1990; Nagata et al., 1999; Tahirov et al., 2001).

1.4.1 Downstream targets of RUNX

In many tissues, RUNX proteins act as transcriptional activators or repressors to regulate gene expression at both genetic and epigenetic levels. A classic example for synergistic regulation is the cooperative DNA-binding and transcriptional activation of T cell receptor and Moloney murine leukemia virus enhancer elements by RUNX1 and Est-1 (Kim et al., 1999; Sun et al., 1995; Wotton et al., 1994). In

addition, RUNX1 also cooperates with Myb, PU.1, and C/EBPα transcription factors to transactivate various promoters and enhancers of the hematopoietic-related genes (Britos-Bray and Friedman, 1997; Hernandez-Munain and Krangel, 1994; Petrovick et al., 1998; Zaiman and Lenz, 1996; Zhang et al., 1996). On the other hand, RUNX proteins recruit co-repressors such as Sin3A or TLE/Groucho to bring about transcriptional repression of hematopoietic- and osteoblastic-related genes (Imai et al., 1998; Javed et al., 2005; Levanon et al., 1998; Wang et al., 1998). Furthermore, RUNX proteins are able to control gene expression through remodelling the chromatin structure. At the epigenetic level, RUNX1 interacts with p300/CREB-binding proteins to recruit histone acetyltransferase, p300/CBP-associating factor, resulting in the de-repression of *myeloperoxidase* gene during myeloid differentiation (Kitabayashi et al., 1998). In a different context, Runx2 interacts with switch/sucrose nonfermentable (SWI/SNF) protein complex and C/EBPβ to epigenetically de-repress *osteocalcin* gene during osteoblast differentiation (Villagra et al., 2006). More recently, RUNX proteins are found to localise in nucleolar organizing regions where they epigenetically silence the expression of *ribosomal RNA* (*rRNA*) genes during interphase and mitosis (Pande et al., 2009; Young et al., 2007a).

RUNX family members play divergent roles in developmental processes and human diseases which are reflected in the distinct subsets of target genes regulated between each RUNX factors. For example, RUNX1 regulates a long list of genes that are involved in haematopoiesis, including *CFS-1 receptor*, *PU.1*, *CCND3* and *IGFBP-3* (Bernardin-Fried et al., 2004; Huang et al., 2008; Iwatsuki et al., 2005; Okada et al., 1998; Zhang et al., 1994). RUNX2 orchestrates bone and cartilage formation through its regulation of key osteogenic genes including *osteocalcin*, *osteopontin*, *collagenase 3* and *MMP9* (Ducy et al., 1999; Ducy et al., 1997; Jimenez

et al., 1999; Pratap et al., 2005). In comparison, the target genes of RUNX3 are relatively less established due in part to its diverse functions in development in multiple tissue contexts. Candidate gene approach has often been applied to study target genes of RUNX3 within a specific cellular context. For example, *CD4* and *TrkB* regulation by RUNX3 were studied in thymocytes and neuronal cells respectively (Inoue et al., 2007; Taniuchi et al., 2002). As *Runx3*-null mice display a hyperplastic gastric epithelium with reduced apoptosis, the growth regulator *p21* and proapoptotic gene *BIM1* were studied and established as RUNX3's target genes in gastric epithelial cells (Chi et al., 2005; Yano et al., 2006). More recently, *Claudin-1* and *AKT1* were also identified as target genes of RUNX3 in human gastric cancer cells (Chang et al., 2010; Lin et al., 2012). Despite these advances, much remains unknown of the full function of RUNX3 that could explain its frequent silencing in human gastric cancers.

1.4.2 Identification of *IL23A* as a novel target gene of RUNX3

As a first step to elucidate the genetic programme maintained by RUNX3 in gastric epithelial cells, an expression microarray study was previously performed (unpublished data). In this study, exogenous RUNX3 was reintroduced into a RUNX3 non-expressing gastric cancer cell line, AGS. Changes in global gene expression were monitored by the analysis of RUNX3- or control-transfected cells across five different time points. The relative expressions of significantly altered genes with a false discovery rate of less than 5% are presented in the form of an expression 'heat map' as shown in Figure 1.3. In this study, 151 genes were found to be differentially expressed in the presence of exogenous RUNX3, of which 70 and 81 genes were upregulated and downregulated, respectively.

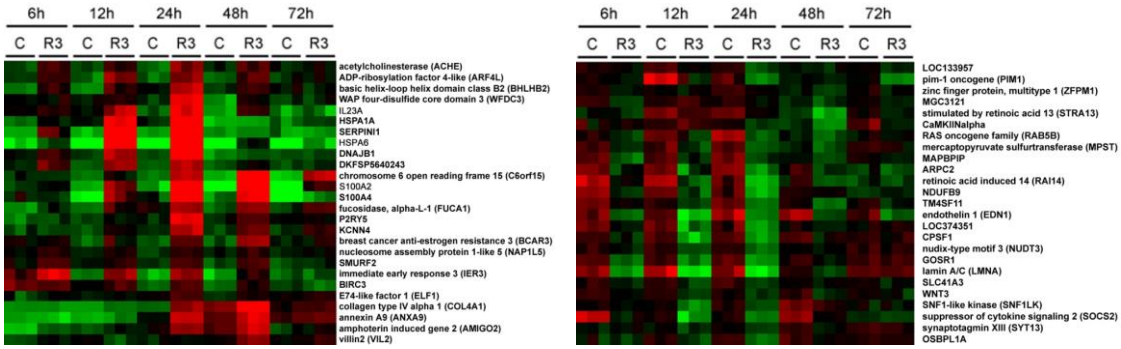


Figure 1.3. Heat map representation of a subset of candidate RUNX3 target genes. Time points of experiment are presented from left to right. 'C' and 'R3' denote control- and RUNX3-transfected cells respectively. Red colour in the heat map represents overexpressed genes; green, underexpressed genes; black, genes showing similar expression levels in RUNX3- verses control-transfected cells.

To extract biological insights from these microarray data, Gene Set Enrichment Analyses (GSEA) were performed (Edelman et al., 2006; Subramanian et al., 2005). Among the gene sets which are significantly correlated to ectopic RUNX3 expression in AGS cells include those involved in the Wnt and apoptotic pathways (Figure 1.4). This is consistent with previous reports of RUNX3's ability to attenuate the Wnt pathway, and its role as a gastric tumour suppressor (Chi et al., 2005; Ito et al., 2008; Li et al., 2002; Yano et al., 2006). In addition, this analysis revealed a group of genes involved in the immune response which are downstream of Interferon, Interkeukin-4 and -6 (Figure 1.4). This hints at a novel function of RUNX3 in the immune response of gastric epithelial cells. Among the top 15 high-confidence candidate target genes, *IL23A* emerged as a prime candidate as it was strongly upregulated by RUNX3 (Table 1.1). With the interest of understanding the immune-related function of RUNX3 in gastric epithelial cells, *IL23A* was chosen for downstream validation. *IL23A* encodes a 19 kDa subunit (p19) of the

proinflammatory cytokine IL-23, which plays a crucial role in host immunity and exhibits the effector functions of a novel class of helper T cells, known as Th17 (Stockinger and Veldhoen, 2007; Volpe et al., 2008; Wilson et al., 2007).

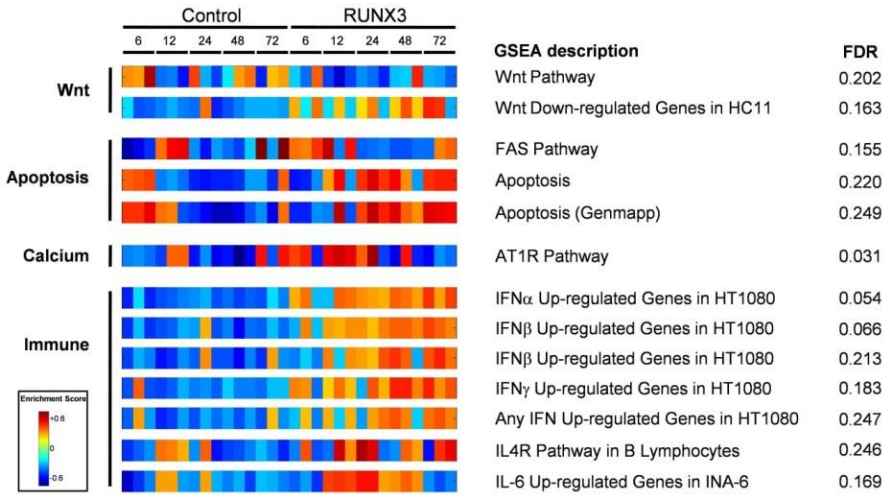


Figure 1.4. Gene set enrichment analysis of the microarray data. Gene sets are grouped into biological pathways in which they are involved. A subset of gene sets that are correlated to the transient expression of RUNX3 is shown (false discovery rate ≤ 0.25).

Gene	Trend in microarray	Biological pathway / function	Description
S100A2	↑	Calcium	Cancer marker
S100A4	↑		Correlates with tumor metastasis
KCNN4	↑		Calcium activated potassium channel
IER3	↑	Stress-induced	Stress-inducible early response protein
HSPA6	↑		Stress-inducible chaperone
FOS	↑		AP1 subunit; interacts with RUNX1/2
CCND3	↑		Cyclin for G1/S transition
IL23A	↑	Immune	Pro-inflammatory protein
IL1RN	↑		Modulates responses of IL1
DKK1	↓	Wnt	Inhibitor of WNT pathway
WNT3	↓		Wnt signalling molecule
MYC	↓		Proto-oncogene
CTGF	↓	Cell adhesion	Secreted growth factor
EDN1	↓	Vascularisation	Promotes for vascularisation
KLF4	↓	Differentiation	Gut tumor suppressor

Table 1.1. Fifteen high-confidence candidate RUNX3 target genes from microarray. '↑' indicates strongly upregulated; '↑' upregulated; '↓' strongly downregulated; '↓' downregulated.

1.5 The biological functions of IL-23

IL-23 is a heterodimeric inflammatory cytokine that belongs to the IL-12 family that has been genetically linked to gastric cancer. IL-23 is composed of a unique IL23A (p19) subunit and a common IL12B (p40) subunit that is shared with IL-12 (Figure 1.5). This cytokine was first discovered in a structure-based bioinformatics search and appeared closely related in structure to the IL12A (p35) subunit of IL-12 (Oppmann et al., 2000). The sharing of subunits of the IL-12 family members is not limited to the ligands but also applied to their receptors. Both IL-23 and IL-12 share a common receptor subunit IL12RB1 that dimerises with either IL23R or IL12RB2 for ligands binding and signaling (Langrish et al., 2004; Parham et al., 2002).

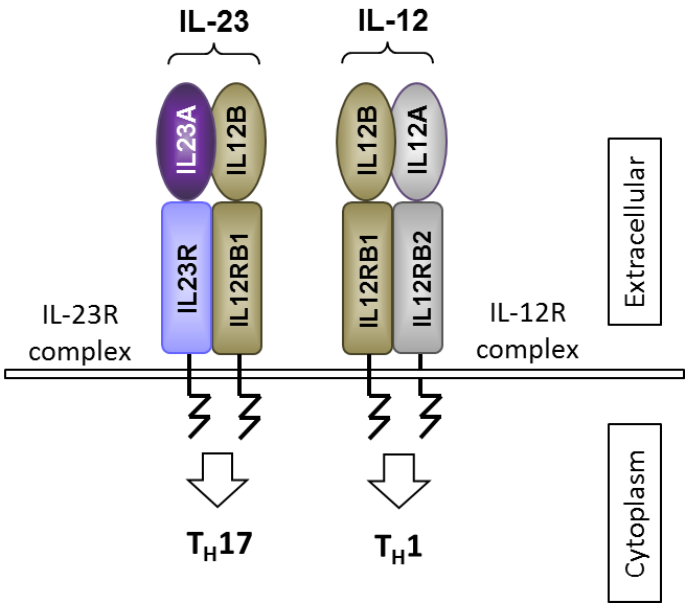


Figure 1.5. Schematic diagram of IL-12 and IL-23: members of a small family of pro-inflammatory heterodimeric cytokines. Both cytokines share a common IL12B subunit that is covalently linked either to IL12A subunit to form IL-12 or to IL23A subunit to form IL-23 (Oppmann et al., 2000; Langrish et al., 2004).

Despite the common characteristics between IL-12 and IL-23, they play profoundly distinct roles in immunity. IL-12 is well-known for promoting the differentiation of type I helper T cells (T_H1) from naïve T cells whereas IL-23 in concert with TGF-β and IL-6 are critical for the expansion of T_H17 cells (Aggarwal et al., 2003; Kobayashi et al., 1989; Langrish et al., 2005; Murphy and Reiner, 2002; Trinchieri et al., 1992).

IL-23 plays a pivotal role in the coordination of organ-specific inflammatory responses. This was revealed in experimental autoimmune and inflammatory studies in mice. Mice that lacked the IL-23-specific component IL23A are resistant to T cell-mediated diseases as summarised in Table 1.2. Cua *et al.* had demonstrated that IL-23 but not IL-12 is essential for the development of central nervous system (CNS) autoimmune inflammation. This was supported by the evidence that intense mononuclear cell infiltration of the spinal cord observed in heterozygous *IL23a*$^{+/-}$ and *IL12a*$^{-/-}$ mice (lack IL-12) but not in mice with *IL23a*$^{-/-}$ or *IL12b*$^{-/-}$ (lack IL-23) background (Cua et al., 2003). Subsequent studies by other research groups also suggest that mice that lack *IL23a* or *IL23r* were resistant to the development of autoimmune inflammation in the joint, gut, kidney and skin (Ghilardi et al., 2004; Hue et al., 2006; Langrish et al., 2004; Murphy et al., 2003; Uhlig et al., 2006; van der Fits et al., 2009). In contrast, widespread expression of IL23a in transgenic mouse model induced multi-organ inflammation, runting, infertility and premature death (Wiekowski et al., 2001). These pro-inflammatory activities of IL-23 have been partly attributed to its ability to support the development of auto-reactive T_H17 cells which are characterized by the production of pro-inflammatory cytokine IL-17 (Bettelli et al., 2006; Langrish et al., 2005; Mangan et al., 2006; Veldhoen et al., 2006) .

Mouse genotype	Mouse phenotype	References
IL23a$^{-/-}$	Defect in DTH responses Resistant to developing EAE and CIA Resistant to bacterial and chemical induced IBD	Cua *et al*., 2003; Langrish *et al*., 2005; Murphy *et al*., 2003; Ghilardi *et al.*, 2004; Mangan *et al.*, 2006; Uhlig *et al.*, 2006; Hue *et al.*, 2006; Cox *et al*., 2012
IL23a transgenic	Develop systemic inflammatory disease	Wiekowski, *et al*., 2001; Kopp *et al*., 2003
IL23a$^{-/-}$. *IL-10*$^{-/-}$	Resistant to T-cell mediated IBD	Yen *et al.*, 2006
IL23r$^{-/-}$	Resistant to developing Lupus Nephritis	Kyattaris et al., 2010
IL23r$^{-/-}$.*Rag2*$^{-/-}$	Susceptible to chemical induced colitis	Cox *et al*., 2012

(DTH: delayed type hypersensitivity; EAE: experimental allergic encephalomyelitis; CIA: collagen-induced arthritis; IBD: inflammatory bowel disease)

Table 1.2. Summary of the knockout and transgenic mouse phenotypes associated with IL23a/IL23r signaling.

A comprehensive sequence analysis revealed that *IL23A* may have gone through positive selection pressure that directs towards inter-population and intra-species sequence conservations, supporting a critical physiological role for IL-23 (Tindall and Hayes, 2010). Although the exact role of IL-23 to the host is obscure, promoting pathogenic autoreactive T cells could not be the primary function of a cytokine that is so conserved in many animals. Therefore, it is more likely that severe autoimmune pathologies associated with IL-23/T_H17 pathway are a reflection of the breakdown of tolerance to "self" tissues and antigens due to their dysregulation (McKenzie et al., 2006). There is ever-growing evidence suggesting that IL-23's primary function was most likely more primitive in origin and related to forming a robust innate immune response for first-line defence against environmental assaults, during infection in the peripheral tissues such as gut, skin and lung (Tato and Cua, 2008). Indeed, further investigations had shed some light for the protective roles of this cytokine. Although the true role of IL-23 awaits to be found but the robust IL-23 response evoked by pathogens strongly suggest that IL-23 is essential for host defence.

In the gastrointestinal tract, bacterial-dependent IL-23 expression has been reported in lamina propria dendritic cells of the terminal ileum (Becker et al., 2003). Although *IL23a*$^{-/-}$ mice are resistant to the development of autoimmune diseases, these mice will eventually succumbed to infection due to impaired bacterial clearance (Happel et al., 2005; Huang et al., 2004; Mangan et al., 2006). It was known that T_H17 is involved in the recruitment of neutrophils to the site of microbial infection (Cruz et al., 2006; Kolls and Linden, 2004; Meeks et al., 2009). Some infectious models with a defective IL-23/T_H17 immunity were reported to have increased disease susceptibility due to reduced neutrophils response thus higher microbial burdens (Happel et al., 2005; Kelly et al., 2005; Khader et al., 2005; Meeks et al., 2009). In the stomach, evidences are accumulating for the involvement of IL-23 in regulating-T_H17 cells, in *H. pylori*-infected gastric mucosa (Caruso et al., 2008). IL-17, a key regulator of neutrophil chemotaxis, was produced in excess in stomachs infected with *H. pylori* (Luzza et al., 2000; Mizuno et al., 2005). These evidences support an important role of IL-23 in mucosal host defence and pathogen surveillance. It is when this response goes unchecked that the beneficial role of IL-23 gets overcome by its pro-inflammatory properties.

While it is well recognised that the main source cells for IL-23 are activated antigen presenting cells (APC) include dendritic cells and macrophages, the possibility of additional cell types that secrete this cytokine has not been ruled out (Oppmann et al., 2000). In fact, more recent study does provide evidence that keratinocytes secrete IL-23 especially in Psoriatic skin (Piskin et al., 2006). Given the important role of IL-23 in pathogen surveillance, it is possible that RUNX3 regulates *IL23A* in the gastric epithelial cells as part of its role as a gastric tumour suppressor to

enable effective surveillance against pathogens as well as neoplasia. Understanding of the transcriptional regulation of RUNX3 on *IL23A* is therefore crucial.

1.6 Project aims and objectives

1. To demonstrate the transcriptional regulation of *IL23A* by RUNX3.

2. To investigate the upstream signals involved in the induction of *IL23A*.

3. To demonstrate the functional effects of RUNX3-induced IL23A production in gastric epithelial cells.

Chapter 2:

Materials and methods

2.1 Mammalian cell culture

2.1.1 Cell culture condition

Cancerous human gastric epithelial cell lines AGS, KATOIII, SNU1, SNU5, SNU16, SNU719, MKN1, MKN7, MKN28, MKN45, MKN74 and non-cancerous human gastric epithelial cell lines HFE-145, GES-1 were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, CA, USA). The same medium was used for culturing the human acute monocytic leukaemia cell line THP-1. Monkey kidney fibroblasts COS-7 and human embryonic kidney cells HEK293T were cultured in 1000mg/L glucose and 4500mg/L glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) respectively. All media were supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific Hyclone, Logan, UT), 100U/ml penicillin, 100µg/ml streptomycin antibiotics (Invitrogen) and 2mM L-Glutamine (Invitrogen). Adherent cells were sub-cultured in 10-cm tissue culture dishes and suspension cells were sub-cultured in non-treated tissue culture flasks (Nunc A/S, Roskilde, Denmark). Cell lines were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2 in the Water-Jacketed CO_2 Incubator (Forma Scientific, OH, USA). All cell lines were stored in multiple aliquots of recovery cell culture freezing medium (Gibco, CA, USA) in liquid nitrogen and passaged for fewer than three months after resuscitation.

2.1.2 Ligands/agonists and inhibitors treatments

To activate the Toll-like receptors (TLR) and NOD-like receptor (NLR) pathways, cells were treated with 1µg/ml LPS, 1µg/ml Zymosan, 1µg/ml Pam3CSK4, 1µg/ml Poly I:C, 1µg/ml R-848, 100µg/ml Flagellin, 10µg/ml NOD1 (C12-iE-DAP)

or 50μg/ml NOD2 (N-glycolyl-MDP) agonists (gifts from Dr. Vinay Tergaonkar, IMCB, A*STAR). In addition, 10ng/ml of human recombinant TNF-α, IL-1α, IL-1β, IL-6, IFN-γ and/or 5ng/ml of TGF-β (Peprotech, NJ, USA) were used to activate immune pathways in gastric epithelial cell lines. Cells were treated with the ligands for the indicated periods prior to quantitative RT-PCR (qRT-PCR) or Western-blot analysis. To block SHP2/Erk signaling pathway, cells were pre-treated with 50μM of SHP2 protein tyrosine phosphatase (PTP) inhibitor, NSC87877 (R&D Systems, Minneapolis, MN) for 3h prior infection with *Helicobacter pylori*. To inhibit protein secretion, cells were treated with 1X Brefeldin A solution (eBioscience, CA, USA) 8h prior harvesting for Western blot analysis.

2.1.3 DNA plasmids transfection and dual-luciferase reporter assay

For the introduction of exogenous DNA plasmids into the cells, cells were transfected at 60-80% confluency. AGS and KATOIII cells were transfected using Lipofectamine 2000 reagent (Invitrogen) in 24-well tissue culture plates. *IL23A* promoter firefly reporter vector (1 – 2μg), promoter-less Renilla luciferase reporter vector pRL-empty (250 – 500ng) and various expression vectors or empty vector (1.5 – 2μg) were co-transfected into the cells for 24 – 48h. Expression vectors used include pcDNA-FLAG encoding a FLAG-epitope as mock control, and pcDNA-RUNX3 encoding amino-terminal FLAG-tagged human RUNX3 (Bae *et al.,* 1995).

Dual-Luciferase Reporter Assay System (Promega, Madision, USA) was used to measure the Firefly and Renilla luciferase activities of transfected cells according to the manufacturer's protocol. Briefly, media were removed and 100μl of 1x Passive Lysis Buffer (PLB) (Promega) was dispensed into each well. The cells were then

subjected to 2 cycles of freeze-thaw to ensure efficient lysis. Subsequently 40µl of cell lysate was loaded on a 96-well assay plate (Nunc A/S), mixed with equal volume of Luciferase Assay Reagent II (LAR II) to quantify firefly luminescence using Veritas Microplate Luminometer (Turner Biosystems, CA, USA). Renilla luminescence was measured by the addition of 40µl Stop & Glo Reagent.

2.1.4 Transient transfection of small interfering RNA (siRNA)

For the introduction of siRNAs into cells, cells were seeded in antibiotics-free media one day before transfection in 24-well or 6-well tissue culture plates. The confluency of the cells during transfection is approximately 50%. Between 5 and 15 pmol of ON-TARGETplus SMARTpool scrambled (control), RUNX3 and/or RUNX1 siRNAs (Thermo Fisher Scientific, MA, USA) were transiently transfected into HFE-145 cells using jetPRIME transfection reagent (Polyplus-transfection SA, Illkirch, France) for 48h followed by TNF-α (10ng/ml) treatment and *H. pylori* (MOI100) infection. Subsequently, cells were harvested for qRT-PCR and/or western-blot analysis.

2.2 *Helicobacter pylori* culture and infection of mammalian cell lines

Helicobacter pylori (*H. pylori*) wild-type strain (NCTC11637) and its CagA-defective isogenic strain (ΔCagA) are kind gifts from Dr. Masanori Hatakeyama (Hokkaido University, Japan). Both strains of bacteria were streaked and cultivated on a Trypticase Soy Agar supplemented with 5% sheep blood (Biomed Diagnostic, USA) to form colonies. *H. pylori* was cultivated at 37°C in a humidified and micro-aerophilic atmosphere with 6–12% O_2 and 5–8% CO_2 generated using Anaero Pack-MicroAero (MGC, Tokyo, Japan) in a sealed container for 3 days. Prior infection of mammalian cells, broth culture was prepared by inoculating colonies of *H. pylori* from agar into Brucella broth (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FBS and grown under the same atmosphere for 24h. Gastric cancer cell lines were seeded in a 6-well or 12-well tissue culture plates and infected with *H. pylori* harvested from the broth culture for indicated period at 100 multiplicity of infection (MOI).

2.3 Lentivirus production and transduction

Lentiviruses were produced according to the protocol described by(Tiscornia et al., 2006)with minor modifications. Briefly, $0.7x10^6$ HEK293T cells were seeded on a 6-well plate pre-coated with 0.001% of poly L-lysine (Sigma-Aldrich) one day before transfection. Lenti-Control, -*RUNX3* or -*RUNX3*^{R178Q} transfer vectors (1µg) were co-transfected into HEK293T cells along with third generation packaging plasmids containing pLP/VSVG (1µg), pLP1 gag/pol (0.66µg) and pLP2 Rev (0.33µg) (ViraPower Lentiviral Expression system; Invitrogen) using 10µl of FuGENE HD (Roche, Basel, Switzerland). The cells were washed with PBS 18h post-transfection and replaced with fresh DMEM supplemented with 15% FBS (Thermo Scientific HyClone). The virus-containing supernatants were harvested at 36h and 48h post-transfection. Viruses harvested at two different time points were pooled before freezing in aliquots at -80°C. For lentiviral transduction, cells cultured in 6-well or 12-well plates at 80% confluency were incubated with 1/3 volume of virus-containing supernatants for 24h before splitting them for downstream experiments. Infected cells were subjected to TNF-α treatment and/or *H. pylori* infection followed by qRT-PCR and/or western-blot analysis. For safety purposes, supernatants from infected cells were collected and filtered with 0.22µm hydrophilic PVDF membrane filter unit (Millipore, Cork, Ireland) before ELISA or immune cell-based assays.

2.4 Generation of wildtype and mutant *IL23A* promoter-reporter constructs

2.4.1 Molecular cloning

Human *IL23A* promoter sequence (Genebank accession no NM_001265) was obtained from UCSC Genome Bioinformatics (http://www.genome.ucsc.edu/). The -1200 to +105 of human *IL23A* promoter was reversed transcribed into cDNA and amplified by polymerase chain reaction (PCR). PCR amplification was performed in a 50µl reaction mixture containing 1.25U of *Pfu* Turbo DNA polymerase (Invitrogen), 1X *Pfu* Turbo amplification buffer, 0.25 mM of dNTPs (Finzymes, Espoo, Finland), 0.8µM of forward primer (5'-GGACAAGTTTCTACGCGTAAAAGGGTCAAC-3') and 0.8µM of reverse primer (5'-GAATCTCTGCCCAGATCTACTTGCTTTGAG-3') using GeneAmp® PCR system 9700 (Applied Biosystems, CA, USA). The PCR cycling parameters consist of 24 cycles at 95^{o}C for 30s, 55^{o}C for 30s and 68^{o}C for 20min. Final extension at 68^{o}C for 20min was performed. PCR products were analysed by agarose gel electrophoresis and the band of correct size was excised and recovered with QIAquick gel extraction kit following manufacturer's protocol (QIAGEN, Hilden, Germany). The PCR products were cloned immediately upstream of *firefly luciferase* gene via 5' *Mlu* I and 3' *Bgl* II sites in the pGL3-Basic plasmid, a firefly luciferase reporter vector (Promega, Madision, USA). The ligation of digested PCR products (3µl) and linearised pGL3-Basic vector (3µl) was performed using T4 DNA ligase (1µl) (NEB, Ipswich, MA, USA) and incubated at room temperature for 1h or 16°C overnight in a 10µl reaction. The cloning of the -1.2kb *IL23A* promoter was performed by Dominic Voon (Cancer Science Institute of Singapore, Singapore).

2.4.2 Site-directed mutagenesis

Mutant *IL23A* promoter constructs were generated using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Wild-type *IL23A* promoter (-1.2 kb) sequence was used as a template and the PCR reaction mixture was set up as described in section 2.4.1. Primers listed in Table 2.1 were used to mutate putative RUNX and NF-κB binding sites in *IL23A* promoter, as well as to generate truncated versions of *IL23A* promoters by PCR amplification. Compound mutants were generated by mutating individual sites in series. The amplified PCR products were subjected to *Dpn* I (Biolabs, UK) restriction enzyme digestion at 37°C for 4 h to completely digest the methylated parental plasmid DNA and the enzyme was subsequently denatured at 72°C for 20 min. The *Dpn* I digestion was visualised by gel electrophoresis in 0.8 % agarose gels.

