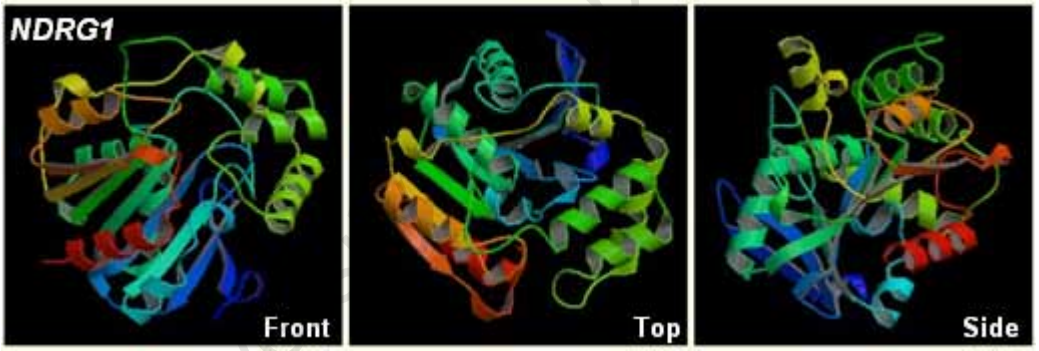
FUNCTIONAL ANALYSIS OF N-MYC DOWNSTREAM REGULATED GENE 1 (NDRG1) IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA

Thesis presented by

WEI WEI



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Thesis supervisor: Dr. Denver T Hendricks

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TITLE IMAGE Predicted comparative 3D structure of NDRG1. Pictures (in .pdb format) were cited from the ModBase database in University of California, San Francisco (http://modbase.compbio.ucsf.edu/modbase-cgi).

As the candidate's supervisor, I have approved this thesis for submission.

Name: Dr. Denver Thomas Hendricks

Signed: _____

Date: 15th February, 2009

DECLARATION

I, Wei Wei, hereby declare that: (1) the contents of this thesis represent my own unaided work, both in conception and execution, except where acknowledgements indicate otherwise; (2) neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I am now presenting this thesis for academic examination towards the Degree of Doctor of Philosophy in Medical Biochemistry.

Signed: _____ **Date:** 15th February, 2009

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ABSTRACT

Oesophageal squamous cell carcinoma (OSCC) ranks as one of the deadliest tumours with a high incidence in developing countries in the areas of Southern Africa, Middle East and Far East. Moreover, its unfavourable prognosis is further complicated by the lack of knowledge about the molecular biology of this disease. In this thesis, we describe our work analysing the function of N-myc downstream regulated gene 1 (NDRG1, also known as Cap43 or Drg-1) in the neoplastic progression and maintenance of OSCC.

Although NDRG1 has previously been implicated in breast, prostate, colon and liver carcinoma, the exact role of NDRG1 in OSCC still remains unclear. According to the immunohistochemical analysis of clinical OSCC tissue samples (n=52), NDRG1 expression was gradually increased in tumour tissue versus normal, indicating the potential involvement of NDRG1 in the neoplastic progression of OSCC. We next performed ectopic NDRG1 gain-of-function and loss-of-function studies using transfectants established from transduced OSCC cell lines (KYSE30 and KYSE150) by lentiviral vector mediated gene delivery. In KYSE30 cells, although no substantial effects on *in vitro* cell proliferation and differentiation were observed with altered NDRG1 expression, the ectopic overexpression of NDRG1 was found to be positively linked to metastasis, angiogenesis and apoptotic evasion as measured in cell culture. Accordingly, in the nude mouse xenograft model system, NDRG1 overexpression promoted the *in vivo* growth and metastasis of KYSE30 derived xenografts, which could be attributed to the reduced apoptotic and enhanced angiogenic activities promoted by this gene. Nevertheless, no significant phenotypic changes were observed in response to NDRG1 knock-down, suggesting that this gene was not essential for the neoplastic progression of OSCC. Moreover, null effect of either ectopic NDRG1 overexpression or knock-down were observed in KYSE150 cells, indicating

that the function of NDRG1 may be largely dependent on the cellular context (Chapter 2).

In addition to direct functional assays, evidence from analysing the regulation pattern of NDRG1 in OSCC cells was also presented to provide clues to indirectly predict the function of NDRG1 in OSCC. In Chapter 3, we demonstrated that NDRG1 could be actively regulated by various oncogenic stimuli such as cellular stress (genotoxicity and hypoxia) and mitogenic factors (EGF and IGF). Although these oncogenic regulatory effects on NDRG1 expression in OSCC cells may be dichotomous, the functional significance of NDRG1 upregulation, especially by hypoxia and EGF signalling, is highlighted. In our studies, the regulatory pattern of NDRG1 in OSCC is highly consistent with its oncogenic function revealed in ectopic studies (Chapter 2), further suggesting that phenotypic changes observed in the functional studies may not be artifactual, but may reflect the role of NDRG1 in the neoplastic progression of OSCC in physiological conditions.

Taken together, our current data implicate NDRG1 as an effective but non-essential promoter in the neoplastic progression of oesophageal squamous cell carcinoma. Although the mechanism still needed to be further explored, our study suggests important clues regarding these mechanistic roles considering the impact of this gene on apoptosis, metastasis and angiogenesis.

Chapter One

Introduction

1.1 Oesophageal Cancer: Overview

1.1.1 Histological classification

With over 300,000 new cases worldwide per year, oesophageal cancer ranks as one of the most aggressive types of common malignancies. Although great efforts have been made in diagnosis and treatment, until recently, oesophageal cancer still presents a poor prognosis with a rising overall incidence [1;2].