Primer	Sequence	Nucleotide position	Description
d1-F	5'-AGGAACTTGAGACCA|GATGCAGGGAGGGGA-3'	-100 to -300	**Regions targeted for deletion**
d1-R	5'-TCCCCTCCCTGCATC|TGGTCTCAAGTTCCT-3'		
d2-F	5'-TCATACTGGCTTCCC|GATTAATTTAAATAT-3'	-300 to -600	
d2-R	5'-ATATTTAAATTAATC|GGGAAGCCAGTATGA-3'		
d3-F	5'-TCCTGGGCTTCCTAG|AGTTCTCCAAGTTCC-3'	-600 to -900	
d3-R	5'-GGAACTTGGAGAACT|CTAGGAAGCCCAGGA-3'		
d4-F	5'-CGAGCTCTTACGCGT|CCATGGGGTCCAAAG-3'	-900 to -1200	
d4-R	5'-CTTTGGACCCCATGG|ACGCGTAAGAGCTCG-3'		
mA-F	5'-GGGAGCCAGCTGTTGGTGCACCGATGGCCT-3'	-1161	**Mutation of putative RUNX sites**
mA-R	5'-AGGCCATCGGTGCACCAACAGCTGGCTCCC-3'		
mB-F	5'-CCAGGCCTCTAGTGTCAGCACACCAAC-3'	-271	
mB-R	5'-GTTGGTGTGCTGACACTAGAGGCCTGG-3'		
mC-F	5'-AGGCCATCGGTGCACCAACAGCTGGCTCCC-3'	-263	
mC-R	5'-GGGAGCCAGCTGTTGGTGCACCGATGGCCA-3'		
mD-F	5'-ACCTGGGCTCCCCCTCGAGGGGGATGATGC-3'	-115	
mD-R	5'-GCATCATCCCCCTCGAGGGGGAGCCCAGGT-3'		
mκB-F	5'-ATGATGCAGGGAGGGACGCGTCACCTGCTGTGAGTC-3'	-90	**Mutation of NF-κB site**
mκB-R	5'-GACTCACAGCAGGTGACGCGTCCCTCCCTGCATCAT-3'		

Table 2.1. List of primers used for mutation and truncation of *IL23A* promoter. Red coloured lines denote the deleted sequences and red coloured letters are the mutated bases. (d: deletion; m: mutation)

2.4.3 Chemical transformation of *Escherichia coli*

Ligated plasmids or *Dpn* I digested products were transformed into 50µl of home-made chemically-competent *Escherichia coli* XL10-Gold strain originally obtained from Stratagene. The mixture was sat on ice for 30min prior heat-shock at 42^{o}C for 80s. The mixture was immediately returned to ice for 5min and shook in 250µl Luria-Bertani (LB) medium at 37^{o}C for 1h. The transformation mixture was subsequently plated onto LB agar plates containing 100µg/ml of ampicillin (Sigma-Aldrich) and incubated overnight at 37^{o}C for selection of successfully transformed bacterial cells. For the screening of positive clones, colonies were picked from the plate and inoculate into 3ml of LB prior shaking at 37^{o}C for 4 hrs. For the purpose of amplifying existing DNA plasmids, the transformed bacteria containing the corresponding plasmids were inoculated directly into conical flasks containing 50ml of LB with ampicillin and incubated overnight for 15h.

2.4.4 Plasmid DNA extraction and purification

Mature cultures of bacteria were harvested by centrifugation at 9000rpm for 15min at 4°C. DNA purification was performed at small-scale of 1.5ml of starter cultures with QIAprep Spin Miniprep columns (QIAGEN) or large-scale of 50ml of overnight cultures with QIAfilter Plasmid Midi/Maxi kit (QIAGEN) according to manufacturer's protocols. DNA was dissolved in 50 µl of elution buffer (10mM Tris-Cl, pH8.5) and quantified using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Plasmid DNA prepared from Midi kits were used for transient transfections subsequently.

2.4.5 Sequencing of plasmid DNA

Clones of recombinant plasmid DNA were fully sequenced using BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems). Each sequencing reaction was made up of 300ng of plasmid DNA, 3.2pmol of the forward or reverse primer (Section 2.4.1), 8µl of Terminator Ready Reaction Mix, and H_2O to a final volume of 20µl. PCR was carried out in 96-well GeneAmp PCR System 9700 (Applied Biosystems). The PCR reaction involves an initial denaturation at 95^{o}C for 3min and 40 cycles at 95^{o}C for 30s, 52.5^{o}C for 10s and 60^{o}C for 4min. Purification and subsequent sequencing of the PCR products were carried out by the Sequencing Facility residing in 1st Base (Singapore).

2.5 Methods for detecting protein-DNA interaction

2.5.1 Electrophoretic Mobility Shift Assay (EMSA)

COS-7 cells were transiently transfected with plasmids encoding Flag-RUNX3, Flag-Empty or CBFβ using FuGENE 6 (Roche) for 24h. Nuclear extracts of transfected cells were prepared using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, MA, USA). The subsequent steps were performed using LightShift® Chemiluminescent EMSA kit (Thermo Scientific). The stated combination of nuclear extracts were incubated with 10μM biotinylated probes containing putative RUNX binding sites (Table 2.2) and 1μg PolydI:dC in 1X EMSA binding buffer (HEPES, pH 7.9) in a 20μl reaction on ice for 30min. For super-shift assays, 0.1 μg anti-FLAG or anti-RUNX3 (5G4) antibody was added in and incubated for another 30min at 4°C. At the end of incubation, loading dye was added to the samples prior electrophoresis in 6% glycerol tolerant polyarylamide gel under native conditions. Next, protein-oligonucleotide complexes were blotted onto Hybond-N+ Nylon membrane (GE Healthcare, UK) followed by UV cross-linking using UVC 500 UV Crosslinker (GE Healthcare). The membrane was blocked by using blocking buffer containing HRP for 30min at RT and washed four times using washing buffer for 10min at RT with shaking. Next, the membrane was incubated in substrate equilibration buffer for 5min prior incubation with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare). Chemiluminescent signals were captured on X-ray medical film (Fuji Photo Film, Tokyo, Japan) using SRX-101A Medical Film Processor (Konica Minolta, USA).

Label	Probe	Oligonucleotide	Sequence
Biotin	Probe_BC	Sense	CTAGCCACAGCAACCACACTACTCATTTCC
		Anti-sense	GGAAATGAGTAGTGTGGTTGCTGTGGCTAG
	Probe_D	Sense	ATACCTGGGCTCCCCACAGAGGGGGATGAT
		Anti-sense	ATCATCCCCCTCTGTGGGGAGCCCAGGTAT
	Probe_mBC	Sense	CTAGTGTCAGCACACCAACTACTCATTTCC
		Anti-sense	GGAAATGAGTAGTTGGTGTGCTGACACTAG
Non-labelled	Probe_BC	Sense	CTAGCCACAGCAACCACACTACTCATTTCC
		Anti-sense	GGAAATGAGTAGTGTGGTTGCTGTGGCTAG
	Probe_mB	Sense	CTAGTGTCAGCAACCACACTACTCATTTCC
		Anti-sense	GGAAATGAGTAGTGTGGTTGCTGACACTAG
	Probe_mC	Sense	CTAGCCACAGCACACCAACTACTCATTTCC
		Anti-sense	GGAAATGAGTAGTTGGTGTGCTGTGGCTAG
	Probe_mBC	Sense	CTAGTGTCAGCACACCAACTACTCATTTCC
		Anti-sense	GGAAATGAGTAGTTGGTGTGCTGACACTAG

Table 2.2. Lists of oligonucleotides designed for EMSA studies. Red coloured letters denote the mutations in the corresponding RUNX sites.

2.5.2 Chromatin immunoprecipitation (ChIP)

Experiment was performed using UpState ChIP Assay Kit (Merck Milipore, Cork, Ireland) following manufacture's protocol. Briefly, THP-1 cells (1 x 10^7) were induced with 1μg/ml of LPS for 24 h and treated with 1% formaldehyde. Cross-linking reaction was quenched by glycine and cells were washed in 1X PBS containing 1X Complete protease inhibitor cocktail (Roche) and 10mM PMSF. Cells were lysed by ionic buffer supplemented with Complete protease inhibitors cocktail and PMSF on ice. The cell lysate was sonicated with 6 sets of 12s on ice using a sonicator (Thermo Fisher Scientific) set at 30% of maximum power, which routinely yields sheared DNA of approximately 500bp in length. Immunoprecipitation (IP) to enrich for RUNX3/DNA complexes was carried out by addition of 5μg of anti-RUNX3 (Active motif, Carlsbad, CA) or anti-CBFβ (kind gift from Dr. Ichiro

Taniuchi) antibodies into the lysate followed by incubation with the pre-cleared Protein G Agarose. Enriched protein/DNA complexes were eluted in $NaHCO_3$ buffer with 20% SDS and reverse cross-linked in 5M NaCl at 65℃ for 4-5h. DNA was purified using QIAGEN spin columns after RNase A (37℃, 30min) and Proteinase K (45℃, 1-2h) treatments. Quantitative RT-PCR was carried out using primer pairs that span across putative RUNX binding sites A, BC, D or BCD, and a region not containing putative RUNX binding site as a negative control. The primers used for ChIP analysis are listed in Table2.3.

Targeted site	Amplified regions	Primers	
RUNX site A	-1211 to -1111	Forward	TTCTCAGAAGAAAAGGGTCAACCT
		Reverse	GGTCCCAGAACAATTTCGAAGA
RUNX site BC	-235 to -145	Forward	CCCCTTCTATAGCTATTTCGATTCCT
		Reverse	GGGAAATGAGTAGTGTGGTTGCT
RUNX site D	-139 to -60	Forward	ACTGAGGCTGCATACCTGGG
		Reverse	AGCAGGTGACTCACAGCAGG
RUNX site BCD	-190 to -119	Forward	TCCCATCCCAGGCCTCTAG
		Reverse	CCCAGGTATGCAGCCTCAGT
Non-specific 1 (NS1)	-708 to -617	Forward	GAGGAGTCAGGTGGTTCTTAGTCAA
		Reverse	GGTCTTATTGCTCCCCATTTCA
Non-specific 2 (NS2)	-2040 to -1975	Forward	GTAGTTCCTGCCACGCAACA
		Reverse	TGCAGGAGTTGGGTAGGAAGA
Non-specific 3 (NS3)	-3169 to -3073	Forward	ATTGGCCCTCTCACTCACTGTAG
		Reverse	GGTGGCCAGTGCCTGTAGTTA

Table 2.3. Oligonucleotide primers and the position of targeted regions for ChIP assay.

2.6 Transcriptomic analysis

2.6.1 Purification of RNA

Cells were washed with phosphate-buffered saline (PBS) and resuspended in 350µl of RLT lysis buffer containing 1% β-mercaptoethanol. RNA extraction was performed using RNeasy Mini Kit and RNase-free DNase Set (QIAGEN) following manufacturer's protocol. Briefly, cell lysates were homogenised by vortexing for 1 minute, followed by addition of 70% ethanol and samples were transferred to RNeasy spin column. On-column digestion of genomic DNA was carried out using 80µl of RNase-free DNase I set (QIAGEN). The spin column was then washed with buffers RW1 and RPE, followed by a final spin to remove residual ethanol. RNA was subsequently eluted in 30-50µl of RNase-free water and quantified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

2.6.2 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Complementary DNA (cDNA) was synthesized from 0.2-1.0µg of total RNA using Omniscript reverse transcription (RT) kit (QIAGEN) in a T3 Thermocycler (Biometra, DE UK). The reaction mixtures were incubated at 37^{o}C for 70min and the Omniscript reverse transcriptase was inactivated at 95^{o}C for 5min. Quantitative PCR was performed in 7500 Real-time PCR system using 1µl of cDNA and TaqMan Universal PCR Master Mix or Power SYBR Green Master Mix (Applied Biosystems). TaqMan gene expression probes for human *IL23A/p19, IL12B/p40, RUNX3, RUNX1* and *GAPDH* (endogenous control) are Hs00900829_g1, Hs99999037_m1, Hs00231709_m1, Hs00231079_m1 and Hs99999905_m1 respectively. The gene-specific oligonucleotide primers used for SYBR Green-based

measurements are listed in Table 2.4. For the analyses of TaqMan and SYBR Green RT-PCR results, the threshold cycle (C_T) values of genes of interest were normalized against corresponding C_T value of *GAPDH* to account for variations in cDNA input.

SYBR Green Primers		
Gene	**Forward**	**Reverse**
IL12A	GCAGGCCCTGAATTTCAACA	CATGAAGAAGTATGCAGAGCTTGATT
IL27A	ATCTCACCTGCCAGGAGTGAA	TGAAGCGTGGTGGAGATGAAG
EBI3	GGGGAACTGAGTGACTGGAG	AGTCGGTCATCTGAGGTTGC
IL8	TCACTGTGTGTAAACATGACTTCCA	TGGCAAAACTGCACCTTCAC
TaqMan Probes		
Gene	**TaqMan ID**	
GAPDH	Hs99999905_m1	
IL23A	Hs00900829_g1	
IL12B	Hs99999037_m1	
RUNX3	Hs00231709_m1	
RUNX1	Hs00231079_m1	

Table 2.4. Gene-specific oligonucleotide primers and TaqMan primers used in quantitative RT-PCR.

2.7 SDS polyacrylmide gel electrophoresis (PAGE) and Western blot

Cells harvested from culture were washed with 1x PBS and spun down as cell pellets. Ionic buffer supplemented with 1x cOmplete protease inhibitor (Roche) and 10mM PMSF was used to lyse the cells by resuspending the pellets. Passive lysis of cells was performed on ice for 15 mins followed by three cycles of freeze-thaw with vortex to completely lyse the cells. Samples were centrifuge at 10,000 rpm for 10mins at 4^{o}C to collect the cell debris. Cell lysates were then transferred into fresh ependoff tubes and the amount of protein was quantified using Bradford Assay. Briefly, 2µl of cell lysate was mixed with 1998µl of 1X Bradford dye in cuvette and incubate at RT for 5min prior assaying at 595nm wavelength using GeneQuant 1300 spectrophotometer (GE Healthcare). After quantification, amount of protein was normalized against the most diluted sample, and SDS-containing loading buffer was added before boiling for 5mins on heat block for protein denaturation. To cool down the samples after boiling, denatured protein lysates were centrifuged at 10,000 rpm for 5min at RT. Using 10 – 15% PAGE gel, 50µg of protein was loaded into each well and electrophoresed at 200V and 40mA (for one gel) for approximately 1h. Precision Plus Protein^{TM} standards (Biorad, CA, USA) was used as protein marker. Proteins were transferred onto a PVDF membrane (Biorad) at constant 100V for 1 h 45 min. After transfer, membrane was blocked using 5% skim milk in PBST (PBS + 0.1% Tween) (Fonterra, AU, New Zealand) for 30 min – 1h before overnight primary antibody incubation at 4 ^{o}C. Detection of IL23A protein was performed using anti-IL23A antibody (eBioscience) at 250x dilution and RUNX3 protein was detected using anti-RUNX3 5G4 antibody at 1µg/ml (kind gift from Kotaro Tada). For the detection of housekeeping protein, α-tubulin specific antibody (Sigma-Aldrich) was used at 50000x dilution. Membranes were washed 3x with PBST buffer at RT

followed by incubation with HRP-conjugated anti-mouse secondary antibody (GE Healthcare) for 1h. After washing off excess secondary antibody, membranes were incubated with ECL reagent (GE Healthcare) for 5mins and chemiluminescent signals were captured as described in section 2.5.1.

2.8 Functional assays

2.8.1 Harvesting mononuclear cells from human peripheral blood

Human peripheral blood was purchased from NUH Blood Donation Centre and different types of blood cells were separated by gradient centrifugation. Briefly, 25ml of blood was carefully layered on Ficoll-Paque PLUS with 1.077g/ml density (GE Healthcare). Blood cells were fractionated by centrifuging at 2400rpm for 30min with slow acceleration and deceleration. Human peripheral blood will be separated into various layers including plasma, mononuclear cells, granulocytes and red blood cells (RBC). After discarding the top-most layer of plasma, peripheral blood mononuclear cells (PBMC) were harvested using Pasteur pipette and transferred into a fresh tube. PBMCs were washed in 1X PBS containing 2mM EDTA, and centrifuged at 2100rpm for 10mins. RBCs were lysed by RBC lysis buffer prior centrifuging at 1500rpm for 5min. Supernatant was discarded and PBMCs were resuspended in RPMI supplemented with 10% FBS and penicillin/streptomycin. Subsequently, 0.1 – 0.2 x 10^6 PBMCs in 100μl of fresh medium were seeded into a 96-well round bottom plate and incubated with equal volume of culture media harvested from gastric epithelial cells together with anti-CD3 antibody (clone OKT3) at 1.1ng/ml (suboptimal dose). PBMC were then cultured for three or seven days before analysis by flow cytometery.

2.8.2 Neutralisation of IL23A in culture supernatant

For the neutralization of secreted IL23A protein, 6-well plates were coated with 2μg/ml of functional grade anti-IL23A antibody (eBioscience) or anti-mouse IgG (Santa Cruz) overnight at 4°C. The coated wells were washed once by 1X PBS to

remove unbound antibodies. Culture supernatants harvested from gastric epithelial cells were incubated on the coated wells for 2h at 37°C. The above step was repeated for another time by transferring the treated supernatants onto a fresh coated well. Supernatants were then collected and stored at 4°C for downstream immunological assays.

2.8.3 Cell proliferation assay

This assay was performed using CellTrace carboxyfluorescein diacetate, succinimidyl ester (CFSE) cell proliferation kit (Invitrogen) following manufacturer's protocol. PBMCs harvested from human peripheral blood were re-suspended in pre-warmed PBS with 0.1% BSA at a final concentration of $1x10^8$ cells/ml. PBMCs were stained with 10μM/ml of CFSE solution at 37°C for 10min. The reaction was quenched by adding five volumes of ice-cold culture media to the cells followed by 5min incubation on ice. Cells were concentrated by centrifugation (1500rpm, 5min) and washed 3 times by re-suspending the pellet in fresh media. *In vitro* culture of the PBMCs was set up and PBMCs were harvested at indicated time points. Cells were analysed using flow cytometer LSRII Special Order (BD Biosciences, San Jose, CA) with 488nm excitation and emission filters appropriate for fluorescein. Flow cytometry data were analysed using FlowJo computer software (Tree Star, OR, USA).

2.8.4 Enzyme-linked immunosorbent assay (ELISA)

Culture media of cells induced by the stated stimulants were harvested and stored at -20°C before use. ELISA was performed using IL-23, IFN-γ and IL-17 ELISA Ready-SET-Go kit (eBioscience) following manufacture's protocol. Briefly, transparent 96-well UV microplate (Corning, NY, USA) was coated with capture

antibody at 250x dilution at 4°C overnight. Unbound antibodies were removed by five washes of 1x PBST (PBS + 0.05% Tween). The wells were then blocked with 200µl 1x assay diluent for 1h at RT. The wells were washed five times using 1x PBST prior incubating with 100 – 200µl of stimulated culture media for 2h at RT. Fresh medium was used as a negative control. After incubation, supernatants were aspirated and wells were washed with 1x PBST followed by incubation with detection antibody at 250x dilution for another hour at RT. After binding of detection antibody, wells were incubated with avidin-HRP at RT for 30 mins in dark. The wells were washed up to seven times with 1x PBST for 2min each. After adding 100µl of Substrate Solution for 15min at RT, the reaction was quenched by adding 50µl of Stop Solution (1M H_2SO_4) and absorbance was measured at 450nm wavelength using microplate reader (Tecan, Männedorf, Switzerland).

2.9 Statistical analysis

The data are presented as with SEM. When two data sets were compared, the Student's *t*-test was used and *p*-values <0.05 are considered significant.

Chapter 3:

IL23A is a genuine target gene of RUNX3 in gastric epithelial cells

3.1 Introduction

3.1.1 Transcription regulatory mechanisms

Transcription initiation is a key node of eukaryotic gene expression. Research in unicellular and multicellular organisms has amply demonstrated the prominence of transcription factors in controlling development and homeostasis (Davidson, 2001). In mammalian system, gene regulation involves a complex interplay between activators, repressors, the general transcription machinery, and chromatin (Oelgeschlager et al., 1996). This intricate task is controlled by multiple different transcription factors that bind to specific DNA sequences and modulate the activity of RNA polymerase. The *cis*-acting regulatory sequences upstream of a gene where transcription factors assemble is termed promoters and enhancers (Blackwood and Kadonaga, 1998). These regulatory regions are encompassed by chromatin in the cells where alteration or "remodelling" of which is required to permit the access of transcriptional regulators.

Transcription factors are often grouped into families according to the sequence and structure of their DNA-binding domains. The RUNX family of transcription factors are one such example where the name originates from its DNA-binding domain termed the Runt domain (Kamachi et al., 1990). This domain defines the binding of RUNX to its unique *cis*-regulatory elements and therefore its contribution to differential gene regulation. Thus, in general, understanding how transcription factors distinguish and bind to specific sites is a crucial step for the investigation of gene expression. However, due to the limitations of current technology in performing a systematic mutational analysis of an endogenous gene *in vivo*, artificial assays are often employed.

The most widely-used functional assay for promoter analysis is genetic reporter assay (Brasier et al., 1989; Bronstein et al., 1996; de Wet et al., 1987; Wood, 1995). Eukaryotic promoter sequences are typically ligated to a reporter gene that encodes an easily detectable enzyme, e.g. the firefly luciferase (*luc*), that acts as a surrogate reporter of transcriptional activity in the form of luminescent signal (Wood, 1995). The expression of the reporter gene following its transfer into cultured cells provides a rapid and sensitive assay that quantifies the ability of the cloned promoter sequences to direct transcription. Through this approach, biologically active regulatory regions can thus be identified, and *in vitro* mutagenesis can be used to determine the roles of specific sequences within the cloned region.

After defining the DNA sequence elements responsible for regulating a gene, one can further demonstrate the specific binding of the transcription factors to the functional sites. One biochemical approach to analyse protein-DNA interaction is to perform electrophoretic-mobility shift assay (EMSA) by using synthesised oligonucleotides comprising the functional binding sites (Fried and Crothers, 1981; Garner and Revzin, 1981). Binding of the protein-of-interest to DNA can be studied by the formation of retardation complexes during electrophoresis in a non-denaturing gel. To determine the specificity of the protein-DNA interaction for the transcription factor-of-interest, an antibody that is specific to the transcription factor can be added to create an even larger complex with a greater retardation or 'shift' in the electrophoresis. This method is referred to as a supershift assay, and is used to unambiguously define the presence of a specific protein-DNA complex.

The approaches described in the preceding paragraphs study are commonly employed to study the transcriptional regulation of genes on naked DNA. However, this is not the case for transcriptional regulation inside the cells where the DNA of all

eukaryotic cells is tightly bound to histones that constitute the chromatin. Thus, reporter gene assay needs to be complemented by studying the direct products of transcription, messenger RNA (mRNA), which are representative of endogenous promoter activity. In addition, EMSA experiments ought to be reinforced by accessing the *in vivo* promoter occupancy at the chromatin level *in vivo*. One of the frequently used assays for such purpose is chromatin immunoprecipitation (ChIP) (Bauer et al., 2002; Gilmour and Lis, 1985; Jackson, 1978; Solomon and Varshavsky, 1985). This method ascertains the physical association of a known transcription factor with a specific genomic region in living cells. The protein-DNA interaction is often more complex at the genomic level as the binding of transcription factors on the regulatory sequences may be influenced by the presence of other transcription factors or co-factors.

3.1.2 RUNX3: a transcription factor with tumour suppressor functions

Cancer is caused by the accumulation of defects in many genes, especially those involved in important pathways, as a function of time, leading to an invasive malignancy. The hallmarks of cancer cells encompass a combination of dysregulated signaling pathways such as those involved in growth and death signals. Ultimately, transcription factors participate in all of these pathways by effecting the upregulation or downregulation of specific genes. Crucially, a lot of proto-oncogenes, oncogenes and tumour suppressor genes encode for transcription factors, for example *p53*, *c-Myc*, *ETS-1*, *STAT* and *AP-1*. The RUNX family proteins, which were discovered as context-specific transcriptional regulators, possess tumour suppressive functions in multiple tissues. Among all three RUNX members, the biological roles of RUNX3 appear to be the most diversified and complex. It is well-established that RUNX3 is

involved in neuronal development and immune cell functions, and acts as an important tumour suppressor in gastric epithelial cells (Section 1.2.3 and 1.2.4).

Similar to other RUNX proteins, RUNX3 achieves its roles by specific interaction and partnership with other transcriptional regulators and signaling pathways. In the gastrointestinal epithelium, RUNX3 functions as an important regulatory node for both the tumour suppressive TGF-β pathway and oncogenic Wnt signaling (Hanai et al., 1999; Ito et al., 2008; Ito and Miyazono, 2003). Being an integral component of the TGF-β signaling pathway, RUNX3 cooperates with SMADs in gastric epithelial cells to activate the transcription of target genes such as $p21^{WAF/Cip1}$ (Chi et al., 2005). On the other hand, RUNX3 acts as an attenuator of the canonical Wnt pathway through direct interaction with β-catenin and TCF4 thereby down-regulating Wnt target genes *c-Myc*, *cyclinD1*, *EphB2* and *CD44* (Ito et al., 2011; Ito et al., 2008). However, due to a restricted number of target genes identified to date, the full spectrum of RUNX3's role as a tumour suppressor remains to be explored (section 1.4.1).

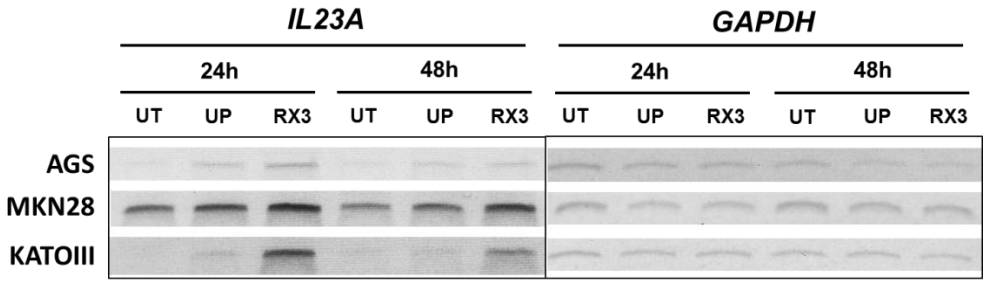
Identification of target genes often expands the understanding of known biological roles of a transcription factor and may reveal unappreciated and unexpected functions. By employing the cDNA expression microarray technology (section 1.4.2), cytokine gene *IL23A* was identified as a high-confidence candidate target gene of RUNX3. This interesting observation points to a novel immune-related function of RUNX3 in gastric epithelial cells. To explore this possibility, experiments described in this chapter were designed to establish the transcriptional regulation of *IL23A* by RUNX3.

3.2 Results

3.2.1 Validation of the regulation of *IL23A* by RUNX3 in gastric cancer cell lines

IL23A was identified as a putative RUNX3 target gene in AGS cells using cDNA expression microarray technology (Section 1.4.2). To validate the above observation, RUNX3 was transiently and ectopically expressed in a panel of three RUNX3-negative gastric epithelial cell lines including KATOIII, MKN28 and MKN7. In addition, AGS cell line was included to act as a biological replicate of the microarray experiment. By semiquantitative and quantitative RT-PCR, the induction of *IL23A* expression by ectopic RUNX3 was observed in five out of six cell lines (Figure 3.1A and 3.1B). The conservation of this regulation in multiple gastric cancer cell lines of heterogenous origins provides compelling evidence that RUNX3 is indeed a positive regulator of *IL23A* in gastric epithelial cells.

A.



B.

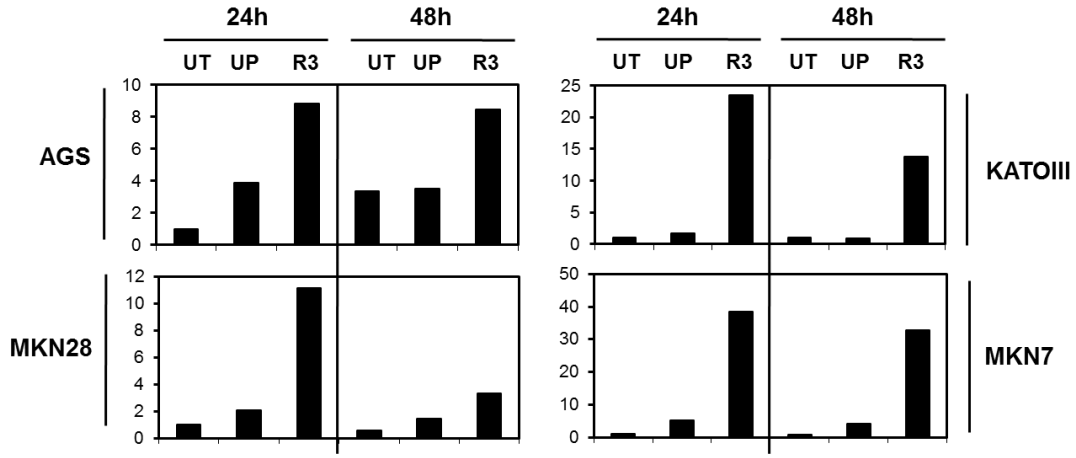


Figure 3.1. RUNX3 upregulates *IL23A* in multiple gastric cancer cell lines. Four RUNX3-null gastric epithelial cell lines were transiently transfected with RUNX3-expression vector for 24 and 48 hours. *IL23A* transcripts were ascertained using (A) semiquantitative and (B) quantitative RT-PCR. The qRT-PCR values of *IL23A* were normalised with those of *GAPDH* and expressed relative to the untransfected control 'UT'. 'UP' denotes 'unrelated protein' and 'R3' denotes RUNX3. (N.A.: not available)

3.2.2 RUNX3 activates *IL23A* in a DNA-binding dependent manner

To further investigate the positive regulation of *IL23A* by RUNX3, exogenous wildtype RUNX3-, RUNX3^{R178Q}- and Control-expressing vectors were stably reintroduced into RUNX3-negative AGS cells by lentiviruses. RUNX3^{R178Q} bears an amino acid substitution of arginine to glutamine in the Runt domain of RUNX3 and is defective in DNA-binding (Inoue et al., 2007). Ectopic expression of wildtype RUNX3 (Lenti-RUNX3) in AGS cells led to a 20-fold increase in *IL23A* mRNA expression relative to the control-infected (Lenti-Control) sample (Figure 3.2). This marked increase was not observed for stable expression of RUNX3^{R178Q} (Lenti-R178Q) in AGS cells, indicating that the induction of *IL23A* is dependent on RUNX3's DNA-binding activity (Figure 3.2). As *IL23A* is a subunit of IL-23 belongs to the IL-12 family and closely resembles *IL12A*, the positive role of RUNX3 on all other IL-12 family members was examined. Surprisingly, the activating effect of RUNX3 was only observed for *IL23A* but no other family members of IL-12 suggesting that RUNX3's effect is unique to *IL23A* (Figure 3.3).

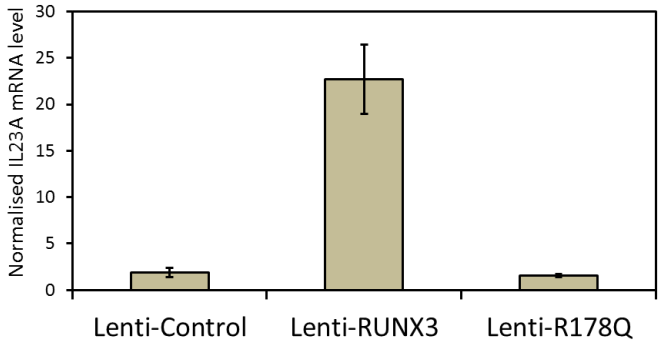


Figure 3.2. RUNX3 activates *IL23A* transcript in a DNA binding-dependent manner. AGS cells were stably transduced with lentiviruses expressing either control (Lenti-Control), RUNX3 (Lenti-RUNX3) or RUNX3^{R178Q} (Lenti-R178Q). Cells were harvested 48h post-infection and subjected to qRT-PCR. The values of *IL23A* transcripts were normalised with those of *GAPDH* and expressed relative to Lenti-Ctrl. Data are presented as means ± SEM (n=3).

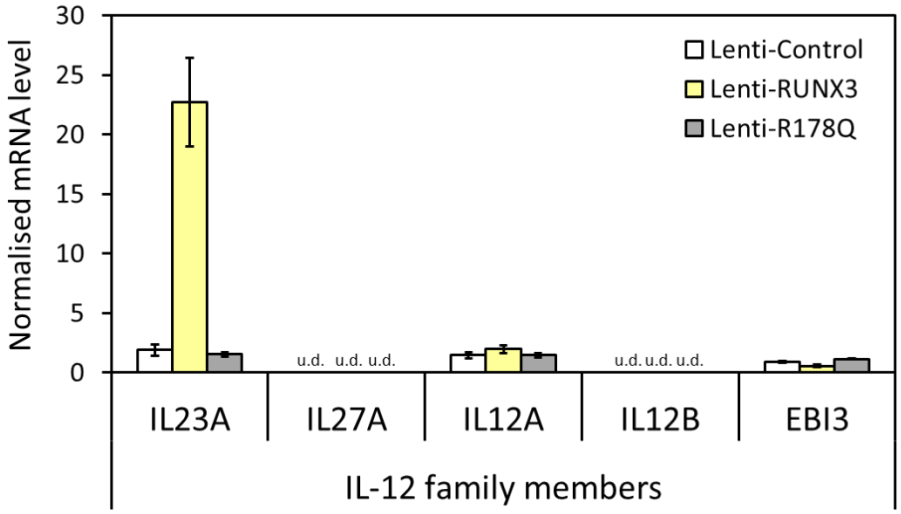


Figure 3.3. The activating effect of RUNX3 is unique to *IL23A*. AGS cells were infected with the same series of lentiviruses described in Figure 3.2. Forty-eight hours post-transduction, cells were harvested for RNA extraction prior to cDNA synthesis. The expression of *IL23A, IL27A, IL12A, IL12B* and *EBI3* transcripts were ascertained by qRT-PCR. The values were normalised with those of *GAPDH* and expressed relative to Lenti-Control calculated by ΔΔCt method. Data are presented as means ± SEM (n=3). (u.d.: undetectable)

3.2.3 Putative RUNX binding sites in *IL23A* promoter

Given the requirement of RUNX3's DNA-binding ability for its activation of *IL23A*, the presence of RUNX3-responsive elements in the *IL23A* gene locus was determined. The promoter region of the *IL23A* gene from nucleotide (nt) -1250 to the transcriptional start site were first analysed using the computer programmes, TESS and TFSearch, against the online transcription factor consensus binding site database TRANSFAC (Heinemeyer et al., 1998). Among all the predicted transcription factor binding sites, four putative RUNX sites from nt-1161 to -1156 (site A), nt-171 to -166 (site B), nt-163 to -158 (site C) and nt-115 to -110 (site D) were identified as shown in Figure 3.4A. These putative RUNX sites were further categorised as "regular" and "sub-optimal" to reflect their resemblance to the strict RUNX consensus binding sequence (Figure 3.4B). To demonstrate the functional relevance of these sites, the -1200 to +105 upstream region of *IL23A* locus was cloned into a firefly luciferase reporter construct as shown in Figure 3.4B.

A.

```
-1250 aatagctcta agggaaggga gacctccatt caggacaagt ttctcagaag
-1200 aaaagggtca acctcttggg ggaggctttg ggagccagct gtgtggtcac
-1150 cgatggcctc attctgacgt cttcgaaatt gttctgggac cctccactgg
-1100 ggtcggggca gtcccggctt tggaccacct tccactccca cgcccaacct
-1050 cacactctta gctgtttcac tcgatgttgc atcatggagg gtgatgaaat
-1000 cggtgtcagt ggattttacc catggatgca acaagctgaa ggaccagcca
 -950 gagtcattga cagtgcacct tcgactaccc agaactcctg ggcttcctag
 -900 ccatggggtc caaagctggg actgccccga ccccagtgga gggtcccaga
 -850 acaatttgga tgacgtcaga atgaggccat gggactaggt gctggaatgt
 -800 ctaagttgaa cttccaggcc ttatttgcac tagtcctgaa aaaaacatca
 -750 tccaactctt atagagccta tgaaatcttg ggccactagg gttgaggagt
 -700 caggtggttc ttagtcaata accctcttcc cacaagagcc tttctaacct
 -650 ccactgtgag gcctgaaatg gggagcaata agacctcata ctggcttccc
 -600 agttctccaa gttccttcat gcgcattctc tcccatgaaa ccaggaccat
 -550 ccagttgaaa taatgttgtt tccaactgag aaaaagaagc ccgtttattc
 -500 ctaatagggg gcatcaggta ggaatcaaac ttcattgcaa acagctcacc
 -450 atcctattgg gagatgaatg gatgtttctc tgttttgctt tttcctcaag
 -400 caggaggaag tgaggaaatt aggtttgggg tggggtaggg gtatagcttt
 -350 gagaggcaaa aagatcaggg aaagatcaaa caggaaggaa cttgagacca
 -300 gattaattta aatatttgtt ctcccttacc cctcccaccc catccccgct
 -250 gtgcccccca tccccgcccc ttctatagct atttcgattc ctggagagca
 -200 ttacacatgt gtcccatccc aggcctctag ccacagcaac cacactactc
 -150 atttcccctg gaactgaggc tgcatacctg ggctccccac agaggggat
 -100 gatgcaggga gggaatccc acctgctgtg agtcacctgc tggTATAAAg
  -50 ggcgggcctt acaatgcagg gaccttaaaa gactcagaga caaagggaga
    1 AAAACAACAG GAAGCAGCTT ACAAACTCGG TGAACAACTG AGGGAACCAA
   51 ACCAGAGACG CGCTGAACAG AGAGAATCAG GCTCAAAGCA AGTGGAAGTG
  101 GGCAGAGATT CCACCAGGAC TGGTGCAAGG CGCAGAGCCA GCCAGATTTG
```

B.

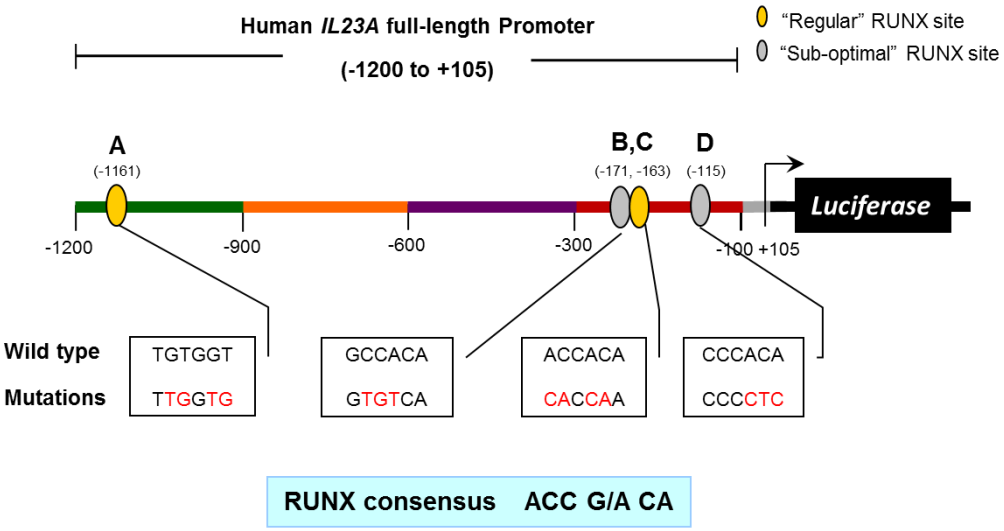


Figure 3.4. Sequences of the human *IL23A* promoter and the cloned *IL23A* promoter region in the luciferase reporter construct. (A) Sequence of the *IL23A* promoter from the -1200 to +150 was shown. Four putative RUNX consensus sites, identified using TFSearch program (Heinemeyer et al., 1998), were boxed in the promoter region. A putative NF-κB site was highlighted in black; a TATA-like sequence was marked in red and a transcriptional start site was highlighted in yellow. (B) Schematic diagram of the *IL23A* promoter firefly luciferase reporter construct. The putative RUNX sites were classified into regular RUNX sites (A and C) and sub-optimal RUNX site (B and D). The RUNX sites were mutated individually and in series as depicted in the boxes. The red letters in the callouts represent the mutated bases.

3.2.4 Mapping of RUNX3-responsive elements in the *IL23A* promoter

Given the clear induction of *IL23A* by RUNX3 observed in the RUNX3-null gastric epithelial cell line, KATOIII (Figure 3.1), this cell line was chosen as a cell model to study the effect of RUNX3 on the cloned full-length (1.2kb) *IL23A* promoter in the luciferase reporter assay. Transient transfection of exogenous RUNX3 led to ~6-fold increase in activity of the full-length *IL23A* promoter (Figure 3.5). This result suggests that functional RUNX binding site(s) are present within this cloned *IL23A* promoter. To identify the RUNX3-responsive elements in the *IL23A* promoter, a series of deletions were performed on the full-length *IL23A* promoter to generate truncated *IL23A* promoter fragments. The full-length promoter was divided into Regions 1, 2, 3 and 4 as depicted by the different colours in which Region 4 contained RUNX site A while the other three RUNX sites were located in Region 1 (Figure 3.5). The results showed that the deletion of Region 1 of the promoter completely abolished the induction of *IL23A* by RUNX3. Conversely, only subtle changes were observed when Regions 2, 3, or 4 were deleted despite the removal of RUNX site A (Figure 3.5). This observation indicates that site A is dispensable to RUNX3's effect on *IL23A* promoter activity in *in vitro* reporter assays. The complete loss of RUNX3-mediated induction following the deletion of the most proximal region (Region 1) clearly indicates that RUNX3 acts via the *cis*-acting elements within this promoter region (Figure 3.5).

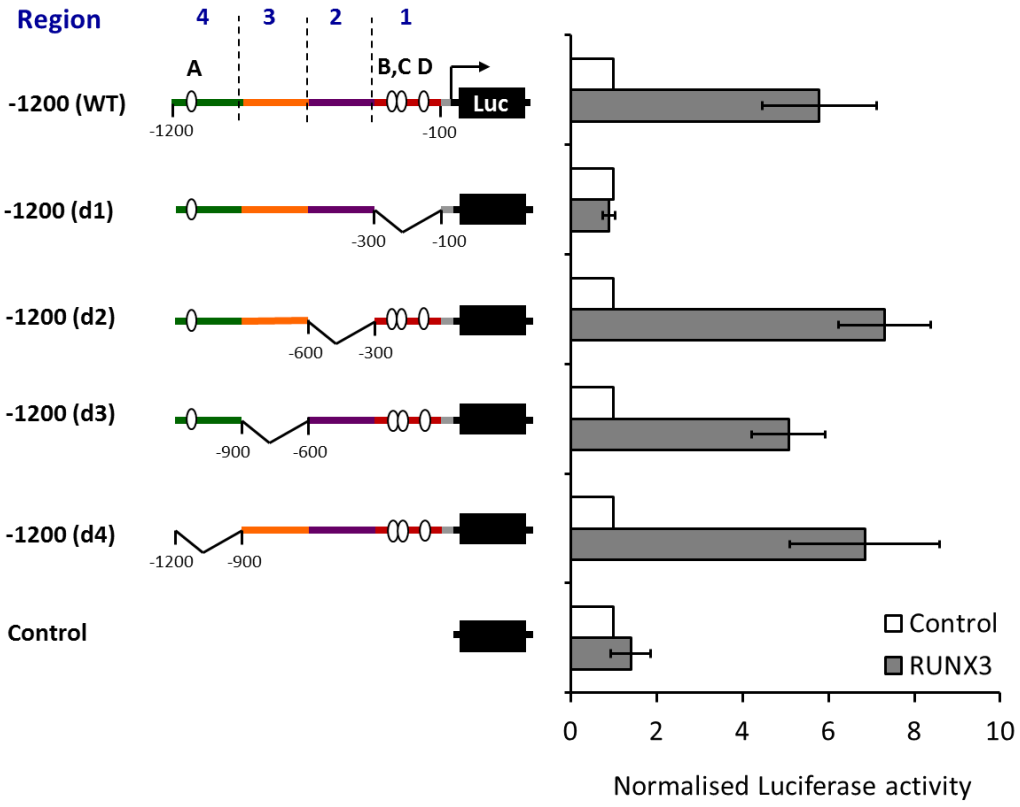


Figure 3.5. RUNX3 mediates its effect through Region 1 (-300 and -100) of *IL23A* promoter. A series of deletions of the -1200 to +105 *IL23A* promoter construct was created as depicted by the schematic diagrams on the left. The truncated promoters were transiently transfected into KATOIII cells together with either control or RUNX3 expression vector for 48 h. The firefly luciferase activities of the transfected constructs were then measured and normalised against the internally co-transfected Renilla luciferase reporter. Oval shape on the promoter indicates the RUNX binding sites and V shape on the promoter indicates deletion of the corresponding region. (d: deletion) (means ± SEM)

3.2.5 Identification of functional RUNX binding sites

To further pinpoint the RUNX sites responsible for transactivation of *IL23A* promoter activity by RUNX3, a series of point mutations were introduced into individual RUNX sites as depicted in Figure 3.4B. These mutations were generated initially into a reporter construct containing only the minimal region (Region 1) needed for RUNX3's transactivating activity. Within the context of this minimal *IL23A* promoter, mutations of sites BC or site D led to a modest decrease in RUNX3-responsiveness (Figure 3.6A). Complete abolishment of RUNX3's activating effect was only observed in the triple RUNX sites mutant promoter. These results clearly suggest that all three RUNX sites were requisite for the activation of the minimal *IL23A* promoter by exogenous RUNX3 (Figure 3.6A). Lastly, the contribution of these three RUNX sites was studied in the context of the full-length *IL23A* promoter. Mutations of the three putative RUNX sites within Region 1 effectively abolished the ~7-fold induction of the *IL23A* promoter activity by RUNX3, which was comparable to the truncated promoter activity bearing a deletion of the Region 1 (Figure 3.6B). These observations are consistent with the changes observed in the minimal *IL23A* promoter (Figure 3.6A and B). In summary, transactivation of the *IL23A* promoter by exogenous RUNX3 is mediated through sites B, C and D within the minimal promoter region (Region 1).

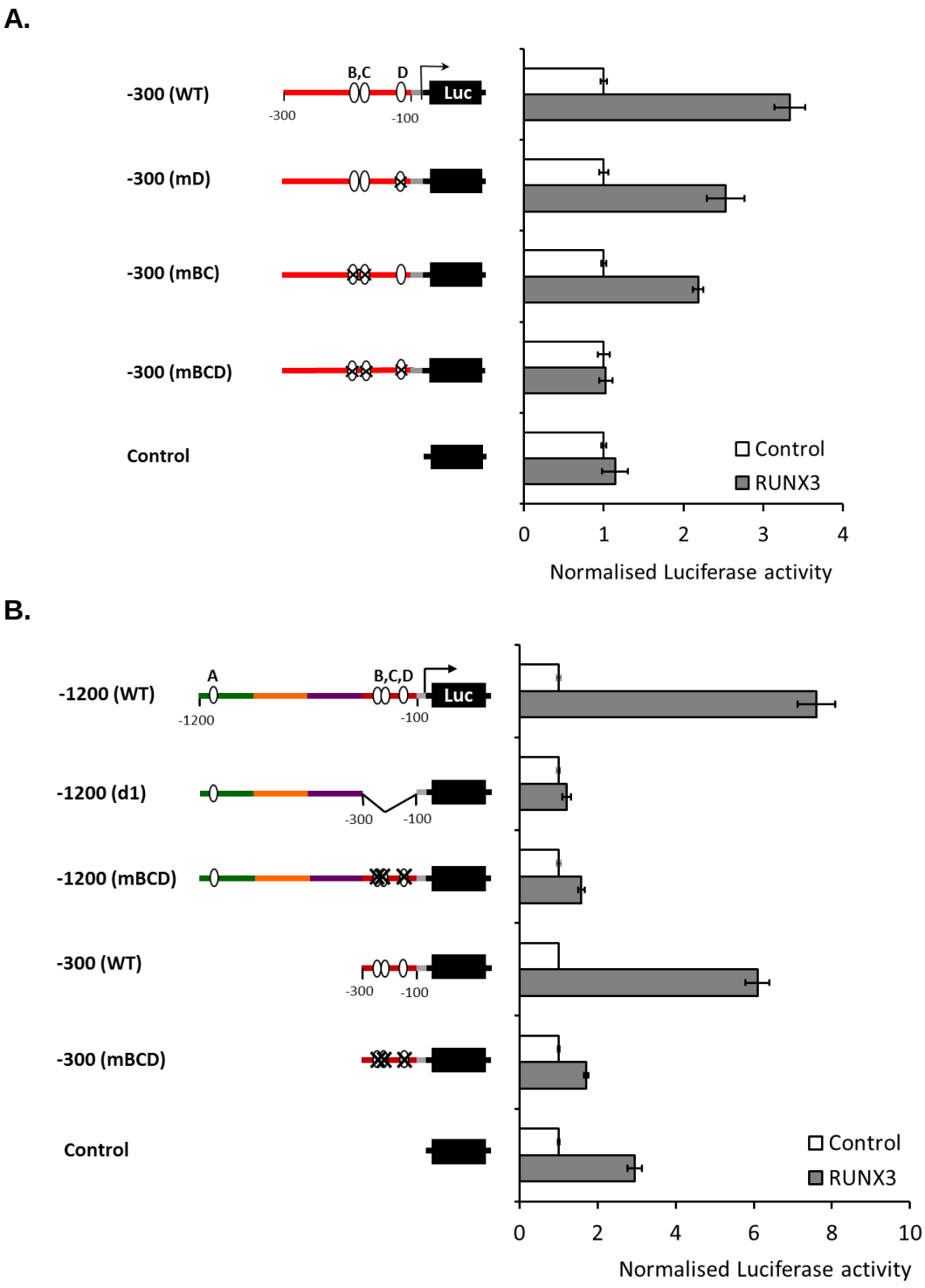


Figure 3.6. RUNX3 transactivates *IL23A* promoter through the three proximal RUNX binding sites. (A) *IL23A* minimal promoter constructs from which mutations of individual or multiple RUNX sites were generated as depicted in the schematic diagram on the left. These constructs were transfected into KATOIII cells together with either Control or RUNX3 expression vector. Cells were harvested 48h post-transfection for the measurment of the luciferase assay. (B) Compund RUNX sites mutants of full-length and minimal *IL23A* promoter constructs generated as shown in the diagram on the left. These constructs were transfected into KATOIII cells together with either Control or RUNX3 expression vector. The firefly luciferase activities of the transfected constructs were measured and normalised against the internally co-transfected Renilla luciferase reporter. The data presented are compilation of three independent experiments. On the promoter, oval shapes indicate the RUNX binding sites, V shape indicates deletion of the corresponding regions and **X** indicates mutation of the respective RUNX site. (d: deletion; m: mutation) (means ± SEM)

3.2.6 Involvement of RUNX1 in the regulation of the *IL23A* promoter

Another RUNX family member that is expressed in normal gastric epithelium is RUNX1 (Sakakura et al., 2005; Usui et al., 2006). Similar to RUNX3, significant downregulation of RUNX1 was observed in gastric tumour tissues compared to the surrounding mucosa (Sakakura et al., 2005). As RUNX proteins share common DNA binding sequences, the potential role of RUNX1 in the regulation of *IL23A* was also examined. Similar to the exogenous RUNX3, ectopic expression of RUNX1 in both KATOIII and SNU16 cells led to an increase in the full-length *IL23A* promoter activity (Figure 3.7). Not surprisingly, RUNX1-mediated activation was ablated when the binding sites were disrupted (Figure 3.7). These results indicate that RUNX1 is a positive regulator of *IL23A* promoter in addition to RUNX3. Interestingly, the co-expression of RUNX1 and RUNX3 led to an additive increase in the induction of *IL23A* promoter activity suggesting that RUNX3 and RUNX1 may play complementary role through the same binding sites (Figure 3.7).

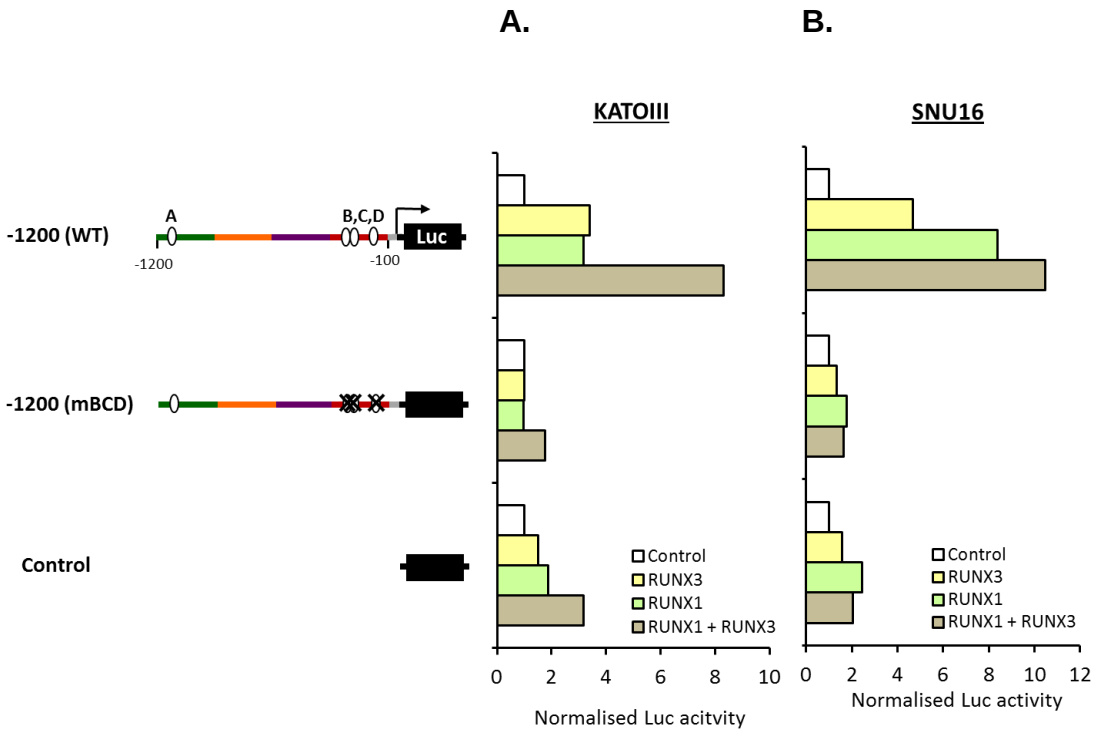


Figure 3.7. Both RUNX1 and RUNX3 transactivate *IL23A* promoter in gastric epithelial cells. Gastric epithelial cell lines (A) KATOIII and (B) SNU16 were transiently transfected with Control, RUNX3 and/or RUNX1 expression vectors together with full-length *IL23A* reporter constructs for 48h. Cells were harvested and the firefly luciferase activities of the transfected constructs were measured and normalised against the internally co-transfected Renilla luciferase reporter.

3.2.7 RUNX3 physically interacts with *IL23A* promoter *in vitro*

Having identified the three functional RUNX sites in *IL23A* promoter, the binding of RUNX3 on these sites *in vitro* were determined by electrophoretic mobility shift assays (EMSA). Biotinylated oligonucleotide probes containing consensus RUNX sites B and C (Probe_BC) and site D (Probe_D) were synthesised and incubated with nuclear extracts of COS-7 cells expressing either exogenous FLAG-RUNX3 or unfused FLAG-tag as a control (i.e. Mock). The incubation of Probe_BC with nuclear extract from untransfected COS-7 cells resulted in the appearance of several retardation (EMSA) complexes (lane 2 in Figure 3.8A), suggesting that endogenous proteins present in COS-7 cells were capable of binding to this probe. As such, these bands were regarded as 'non-specific' bands, though the possibility that

they contained endogenous RUNX proteins cannot be ruled out. When Probe_BC was incubated with COS-7 nuclear extracts overexpressing exogenous RUNX3, additional retardation complexes were observed (lanes 2 and 3 in Figure 3.8A). This shows that exogenous RUNX3 proteins display an affinity to sites B and C.