Oesophageal cancer usually originates in the mucosa of the oesophagus and can occur in any section of the organ. More than 90% of oesophageal cancers fall into one of two classes: squamous cell carcinoma and adenocarcinoma. These two histological subtypes are viewed as separate disease entities due to significant differences in their pathological and epidemiologic profiles. Adenocarcinoma is often associated with oesophageal reflux which can result in a metaplastic change of oesophageal squamous mucosa to "intestine-like" columnar epithelium, called Barrett's oesophagus. Oesophageal adenocarcinoma

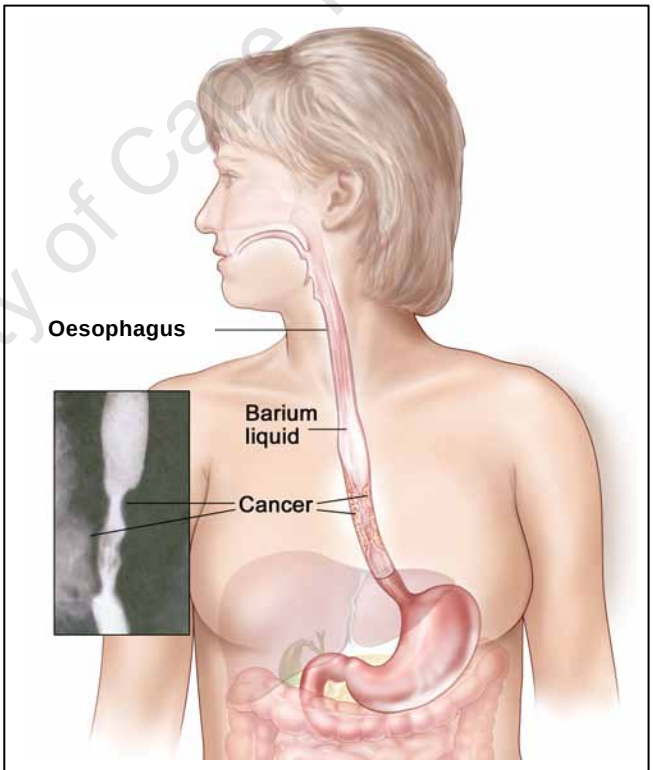


Fig. 1-1: Oesophageal cancer, sketch map and real image of an oesophagogram from barium-swallow examination. (Figure adapted from the NCI.gov database redistributed by University of Bonn, http://www.meb.uni-bonn.de/cancer.gov, © 2005 Terese Winslow by the U.S. government)

usually develops in the lower third of oesophagus and gastro-oesophageal junction. In contrast, squamous cell carcinoma primarily occurs in the middle third of oesophagus, with only 30% in the lower third [1;3].

1.1.2 Epidemiology

According to the International Agency for Research on Cancer (IARC), in the year 2005, oesophageal cancer was reported as the ninth most common malignancy (third of the digestive tract) which ranked the sixth leading cause of cancer-related deaths worldwide. The annual incidence is 3-11 per 100,000 for males and 0.6-6 per 100,000 for females. High incidence areas of oesophageal cancer include: China (21 per 100,000), Iran (16 per 100,000) South America (13 per 100,000), Western Europe (11 per 100,000), South Africa (10 per 100,000) and Japan (9 per 100,000) [2;4;5].

Although adenocarcinoma is more common in westernized countries, squamous cell carcinoma is the most prevalent histological type of oesophageal cancer, especially in developing countries [6-8]. In South Africa, oesophageal squamous cell carcinoma (OSCC) ranks the second most common cancer among the South African people and one of the most common cancers in black males. Regions of South Africa, like the Eastern Cape, have a particularly high incidence of OSCC at 40 per 100,000 [9;10]. Considering the high incidence of this disease in South Africa, oesophageal squamous cell carcinoma (OSCC) will be the focus in this thesis.

1.1.3 Aetiology

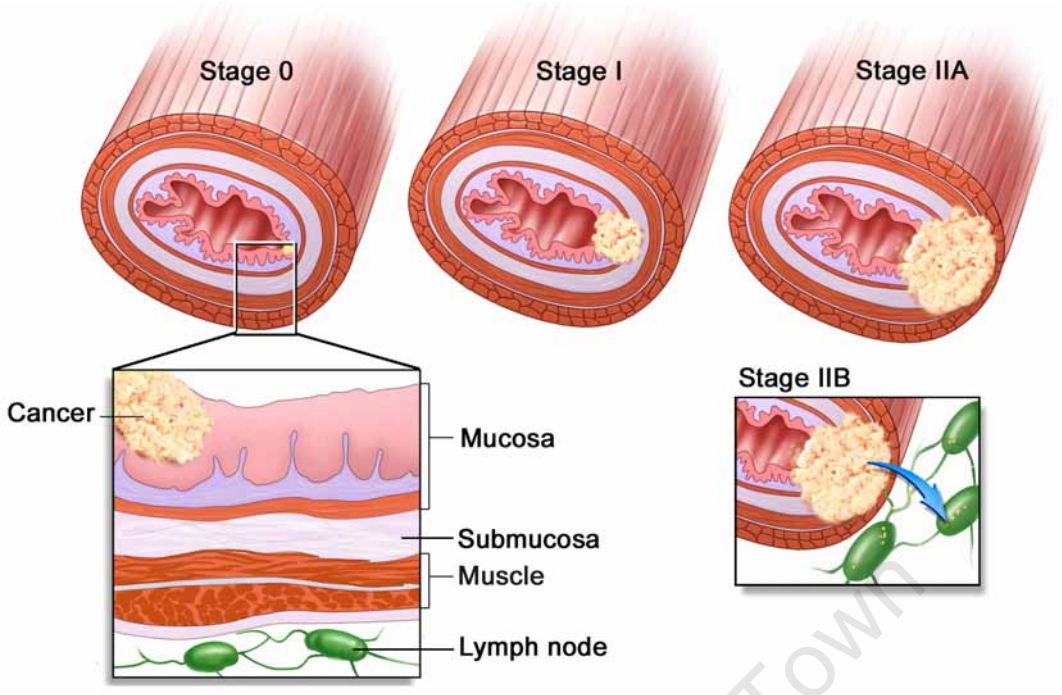
During the past few decades, several risk factors for oesophageal squamous cell carcinoma (OSCC) have been described. These include tobacco and alcohol consumption [11;12], chronic injury to the oesophageal epitheliua (e.g. extremely hot beverage intake) [1;3], nutritional imbalance or deficiency and consumption of mycotoxin contaminated food [13-15]. Recently, viral etiopathogenesis such as human papillomavirus and Epstein-Barr virus infection have also been linked to the risk of developing oesophageal squamous cell carcinoma [6;16;17].