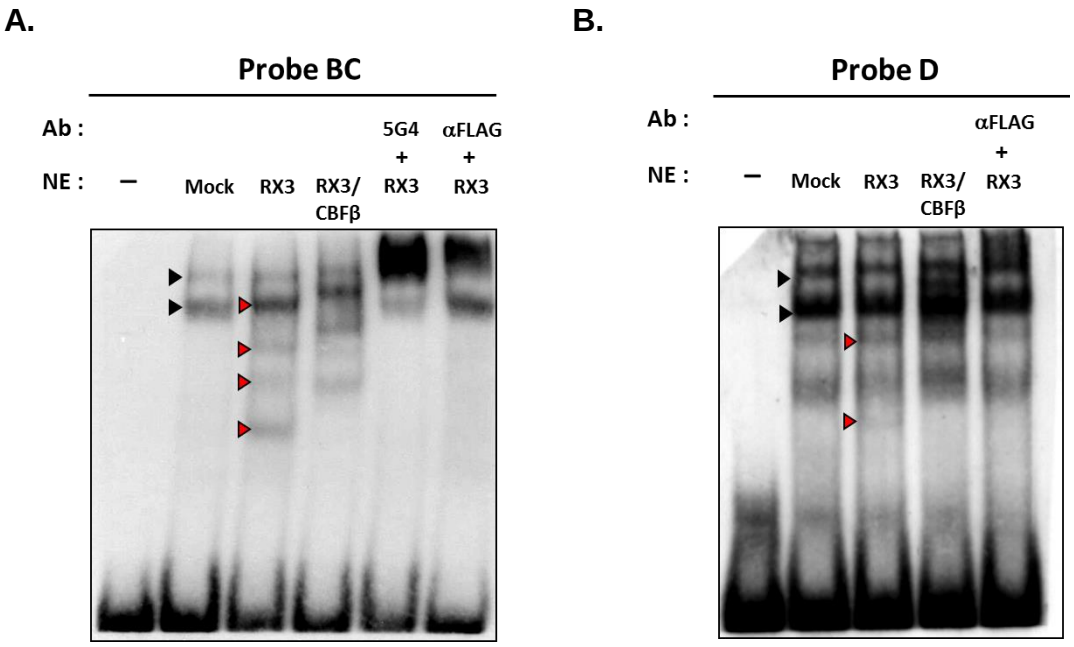


Figure 3.8. The bindng of RUNX3 to RUNX sites B, C and D of the *IL23A* promoter determined by EMSA. (A) Biotinylated Probe_BC and (B) Probe_D were mixed with nuclear extracts of COS-7 cells expressing exogenous FLAG-RUNX3 or FLAG-tag as a control (Mock). Supershift assays were performed by incubating the probes with nuclear extracts of COS-7 cells expressing FLAG-RUNX3 in the presence of exogenous CBFβ, anti-RUNX3 (5G4) and anti-FLAG monoclonal antibodies. The above samples were resolved in a non-denaturing polyacrylamide gel for analysis of the presence of protein-DNA complexes. (◗ :RUNX3-specific complex; ◗ non-specific complex)

To verify the identity of the RUNX3-related EMSA complexes, super-shift assays were performed. The addition of COS-7 nuclear extracts containing the binding partner of RUNX proteins, CBFβ together with RUNX3, resulted in a further retardation (i.e. super-shift) of the RUNX3-related EMSA complexes (lanes 3 and 4 in Figure 3.8A). This is consistent with the formation of a larger protein-DNA complex containing the RUNX3/CBFβ heterodimer. To demonstrate the proteins that interact

with sites BC is RUNX3, a monoclonal antibody against RUNX3 (5G4) was used. This resulted in the super-shifting of all RUNX3-related bands due to the formation of larger EMSA complexes with the antibody (lane 5 in Figure 3.8A). Moreover, the addition of anti-FLAG antibody that recognised FLAG-RUNX3 resulted in a very similar pattern of super-shifts (lane 6 in Figure 3.8A), further confirming the RUNX3-specific binding on sites B and C. Lastly, the 'non-specific' bands appeared to be affected by CBFβ and 5G4 antibody, suggesting a possible involvement of endogenous RUNX proteins.

Similar results were observed when Probe_D was used in EMSA; RUNX3-related complexes formed migrated up modestly with CBFβ, and super-shifted in the presence of anti-FLAG antibody (Figure 3.8B). However, it was clear that RUNX3 had a weaker binding affinity for Probe_D, as the intensity of the RUNX3-related bands was much lower relative to the non-specific complexes. This lower affinity of RUNX3 protein for Probe_D is consistent with the suboptimal nature of site D (Figure 3.4B). There is a possibility that additional transcription factors or cofactors are involved to enhance the binding of RUNX3 on this site. However, this event could not be recapitulated within a short and 'naked' EMSA probe (~20 nt).

Together, the significant loss of RUNX3-responsiveness observed in luciferase reporter assays following the mutations of these sites is likely due to the ablation of direct RUNX3 binding on these sites as revealed by EMSA. To confirm these observations, interrogation of the binding of RUNX3 to the endogenous *IL23A* promoter at the level of chromatin is required.

3.2.8 Establish a cell model for *in vivo* binding of RUNX3 on the *IL23A* locus

To study the binding of endogenous RUNX3 on *IL23A* promoter *in vivo*, a cell model expressing both RUNX3 and IL23A proteins was first established. It has been reported that monocytes express IL23A and secrete the heterodimeric IL-23 (IL23A/IL12B) in response to bacteria endotoxin lipopolysaccharide (LPS) (Carmody et al., 2007). Thus, two acute monocytic leukaemia cell lines KG-1 and THP-1 were tested with the same stimulant. Using a sandwich ELISA containing anti-IL23A-specific capture antibody and anti-IL12B detection antibody, secretion of IL-23 was only detected in LPS-stimulated THP-1 cells but not in KG-1 cells (Figure 3.9A). Therefore, THP-1 cell line was chosen for analysis of the expression of endogenous RUNX3.

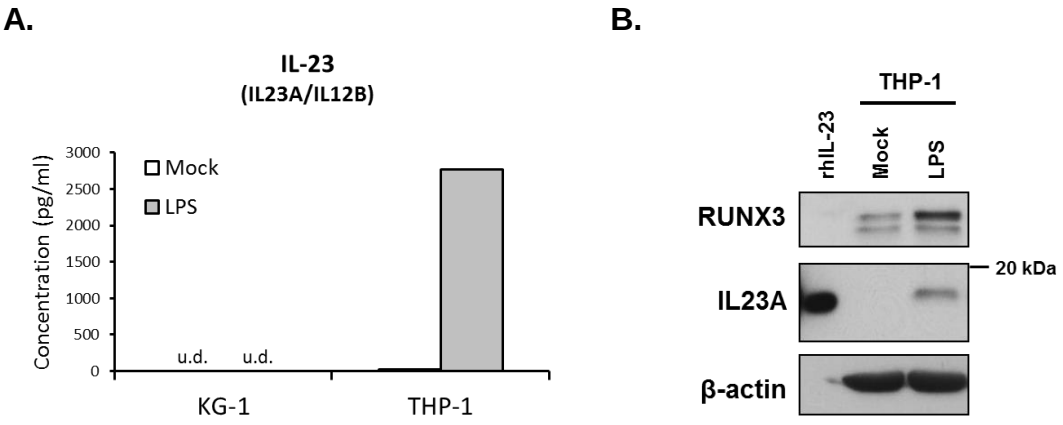


Figure 3.9. The expression of endogenous RUNX3 and IL23A proteins in monocytic cell lines. (A) KG-1 and THP-1 cells were stimulated with 1μg/ml of LPS for 6h. Supernatants collected from these cells were subjected to IL-23 ELISA for quantification of the secreted amount of IL-23. (B) THP-1 cells were cultured in the presence or absence of 1μg/ml LPS for 6h. Whole cell lysates were then prepared and analysed for RUNX3, IL23A and β-actin (loading control) protein expression using Western blot together with 5μg of human recombinant IL-23 which acts as a positive control for endogenous IL23A in THP-1 cells. (u.d.: undetectable)

Western blot analysis showed that RUNX3 was detected in resting THP-1 cells and its expression was increased modestly upon treatment with LPS (Figure 3.9B). In the case of IL23A, a ~19kDa band was detected in LPS-stimulated THP-1 cells that corresponded well with the band of recombinant human IL-23 (rhIL-23) (Figure 3.9B). This is consistent with the results obtained from ELISA whereby the secretion of IL-23 was only observed in THP-1 cells treated with LPS. Although it appears that the latter method is more sensitive for the detection of IL23A. These results confirmed that THP-1 cells express endogenous RUNX3 and LPS-induced IL23A and is therefore a suitable cell model for studying *in vivo* binding of RUNX3 on *IL23A* promoter.

3.2.9 RUNX3 occupancy on *IL23A* promoter at the chromatin level

After establishing THP-1 as a suitable cell model, chromatin immunoprecipation (ChIP) analysis was performed in the presence of LPS to determine the *in vivo* binding of RUNX3 on RUNX sites B, C and D in the *IL23A* promoter. Anti-RUNX3 and anti-CBFβ antibodies were used to pull-down RUNX3-DNA complexes in formaldehyde cross-linked THP-1 cells. A schematic diagram that illustrates the target regions in *IL23A* promoter which were spanned by the corresponding primers was presented in Figure 3.10A. Using primers encompassing the various RUNX sites, the enrichment of genomic DNA fragments containing sites BC, D and BCD was observed (Figure 3.10B). Such enrichment was not observed in control samples that were incubated with anti-IgG antibody and no antibody, indicating that the enrichment is RUNX3-specific. In contrast, no precipitation was observed for the DNA fragment containing RUNX site A that was similar to the non-

specific site control (Figure 3.10B). These results further support the earlier observation in reporter assay studies that site A is not involved in the regulation of *IL23A* by RUNX3 (Figure 3.5). Similar result was obtained when ChIP analysis was performed in AGS cells that had been transduced with Lenti-RUNX3 (Figure 3.10C). In this case, strong enrichments of genomic DNA fragments bearing Site B, C and D were observed for both polyclonal and monoclonal anti-RUNX3 antibodies. In summary, the data from EMSA and ChIP analyses demonstrate that the physical binding of RUNX3 to sites B, C and D of the proximal *IL23A* promoter is in concordance to their functional characterisation by reporter assays.

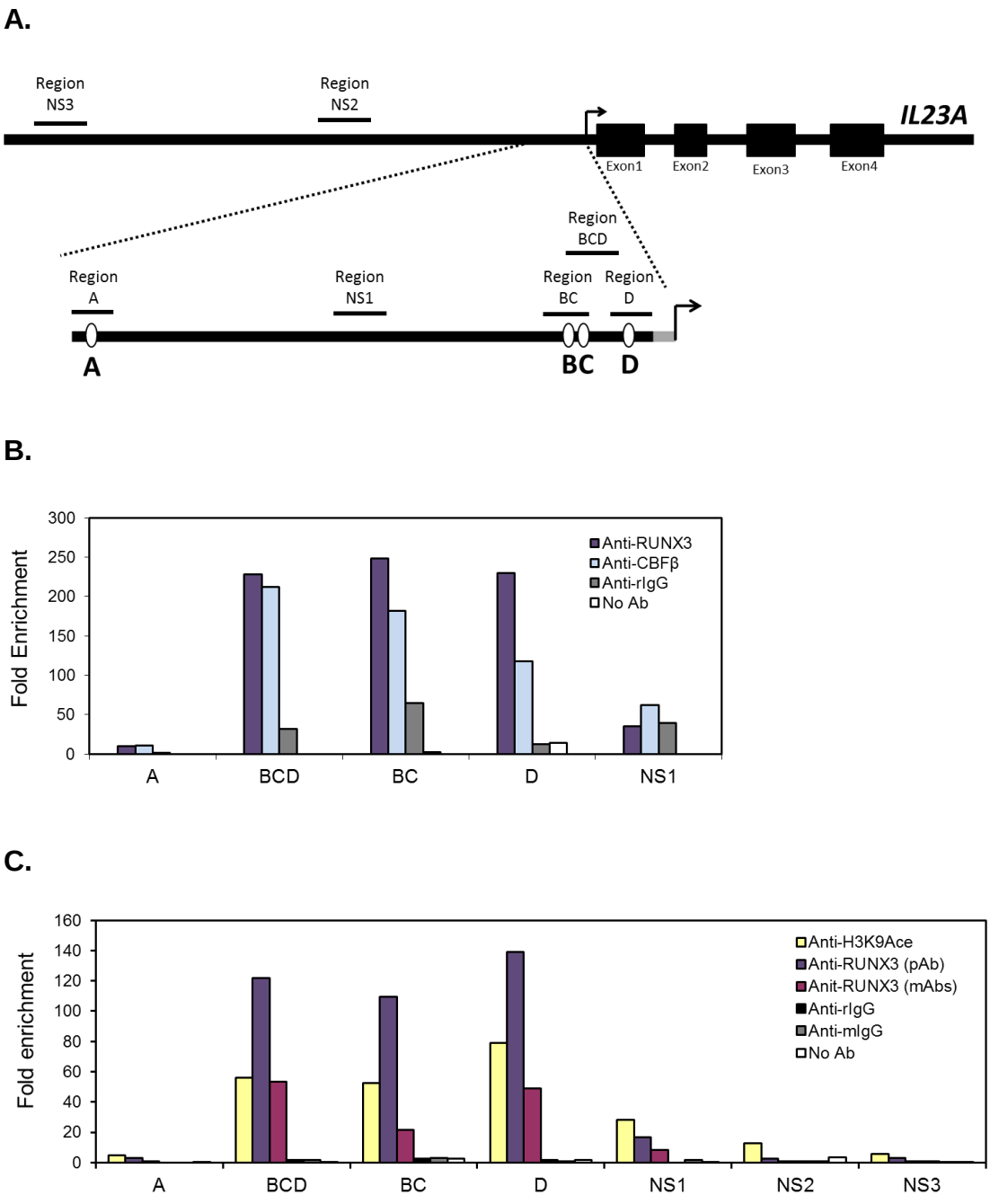


Figure 3.10. *In vivo* occupancy of RUNX3 on *IL23A* promoter in THP-1 and AGS cells. (A) Schematic diagram of *IL23A* gene locus and regions containing RUNX site or non-specific site spanned by corresponding set of primers (Table 2.3) (B) Chromatin immunoprecipitation (ChIP) was performed using THP-1 cells stimulated with LPS (1μg/ml) for 24 hours. Anti-CBFβ and anti-RUNX3 antibodies were used to immunoprecipitate DNA fragments that were cross-linked with RUNX3. Anti-IgG and no antibody were included as negative controls. PCR amplification of the DNA fragments were performed using primers designed to amplify the corresponding regions shown in (A) and quantitated using qRT-PCR method. The values were expressed relative to those of Region A in the presence of anti-IgG antibody.(C) The chromatin of AGS cells transduced with Lenti-RUNX3 virus was crosslinked and immunoprecipitated with rabbit IgG (rIgG) control, mouse IgG (mIgG) control, RUNX3 polyclonal, RUNX3 monoclonal (5G4 + 6E9) or H3K9Ace antibodies. The degrees of enrichment were measured using real-time PCR and expressed relative to rIgG control of NS3 sample. (NS: non-specific)

3.2.10 LPS is not a potent inducer for IL-23 in gastric epithelial cells

Gastric epithelial cells are not known to express IL23A or secrete IL-23. To demonstrate that the transcriptional regulation of *IL23A* by RUNX3 was translated into an increase in its protein expression, the level of IL23A was first assessed in the supernatants of gastric epithelial cell lines SNU16, MKN45, MKN1, KATOIII and AGS using IL-23 ELISA. As IL-23 was only detectable in THP-1 cells post-stimulation with LPS (Figure 3.9), the same stimulant was applied to gastric epithelial cell lines. However, the secretion of IL-23 in gastric epithelial cell lines could not be readily detected even in the presence of LPS, in stark contrast to THP-1 cells (Figure 3.11). Given the tight regulation of IL23A observed in THP-1 cells, it is possible that gastric epithelial cells are hyporesponsive to LPS and additional signals relevant to this cell type may be required for IL-23 production.

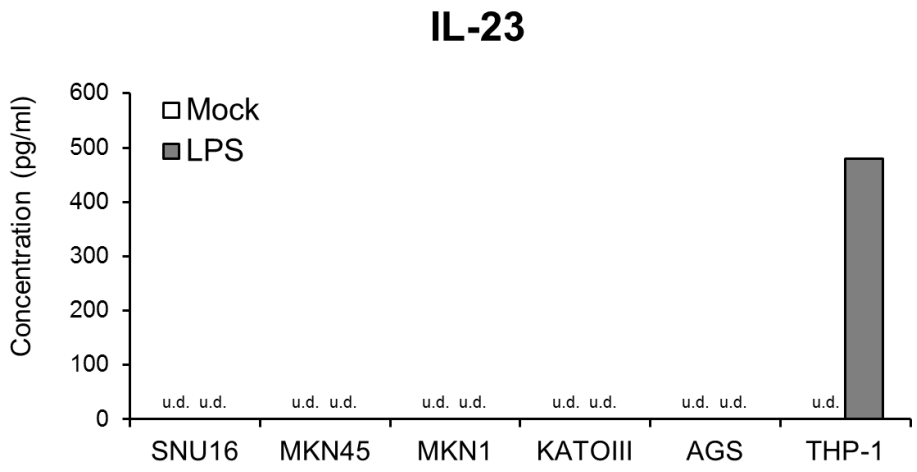


Figure 3.11. The secretion of IL-23 induced by LPS in gastric epithelial cell lines. A collection of five gastric epithelial cell lines and THP-1 cells were stimulated with 1mg/ml of LPS for 24h. Supernatants collected from these cells at the end of stimulation were subjected to sandwich ELISA for quantification of IL-23 secretion. The amount of cytokine was calculated based on the standard curve obtained from the recombinant human IL-23. (u.d.: undetectable)

3.3 Discussion

The importance of IL-23 has to date been focused on its involvement in immunity against pathogens and autoimmunity against 'self'. The remarkable phenotypes observed in *IL23A* knockout and transgenic mouse models (section 1.5) had revealed that it is critically important for this cytokine gene to be maintained at a homeostatic level. As a first step towards understanding the link between RUNX3 and *IL23A*, the direct regulation of RUNX3 on *IL23A* is addressed in this chapter.

IL23A was first identified from a microarray study of RUNX3 target genes using gastric cancer cell line, AGS as a cell model (section 1.4.2). In validation studies, *IL23A* was found to be frequently upregulated in multiple gastric cancer cell lines (Figure 3.1). The variation for the basal levels of *IL23A* transcript in different gastric cancer cell lines could reflect the different driver mutations each line has inherited from the different gastric carcinomas from which they were derived. It is well-known that cancer is a heterogeneous disease which is reflected by their phenotypic and genomic diversities (Marusyk et al., 2012). Tremendous amounts of studies have shown that cell lines derived from tumours not only recapitulate the phenotypes of primary tumours but their genetic landscape is remarkably similar to that of the primary tumours from which they originated (Lin et al., 2008; Neve et al., 2006; Sharma et al., 2010; Sos et al., 2009). Therefore, the heterogeneity of cancer is preserved in cancer cell lines which serve as a useful tool for *in vitro* studies. Given the heterogeneity of gastric cancer cell lines, the remarkable conservation of RUNX3's positive regulation on *IL23A* in multiple cell lines implicates that this may be of central importance to the basic functions of gastric epithelial cells (Figure 3.1).

In line with the transcriptional function of RUNX3, the activation of *IL23A* was found to be dependent on its DNA binding ability (Figure 3.2). This is clearly

reflected in the lack of activation of *IL23A* by its DNA-binding defective mutant of RUNX3, RUNX3^{R178Q}. The DNA-binding dependent transactivation of *IL23A* by RUNX3 supports a direct role of RUNX3 in the regulation of *IL23A* in human gastric cancer-derived cell lines. In addition, this positive role of RUNX3 is not observed in other close family members of IL-12 (Figure 3.3). This highlights the specificity and uniqueness of RUNX3's effect on *IL23A*. Through a combination of promoter deletion and mutation analyses, RUNX sites B, C and D were shown to functionally contribute to the transactivation of *IL23A* promoter by RUNX3 (Figures 3.5 and 3.6). The presence of multiple functional RUNX sites in *IL23A* promoter implicates that RUNX3 is an important regulator of this gene. Interestingly, less than 50% decrease in RUNX3-responsiveness was observed for mutation of either sites B and C or site D in *IL23A* promoter (Figure 3.6A). The above observation implicates that these RUNX sites are interdependent for RUNX3's function on *IL23A*. Such regulatory mechanisms revealed a tight regulation of *IL23A* by RUNX3 as mutations of all these sites are necessary to impair RUNX3's activation. Furthermore, RUNX1 was found to be another positive regulator of *IL23A* promoter (Figure 3.7). The involvement of RUNX1 in this regulation through the same RUNX binding sites revealed a certain degree of overlapping functions for RUNX proteins in this regard. Such phenomenon is not uncommon and has been observed for other RUNX target genes such as *IgC*α, *Defensin-3* and *MDR1* (Hanai et al., 1999; Javed et al., 2000; Westendorf et al., 1998). Furthermore, the additive effects of RUNX3 and RUNX1 on *IL23A* promoter activity revealed that they play similar but not identical roles in this regulation. However, the consensus sequence is not the sole determining factor that directs RUNX binding, as the involvement of other transcription factors or co-factors in the cells will also influence their affinity to the binding site (Carey, 1998; Joshi et al.,

2007; Mann et al., 2009; Merika and Thanos, 2001; Stein and Baldwin, 1993). As such, a detail study into the preference of RUNX3 and RUNX1 on the different functional binding sites in *IL23A* promoter may reveal more insights on the additive increase observed for *IL23A* promoter.

In EMSA analyses, the same proximal RUNX sites B-D were shown to be critical for the binding of RUNX3 on *IL23A* promoter. The appearance of RUNX3-related EMSA complexes reflects the affinity of RUNX3 on sites B and C (Figure 3.8A). Although the traditional view of specific DNA recognition is relied on static contacts with the bases but more recent discoveries reveal that it is a dynamic process influenced by the interaction with other proteins in the cells (Fuxreiter et al., 2011). The formation of multiple RUNX3-related EMSA complexes on sites B and C suggests that other transcription factors and co-factors in COS-7 nuclear extracts are involved in the binding (Figure 3.8A). Factors that have lower affinity may dissociate from the oligonucleotides during the process of electrophoresis leading to different retardation complexes. More importantly, all of these RUNX3-related EMSA complexes were super-shifted by RUNX binding partner, CBFβ and further shifted by antibodies that recognised RUNX3, thus clearly demonstrates the specificity of RUNX3 binding on sites B and C. In the case of RUNX site D, although EMSA analysis showed certain degree of specificity of RUNX3 binding, the affinity of RUNX3 on this site is low (Figure 3.8B). One possible explanation is the suboptimal RUNX consensus sequence for site D and additional transcription factors or co-factors may be required to enhance RUNX3 binding.

The induction of IL23A protein by LPS in THP-1 cells suggests that *IL23A* is a transcriptionally active locus that is accessible by transcription factors and co-factors binding (Figure 3.9). Together with the increased expression of RUNX3 in this

cell line upon LPS treatment, THP-1 is a useful cell model to assess the association of RUNX3 with *IL23A* promoter *in vivo*. In ChIP assay, precipitation of genomic DNA fragments containing RUNX sites B, C and D by antibodies against RUNX3 and CBFβ confirmed the endogenous RUNX3's binding on these sites (Figure 3.10B). ChIP measures the interaction of proteins with their target DNA sequences in living cells which is a dynamic process involving chromatin remodelling, as well as positioning and modification of nucleosomes (Pascual-Ahuir and Proft, 2012). Given the complexity of the above process, the clear enrichment observed for all three RUNX sites further supports the specificity of endogenous RUNX3 binding on *IL23A* at the chromatin level.

Through a combination of quantitative real-time PCR, sequence analysis, reporter gene assays, EMSA and ChIP, this chapter uncovers the molecular mechanisms underlying the transcriptional regulation of *IL23A* by RUNX3. In THP-1 cell model, the lack of IL23A expression in the absence of LPS suggesting that IL23A is specifically and stringently regulated by inflammatory stimulus. However, unlike THP-1 cells, LPS is not a potent inducer for IL-23 in gastric epithelial cell lines (Figure 3.11). Given the significance of RUNX3 role in the regulation of this gene, the regulatory machinery for IL23A expression in gastric epithelial cells warrants further investigation.

Chapter 4:

RUNX3 is a critical requirement for the induction of IL23A expression by inflammatory stimuli in gastric epithelial cells

4.1 Introduction

4.1.1 NF-κB is a positive regulator of *IL23A*

As with many cytokine genes, especially those involved in inflammatory responses, *IL23A* is a direct target gene of the NF-κB (nuclear factor kB) pathway that plays a pivotal role in host immunity and inflammation. This was amply supported by data from mouse gene targeting experiments in which components of the NF-κB pathway were targeted. NF-κB-deficient monocytes isolated from *c-rel*$^{-/-}$ and *relA*$^{-/-}$ mice were defective in expressing *IL23a* in response to TLR (Toll-like receptor) ligands, indicating that NF-κB is essential for the regulation of this gene (Carmody et al., 2007; Mise-Omata et al., 2007). This phenomenon was subsequently attributed to a proximal κB binding site in murine *IL23a* promoter, which was requisite for induced *IL23a* expression (Mise-Omata et al., 2007). This regulatory mechanism appears to be conserved across species and NF-κB induction of *IL23A* has been reported in human macrophages (Garrett et al., 2008). In the current study, this phenomenon is supported by the strong induction of *IL23A* in THP-1 cells by LPS, a potent activator of NF-κB signaling (Figure 3.9). Given the constitutive expression of NF-κB family members in a variety of cell types, it is likely that NF-κB plays an important role in the regulation of *IL23A* in many tissue contexts, including the gastric epithelium.

4.1.2 Activating the NF-κB pathway in gastric epithelial cells

In the stomach, a single layer of epithelial cells that separates the luminal contents of the mammalian stomach from the underlying tissues often represents the first line of defence of the body. Gastric epithelial cells, besides acting as a physical

barrier to protect the body from adverse environmental agents, have important immunological properties. The strategic location of gastrointestinal mucosal epithelial cells allows them to simultaneously interact with both luminal antigens and resident lamina propria immune cells which is important for mucosal immunity. A wide variety of stimuli, including pathogens, inflammatory cytokines, antigens and stress factors activate the canonical NF-κB pathway in gastric epithelial cells (Pasparakis, 2008).

Gastrointestinal epithelial cells express a range of cytokine receptors that enable them activate the NF-κB pathway in response to inflammatory signals, such as injury and infection of pathogens (Panja et al., 1998; Reinecker and Podolsky, 1995). This would trigger a cascade of cellular events that includes the secretion of chemokines for the recruitment of leukocytes; and cytokines for the amplification of the inflammatory signal and activation of host defence mechanisms (Burchett et al., 1988; Elkon et al., 1997; Yamada et al., 2000). The most well-known inflammatory cytokines that activate NF-κB signal transduction pathway in gastric epithelial cells are TNF-α and IL-1β (Pasparakis, 2008). Aberrant production of these cytokines were linked with gastric inflammation and cancer as discussed in section 1.2.6 (El-Omar et al., 2000; El-Omar et al., 2003; Oshima and Oshima, 2012). These highlight the importance of cytokine signaling pathways for the regulation of host immune response of epithelial cells in the stomach.

In addition to the ability to response to cytokines, gastric epithelial cells are equipped with numerous germline-encoded pattern recognition receptors (PRR) to sense the presence of microbes via recognition of the conserved molecular structures known as pathogen-associated molecular patterns (PAMP). The best characterised

PRR are the Toll-like receptors (TLR) and NOD-like receptors (NLR), which play critical roles in distinguishing pathogens from commensal bacteria, and mounting a precise immune response (Kumar et al., 2009; Martinon et al., 2009; Wells et al., 2011). TLR are localised on the cell membrane and/or endosomal membrane components to recognise extracellular and endocytosed PAMPs. In human, the majority of TLR identified to date have been shown to be present in the gastrointestinal epithelial transcriptome with the most well-studied being TLR4. However their spatial distribution in the epithelium, cell lineage specificity, and specific functions in different part of the gut remain unclear (Abreu, 2010; Ishihara et al., 2004; Schmausser et al., 2004; Schmausser et al., 2005; Wells et al., 2010). Unlike TLRs, which are membrane associated, NLRs are cyotosolic and responds to a wide range of bacterial ligands and toxins; as well as certain damage-associated molecular patterns (DAMP) of the host cell (Martinon et al., 2009; Williams et al., 2010). To date, the NLRs that have been well characterised in the gut are NOD1 and NOD2 (NOD: nucleotide-binding oligomerisation domain protein) (Hirata et al., 2006; Hisamatsu et al., 2003; Kobayashi et al., 2005; Viala et al., 2004).