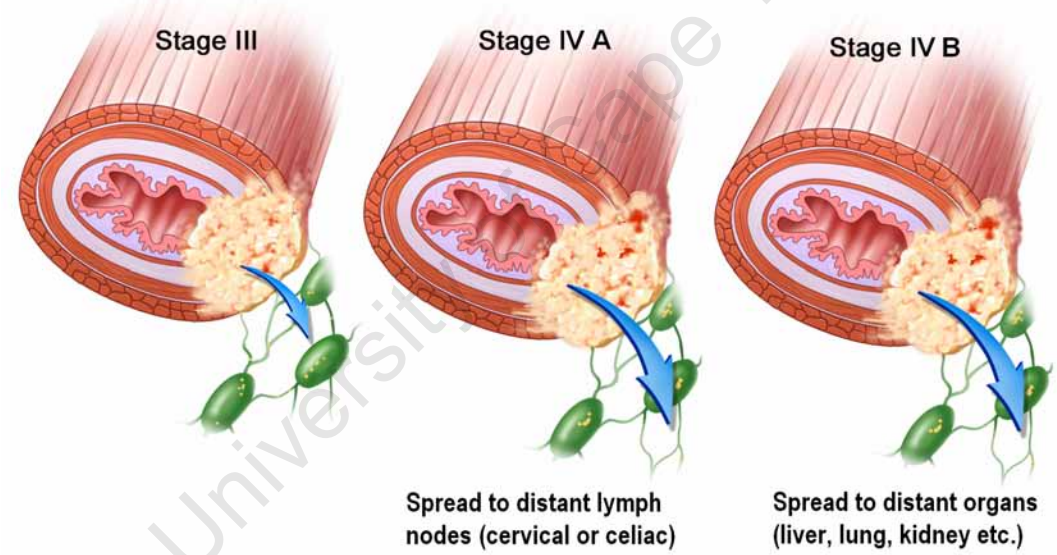
1.1.4 Tumour progression and prognosis

Pathologically, the neoplastic progression of squamous cell oesophageal carcinoma (OSCC) is described based on the size of the tumour (**T**, reflected by the depth of oesophageal wall penetration, not the percentage of oesophageal wall circumference affected), the extent of adjacent lymph node involvement (**N**), and the evidence of metastatic disease (**M**). A detailed tumour staging system updated by the American Joint Committee on Cancer in 2002 is shown in Fig 1-2 [1;3].

Although asymptomatic at the early stage when the tumour is still superficial and confined *in situ*, OSCC rapidly develops to a highly aggressive and invasive malignancy. Once the neoplasm penetrates the submucosal layer, dysphagia (difficulty with swallowing) arises, due to extensive tumour infiltration of the oesophageal lumen. Dysphagia often heralds unresectable lesions due to the presence of local lymph node metastases. Compared to other types of carcinoma, lymphatic spread is the most common way for the metastatic dissemination of OSCC. Since lymphatic fluid eventually joins into the blood circulation, in the late stage, distant metastases occur hematogeneously. Liver (32%) and lung (21%) are the most common targets, while cases of bone, kidney and adrenal gland metastases also exist [18].

Fig 1-2 (overleaf): Neoplastic progression of squamous cell oesophageal carcinoma and the tumour staging system of primary tumour, regional lymph-node metastasis and distant metastasis. The primary tumour (**T**) is classified as follows: **Tis**, carcinoma *in situ*; **T1**, invasion of lamina propria or submucosa; **T2**, invasion of muscularis propria; **T3**, invasion of adventitia; and **T4**, invasion of adjacent structures. Regional lymph-node metastases (**N**) are classified as follows: **N0**, no regional lymph-node metastases; and **N1**, regional lymph-node metastases. Distant metastases (**M**) are classified as follows: **M0**, no distant metastases; **M1a**, metastasis to cervical nodes or celiac nodes in the cases of cancer in the upper or lower oesophagus respectively; and **M1b**, other distant metastases. (Figure adapted from the NCI.gov database redistributed by the Medical Centre of University of Bonn, http://www.meb.uni-bonn.de/cancer.gov, © 2005 Terese Winslow by the U.S. government; Table adapted from Ref. [1])





Tumour staging according to the American Joint Committee on Cancer

Stage	Tumour	Node	Metastasis	5 Yr Survival
0	Tis	N0	M0	>95%
I	T1	N0	M0	50-80%
II-A	T2-3	N0	M0	30-40%
II-B	T1-2	N1	M0	10-30%
III	T3	N1	M0	10-15%
	T4	Any N	M0	10-15%
IV-A	Any T	Any N	M1a	<5%
IV-B	Any T	Any N	M1b	<1%