At the onset of inflammation, this diverse range of stimuli that includes proinflammatory cytokines and bacterial components that triggers TLR and NOD signaling pathways, converge to activate IκB kinase (IKK) complex (Abreu, 2010; Kim et al., 2008; Pasparakis, 2008). The activation of IKK complex will result in the phosphorylation and degradation of the inhibitory protein for NF-κB (I-κB) I-κB, thereby releasing the NF-κB into the nucleus and to activate the transcription of its target genes as depicted in Figure 4.1. (Abreu, 2010; Hayden and Ghosh, 2004; Kim et al., 2008; Verma et al., 1995; Wells et al., 2010). NF-kB is a heterodimeric transcription factors composed of various combinations of the Rel family of

transcription factors: c-Rel, RelA (p65), RelB, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). The most studied NF-κB heterodimer is composed of p65 and p50. It is known that specific combination of these factors would confer variations such as induction kinetics, binding site and partner specificity that enables the fine-tuning of the specific functions of NF-κB in a particular tissue type (Pasparakis, 2009).

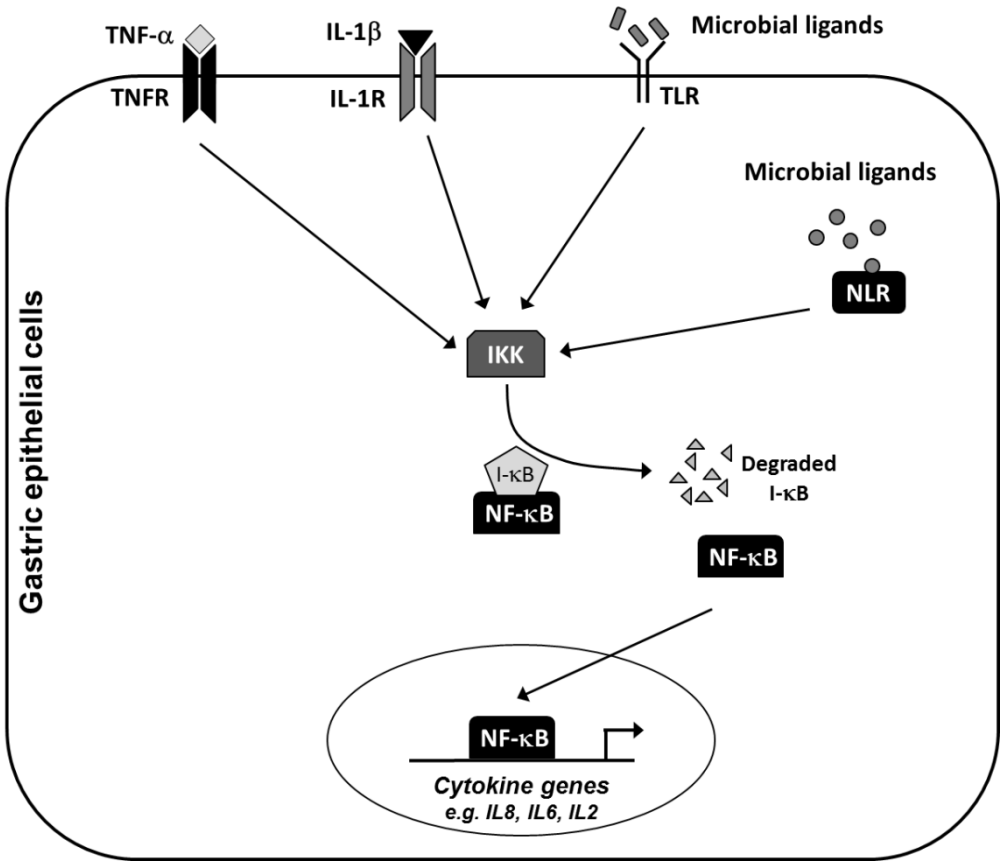


Figure 4.1. NF-κB signal transduction pathway initiated by proinflammatory cytokines and pathogens in gastric epithelial cells. Proinflammatory cytokine such as TNF-α and IL-1 as well as microbial components that bind TLR and NLR activate IKK (I-kB kinase). This activation event leads to phosphorylation and degradation of I-κB (Inhibitory protein for NF-κB) and translocation of NF-κB into nucleus for activation of target genes.

4.1.3 Signaling events triggered by *H. pylori* infection in gastric epithelial cells

Helicobacter pylori (*H. pylori*), a human stomach pathogen that infects half of the world's population is a major risk factor for gastric inflammation and malignancies (Peek and Crabtree, 2006). It is now recognised that *H. pylori* infection triggers majority of the inflammatory cascades that have been implicated in gastric pathologies. The association of *H. pylori* CagA-positive strains with higher grades of gastric inflammation and increased risk of gastric cancer highlights the role of CagA in *H. pylori*-mediated inflammatory response (Blaser et al., 1995b; Parsonnet et al., 1997).

During *H. pylori* infection, the CagA protein is injected into host gastric epithelial cells through a type IV secretion system, which is often followed by its phosphorylation by host Src kinase that enables CagA to target host proteins and alter cellular processes (Hatakeyama, 2008; Peek, 2005). An important interaction appears to be that between phosphorylated CagA and a host phosphatase known as Src homology domain-containing protein tyrosine phosphatase (SHP2), which promotes the activation of extracellular signal regulated kinase (ERK) signaling pathway, leading to the aberrant proliferation of infected cells (Asahi et al., 2000; Hatakeyama, 2004; Higashi et al., 2002; Stein et al., 2000). The phosphorylation of CagA appears to be a key regulatory node for the action of this protein, as hypophosphorylated CagA is shown to interact with the Interleukin 6/11 receptor gp130 to transactivate the JAK/STAT pathway (Lee et al., 2010).

In addition to CagA, the type IV secretion system also delivers *H. pylori*-derived peptidoglycan (ie. iE-DAP) into host cells. This leads to the stimulation of intracellular NOD1, as it recognises *H. pylori*-derived iE-DAP, resulting in the

activation of NF-κB (Viala et al., 2004; Watanabe et al., 2010). It has been reported that *H. pylori*-mediated activation of NOD1 results in robust production of bactericidal *β-defensin* and pro-inflammatory cytokines from gastric epithelial cells (Hishida et al., 2010). It is held that these early responses are crucial for effective host defence and bacterial clearance (Viala et al., 2004; Watanabe et al., 2010). The signaling pathways activated in gastric epithelial cells during *H. pylori* infection is depicted in Figure 4.2. Given the prominent involvement of the NF-κB pathway activated by pathogens or host cell factors in gastric epithelial cells, the emphasis of this chapter is to establish if these pathways are responsible for the regulation of IL23A.

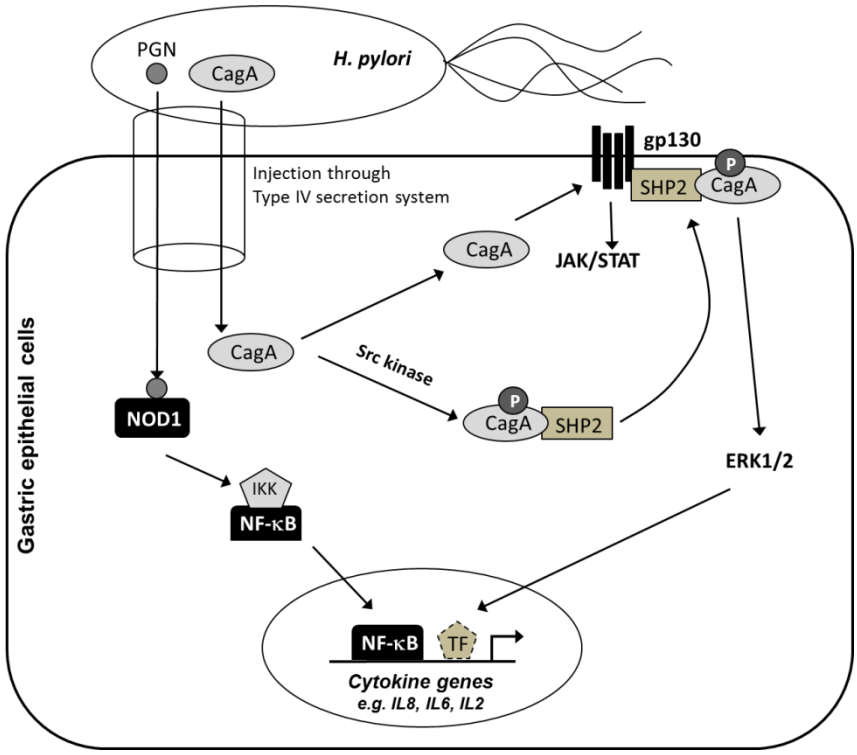


Figure 4.2. Alteration of gastric epithelial cellular signaling by *H. pylori*. *H. pylori* delivers peptidoglycan (PGN) and CagA into gastric epithelial cells via Type IV secretion system. Peptidoglycan enters into the host cells binds onto NOD1 for activation of NF-κB. The injected CagA is phosphorylated by Src kinase that leads to activation of SHP2/ERK signaling whereas the hypophosphorylated CagA activates JAK/STAT signaling pathway. (gp130: IL-6 receptor; IKKs: Inhibitory κB kinase complex; JAK/STAT: Janus kinase/signal transducer and activator of transcription; TF: unknown transcription factor)

4.2 Results

4.2.1 The induction of *IL23A* by inflammatory cytokines

To determine if IL23A is regulated by the NF-κB pathway in gastric epithelial cells, the gastric carcinoma cell line AGS was subjected to treatment with several NF-κB activating cytokines as well as other cytokines that are relevant to the regulation of this gene. Quantitative PCR analyses revealed that NF-κB-activating cytokines TNF-α, IL-1α and IL-1β induced a 20-fold, 5.5-fold and 6-fold increase in *IL23A* transcripts relative to the unstimulated control (Mock), respectively (Figure 4.3). In contrast, no appreciable induction of *IL23A* was observed upon treatment with IFN-γ and IL-6, while TGF-β had only marginal effects (Figure 4.3). In addition, a potential cooperation between TNF-α and other cytokines was also investigated.

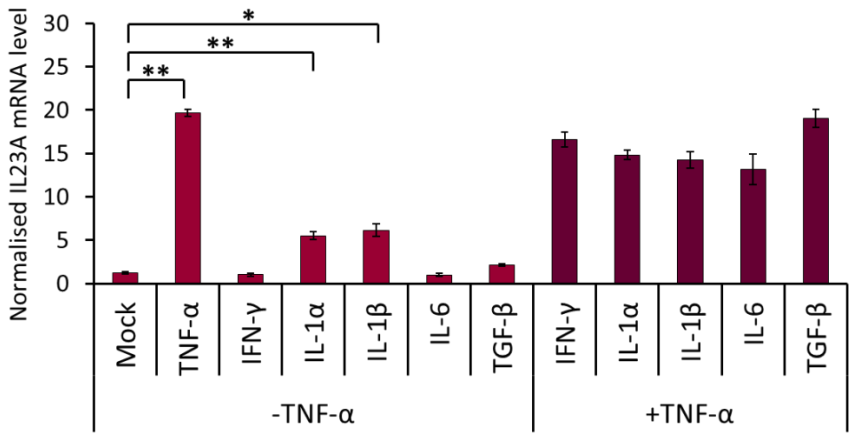


Figure 4.3. The effects of various inflammatory cytokines on the expression of *IL23A*. AGS is singly treated with 10ng/ml of human recombinant IFN-γ, IL-1α, IL-1β, IL-6 and TGF-β (5ng/ml), and co-treated with 10ng/ml of TNF-α for 6h prior to the quantification of *IL23A* mRNA using qRT-PCR. The values were normalised with those of *GAPDH* and were expressed relative to the Mock control. Data are presented as means ± SEM. Data presented are a compilation of three separate experiments and Student t-tests were performed (*p<0.05; **p<0.01).

However, no significant cooperation in *IL23A* induction was observed when AGS cells were co-treated with TNF-α and various cytokines. Interestingly, the lack of additive effect also applied to IL-1α and IL-1β, which resulted in significant activation of *IL23A* on their own (Figure 4.3). This observation suggests that TNF-α and IL-1 are acting on a common pathway, most likely through the activation of NF-κB, with TNF-α being the more potent activator. The lack of significant induction of *IL23A* by the other cytokines indicates that the TNF-α signaling axis is the primary activator for this gene in AGS gastric epithelial cells.

4.2.2 RUNX3 and TNF-α cooperatively activates IL23A

To investigate a potential involvement of RUNX3 in TNF-α-mediated induction of *IL23A*, AGS cells were transduced with lentiviruses to stably express wildtype RUNX3 (Lenti-RUNX3), $RUNX3^{R178Q}$ (Lenti-R178Q) or no cDNA (Lenti-Control) before treatment with TNF-α. The results showed that TNF-α treatment on Lenti-Control or -R178Q infected AGS cells resulted in ~20-fold induction of *IL23A* mRNA levels similar to the previous observations (Figure 4.3 and Figure 4.4). This induction was markedly augmented to 100-fold relative to that of Lenti-Control unstimulate levels in cells expressing wild type RUNX3 (Figure 4.4A). Importantly, this synergistic cooperation was consistently observed at the level of IL23A protein expression, whereby the highest level of IL23A protein expression was detected in RUNX3-expressing cells treated with TNF-α (Figure 4.4B).

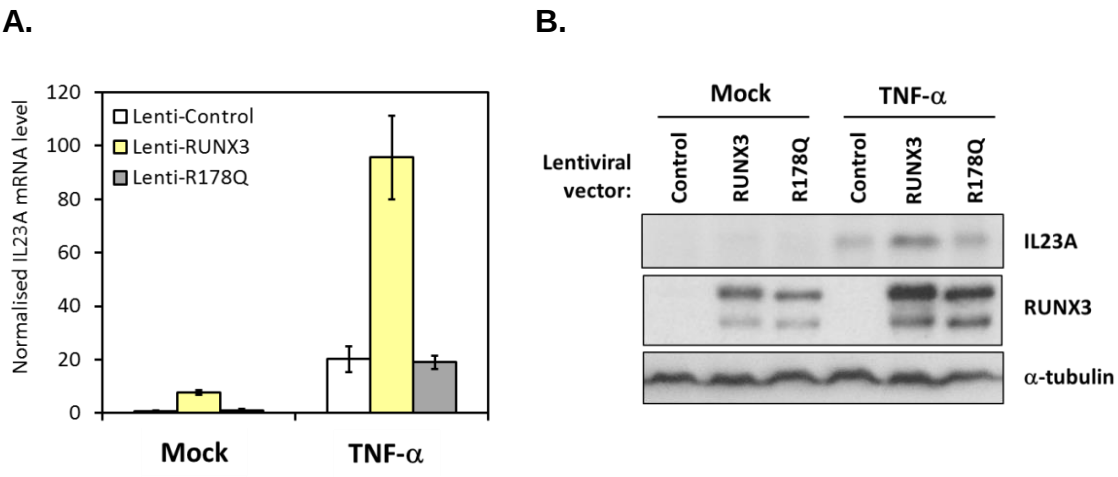


Figure 4.4. The expression of IL23A in the presence of RUNX3 and TNF-α. (A) AGS cells were transduced with Lenti-Control, -RUNX3 or -RUNX3^{R178Q} for 48h preceding treatment with 10ng/ml of TNF-α for 6h. Transcript expression of *IL23A* was determined by qRT-PCR and the values were normalised against those of *GAPDH* and were expressed relative to the Lenti-Control (Mock) sample. The data presented are a compilation of three independent experiments (means ± SEM). (B) AGS cells transduced with the same series of lentiviruses were cultured with 10ng/ml of TNF-α for 18h. Whole cell lysates were then prepared and analysed for IL23A, RUNX3 and α-tubulin (loading control) protein expression using Western blot.

The induction of IL23A transcript and protein by TNF-α and the cooperative effects observed for TNF-α and RUNX3 were recapitulated in the established promoter reporter system (Figure 4.5A). The necessity of RUNX3 was demonstrated by the abolishment of cooperativity on the full-length *IL23A* promoter when the proximal RUNX sites (B, C and D) were mutated (Figure 4.5A). Furthermore, these observations could be reproduced in another RUNX3-negative gastric carcinoma cell line, KATOIII (Figure 4.5B). These data collectively indicate a functional cooperativition between RUNX3 and TNF-α/NF-κB, which was dependent on the proximal RUNX sites within the full-length *IL23A* promoter. It is important to note that the mutation of RUNX sites had no impact on the activation of *IL23A* promoter by TNF-α *per se*, indicating that TNF-α acts via a distinct cis-acting element (Figure 4.5A and 4.5B).

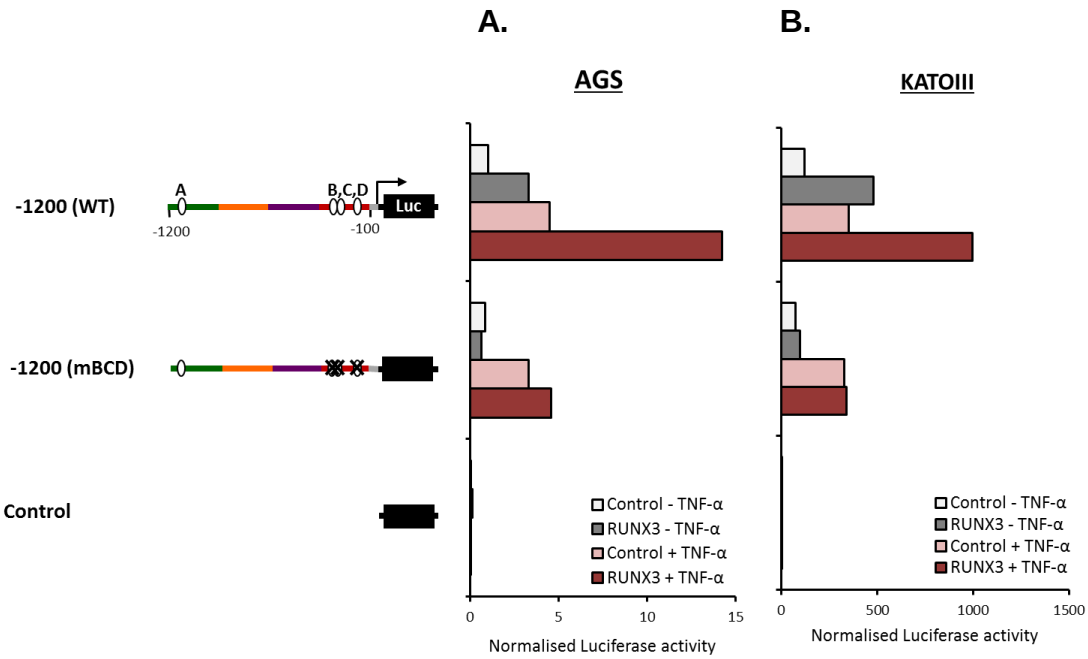


Figure 4.5. Changes in *IL23A* promoter activity in response to RUNX3 and TNF-α. Left panel depicts the schematic diagrams of wiltype and mutant *IL23A* promoter luciferase reporter constructs. Oval shapes on the promoter indicate the RUNX binding sites and **X** indicates mutation of the corresponding RUNX site. These promoter reporter constructs were transiently transfected into the cells together with either control- or RUNX3-expression vector for 24h followed by TNF-α (10ng/ml) treatment for 24h. The luciferase activities of the constructs were then measured and normalised against those of Renilla luciferase activities. These values were expressed relative to the Control untreated sample. Experiments were performed in (A) AGS and (B) KATOIII cells.

4.2.3 TNF-α activates *IL23A* promoter via the NF-κB site

To further demonstrate the activation of *IL23A* promoter by NF-κB in gastric epithelial cells, a reported NF-κB binding site in the *IL23A* minimal promoter reporter construct was mutated (Garrett et al., 2008). The result showed that a strong reduction in the TNF-α-responsiveness of *IL23A* promoter activity was observed in the NF-κB-mutated reporter construct (Figure 4.6). This result indicates that the induction of *IL23A* promoter activity by TNF-α is at least mediated through the NF-κB binding site. Interestingly, the mutation of NF-κB binding site diminished the activating effects of RUNX3 (Figure 4.6).

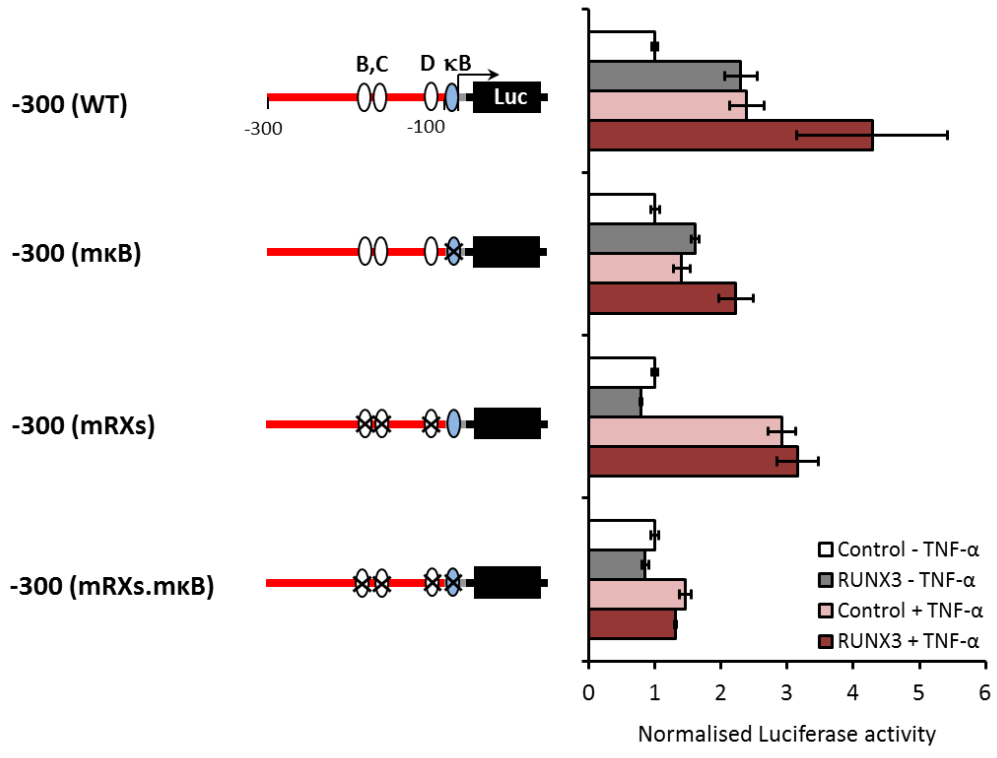


Figure 4.6. The activation of *IL23A* promoter by TNF-α is mediated through NF-κB binding site. Left panel depicts the schematic diagram of a series of wildtype and mutant *IL23A* minimal promoter reporter constructs. White ovals on the promoters indicate RUNX binding sites and the yellow oval indicates B binding site. These constructs were transfected into AGS cells before stimulation with TNF-α (10ng/ml) for 24h. The promoter activities were evaluated with luciferase assays as described in Figure 3.5. The data are representative of experiments performed in triplicate and presented as means ± SEM. (*p<0.05). (κB: NF-κB consensus site)

The necessity of an intact NF-κB site for the full effect of RUNX3 indicates that RUNX3's effect is at least in part coupled to NF-κB. Similar observation could be made in the context of the minimal promoter, where the *IL23A* promoter was induced strongest in the presence of RUNX3 and TNF-α treatment (Figure 4.6). Accordingly, the synergistic effect between RUNX3 and TNF-α was abolished when RUNX or NF-κB sites was mutated alone or in combination (Figure 4.6).

4.2.4 The involvement of TLR and NLR pathways in the regulation of *IL23A*

The involvement of Toll-like receptors (TLR) and NOD-like receptors in the transcriptional regulation of *IL23A* in leukocytes has been reported previously. In the current study, such regulation was also observed in monocytic cell line, THP-1 as reported in the previous chapter (Figure 3.9). To investigate the relevance of these pathways in the regulation of *IL23A* in gastric epithelial cells, a screen was performed using a combination of TLR/NOD ligands or agonists listed in Table 4.1.

Ligand	Receptors	Origin of ligand
Pam_3CSK4	TLR 1/2	Bacteria and mycobacteria
Poly I:C	TLR 3	Analog of dsRNA from virus
LPS	TLR 4	Gram negative bacteria
Flagellin	TLR 5	Bacteria
Zymosan	TLR 2/6	Fungi
R-848	TLR 7/8	Viruses
iE-DAP	NOD1	Gram negative and some Gram positive bacteria
MDP	NOD2	Bacteria

Table 4.1. Summary of the ligands and their origins for different pattern recognition receptor (PRR) including Toll-like receptors (TLR) and NOD-like receptors (NLR).

The changes in *IL23A* mRNA levels following treatment with ligands of different receptors were shown in Figure 4.7. Unexpectedly, the TLR ligands did not have significant effects on *IL23A* expression in AGS cells (Figure 4.7). On the other hand, activating NOD1 using iE-DAP led to a 4-fold induction of *IL23A* (Figure 4.7). These observations indicate that the NOD1 pathway, most commonly activated by bacterial pathogens, has a role in the regulation of *IL23A* in gastric epithelial cells.

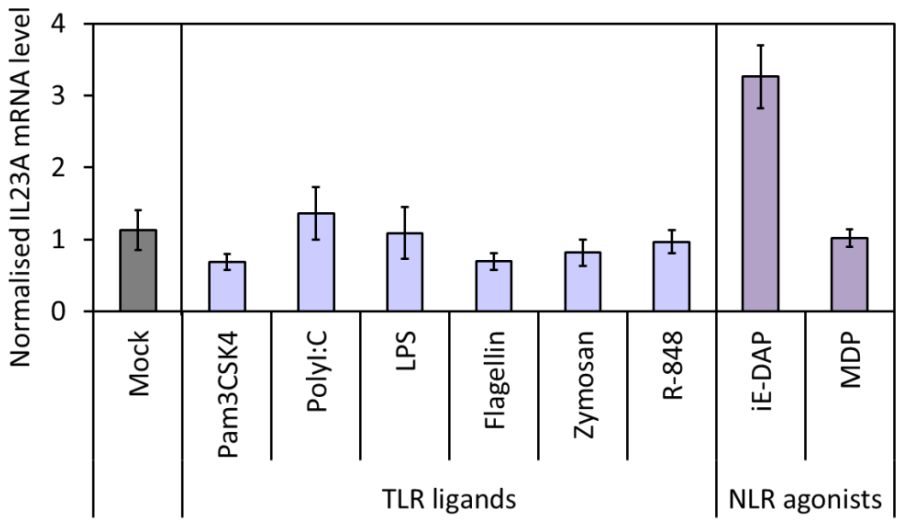


Figure 4.7. Changes in *IL23A* expression in response to several ligands and agonists of TLR and NLR pathways. AGS cells were incubated with 1μg/ml of Pam3CSK4, PolyI:C, LPS, Zymosan, R-848, Flagellin (100μg/ml), iE-DAP (10μg/ml) and MDP (50μg/ml) for 6h. Cells were harvested and *IL23A* mRNA was measured using qRT-PCR. The values were normalised with *GAPDH* and expressed relative to the mock control calculated by ΔΔCt method. Data are presented as means ± SEM (n=3).

4.2.5 *H. pylori* infection induces *IL23A* expression

To further explore the involvement of NOD1 pathway in the activation of *IL23A*, we infected gastric epithelial cells with *H. pylori*, as it is a known activator of the NOD1 pathway and a major bacterial pathogen in the human stomach. To establish a suitable bacterial titre for this study, AGS cells were infected with *H. pylori* at different multiplicities of infection (MOI). Figure 4.8 shows that increasing MOI of *H. pylori* resulted in progressively stronger induction of *IL23A* transcript. This result reveals a positive correlation between *H. pylori* infection and *IL23A* expression in AGS cells. Consistent with the intracellular distribution of NOD1 and the need of an active Type IV secretion system for the delivery of iE-DAP, heat-killed *H. pylori* had no effect on *IL23A* levels.