Oesophageal squamous cell carcinoma (OSCC) is one of the most deadly forms of gastrointestinal cancer. The overall 5-year survival rate (5YSR) is less than 15% [1;18]. Surgery remains the most effective therapeutic approach compared to chemo- and radio-therapy, but prognosis depends on the stage of the disease. Complete surgical resection only offers a significant cure rate if the tumour is entirely restricted to the oesophageal mucosa. The 5YSR for stage 0 is >90% but dramatically drops to 50-80% for stage I when tumour reaches submucosal layer. However, due to the asymptomatic progression of OSCC, early diagnosis rarely occurs. More than 50 percent of patients are diagnosed with metastatic, unresectable disease for which the prognosis is quite poor. Even with palliative surgery, muscular layer involvement (stage II) brings the 5YSR down to less than 30% and outer membrane (adventitia) penetration (stage III) is associated with a 10% 5YSR. Lymphatic micrometastases (identified by immunohistochemical analysis) and lymphatic invasion to the adjacent structure is associated with less than 5% 5YSR while patients with distant metastases (stage IV) who are not candidates for surgery have a median survival of less than one year (Fig. 1-2) [1;2;4;5;9;18;19].

1.2 Introduction of N-myc downstream regulated gene 1 (NDRG1)

The high incidence and mortality of oesophageal squamous cell carcinoma (OSCC) and the comparatively limited methods of diagnosis and treatment currently consist of the major characteristic of this disease [2]. Nevertheless, the thriving research activities examing the molecular mechanisms of OSCC greatly contributed our understanding, which in turn, provided us more opportunities for the identification of diagnostic markers, prognostic indicators as well as therapeutic markers [20]. Through investigating the gene expression profile of OSCC tissue from patients by differential display analysis, we previously observed aberrant expression of N-myc downstream regulated gene 1* (or N-myc down-regulated gene 1, NDRG1) in tumour tissue versus normal. This observation suggests NDRG1 as a potential candidate involved in the development and maintenance of OSCC.

* The only official full name for NDRG1 gene accepted by NCBI and EMBL

1.2.1 Protein structure of NDRG1

NDRG1 (N-myc downstream regulated gene 1) was isolated as a gene upregulated in N-myc mutant mouse embryos, and was named after its repression by N-myc and c-myc [21]. In humans, NDRG1 was first identified as a homocysteine and tunicamycin responsive protein (RTP) in human umbilical vein endothelial cells (HEUVC) [22]. Identified independently and reported according to its perceived function by other research groups, NDRG1 is also known as Cap43 [23], Drg-1 [24], RIT42 [25] and PROXY-1 [26]. Mapped to the end of the long arm of chromosome 8 (8q24.2) of the human genome, a region highly susceptible to alteration during carcinogenesis, human NDRG1 gene encodes an mRNA about 3.0 kb, which is translated to a 43 kDa protein with 394 amino acids and an isoelectric point of 5.7 [22-24].

NDRG1 is the most studied member of an evolutionary conserved, four-member protein family [27-30]. Highly homologous NDRG1 analogues are found in variety of species including mouse [21;31], rat [32], chicken [33], *Xenopus* [34], nematode (*C. elegans*) [35] and even sunflowers [36]. On the other hand, members of the human NDRG family, named NDRG1 to NDRG4, share an amino acid sequence identity of 53-65% with each other. Recently, homologues of NDRG2, 3, and 4 were also reported in mouse [37;38], rat [39], chicken [33] and *Xenopus* [39]. Therefore, NDRG family members are highly conserved proteins, which suggest their important roles in some cellular process.

Motif analysis revealed each of the NDRG family members contains an α/β hydrolase fold and an esterase/lipase/thioesterase active serine. Although both structures are similar to those in the human lysosomal acid lipase, unlike most of the other 900 α/β hydrolase superfamily members, no hydrolysis catalytic activities are observed in any of the NDRG proteins [40]. Considering this, it may be suggested that the NDRG family is evolved from hydrolytic enzyme ancestors, and that the α/β hydrolase fold is eventually functionally inactivated or changed for some different purposes. Unique to NDRG1, three-tandem-repeats of 10 hydrophilic amino acids (TRSRSHTSEG) are located at the C-terminus [27]. This may indicate that NDRG1 has some special function among the family members, although the function of these three

tandem-repeats is presently unclear, except for their interesting capability of binding to divalent nickel and copper ions [41;42]. A potential cytochrome c family heme-binding motif and a phosphopantethein attachment motif are also predicted [43], however, no susceptible peptide motifs have yet been discovered to give indication of the NDRG1 biological function (Fig. 1-3).

NDRG1 is capable of being phosphorylated physiologically by several serine/threonine kinases. Tyrosine phosphorylation, until now, has not been observed. *In vivo* experiments revealed that NDRG1 is the substrate of protein kinase A (PKA) [44-46], caldium-calmodulin kinase II (CaMK-II) [44;45], serum and glucocorticoids-induced kinase1 (SGK-1) [47;48], and glycogen synthase kinase 3 (GSK-3) [47]. On the other hand, kinase inhibitor studies showed kinases such as protein kinase B (PKB), conventional and novel protein kinase C (PKC), p70 S6 kinase ($p70^{S6K}$), p90 ribosomal S6 kinase ($p90^{RSK}$), mitogen activated protein kinase (MAPK), cyclin-dependent kinase (CDK) and casein kinase II (CK-II) might not be able to phosphorylate NDRG1 physiologically [44-48] (Fig. 1-3). Two-dimensional electrophoresis revealed a heterogeneous migration pattern for NDRG1 (more than 8 spots), suggesting that NDRG1 is partially phosphorylated *in vivo* with more than seven phosphorylation sites on the protein [44]. Nevertheless, although some evidence suggested that NDRG1 phosphorylation might be linked to the proliferation and exocytosis activities in endothelial (HEUVC) and mast cells respectively [44;45], the significance of phosphorylation on NDRG1 function as well as its subcellular localization in cancer cells is still not well established.

Besides phosphorylation, other post-translational modification of NDRG1 such as glycosylation has not been observed [44].

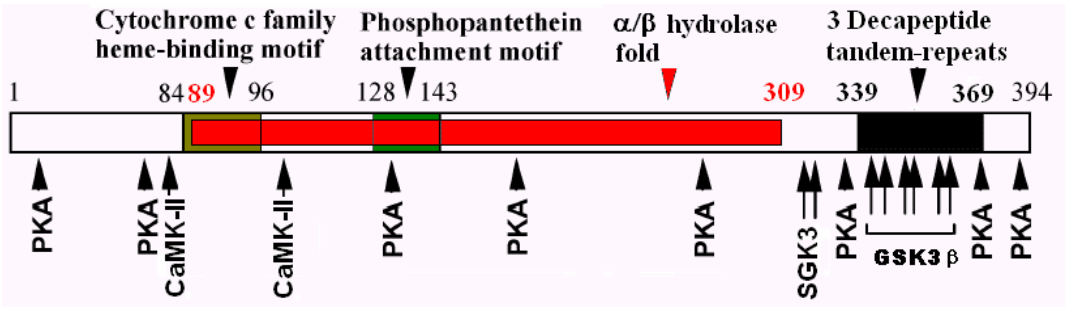


Fig 1-3: Scheme map of the NDRG1 protein, showing the alignment of the potential domains as well as the putative phosphorylation sites.

1.2.2 Cellular and tissue distribution of NDRG1

Unlike the limited distribution of other NDRG members, NDRG1 is ubiquitously expressed in most human tissues, especially in the central and peripheral nervous system, digestive tract, heart, spleen, kidney, prostate, ovary, uterus and placenta [24;49-51]. Despite its wide tissue distribution, NDRG1 protein is mainly found in epithelial cells. In general, mesenchymal and endothelial cells are negative for NDRG1 expression [50].

Although predominantly detected in the cytoplasm, NDRG1, in some cases, may also partially or even exclusively associate with the plasma membrane and nucleus [50;51]. Examples of intense NDRG1 membrane localization include the Schwann cells, epithelia of small and large intestine, the acinar cells of lactating breast, hepatocytes and biliary ducts in liver, as well as the basal cells of the squamous epithelia of skin and oesophagus. In contrast, predominant nuclear staining was detected in prostate epithelia. Nevertheless, in other cell/tissue types, NDRG1 displays cytoplasmic localization with a distinct granular pattern reminiscent of mitochondrial localization [50]. In fact, by immunoelectron microscopic technique, NDRG1 was shown to be associated with the mitochondrial inner membrane in the kidney proximal tubule cells [50] which is in agreement with other reports showing the mitochondrial localization of NDRG1 [25;44]. Although no known mitochondrial targeting sequence has been yet identified, however, the potential cytochrome c family heme-binding motif and the phosphopantethein attachment motif within the NDRG1 protein are compatible with mitochondrial location. In addition, it may also be proposed that NDRG1 could be relocated to mitochondria through protein binding, such as the voltage-dependent anion channel 1 (VDAC1), cytochrome c oxidase subunit II (Cox2) or others [43].

While NDRG1 has been shown capable to localize in membranes, no trans-membrane domain or nuclear localization signal (NLS) has been yet detected in the protein. Hence, protein-protein interaction is suggested to account for the membrane association reported for this protein. Electron microscopy studies of prostate, colon and kidney epithelial cells revealed that membrane associated NDRG1 was predominantly present adjacent to the

cytoplasmic side of adherens junctions and desmosomes as well as in the intermediate and microfilament bundles inserting into the above-mentioned connection structures. This suggests a potential role for NDRG1 in cell-cell adhesion. In fact, proteomics based analysis of the NDRG1 interactome (binding proteins) in the prostate cancer cell line LNCap demonstrated its direct interaction with E-cadherin and β-catenin, suggesting the probable participation of NDRG1 in the formation or maintenance of adherent junctions [46]. Evidence also comes from a demyelinating neuropathy syndrome affecting the Schwann cells in the peripheral nervous system, the Charcot-Marie-Tooth type 4D disease (CMT-4D, or hereditary motor and sensory neuropathy-Lom syndrome, HMSNL), in which NDRG1 is mutated as a truncation (R148X) [52;53]. The dysfunction of NDRG1 in CMT-4D results in decompaction of the myelin sheath, presumably due to disturbance of adherens junctions in the E-cadherin abundant Schmidt-Lanterman incisures [54;55]. Moreover, NDRG1 ectopic overexpression was shown to upregulate the level of E-cadherin, transcriptionally or post-translationally, in colon [49] and prostate [56] tumour cells, that further substantiates the hypothesis of its association with cell membrane. Nevertheless, in other prostate cancer cell lines DU145 and CWR22R, none of the proteins of the E-cadherin complex (E-cadherin, β-catenin, γ-catenin and p120) was found to interact with NDRG1 [56], suggesting the probable interaction between E-cadherin and NDRG1 may be tissue or even cell type specific.