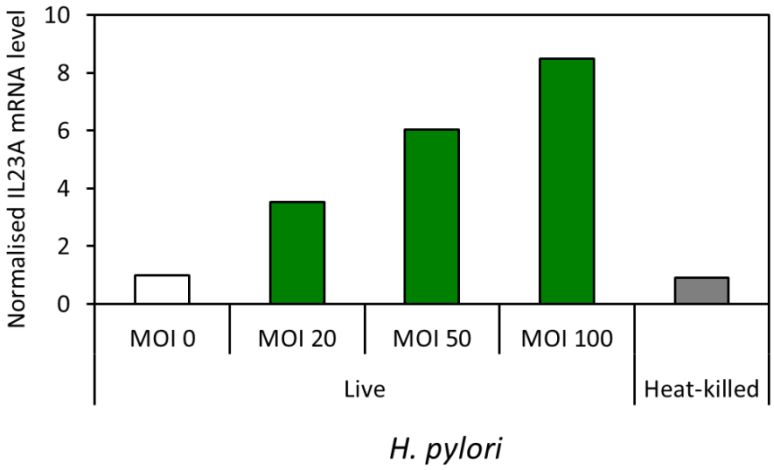


Figure 4.8. *H. pylori* activates *IL23A* in a dose-dependent manner. AGS cells were co-cultured with different MOIs of live and heat-killed *H. pylori* for 6h prior quantification of *IL23A* transcript via qRT-PCR. The values were normalised with *GAPDH* and expressed relative to the MOI0 control calculated by ΔΔCt method.

4.2.6 CagA-status of *H. pylori* determines the outcome of *IL23A* induction

Together with the introduction of iE-DAP into gastric epithelial cells, which actives NOD1, *H. pylori* also delivers the virulence factor CagA (section 4.1.2). To examine the effect of CagA on *IL23A* transcription, AGS cells were infected with either a wild type (WT) *H. pylori* or its CagA-defective isogenic strain (ΔCagA) at four different time points. A comparison of the kinetics of *IL23A* induction resulting from the infection of WT and ΔCagA *H. pylori* revealed that both strains activate *IL23A* mRNA maximally at 6h (Figure 4.9A). However, continual induction of *IL23A* was observed only in the samples treated with wild-type *H. pylori* at 24h suggesting that CagA is critical for sustained induction of this gene (Figure 4.9A). The induction kinetics of *IL23A* in response to *H. pylori* was compared with that of interleukin-8 mRNA (*IL8*), a classical host target gene of CagA (Sharma et al., 1995). This reveals that the induction kinetics of *IL23A* closely resembled that of *IL8*, suggesting that these two cytokine genes may be part of a common pathway of host immune response (Figure 4.9B).

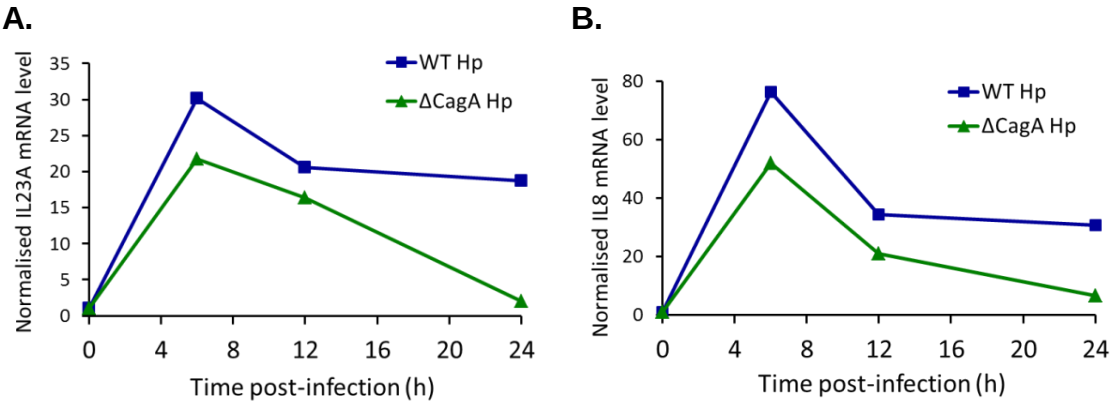


Figure 4.9. Wildtype and ΔCagA strains of *H. pylori* induction time-course for *IL23A* and *IL8*. AGS cells were infected with either wildtype (WT) or ΔCagA strains of *H. pylori* (Hp) at MOI 100 for 0h, 6h, 12h and 24h prior to the assessment of *IL23A* (A) and *IL8* (B) transcripts by qRT-PCR. The values of each gene were normalised with *GAPDH* and expressed relative to those of 0h control.

To demonstrate the specificity of RUNX3's action, changes in the expression of *IL8* were compared against that of *IL23A*. Although the induction of *IL8* by *H. pylori* followed very similar kinetics to that of *IL23A*, it was clearly not dependent on RUNX3 (Figure 4.10). This suggests that RUNX3 may be involved in the regulation of a distinct subset of genes during *H. pylori* infection.

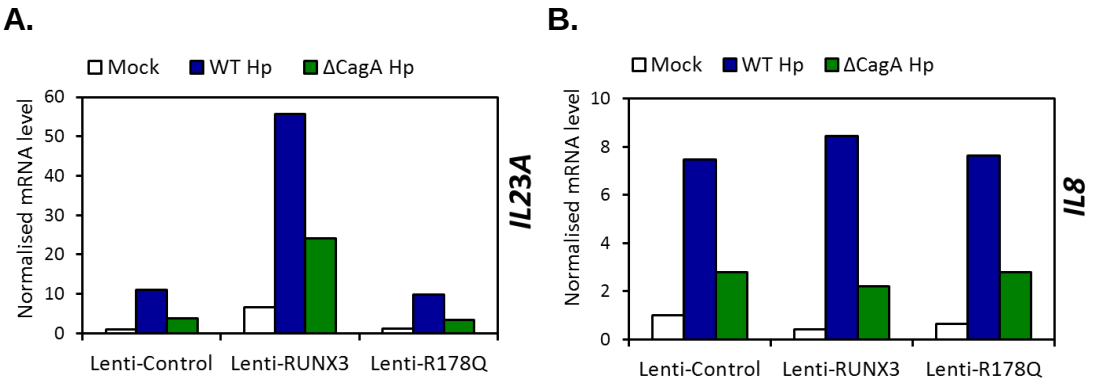


Figure 4.10. RUNX3 specifically enhanced *H. pylori*-induced *IL23A* but not *IL8* in gastric epithelial cells. AGS cells were infected with either WT or ΔCagA *H. pylori* for 6h prior measurement of *IL23A* and *IL8* transcripts by qRT-PCR. The normalised values are expressed relative to uninfected control.

As CagA is known to interact with and acts through SHP2 in gastric epithelial cells, its involvement was examined using specific inhibitor. Treatment with 50μM of SHP2 inhibitor (NSC87877) abolished CagA-dependent activation of *IL23A* by wildtype *H. pylori* (Figure 4.11). These data indicate that prolonged activation of *IL23A* by live *H. pylori* requires the activation of SHP2 pathway by the CagA oncoprotein.

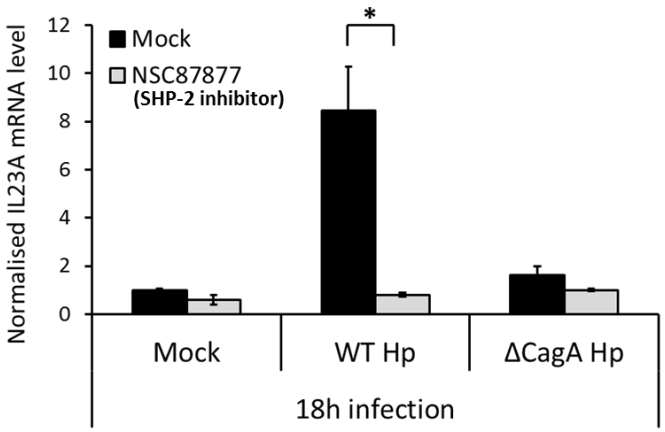


Figure 4.11. *IL23A* induction by *H. pylori* requires activation of SHP-2/ERK pathway by oncoprotein CagA. AGS cells were incubated with 50μM of SHP2 inhibitor (NSC87877) for 3h prior to the infection with wildtype (WT) or ΔCagA strains of *H. pylori* (Hp) at MOI100 or vehicle (Mock) for 18h. The qRT-PCR values of *IL23A* were normalised with *GAPDH* and were expressed relative to the basal values of mock-infected samples (means ± SEM). Data presented are collected from three separate experiments and Student t-test were performed (*p<0.05).

4.2.7 Induction of *IL23A* is observed in multiple gastric epithelial cell lines

Having established that TNF-α and *H. pylori* are the activating stimuli for *IL23A* in AGS cells, a collection of nine gastric epithelial cell lines were next tested to determine if these observation are reproducible. Remarkably, TNF-α and *H. pylori* induce *IL23A* in a majority of these gastric cancer cell lines (Figure 4.12). Furthermore, similar effects were also observed in two non-cancerous gastric epithelial cell lines, HFE-145 and GES-1 (Figure 4.12). The conservation of these regulatory mechanisms across different gastric epithelial cell lines strongly suggests the central roles for *H. pylori* and TNF-α in the regulation of *IL23A* in this cell type. These results implicate that *IL23A* plays a significant role in the inflammatory cascade downstream of TNF-α and *H. pylori* infection.

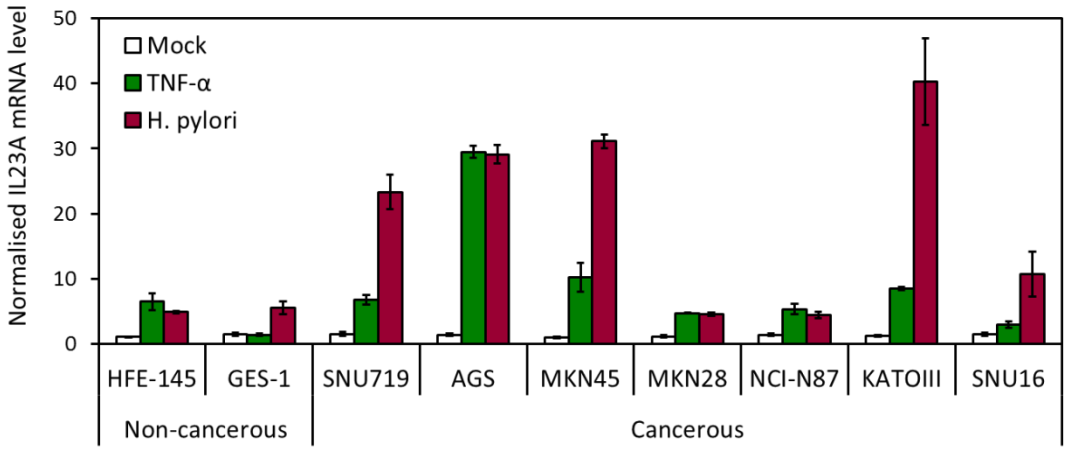
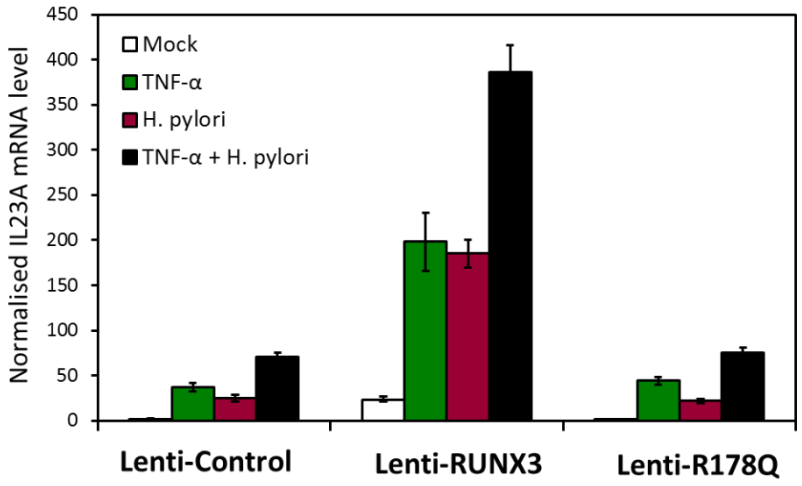


Figure 4.12. TNF-α and *H. pylori* upregulate *IL23A* over multiple gastric epithelial cell lines. Two non-cancerous and seven cancerous gastric epithelial cell lines were stimulated with TNF-α (10ng/ml) or infected with wildtype *H. pylori* (MOI100) for 6h. *IL23A* mRNA levels were determined by qRT-PCR. The values were normalised with *GAPDH* and expressed relative to the Mock control of each cell line. Data are presented as means ± SEM (n=3).

4.2.8 Inflammatory stimuli-induced IL23A is significantly enhanced by RUNX3

Given the significance of TNF-α and *H. pylori* on *IL23A* activation, the cooperation of these signals with RUNX3 was investigated. The results showed that in addition to the cooperation between TNF-α and *H. pylori*, the presence of RUNX3 resulted in a marked enhancement in the induction of *IL23A* (Figure 4.13A). This synergistic effect between RUNX3, TNF-α and *H. pylori* on IL23A was particularly clear at the protein level (Figure 4.13B). Notably, this cooperative effect was entirely absent in the AGS cell line that ectopically expressed the RUNX3^{R178Q} DNA-binding defective mutant. These results indicate RUNX3 is a critical requirement for a strong induction of IL23A when AGS gastric epithelial cells are challenged by the pro-inflammatory cytokine TNF-α and the infection of *H. pylori*.

A.



B.

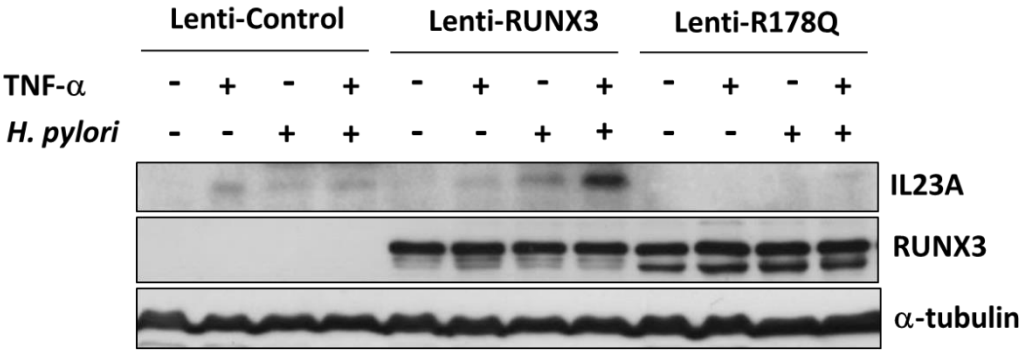


Figure 4.13. The expression of IL23A in response to RUNX3, TNF-α and *H. pylori*. (A) AGS cells were transduced with Lenti-control, RUNX3 or RUNX3^{R178Q} lentiviruses for 48h preceding the treatment with 10ng/ml of TNF-α and/or infection with *H. pylori* at MOI100 for 6h. Transcripts of *IL23A* were determined by qRT-PCR and the data presented are from three independent experiments (means ± SEM). (B) AGS cells transduced with the same series of lentiviruses were cultured with TNF-α and/or infected with *H. pylori* (MOI100) for 18h. Whole cell lysates were then prepared and analysed for IL23A, RUNX3 and α-tubulin (loading control) protein expression using Western blot.

4.2.9 Knockdown of RUNX3 and RUNX1 confirm their positive roles on *IL23A*

In the previous chapter, promoter reporter study revealed that RUNX1 is also a transactivator of *IL23A* promoter (Figure 3.7). Therefore, to validate the role of RUNX proteins in mediating TNF-α/*H. pylori* induction of *IL23A*, an RNAi knockdown experiment targeting RUNX3 and RUNX1 was performed in an

untransformed gastric epithelial cell line, HFE-145 that expresses both RUNX proteins. The results reveal that RNAi targeting of either *RUNX3* or *RUNX1* led to a significant reduction in *IL23A* induction in HFE-145 cells co-treated with TNF-α and *H. pylori*, compared with the cells targeted by control siRNA (Figure 4.14A). Concurrent targeting of *RUNX3* and *RUNX1* further reduced the response of *IL23A* to TNF-α/*H. pylori*, confirming that both RUNX members play overlapping roles in the mediation of TNF-α/*H. pylori* regulation of *IL23A* (Figure 4.14). The RNAi knockdown of RUNX3 and RUNX1 were confirmed by quantitative RT-PCR and Western blot (Figures 4.15A and 4.15B). Taken together, these studies support a positive role of RUNX3 and RUNX1 on the regulation of *IL23A* when gastric epithelial cells are challenged by inflammatory stimuli.

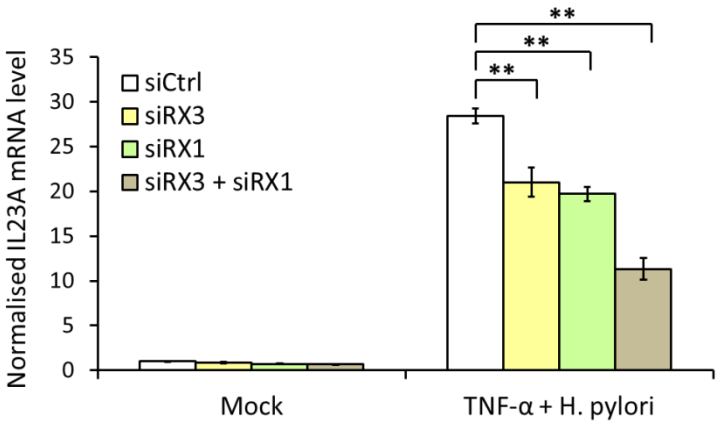
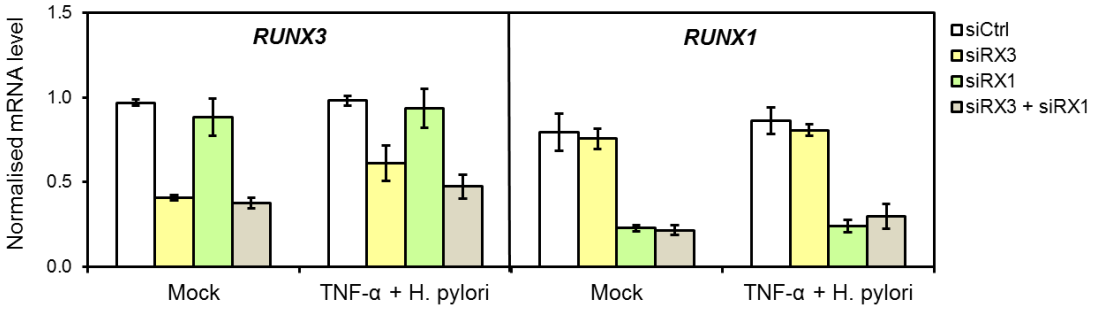


Figure 4.14. The effects of RNAi knockdown for *RUNX3* and *RUNX1* on *IL23A*. HFE-145 cells were transiently transfected with 20pmoles control siRNA (siCtrl) or 5pmoles RUNX3 siRNA (siRX3) and/or 15pmoles RUNX1 siRNA (siRX1) for 48h preceding infection with *H. pylori* (Hp) at MOI100 and stimulation with TNF-α (10ng/ml) for 6h. The transcripts of *IL23A* were measured by qRT-PCR. The values were normalised to those of *GAPDH* and were expressed relative to siCtrl Mock sample. (means ± SEM; n=3). Data are compiled from three separate experiments and Student t-tests were performed (**p<0.01).

A.



B.

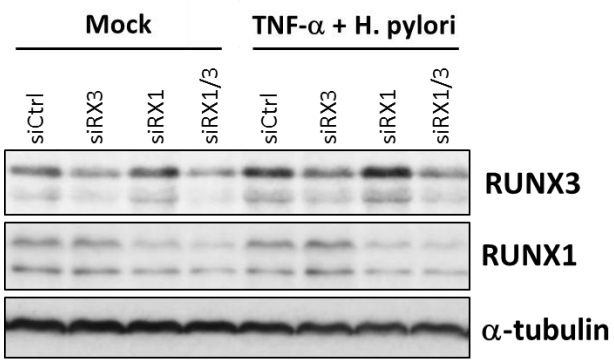


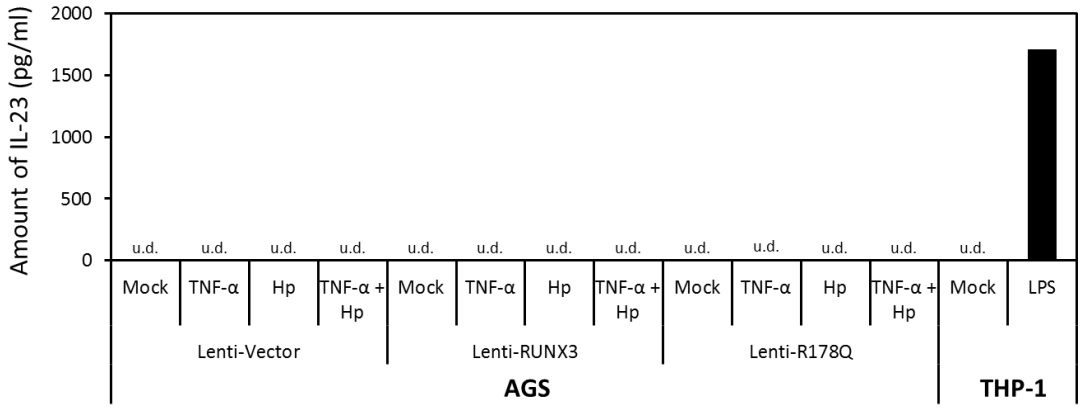
Figure 4.15. The degree of RNAi knockdown for *RUNX3* and *RUNX1* in HFE-145 cells. (A) The normalised mRNA levels of *RUNX3* and *RUNX1* in the same samples described in *Fig. 4.14* were measured by qRT-PCR and expressed relative to the basal values of siCtrl sample (means ± SEM; n=3). (B) Corresponding changes in RUNX3, RUNX1 and α-tubulin (loading control) protein expression were analysed from whole cell lysates by Western blot.

4.2.10 The induction of IL23A in AGS does not result in the secretion of IL-23

To investigate if the expression of IL23A in AGS cells is translated to the secretion of the heterodimeric cytokine IL-23, of which IL23A is a subunit, the supernatants collected from resting and stimulated AGS cells were subjected to enzyme-linked immunosorbent assay (ELISA). Strikingly, in stark contrast to the robust secretion of IL-23 in LPS-stimulated THP-1 cells, the levels of IL-23 in AGS-derived supernatants could not be detected even in the presence of all identified activating signals (Figure 4.16A). A possible explanation for the absence of secreted

IL-23, despite robust expression of IL23A, is the insufficient expression of its partner subunit, IL12B. To investigate this, the mRNA level of *IL12B* was measured in resting and treated AGS cells. Indeed, the partner subunit *IL12B* is neither expressed nor induced by any of the signals (Figure 4.16B). Together, these observations provide firm evidence that IL23A expressed in AGS cells is not secreted in the form of IL-23.

A.



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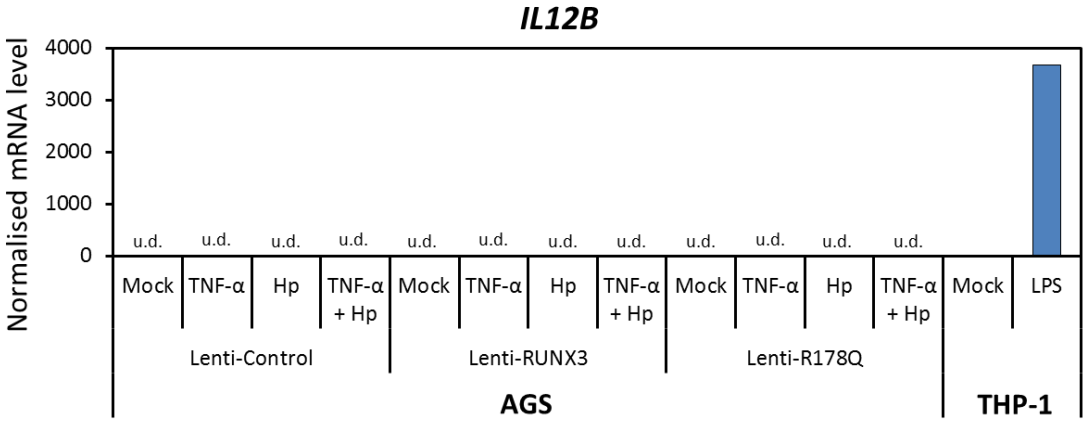


Figure 4.16. The expression of the heterodimeric IL-23 (IL23A/IL12B) in AGS cells. (A) AGS cells were transduced with lentiviruses encoding Lenti-Control, Lenti-RUNX3, Lenti-RUNX3^{R178Q} for 48h preceding TNF-α (10ng/ml) and/or *H. pylori* (MOI100) stimulation for 24h. Supernatants were then harvested and subjected to sandwich ELISA for the quantification of IL-23 secretion. (B) The infected AGS cells as described above were stimulated with TNF-α/or *H. pylori* for 6h. *IL12B* mRNA was quantitated using qRT-PCR and the values were normalised with *GAPDH* and expressed relative to the Mock control in THP-1 cells. (u.d.: undetectable)

4.2 Discussion

As an extension from the earlier observation that *IL23A* is a target gene of RUNX3, this chapter establishes the physiological signals that regulate IL23A expression in gastric epithelial cells. In line with the findings by other groups, *IL23A* is positively regulated by NF-κB signaling in THP-1, human acute monocytic leukemia cell line (Figure 3.9). Thus, different inflammatory stimuli that activate the NF-κB pathway were explored in gastric epithelial cell lines.

NF-κB-activating cytokines TNF-α, IL-1α and IL-1β were found to be inducers of *IL23A* (Figure 4.3). TNF-α and IL-1 are produced in response to bacterial infection, inflammatory and other stimuli primarily by cells of the immune system such as macrophages and T and B lymphocytes (Burchett et al., 1988). These cytokines are involved in coordinating inflammatory response and host defence mechanisms against pathogens (Elkon et al., 1997; Sambhi et al., 1991; Wong and Goeddel, 1986; Yamada et al., 2000). Furthermore, TNF-α and IL-1 belong to a group of cytokines that stimulate acute phase reaction and initiate a cascade of cytokine production for the recruitment of immune cells for host defence (Warren, 1990). The activation of *IL23A* by these upstream signals implicates a role for *IL23A* in host acute inflammatory responses against pathogen challenge. On the other hand, the lack of induction for *IL23A* observed for IFN-γ, IL-6 and TGF-β does not rule out their involvement in this regulation (Figure 4.3). It is possible that the corresponding cytokine receptors are absence in gastric epithelial cells which causes them hyporesponsive to the signals. To rule out the involvement of other cytokines in the regulation of *IL23A*, the positive target genes for each cytokine signal should be analysed.

An intriguing observation is that RUNX3 cooperates strongly with TNF-α in the induction of *IL23A* in gastric epithelial cells (Figure 4.4 and 4.5). Together with the analysis of proximal NF-κB and RUNX consensus binding sites in *IL23A* promoter (Figure 4.6), the possibility of cooperativity between RUNX3 and TNF-α/NF-κB on the transcription of *IL23A* is further strengthened. In addition, the robust expression of IL23A protein induced by RUNX3 and TNF-α underscores the importance of this synergistic cooperation (Figure 4.6). Critically, the above findings revealed that gastric epithelial cells are a novel cell type that expresses IL23A.

Given that NF-κB family members are nuclear effectors of PRRs pathways, the inability of various ligands of these receptors to activate *IL23A* is surprising (Figure 4.7) (Abreu, 2010; Wells et al., 2010). The reason for this could be the expression of PPRs in gastric epithelial cells requires additional challenges, e.g. pathogen infection. The lack of co-receptors such as CD14 and MD2 in gastric epithelial cells may also render them hyporesponsive to TLR ligands (Ferrero, 2005; Watanabe et al., 2010). The NOD1 agonist, iE-DAP, was the exception among all ligands tested in its ability to activate *IL23A* in AGS cells (Figure 4.7). Unlike NOD2 which is a general sensor for bacteria, NOD1 recognises a subset of microbes that contain the dipeptide iE-DAP, such as *H. pylori*, an important pathogen in the stomach (Boughan et al., 2006; McDonald et al., 2005; Viala et al., 2004; Watanabe et al., 2010). The specific induction of *IL23A* by NOD1, together with the notion that the same pathway is required for activation of the classical host immune cytokine IL-8, implicate that *IL23A* belongs to part of a general immune response that targets specific subset of pathogens, e.g. *H .pylori* (Grubman et al., 2010).

The positive correlation between the degree of *H. pylori* infection and *IL23A* mRNA levels implicates that *IL23A* may be required for evoking a strong immune response for bacteria clearance (Figure 4.8). Notably, the lack of induction of *IL23A* in AGS cells by heat-killed *H. pylori* suggests that it is an active and energy-dependent process (Figure 4.8). However, the possibility that heat denatures some bacterial agonists require for the induction of *IL23A* cannot be ruled out. Nevertheless, the delivery of bacterial products into host cells requires adhesion of live *H. pylori* to the host cells for the insertion of the Type IV secretion system (Kwok et al., 2007; Rohde et al., 2003). The active induction of *IL23A* by *H. pylori* indicates the involvement of Type IV secretion system (Figure 4.8).

CagA protein delivered by *H. pylori* into the host cells was crucial for sustained induction of *IL23A* (Figure 4.9). As discussed in Section 1.3.1, CagA-positive strain of *H. pylori* is more virulent compared to CagA-negative strain. The prolonged induction of *IL23A* in gastric epithelial cells may be necessary to mount a proper immune response against CagA-positive *H. pylori*. The CagA-dependent induction of *IL23A* was suppressed when SHP2 activity was inhibited implicating that injected CagA protein is phosphorylated in AGS cells that leads to preferential activation of ERK1/2 signaling (Figure 4.11). Although, the transcription factors that are activated downstream of CagA/SHP2/ERK pathway remains unknown, ETS family of transcription factors which are well-known to be activated by ERK could be one of them (Foulds et al., 2004; Paumelle et al., 2002). Interestingly, ETS1 is also known to interact with RUNX1 protein on Mo-MLV and TCRβ enhancers in immune cells (Sun et al., 1995; Wotton et al., 1994). Therefore, the involvement of ETS1 and its cooperation with RUNX proteins in CagA/SHP2-dependent activation of *IL23A* in gastric epithelial cells will be worth investigating in the future. As inhibitor often has

broad spectrum effects, the involvement of SHP2 in the induction of IL23A by CagA requires further confirmation using RNAi specific targeting of SHP2 in the cells.

The conservation of the effects for TNF-α and *H. pylori* on *IL23A* observed in different gastric epithelial cell lines implicates that IL23A is part of the natural host immune response of gastric epithelial cells (Figure 4.12). More importantly, the induction of IL23A by TNF-α and *H. pylori* was markedly potentiated by RUNX3, indicating a critical role of RUNX3 for the cooperative activation of IL23A expression (Figure 4.13). The ineffectiveness of $RUNX3^{R178Q}$ suggests that the synergistic cooperation between RUNX3 and inflammatory stimuli happens at the level of transcriptional activation and requires the binding to DNA. Reduction of *IL23A* levels upon the knockdown of RUNX3 further confirmed its positive role in the regulation of *IL23A* in the presence of TNF-α and *H. pylori* (Figure 4.14). These observations implicate the importance of RUNX3 in the regulation of *IL23A* during inflammation and infection.

An intriguing observation is that IL23A expressed in AGS cells was not secreted in the known heterodimeric form, IL-23 (Figure 4.16A). This was likely due to the absence of the partner subunit IL12B (Figure 4.16B). A similar observation has been reported in intestinal epithelial cells where *IL23A* was expressed in the absence of *IL12B* (Maaser et al., 2004). Other studies have shown that IL23A was up-regulated in synovial fibroblasts and colonic subepithelial myofibroblasts, especially in disease states (Kim et al., 2007a; Liu et al., 2007; Zhang et al., 2005). However, little is known about the function of IL23A in these tissue contexts. In mice, gene targeting of the cytokine and its receptor generally have same phenotypes. However, Ghilardi and co-workers found an unexpected uncoupling of the phenotypes of *Il23a*$^{-/-}$

and *Il23R*$^{-/-}$ mice. Specifically, they reported that the histological scores of *Il23a*$^{-/-}$ mice were reproducibly less severe than those of *Il23R*$^{-/-}$ mice in chemical-induced colitis (Cox et al., 2012). These observations, together with those observed in the current study suggest that IL23A may functionally interact with proteins other than IL12B to mediate signals distinct from that of IL-23, possibly through another IL-23R-associated heterodimeric receptor.

Findings in this chapter revealed that *H. pylori* infection and TNF-α are two major upstream signals for regulating IL23A expression in gastric epithelial cells. Interestingly, the cooperation between RUNX3, TNF-α and *H. pylori* significantly enhanced IL23A expression in gastric epithelial cells. The regulation of IL23A by TNF-α and *H. pylori* in these cells implicates that this protein is part of the host defence mechanisms. Inactivation of RUNX3 that is frequently observed during gastric carcinogenesis may result in lower production of IL23A by gastric epithelial cells leading to defective host immune response against pathogen infection. The data presented in this study further suggests that IL23A is not secreted as the known heterodimeric form (IL-23) in AGS cells. There is a possibility that this protein is not secreted by gastric epithelial cells, or it might be secreted in an unknown form. Thus, the secretion of IL23A protein and the functional consequences of the above regulation in gastric epithelial cells will be further investigated in the following chapter.

Chapter 5:

The functional effects of gastric epithelial cell-derived IL23A on immune cells

5.1 Introduction

5.1.1 The discoveries and known functions of IL23A/IL-23

IL23A was discovered on the basis of the structural resemblance of its predicted tertiary structure to that of IL12A, which together with IL12B forms the heterodimeric cytokine, IL-12 (Oppmann et al., 2000). Further characterisation revealed that IL23A has no biological activity of its own, but when dimerised with IL12B they form a novel cytokine, IL-23 with distinct biological activities (Figure 1.5). Although IL23A possesses a signal peptide within its N-terminal region that is characteristic of a secretory protein, it was demonstrated that the secretion of IL23A is only efficient in the presence of its partner subunit, IL12B (Oppmann et al., 2000). Therefore, it was suggested that cellular secretion of IL23A takes place only following dimerisation with an interacting partner protein (Oppmann et al., 2000).

Similar to IL-12, IL-23 is usually secreted by activated antigen presenting cells (APC), in particular dendritic cells (DC) and macrophages (Hsieh et al., 1993; Macatonia et al., 1995; Oppmann et al., 2000). Studies have defined IL-12 as an important factor for the differentiation of IFN-γ producing Type I-helper T cells (T_H1) cells from naïve T cells (Gazzinelli et al., 1993; Hsieh et al., 1993; Manetti et al., 1993; O'Garra and Arai, 2000; Seder et al., 1993; Tripp et al., 1993). Therefore, the physiological function of IL-23 was first examined in the context of T cell proliferation and IFN-γ secretion. It was found that recombinant human IL-23 induced the proliferation of CD45RO memory T cells and the secretion of IFN-γ after prolonged CD3 and CD28 co-activation (Oppmann et al., 2000). These immune responses were effectively blocked by incubation with neutralising antibodies against the IL12B subunit (common partner for IL-23 and IL-12) and its receptor IL12RB1

but not IL12A (the unique subunit for IL-12) suggesting the above effects are specific to IL-23 (Oppmann et al., 2000).

Further insights into the unique function of IL-23 were generated in studies of the *IL23a* knockout mouse which revealed a crucial role in the development and effector functions of T_H17, a novel helper T cell subset characterised by its secretion of IL-17 (Langrish et al., 2005; McGeachy et al., 2007; McGeachy et al., 2009). This prompted the purification of human T_H17 cells for further characterisation of IL-23 function. It is now well established that IL-23 is critical for the survival and proliferation of human T_H17 cells and their production of IL-17 for the recruitment of neutrophils to the site of microbial infection (Acosta-Rodriguez et al., 2007; Cruz et al., 2006; Kolls and Linden, 2004; Meeks et al., 2009; Stockinger and Veldhoen, 2007; Volpe et al., 2008; Wilson et al., 2007).

Given the importance of IL-23's effects on T cells, the functional consequences of gastric epithelial cell-derived IL23A will be tested on human (peripheral blood mononuclear cells) PBMC-derived T cells by employing a similar approach adopted by earlier studies on the characterisation of the heterodimeric cytokine IL-23 (IL23A/IL12B).

5.2 Results

5.2.1. Accumulation of intracellular IL23A in AGS cells

In the previous chapter, it was revealed that despite a robust expression of IL23A following its induction by RUNX3, TNF-α and *H. pylori*, no secretion of IL-23 was observed, possibly due to the absence of IL12B. To confirm the above observation, a panel of gastric epithelial cell lines were tested for the secretion of IL-23 upon TNF-α treatment with ELISA. In line with the previous observation, the absence of secreted IL-23 was not restricted to AGS cells, but consistently observed in multiple gastric cancer cell lines (Figure 5.1). However, these assays were performed using a 'sandwich' ELISA specific for the detection of IL23A/IL12B (IL-23) heterodimer. Therefore, the possibility of the secretion of IL23A from gastric epithelial cells could not be excluded.

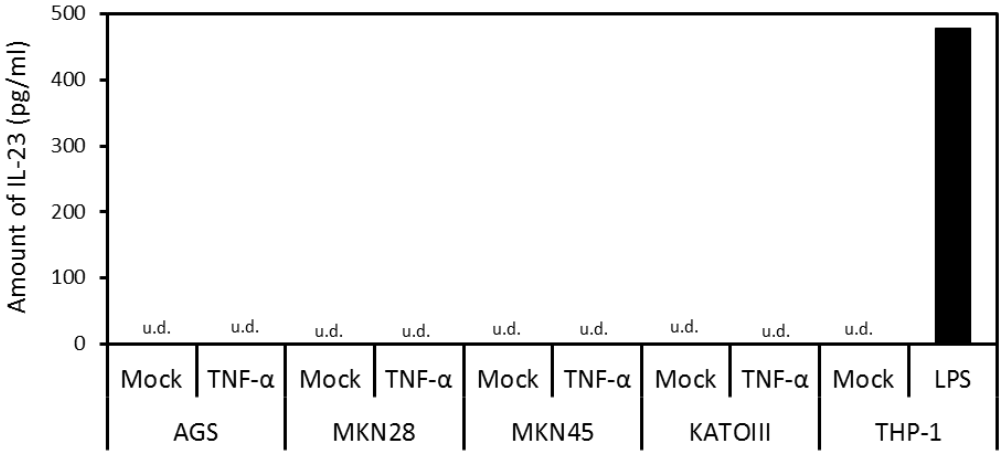


Figure 5.1. The induction of heterodimeric IL-23 by TNF-α in multiple gastric epithelial cell lines. Gastric epithelial cell lines AGS, MKN28, MKN45 and KATOIII were treated with 10ng/ml of TNF-α whereas THP-1 was treated with 1μg/ml of LPS for 24h. Supernatants collected from these cells were subjected to IL-23 ELISA. (u.d.: undetectable).

To further investigate the above possibility, AGS cells were stimulated to express IL23A in the presence or absence of a secretion-blocking agent, fungal macrocyclic lactone Brefeldin A (BfdA). The BfdA has proved to be a potent inhibitor of protein secretion in the endomembrane system of mammalian cells. (Dinter and Berger, 1998; Fujiwara et al., 1988; Klausner et al., 1992; Misumi et al., 1986; Nylander and Kalies, 1999; Sciaky et al., 1997). Newly synthesised secretory proteins are docked to the rough endoplasmic reticulum (ER) and transited through the Golgi apparatus before being exported to the cell surface (Palade, 1975). Treatment with BfdA causes the *trans*-Golgi network to collapse and thus the accumulation of proteins inside the rough ER (Lippincott-Schwartz et al., 1991; Reaves and Banting, 1992).

To assess the secretion of IL23A, AGS cells transduced with lentiviruses encoding Lenti-Control, Lenti-RUNX3 or Lenti-RUNX3^{R178Q} were stimulated with TNF-α and *H.pylori*, before blocking with BfdA for duration of 8h. The results presented in Figure 5.2 revealed that inhibition of protein secretion by BfdA led to a clear induction of IL23A in Lenti-RUNX3 infected AGS cells even in the absence of inflammatory stimuli, compared with the unstimulated control (-BfdA) (Figure 5.2, lanes 3 and 4). Furthermore, a clear increase in intracellular IL23A protein was observed in the *H. pylori*- and TNF-α-stimulated Lenti-Control and Lenti-RUNX3^{R178Q} samples (Figure 5.2, lanes 7, 8, 11 and 12). Consistent with this trend, the accumulation of IL23A in the cells was markedly higher in the presence of RUNX3, TNF-α and *H. pylori*, reflecting their cooperative activation of IL23A (Figure 5.2, lanes 9 and 10). Together, these data reveal that the accumulation of IL23A following blockade of protein export, hence suggesting its secretion by AGS cells when induced by RUNX3, TNF-α and *H. pylori*.

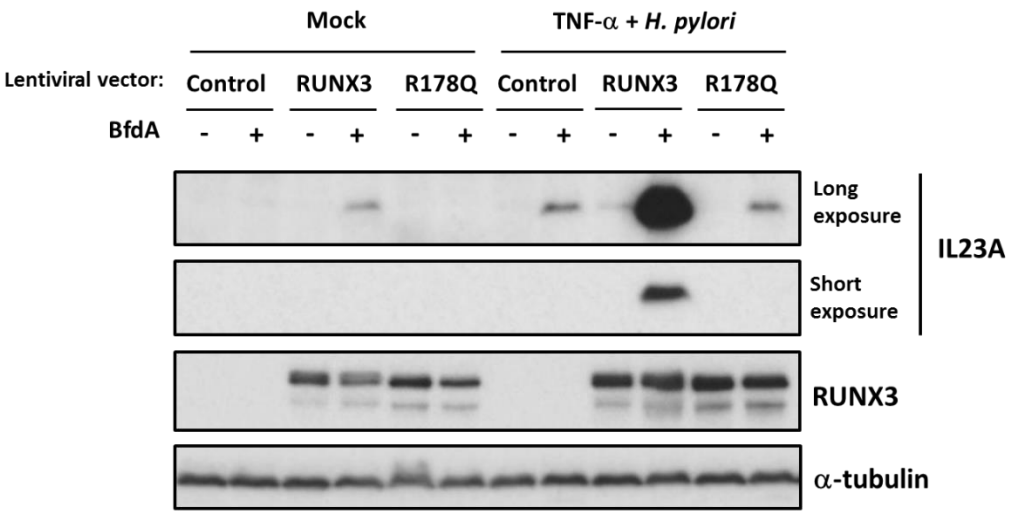


Figure 5.2. The accumulation of intracellular IL23A in AGS cells. AGS cells were transduced with lentiviruses expressing Control, RUNX3 or RUNX3^{R178Q} for 48h preceding TNF-α (10ng/ml) treatment and *H. pylori* (MOI100) infection for 9h. Brefeldin A (BfdA) solution (1:1000) was added into the culture medium at the last 8h before the cells were harvested. Whole cell lysates were then prepared and analysed for the expression of IL23A, RUNX3 and α-tubulin (loading control) using Western blot. A shorter exposure for IL23A protein was included to show the band morphology of IL23A induced by RUNX3, TNF-α and *H. pylori* in the presence of BfdA.

5.2.2 The accumulation and secretion kinetics of IL23A

To better understand the kinetics of IL23A accumulation and secretion, AGS cells infected with Lenti-RUNX3 virus were treated with TNF-α and *H. pylori* for different durations before the blockade of protein export by BfdA in the last 8h. The results showed that the accumulation of IL23A protein resulting from the action of BfdA could be observed over multiple time points (Figure 5.3). The highest accumulation of IL23A protein was observed at 9h following TNF-α and *H. pylori* stimulation indicating that the protein was synthesised and secreted within a short period of time (Figure 5.3, lanes 3 and 4). These results provide strong evidence for the secretion of IL23A by AGS cells upon induction. Furthermore, they revealed that the secretion of IL23A occurs transiently where maximal secretion occurs within 9h.

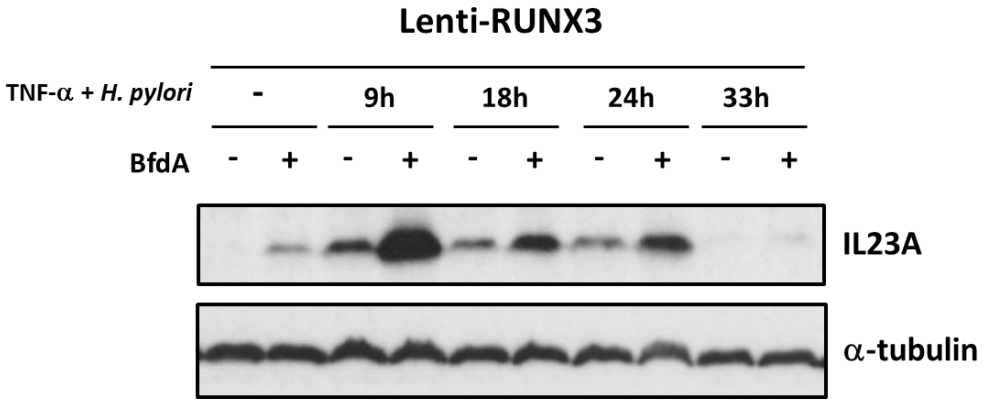


Figure 5.3. The accumulation and secretion kinetics of IL23A in AGS cells. AGS was transduced with Lenti-RUNX3 virus as described in *Fig 5.2* prior TNF-α (10ng/ml) and *H. pylori* (MOI100) stimulation for 1h, 10h, 16h, and 25h followed by addition of Brefeldin A (BfdA) solution for 8h before the cells were harvested. Whole cell lysates were then prepared and analysed for the expression of IL23A and α-tubulin (loading control) using Western blot.

5.2.3 Neutralising antibody effectively depletes IL23A from culture supernatant

As a first step towards the functional characterisation of secreted IL23A in the supernatant of AGS cells, a monoclonal IL23A-specific antibody was tested for its ability to immunodeplete IL23A. The effectiveness of this antibody was assessed on its ability to deplete (or "neutralise") secreted IL-23 in the supernatant of LPS-activated THP-1. The extent of depletion was measured by IL-23-specific ELISA and results revealed a marked decrease in the secreted IL-23 following pre-incubation with the anti-IL-23A antibody, compared with an anti-IgG antibody (Figure 5.4). This result demonstrates that the anti-IL23A neutralising antibody was effective in depleting secreted IL23A and is therefore suitable for functional study of this protein in the subsequent assays.

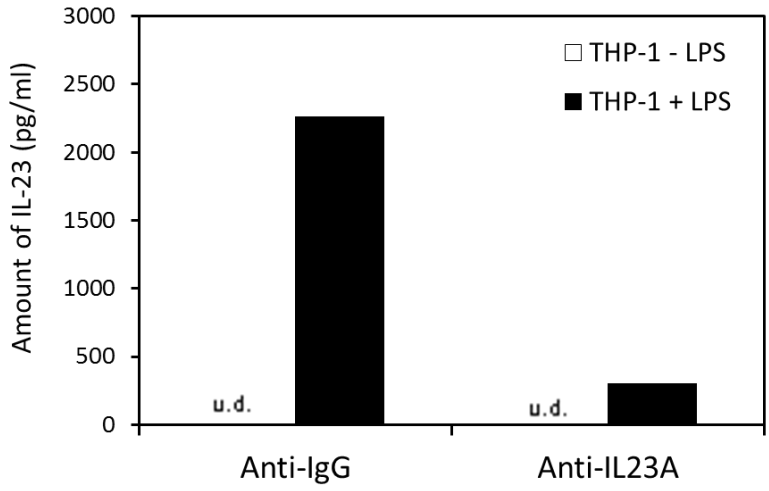


Figure 5.4. The effects of IL23A-specific neutralising antibody on secreted IL-23. THP-1 cells were stimulated with 1μg/ml of LPS for 6h. Supernatants from both stimulated and non-stimulated THP-1 cells were harvested and incubated with either 2μg/ml of anti-IL23A or IgG control antibody for 4h at 37°C. The treated supernatants were then subjected to sandwich ELISA for the quantitation of IL-23 in the supernatants. (u.d.: undetectable)

5.2.4 The activation of T cell proliferation by anti-CD3 antibody

To establish a T cell model for studying the functional effects of the secreted IL23A from gastric epithelial cells, human peripheral blood mononuclear cells (PBMC) were treated with an anti-CD3 monoclonal antibody to activate T cells. For the purpose of optimising the time frame for T cell proliferation, PBMC were pulsed with a fluorescence dye CFSE (carboxyfluorescein diacetate, succinimidyl ester) followed by the activation with anti-CD3 in *ex vivo* culture for three and seven days. The results showed that there was no obvious difference in CFSE fluorescence intensity in PBMC three days after anti-CD3 activation compared to those at resting state (Figure 5.5). In contrast, a clear decrease in CFSE fluorescence was observed at day 7 in anti-CD3 activated T cells (Figure 5.5). The shift of cell population towards lower CFSE fluorescence intensity indicates that T cell division occurred seven days post-stimulation with anti-CD3. Having established seven days is required to achieve robust T cells proliferation *ex vivo* following anti-CD3 activation, this regime will be used for investigating the effects of IL23A induced by ectopic RUNX3 in gastric epithelial cells.

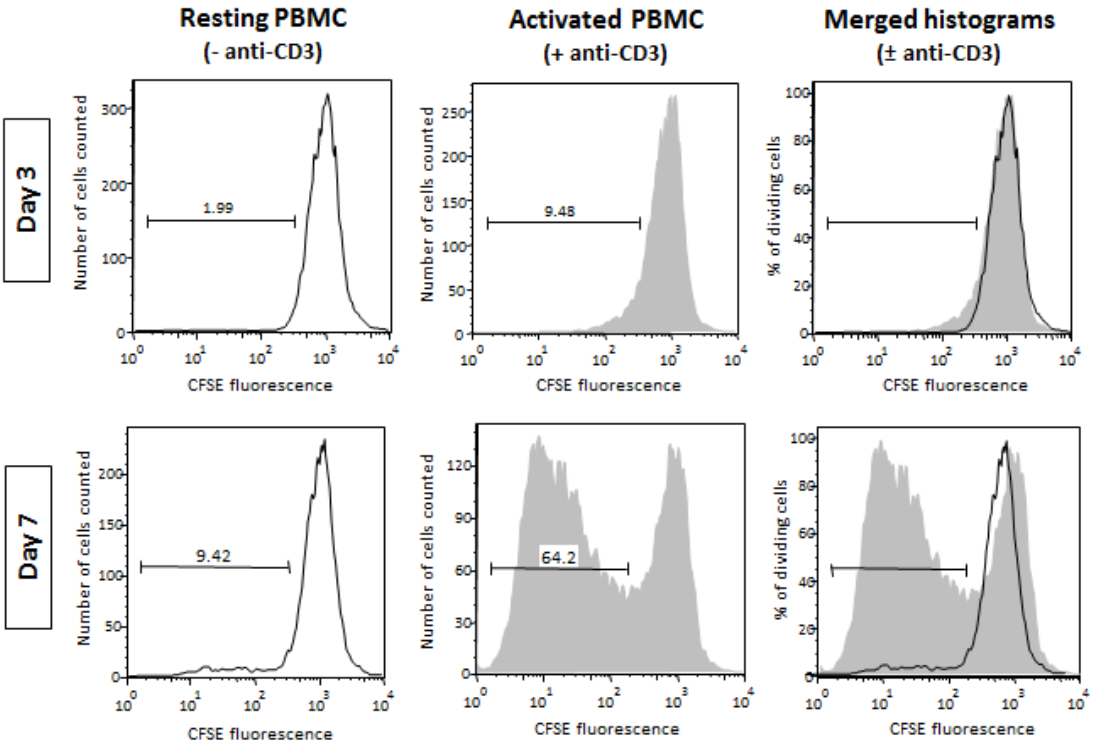
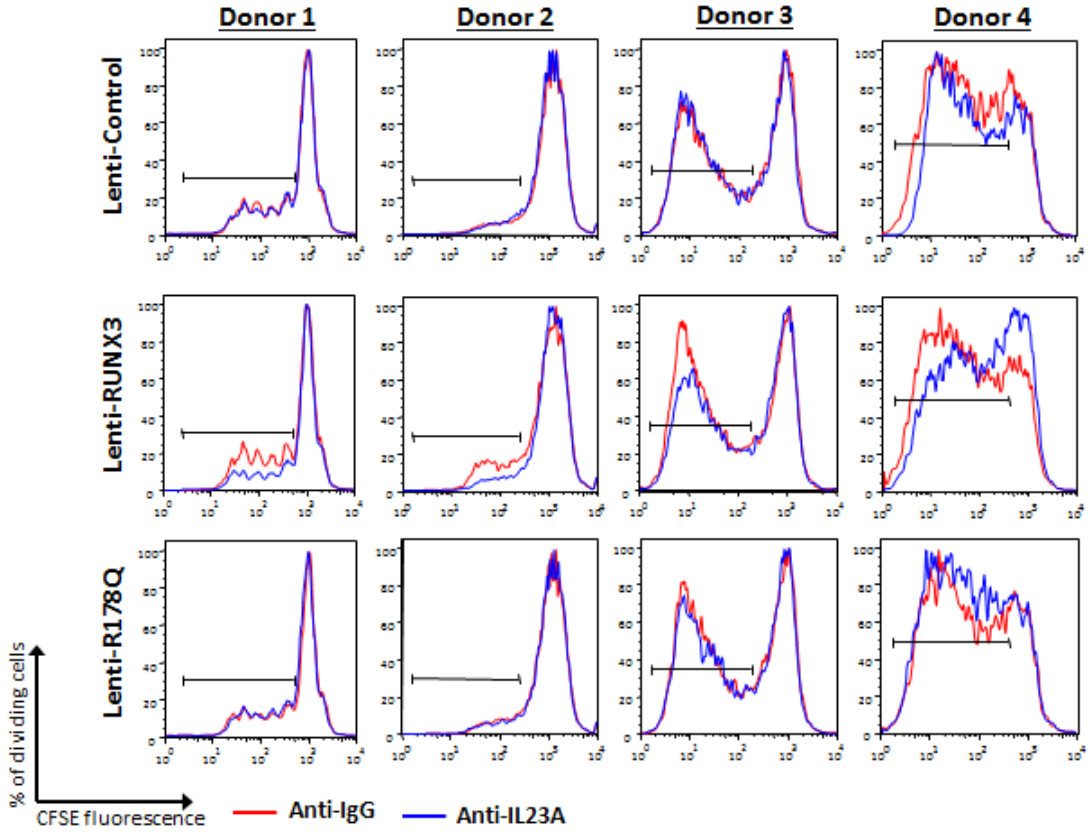


Figure 5.5. The proliferation of anti-CD3 activated T cells derived from human PBMC. PBMC harvested from human blood sample was stained with 5μM CFSE dye prior to the activation by 1.1ng/ml of anti-CD3 antibody to stimulate T cell proliferation as described in materials and methods (section 2.9.2). Next, 0.2 x 10^6 of cells were seeded into each 96-well U bottom plate in fresh medium and cultured for three and seven days. Analysis of CFSE fluorescence was performed by flow cytometry. A gate was set to demarcate the proliferative T cells based on the profile in resting PBMC of day 3 and day 7 respectively.

5.2.5 Secreted IL23A stimulates T cell proliferation

To assess the biological activity of secreted IL23A on T cell proliferation, anti-CD3 activated PBMC from four different donors were harvested and co-cultured with supernatants of AGS cells treated with either anti-IL23A or anti-IgG neutralising antibody. In these four independent experiments, significant variations in the proliferative potential of PBMC-derived T cells from different donors were observed (Figure 5.6A). This variation could be attributed to the different health statuses of the individual donors, which may affect the concentration of different mononuclear cells in the PBMC. Notwithstanding these fluctuations, a modest increase in T cell proliferation could be observed when cultured in the supernatants derived from AGS cells stably expressing RUNX3 in these four donors (Figure 5.6A). Importantly, the increase in T cell proliferation was specifically blocked in the same supernatants that were neutralised with anti-IL23A antibody, but not in anti-IgG control treated samples (Figure 5.6A). In contrast, the proliferative populations in anti-IgG- and anti-IL23A-treated supernatants were indistinguishable in the cases of Lenti-Control and Lenti-RUNX3^{R178Q} infected AGS cells, which is consistent with the lack of secreted IL23A in these samples (Figure 5.6A). The T cell proliferation assay for each PBMC donor was performed in triplicates and the data from one representative donor were presented in Figure 5.6B. Together, these data suggest that RUNX3-induced IL23A in gastric epithelial cells play a positive role in T cell proliferation.

A.



B.