Besides the proposed interaction between NDRG1 and the E-cadherin complex, potential binding between NDRG1 and other membrane proteins have also been reported, which may as well explain the membrane localization of NDRG1. Screening of a mouse kidney yeast two-hybrid library detected the association of NDRG1 with membrane trafficking proteins such as Hsc70, PICK-1, and p47 [43]. In another study, NDRG1 was identified as the binding partner of prenylated rab acceptor protein 1 (Pra1) and reticulon 1C (RTN1C) [57]. All of these five proteins are converged with the SNARE (soluable *N*-ethyl-maleimide-sensitive factor attachment protein receptor) membrane fusion machinery, which indicates the possible participation of NDRG1 in exocytosis (Section 1.2.5).

Similarly, since NDRG1 displays no nucleus localization signal (NLS), protein interaction is also proposed to explain the nuclear localization of the NDRG1 protein. Molecular chaperons such as heat-shock cognate protein 70 (Hsc 70) and heat-shock protein 90 (HSP90) are considered potential candidates for this phenomenon [43;46]. Nuclear localization of NDRG1 has also been observed when cells are treated with the DNA damaging agent mitomycin C [25]. Recently, the association between NDRG1 and nuclear protein Ku70 or RUVBL2 was demonstrated by co-immunoprecipitation analysis [46]. Since Ku70 is one of the key components for non-homologous end joining recombination of double-strand DNA break repair [58], while RUVBL2 participates in DNA repair by driving the branch migration of the Holliday junction [59], this observation could suggest the participation of NDRG1 in the maintenance of genome stability. Finally, although the sequence does not predict its function as transcription factor, interaction between NDRG1 and some transcription factors was also observed, raising the possibility that NDRG1 may be implicated in transcriptional regulation [46]. Nevertheless, due to the insufficiency of literature, the biological role of NDRG1 as a nuclear protein still requires further examination.

Analysing NDRG1 subcellular localization using PSORT II software also predicted its association with the cytoskeleton (http://psort.hgc.jp). In fact, experimental evidence also suggests that NDRG1 is associated with microtubules and centrosomes. However, this association was only demonstrated through co-localization by immunofluorescence and co-sedimentation by ultra-centrifugation; no direct evidence of the interaction between NDRG1 and α-/β-tubulin or γ-tubulin were provided through co-immunoprecipitation in this report [60].

In summary, the ubiquitous tissue distribution as well as various subcellular localizations consists of the major characteristic of the NDRG1 expression profile, which in turn, may implicate versatile functions of this protein. Considering the lack of localizing signal peptide sequences, it is suggested the distribution of NDRG1 may be largely determined through protein-protein interactions with binding partners. Apparently, these binding partners define

the potential role of NDRG1 protein under certain subcellular localization patterns such as cell adhesion, membrane trafficking, DNA damage repair and cytoskeleton dynamics.

Nevertheless, our understanding of the cellular and tissue distribution of NDRG1 still remains quite obscure. Comparing the substantial evidence suggesting the undefined cytoplasmic localization of NDRG1, information from the above studies showing non-random cytoplasmic localization are all limited to cell or tissue types. Moreover, until recently, nothing has been described for the potential regulating mechanism of NDRG1 redistribution through subcellular compartments, which further impedes the establishment of clear correlation between NDRG1 distribution and its function.

1.2.3 NDRG1 in cell differentiation and tissue development

Differentiation is a complex multistep process of cell specialization resulting in the production of cells with distinctive biological functions. Representing a form of negative control of growing, in most of cases, differentiation eventually diminishes the cellular proliferative capacity irreversibly and may sometimes act as an apoptosis initiator (e.g. in the maturation of squamous epithelium).

A role for NDRG1 as a developmental gene has been documented considering that the expression of NDRG1 is actively modulated during the cellular differentiation or tissue maturation of Schwann cells [52;61], mast cells [45;62;63], macrophages/monocytes [64], endometrium [65], placenta (trophoblasts) [66-68], as well as the foetal and postnatal development of kidney, brain, liver and gut in mouse and rat [21;37;69;70]. In particular, the importance of NDRG1 in the development of Schwann cell is reflected by Charcot-Marie-Tooth type 4D disease, in which the truncated NDRG1 gene mutation (R148X) is linked to the demyelinating neuropathy of the peripheral nervous system [53;71]. Consistently, NDRG1 double knock-out mice showed similar phenotype of myelin sheath failure due to impaired development of Schwann cells [61]. In another relevant study, NDRG1 is shown to be essential for the regeneration of the peripheral nervous system [72].

The first report describing the relationship between NDRG1 and cell differentiation can be dated back to when the NDRG1 gene was independently identified through differential display technique during the *in vitro* differentiation of the HT29-D4 colon carcinoma cell line [24]. A number of subsequent studies also indicate increasing NDRG1 expression in response to cell differentiation signals. For instance, ligands of nuclear transcription factors involved in cell differentiation, such as PPARγ [49;73], vitamin A (all trans-retinoic acid) and vitamin D (1,25-$(OH)_2$ vitamin D_3) [73;74], which inhibit cell growth and induce differentiation, also markedly upregulate NDRG1 at the same time. Likewise, similar phenomena are observed when cells are treated with phorbol ester, a potent differentiation inducer [64;68;73]. Stimulated by nerve growth factor (NGF) and transforming growth factor-β (TGF-β) respectively, PC12 pheochromocytoma cells and trophoblasts develop differentiated phenotypes, with a simultaneous marked induction of NDRG1 [67;75]. Moreover, bone marrow derived mast cells, when co-cultured with Swiss 3T3 fibroblasts, strongly induce the protein level of NDRG1, accompanying the transformation of the mast cells from an immature to mature phenotype marked by degranulation and enhanced exocytosis activity [45;62]. Accordingly, in NDRG1 double knock-out mice, mast cell maturation and degranulation are impaired, resulting in attenuated allergic responses. This phenomenon suggests that NDRG1 is involved in the terminal maturation and effecter function (degranulation) of mast cells [63]. Finally, NDRG1 also significantly increases during the differentiation of Schwann cells compared with their proliferating counterparts [72].

NDRG1 expression has also been shown to be controlled by sexual hormones, some delicate regulators of both differentiation and proliferation. An association of NDRG1 induction with growth arrest and differentiation was observed by treating several androgen receptor positive prostate cancer cell lines with relatively high concentration of synthetic or natural androgenic ligands such as methyltrienolone (R1881), 5α-dihydrotestosterone (DHT), and testosterone [76]. Interestingly, low concentration of R1881, which is optimal for proliferation rather than differentiation, has minimal effect of NDRG1 induction. Therefore, NDRG1 is suggested to be a marker of

androgen-induced differentiation in human prostate in this research. Conversely, it was also shown that 17β-estradiol administration decreased the NDRG1 levels in several estrogen receptor positive breast cancer cell lines, with a marked enhancement of cell proliferation [77]. Consistently, investigation of the endometrium during the menstrual cycle demonstrated an elevation of NDRG1 mRNA and protein from the proliferative phase in which different cell types in the endometrium undergo extensive growth in response to estrogens and proliferative cytokines to secretory phase characterised by global tissue maturation and differentiation mediated by progesterone [65]. This observation further suggests the differentiation-related function of NDRG1 under the control of sexual hormones.

Furthermore, ectopic NDRG1 overexpression in metastatic colon cancer cell line SW620 induces differentiation-specific morphological changes that are similar to changes induced by known differentiating reagents such as retinoids. Consistently, several colonic specific differentiation markers such as E-cadherin, alkaline phosphatase, and carcinoembryonic antigen were observed to be induced by NDRG1 overexpression. This observation further substantiates the hypothesis that NDRG1 is capable of directly inducing colon cancer cell differentiation [49].

Interestingly, NDRG1 up-regulation observed during cellular differentiation and tissue maturation is often accompanied with an inverse regulation of c-myc or N-myc protein levels [21;73-75]. On the other hand, down-regulation of NDRG1 with an enhanced proliferative activity by 17β-estradiol was observed with simultaneous c-myc induction. Since repression of c-myc and N-myc is required for terminal differentiation of many cell types [78;79], it has been proposed that Myc may be pivotal for the NDRG1 function in mediating cell differentiation and tissue development.

Although there is substantial evidence supporting the hypothesis that the regulation of NDRG1 is part of the cellular programme of differentiation and related growth arrest, discrepancies do exist. NDRG1 induction is absent in growth-inhibited A431 epidermoid carcinoma cells, suggesting the

upregulation of NDRG1 by *in vitro* differentiation may not be a result of decreased proliferation [24]. On the other hand, although ectopic NDRG1 overexpression in SW680 colon cancer cells lead to a more differentiated phenotype, no impact of cell growth rate is observed in these cells [49]. Moreover, NDRG1 induction in differentiation also seems to be largely dependent on the cellular context, as well as the particular type of differentiating stimuli. For example, differentiation stimuli such as ipopolysaccharide, IFN-α, and IFN-γ failed to induce NDRG1 in macrophage/monocyte cells [64]. Similarly, interleukins as well as stem cell factor (SCF) have no effect on NDRG1 expression in mast cells, despite their potent effects on the maturation of mast cells [62]. Discrepancies also exist in sexual hormone mediated NDRG1 regulation, which is potentially linked to differentiation. In a murine T cell hybridoma cell line, treatment with the same concentration of 5α-dihydrotestosterone (DHT), and testosterone as in Ref. [76] repressed the expression level of mouse homologue of NDRG1 (TDD5), while 17β-estradiol showed no effect of TDD5 expression in these cells [31]. Finally, rather than upregulated as in several differentiation processes, NDRG1 was shown downregulated in EGF mediated trophoblast differentiation [67].

Although inconsistency does exist, considering the active upregulation of NDRG1 expression in most of the cases, it still suggests a potential involvement of NDRG1 in cell differentiation and tissue development. Nevertheless, due to the lack of functional studies (e.g. through ectopic alteration of NDRG1 expression), whether differentiating stimuli modulated NDRG1 expression is directly linked with enhanced differentiation or reduced proliferation still remains obscure. Since Ref. [49] is the only information dealing with this issue, much more work is still needed to argue the possibility that modulation of NDRG1 expression level may be just a by-product of differentiating process, and thus irrelevant to the cellular differentiation status.

1.2.4 NDRG1 and endoplasmic reticulum stress

The endoplasmic reticulum (ER) is critically involved in protein metabolism, including the correct folding of many proteins and their post-translational modifications, such as glycosylation and disulfide bond formation. Compounds that interfere with the normal function of the ER by altering the calcium homeostasis, redox status, or protein glycosylation and transportation to Golgi complex will induce ER stress [80]. Typical chemicals that induce ER stress include the calcium ionophore A23187 (calcium homeostasis disturbance), tunicamycin (glycosylation suppression) and sulfhydryl reagents such as homocysteine, cysteine and ß-mercaptoethanol (affect disulfide bond formation due to altering redox potential inside cells). All these chemicals cause protein folding dysfunction, and the accumulated misfolded/unfolded proteins induce ER stress. Since excessive stress may lead to cell death, the ER stress response includes the assistance of protein folding by elevating ER protein chaperone levels as well as the enhanced degradation of misfolded proteins via the regulation of molecules involved in the ER-associated protein degradation pathway [80-82].