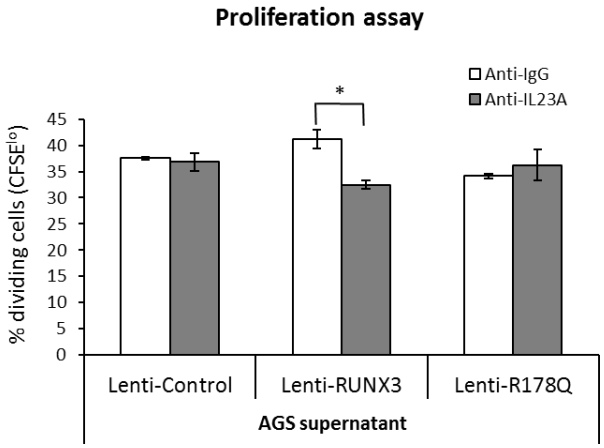


Figure 5.6. The effects of supernatants derived from AGS cells on T cell proliferation. (A) CFSE-stained PBMC cultured in the presence of anti-CD3 (1.1ng/ml) was incubated with the supernatants harvested from AGS cells infected with Lenti-Control, Lenti-RUNX3 or Lenti-RUNX3^{R178Q} viruses that were treated with either 2μg/ml of anti-IgG or anti-IL23A antibody. PBMC was cultured in AGS-derived supernatants for 7 days prior to analysis by flow cytometry. Due to the large variation in the extent of T cell proliferation between donors, the gating for proliferative T cells was set based on the Lenti-Control sample within each donor. The experiments for each PBMC donor were performed in triplicates and the flow cytometry plots from one replicate were presented. (B) Percentage of proliferating cells in response to AGS-derived supernatants. Data from a representative donor was presented and expressed as percentage of dividing cells. Data are presented as means ± SEM (n=3). * $p < 0.05$

5.2.6 IFN-γ production of T cells is modulated by secreted IL23A

To gain further insights into the effects of AGS-derived IL23A on activated T cells, the culture supernatants of T cells were analysed for cytokine contents seven days after *ex vivo* culture. As IFN-γ is a signature cytokine produced by T_H1 cells and IL-17 marks T_H17 cells, the secretion of these two cytokines were measured in our study. As shown in Figure 5.7A, the level of IFN-γ in the T cell supernatant stimulated with Lenti-RUNX3 sample was significantly reduced following the depletion of IL23A by anti-IL23A antibody. Such a reduction was not observed in control samples in which activated T cells were cultured with supernatants from Lenti-Control and Lenti-RUNX3^{R178Q} samples (Figure 5.7A).

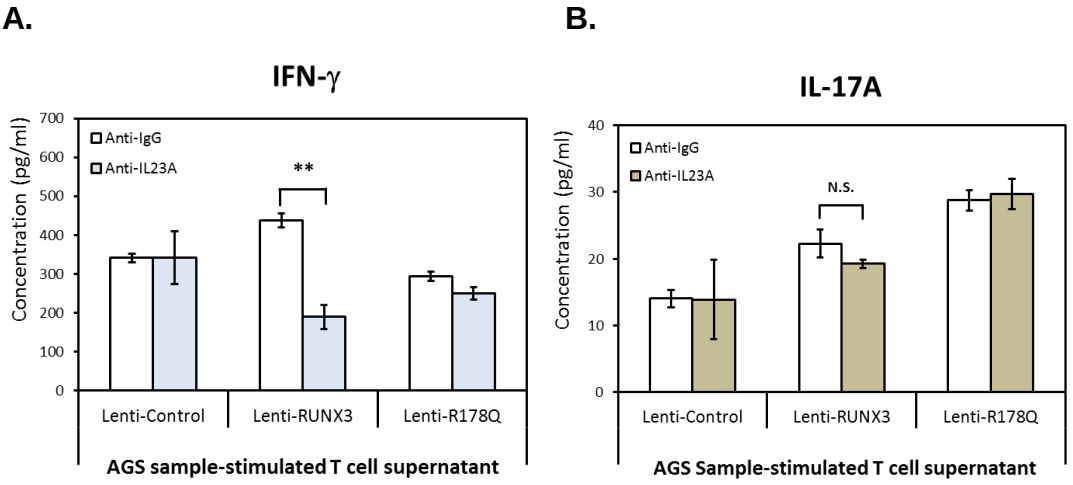


Figure 5.7. The secretion of IFN-γ and IL-17 by T cells in response to various supernatants of AGS cells. The supernatants from T cells at seven days post-stimulation by the supernatants derived from AGS cells infected with Lenti-Control, Lenti-RUNX3 and Lenti-RUNX3^{R178Q} viruses that were pre-treated with 2μg/ml anti-IgG or anti-IL23A antibody. The supernatants from cultured T cells were collected and subjected to ELISA for quantification of (A) IFN-γ and (B) IL-17. Data were expressed relative to anti-IgG of each donor and presented as means ± SEM (n=3). ** $p < 0.01$, (N.S.: not significant.)

Notably, the secretion of IL-17 by T cells was not altered in the presence of IL23A-specific neutralising antibody in the Lenti-RUNX3 sample (Figure 5.7B). This is surprising as IL-17 is normally produced by T cells in response to stimulation by the heterodimeric IL-23. The changes in the production of IFN-γ and IL-17 were consistently observed in PBMC derived from four independent donors (Figure 5.8A and 5.8B). The data provides evidence that AGS-derived IL23A plays a role in effecting a change in the T cell behaviour towards an IFN-γ producing phenotype.

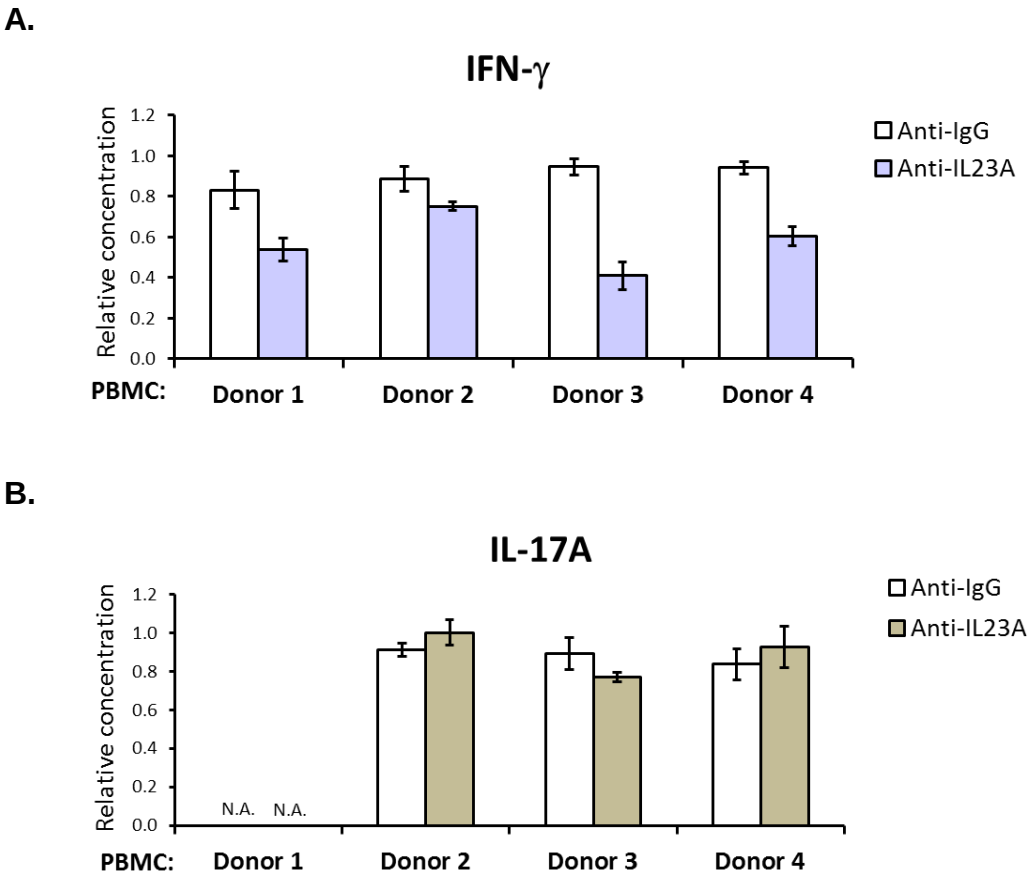


Figure 5.8. The secretion of IFN-γ and IL-17 by T cells derived from different donors in response to supernatants from Lenti-RUNX3 sample. T cells derived from PBMC of different donors were stimulated with supernatants derived from AGS cells infected with Lenti-RUNX3 viruses for 7 days. These supernatants were pre-treated with 2μg/ml anti-IgG or anti-IL23A antibody. The supernatants of cultured T cells were collected and subjected to ELISA for quantification of (A) IFN-γ and (B) IL-17. Data presented were expressed relative to those of anti-IgG of each donor as means ± SEM (n=3). (N.A.: not available)

5.3 Discussion

The discovery of the expression of IL23A in gastric epithelial cells upon synergistic induction by RUNX3, TNF-α and *H. pylori* raises important questions. *H. pylori* induces stomach inflammation by triggering host immune response whereas TNF-α is a host cell factor activated during infection and is crucial for coordinating immune response (Elkon et al., 1997; Naumann and Crabtree, 2004; Pasparakis et al., 1996; Peek and Crabtree, 2006; Sambhi et al., 1991; Wong and Goeddel, 1986). The cooperation of RUNX3 with these key inflammatory signals implicates a potential communication between epithelial cells and immune cells through IL23A. This chapter addresses the functional effects of IL23A regulated by RUNX3 and its contribution to T cell response.

The lack of secretion of IL-23 by gastric epithelial cell lines has prompted the investigation of IL23A secretion using a protein secretion blocker (Figure 4.16A and 5.1). Inhibition of intracellular IL23A protein trafficking by BfdA resulted in a robust accumulation of this protein, suggesting that IL23A is targeted for secretion in AGS cells (Figure 5.2 and 5.3). However, the above observation does not rule out the possibility of IL23A being targeted to the plasma membrane of the cells as both secretory and transmembrane proteins share the same transportation pathway that is targeted by BfdA. Nevertheless, solubilisation of the membrane proteins using a stronger lysis method did not result in greater level of IL23A protein detected compared to current lysis method (data not shown). Moreover, it was reported that IL23A contains a secretory signal peptide but lack transmembrane domain (Inoue, 2010). Therefore, our results suggest that AGS-derived IL23A is most likely a secretory protein rather than a membrane-bound surface protein. The induction time-

course experiment showed that IL23A was expressed and secreted at an early time point (Figure 5.3). This temporal pattern of IL23A secretion implicates that this protein is part of the acute inflammatory response against microbial infection such as *H. pylori*. Given the paradoxical roles of IL23A in host defence mechanism and autoimmunity in the gastrointestinal tract (section 1.5); it is of utmost importance to explore the functional relevance of this protein in gastric epithelial cells.

The immunoregulatory effects of the secreted IL23A in gastric epithelial cells was investigated in an *ex vivo* culture system that employs T lymphocytes as responder cells. This approach will shed light on the existence of IL23A by the measureable function on T cell response. PBMC was chosen in this study as it contains a mixture of T and B lymphocytes, granulocytes and monocytes. The presence of these cells may be necessary for the amplification of T cell response, and is therefore a suitable platform for the exploration of IL23A's effect. Using anti-CD3 activated T cells derived from PBMC, a proliferative signal on these cells was observed from IL23A-containing supernatants derived from gastric epithelial cells (Figure 5.6A and 5.6B). Importantly, this enhanced proliferation could be blocked by an anti-IL23A neutralising antibody, hence confirming the specificity of IL23A's effect. Unlike anti-CD3 which is a general inducer for T cell proliferation, RUNX3-induced IL23A resulted in a modest increase in the percentage of proliferative T cell population in addition to the dramatic elevation caused by anti-CD3 (Figure 5.5 and 5.6A) (Frauwirth and Thompson, 2002; Trickett and Kwan, 2003). This interesting observation suggests that the modest increase in the percentage of proliferative population may be attributed to a unique subset of responsive T cells. Thus, identification and purification of this specific subset of responder T cells would potentially demonstrate a clearer effect of IL23A derived from AGS cells.

As IL23A secretion has been shown to be dramatically induced by RUNX3, TNF-α and *H. pylori*, supernatants from cells stimulated by these three activating signals would result in greater responsiveness by T cells. However, the drawback of using PBMC as a model is that these cells respond strongly to inflammatory stimuli (e.g. bacteria) especially in the presence of monocytes (Zareie et al., 2001; Ziegler-Heitbrock, 2007). This strong immune response caused by bacteria may mask the true biological effects of IL23A. Consequently, TNF-α- and *H. pylori*-treated samples could not be included in the current experimental setup; the modest differences observed for T cell proliferation could be attributed to the low level of IL23A secreted by AGS cells in the absence of these inflammatory stimuli (Figure 5.2). To enhance the level of IL23A and avoid non-specific effects from PBMC, purification of IL23A from the supernatant of gastric epithelial cells will be required to concentrate the amount of secreted IL23A in order to induce greater T cell response.

In addition to the proliferative properties of T cells, measurement of cytokine secretion from T cells will also shed light on the effects of IL23A on the different subsets of T lymphocytes. Intriguingly, a significant difference was observed for the secretion of IFN-γ but not IL-17 by T cells in response to IL23A (Figure 5.7 and 5.8). This is unexpected as IL-23 is critical for the differentiation of IL-17 secreting T_H17 cells (Langrish et al., 2005; McGeachy et al., 2007; McGeachy et al., 2009). These data provide further evidence that gastric epithelial cell-derived IL23A plays a different role in eliciting T cell response as compared to IL-23 secreted by monocytes.

In summary, these functional studies further strengthen the secretion of IL23A and therefore the importance of its regulation by RUNX3, TNF-α and *H. pylori* in gastric epithelial cells. It is possible that RUNX3 is involved in the host inflammatory

response against pathogens through the regulation of IL23A secreted by gastric epithelial cells. The above phenomenon observed in T cells may reflect the communication between gastric epithelial cells and immune cells via IL23A. Therefore, we postulate that in addition to its previously reported effects on epithelial cell growth and apoptosis, RUNX3 promotes an effective inflammatory response by activating IL23A in response to inflammatory signals and infection. Studies have reported that efficient secretion of IL23A requires dimerisation with a partner subunit (Kopp et al., 2003; Oppmann et al., 2000). For instance, secretion of IL-23 by keratinocytes was promoted in transgenic mice that constitutively express IL12B (Kopp et al., 2003). However, there is no report to date about the association of IL23A with other subunits of the IL-12 family other than IL12B. It is possible that the partner subunit of IL23A in gastric epithelial cells has yet to be discovered and the identity of this unknown cytokine awaits further investigation.

Chapter 6:

General discussion and future works

6.1 Summary of findings

The current study establishes *IL23A* to be a novel target gene of the transcription factor and gastric tumour suppressor RUNX3 in gastric epithelial cells. RUNX3 was found to promote the transcription of *IL23A* in the majority of gastric cancer cell lines tested in this study. Through a combination of biochemical and cell biological studies, it demonstrated that RUNX3 binds to three RUNX binding site in the proximal *IL23A* promoter to transactivate *IL23A* transcription. In elucidating the upstream signals that regulates this gene in gastric epithelial cells, it was found that the proinflammatory cytokine TNF-α and gastric pathogen *H. pylori* act as potent activating signals for *IL23A* in a wide range of gastric epithelial cells. It was shown that TNF-α mediates its activation of *IL23A* via a conserved NF-κB site at the proximal promoter. Activation of the NF-κB pathway by NOD1 also appears to play a role in the transient activation of *IL23A* transcription following *H. pylori* infection. However, sustained induction of this gene is determined by the activation of SHP2 pathway and the presence of oncoprotein CagA, thereby revealing an intriguing two-phase induction mechanism. Importantly, RUNX3 is a critical requirement for a synergistic induction of *IL23A* transcript and protein by TNF-α and *H. pylori*. Furthermore, promoter studies and RNAi experiments revealed that RUNX1 plays similar but non-identical role as RUNX3 in the regulation of *IL23A* in gastric epithelial cell lines. Although the robust expression of IL23A promoted by RUNX3, TNF-α and *H. pylori* identifies gastric epithelial cell as a novel source cell of IL23A, it does not appear to be secreted in its known heterodimeric form (i.e. IL-23). Nevertheless, the secretion of IL23A is supported by its rapid accumulation following the blockade of cellular protein export by Brefeldin A. Furthermore, stimulating the human T cells with secreted IL23A derived from AGS cells reveals putative functions

for this protein in promoting T cell proliferation and IFNγ production. Collectively, these findings indicate that RUNX3 as well as RUNX1 may play a hitherto unappreciated role in modulating mucosal immune response via a direct regulation of IL23A during gastric inflammation and infection by *H. pylori*. As *H. pylori*-induced inflammation represents a major risk factor in gastric cancer, these findings suggest RUNX3 may exert its tumor suppressive effect in part through an influence in gastric mucosal immunity and inflammation. Based on the data presented in this study, a model of RUNX1/3 regulation of IL23A is depicted in Figure 6.1.

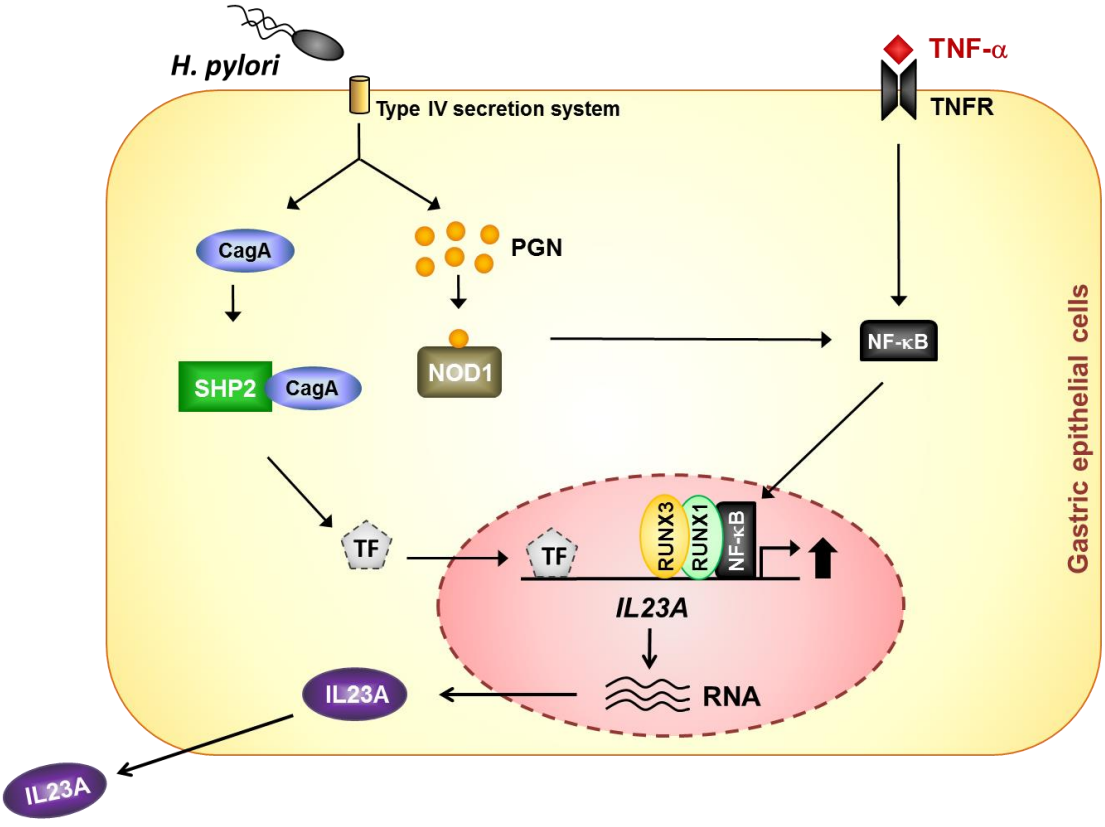


Figure 6.1. A model illustrating the regulation of IL23A by RUNX3 and inflammatory signals triggered by TNF-α and *H. pylori* in gastric epithelial cells. 'TNFR' and 'PGN' denote tumour necrosis factor receptor and peptidoglycan, respectively; "⬆" denotes transcriptional activation.

6.2 Significance of findings and future works

Host immunity and inflammation are intimately linked with cancer. The long-held theory of immune surveillance proposes that cells and tissues are constantly monitored by an ever-alert host immunity that is responsible for identifying and destroying the vast majority of incipient cancer cells, and thus nascent tumour (Burnet, 1970; Thomas, 1959). This theory was firmly supported by substantial studies from genetically engineered mice and clinical epidemiology, which showed that host immunity operates as a significant barrier to tumour formation and progression (Bindea et al., 2010; Ferrone and Dranoff, 2010; Kim et al., 2007b; Nelson, 2008; Smyth et al., 2006; Teng et al., 2008). On the other hand, tumour-associated inflammatory response had the unanticipated, paradoxical effect of enhancing tumorigenesis, in effecting incipient neoplasias to acquire the hallmark capabilities of cancer. Subsequent studies on the intersections between inflammation and cancer have produced abundant and compelling evidence of the functionally important tumour-promoting effects of host immunity (Colotta et al., 2009; DeNardo et al., 2010; Grivennikov et al., 2010; Qian and Pollard, 2010). Given the significance of this causal relationship, tumour-promoting inflammation and evasion of tumour immunity are now regarded as emerging hallmarks of cancer (Hanahan and Weinberg, 2011). The growing appreciation of the immunological dimensions of cancer reflects the fact that host immunity is a double-edge sword and homeostatic balance of immune response is therefore important.

The strong connection between chronic inflammation and cancer is particular apparent in the gastrointestinal tract, where microbial challenge is frequent. The monolayer of epithelial cells that lines the gastrointestinal tract not only provides a physical barrier but also serves immunoregulatory functions. As gastric cancer is an

epithelial-derived disease, understanding the immune-related function of gastric epithelial tumour suppressor RUNX3 is therefore of specific relevance. The findings presented in this thesis reveal a novel role for RUNX3 in the regulation of the cytokine gene *IL23A* in gastric epithelial cells, thereby implicating a role for RUNX3 in the modulation of gastric mucosal immunity.

In elucidating the molecular machinery for the transcriptional regulation of *IL23A*, RUNX3 was found to be critical for the amplification of TNF-α activation of NF-κB pathway. Conversely, RUNX3's own effect in the absence of exogenous stimulation also appears coupled with NF-κB. Together; these observations signify a strong cooperation between NF-κB and RUNX3 as well as RUNX1. Indeed, cooperation between classical tumour suppressor such as p53 and immune signaling has recently been reported (Puszynski et al., 2009; Schneider et al., 2010; Tergaonkar and Perkins, 2007). While the precise mechanism remains obscure, it is noteworthy that the RUNX- and NF-κB-responsive elements are in close proximity on the *IL23A* promoter, which may enable direct interaction. In future studies, this possibility could be addressed by employing a combination of biochemical analyses such as co-immunoprecipitation of NF-κB and RUNX3 proteins, together with functional assays such as reporter gene assays. Recent studies have shown that the inhibition of NF-κB in epithelial cells has an unexpected pro-inflammatory effects and contributes to carcinogenesis (Lind et al., 2004; Nenci et al., 2007; Pasparakis et al., 2002; van Hogerlinden et al., 1999). Such emerging evidence highlights the intricacies that exist within host immunity where the modulating role of RUNX3 on key NF-κB targets could prove very significant.

During gastric carcinogenesis, host immune response and inflammation are often initiated by wounding of tissues due to pathogen invasion. Wound healing involves not only the clearance of microbes but also enhanced cell proliferation for tissue repair and the reconstitution of the barrier system. Failure to do so may perturb the homeostatic balance which leads to chronic inflammation, hyperproliferation and formation of neoplastic lesions (section 1.2.6); (Coussens and Werb, 2002). A major culprit for gastric inflammation and gastric cancer is *H. pylori* (Suerbaum and Michetti, 2002). The positive correlation observed for *H. pylori* infection and *IL23A* expression in this study implicates *IL23A* as a component of the innate host defense mechanism. This notion is further strengthen by the prolonged induction of *IL23A* by the *CagA*-positive strain of *H. pylori*, which confers a higher risk of gastric cancer and ulceration (Suerbaum and Michetti, 2002).

In addition to the gastric epithelial cell-autonomous regulation, the balance of host immunity *in vivo* is delicately maintained by the communication between epithelial cells, immune cells and the surrounding stroma (Izcue et al., 2009; Shaykhiev and Bals, 2007). A growing body of evidence indicates a pathophysiological background for cancer is constituted by the impaired communication between immune and epithelial cells of the tissues (Shaykhiev and Bals, 2007). The observation that RUNX3-induced IL23A derived from AGS cells modulates T cells proliferation and differentiation point to a potential involvement of RUNX3/IL23A in the complex communication between epithelial and immune cells. In addition, AGS-derived IL23A was shown to modulate the production of IFN-γ by T cells suggests that it may promote the proliferation and differentiation of Type I helper T cells (T_H1). In future studies, a detailed characterisation of the surface and intracellular markers expressed by the proliferating T cells fraction is necessary for

the identification of the specific subsets of T cells that respond to IL23A. Their identification will in turn enable the purification of the responsive subset of T cells to further elucidate the biological effect of gastric epithelial-derived IL23A. This should be complemented with assays based on a co-culturing of epithelial cells and immune cells, as it mimics the *in vivo* communication between these cell types that may further potentiate the effects of secreted IL23A (Bernhardt et al., 2010; Duell et al., 2011). Lastly, future studies employing these approaches would be greatly advanced by the identification of the putative partner for IL23A. Given the body of evidence presented here, a proteomic approach using mass spectrometry can be employed to profile the secretome of AGS cells enriched with IL23A-specific antibody to reveal the identity of this putative cytokine.

In addition to the data from human gastric cell lines, murine primary gastric epithelial cells were shown to express *IL23a* transcript upon TNF-α treatment (Jason Koo, unpublished data). The conservation of this regulatory mechanism in both mouse and human strengthens its physiological relevance and produces an additional means for downstream investigation of the *in vivo* function of IL23a in mouse models. Of particular interest would be a stomach-specific knockout of *IL23a* to ascertain the *in vivo* role played by IL23a, which will also shed new light on the function of Runx3. In addition, *H. pylori*'s effects on *IL23A* in human gastric epithelial cell lines also await further validation through the use of murine *Helicobacter* infection models. Two strains of *Helicobacter*, *H. felis* and *H. pylori* have been proven to colonise mouse stomachs and lead to chronic inflammation (Chen et al., 1992; Ferrero et al., 1995; Marchetti et al., 1995; Michetti et al., 1994). By establishing a model of *Helicobacter* infection, the role of IL23a in anti-bacteria immunity and inflammation in the stomach could be further established. Lastly, to demonstrate the functional

importance of RUNX1 and RUNX3 in the regulation of *IL23A in vivo*, a transgenic mouse model should be generated in which the Runx3-responsive element in *IL23a* promoter could be conditionally deleted using stomach-specific Cre/loxP recombination system.

To complement the functional study of Runx1/3 regulation of *Il23a* in mice, the clinical significance of this relationship should also be established. This would include clinicopathological analysis of human gastric tissues by immunohistochemical (IHC) staining to demonstrate the expression of IL23A in the human gastric epithelium. Furthermore, as the loss of RUNX3 function has been reported in up to 80% of gastric cancer cases (section 1.2.4), it will be of clinical relevance to investigate if the expression of IL23A in human samples correlates with that of RUNX3 expression, in normal and diseases states; and whether it co-occurs with the infiltration of leukocytes. Lastly, a comparison between the expression of RUNX3 and IL23A in *H. pyrori*-positive and -negative gastric patients would provide further evidence for a role of this pathway in host response against this important gastric pathogen.

In conclusion, the findings in this study reveal an immunological aspect of RUNX3 function in gastric epithelial cells through the direct regulation of *IL23A* and cooperation with TNF-α and *H. pylori*. It suggests that the loss of RUNX3's function during gastric carcinogenesis may impair the ability of gastric epithelial cells to produce IL23A as part of an innate host immune response against pathogen-induced inflammation. This would in turn result in ineffectual pathogen immunity, chronic inflammation and ultimately gastric neoplasia. As such, a comprehensive understanding of biological function of IL23A and the pivotal role of RUNX proteins

play may offer a novel therapeutic strategy in preventing *H. pylori*-induced gastric cancer and inflammatory pathologies in the stomach.

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