Interestingly, NDRG1 expression has been observed to be upregulated by all of the above ER stress inducers in HEUVC cells and cancer cells (by A23187) [22;23;44;83;84]. Although the punctate cytoplasmic subcellular localization of NDRG1 does not predict its ER localization [44], the proteomic analysis of the NDRG1 interactome suggest the ability of NDRG1 to complex with ER chaperones such as heat shock protein 90-α (GRP94), heat shock 70 kDa protein 5 (GRP78), heat shock cognate protein 70 (Hsc70), calnexin and transitional endoplasmic reticulum ATPase (VCP) [43;46]. Moreover, it has been demonstrated that knock down of NDRG1 in LNCaP cells decreased the protein level of GRP94, further suggesting a link between NDRG1 and ER chaperons [46].

As mentioned, proteolysis is another way to relieve the overwhelmed burden of misfolded proteins in the endoplasmic reticulum. The 26S proteasome, composed of a 20S catalytic subunit and a 19S regulatory unit, is the main site for proteolysis. Although NDRG1 has been identified to interact with several

19S subunit proteins, suggesting its suspected involvement in relieving ER stress through facilitating the substrate entry into the proteasome, further investigation using NDRG1 knock-down cells shows increased proteasomal activity [46], suggesting its negative regulatory effect of proteolysis as well as the rejection of the above hypothesis.

Similar as the status of NDRG1 in differentiation, the position of NDRG1 in ER stress is also limited to "undefined potential" through the data merely showing regulatory involvement. However, the revealed interaction of NDRG1 with several ER chaperones shed some light on the possibility of direct modulation of ER stress by NDRG1, although much still need to be done in the future, especially for the mechanism. Considering the negative effect of NDRG1 in proteolysis regulation, it is tempting to speculate the participation of NDRG1 in the release of ER stress is via re-folding or retention (through HSP family members or VCP) but not proteasome based degradation of incorrectly or incompletely folded proteins.

1.2.5 NDRG1 in protein transport and exocytosis

Another important function of the endoplasmic reticulum is protein transport. In this process, proteins destined outside the ER are packed into transport vesicles and moved along the cytoskeleton toward their destination (e.g. plasma membrane, nucleus, lysosome). Based on the potential binding ability with ER chaperons, NDRG1 may also be a candidate participant in the vesicular transportation. In fact, in mast cells, NDRG1 is shown to be associated with heat shock cognate protein 70 (Hsc70) enabling itself to be translocated to the nucleus [43], since Hsc70 is a nucleo-cytoplasmic transport protein [85]. Finally, the interaction between NDRG1 and the heavy chain of kinesin [46], a motor protein responsible for vesicular movement along the microtubule network, further supports the potential role of NDRG1 in vesicular transport.

More specifically, NDRG1 is apparently involved in the regulation of exocytosis. Direct evidence has shown that NDRG1 overexpression induced mast cells to degranulate more rapidly, leading to an enhanced exocytotic

response to various stimuli [43]. In addition, as shown in Section 1.2.2, NDRG1 is capable of interacting with SNARE related proteins such as Hsc70, PICK-1, p47, Pra1 and RTN1C [43;57], all of which are involved in the trafficking, docking and fusion of transporting vesicles [86-90]. Moreover, since Pra1 interacts with a number of Rab proteins, a family of small GTPase in charge of targeting the destination of transporting vesicles, it may be worthwhile to consider the probable relationship between NDRG1 and Pra1-Rab system [91;92].

Rab proteins are small GTPases involved in the regulation of intracellular vesicle budding, tethering, and fusion in mammalian cells which act as molecular switches that spatially and temporally regulate protein trafficking, recycling, and degradation [91;93]. As endocytosed surface molecules and receptors pass through vesicles decorated by different Rab proteins and effectors before they are degraded or recycled back to the cell surface, Rab GTPases regulate a broad range of cellular processes, including cell growth, differentiation, and movement. Classic Rab targets include epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI-3K), phosphatidylinositol 4-kinase (PI-4K) and E-cadherin [93;94]. In fact, NDRG1 was shown to be associated with several kinds of Rab GTPases in HEK293 cells (including Rab 4a, 7 and 11) and participate in the recycling of E-cadherin [56]. The discovery of NDRG1 as a Rab effecter together with the potential involvement of NDRG1 in vesicular transport, probably implicate the indirect role of NDRG1 in regulating signal transduction, since the spatiotemporal cellular loci of receptors and signalling molecules are tightly regulated for appropriate signal transduction [95;96].

1.2.6 NDRG1 and lipid metabolism

There is also evidence to suggest that NDRG1 may be involved in the regulation of high-density lipoprotein-cholesterol (HDL-C) level since yeast two hybridization assay demonstrated an interaction between NDRG1 and apolipoprotein A-I (Apo A-I) or apolipoprotein A-II (Apo A-II) [57]. In fact, in patients with CMT-4D disease, a peripheral demyelinating neuropathy caused

by Schwann cell dysfunction due to the homozygous NDRG1 mutation (R148X), a low HDL-C/total cholesterol ratio is observed. In addition, it is also suspected that the presence of the phosphopantethein-binding domain, as an interacting site of APO A-I and APO A-II, may point to its possible involvement in the lipid biosynthetic pathways operating in the myelinating Schwann cells [52].

1.2.7 Summary: NDRG1 in physiological processes

Considering the high degree of evolutional conservativeness of NDRG1, it is easy to speculate the importance of this protein in some certain physiological processes. Indeed, data described in this section clearly indicate the involvement of NDRG1 in cell differentiation and tissue development, lipid metabolism, endoplasmic reticulum stress, vesicular protein transport and exocytosis. Although much still need to be done to reveal the potential mechanism of NDRG1 in these physiological processes, the various NDRG1

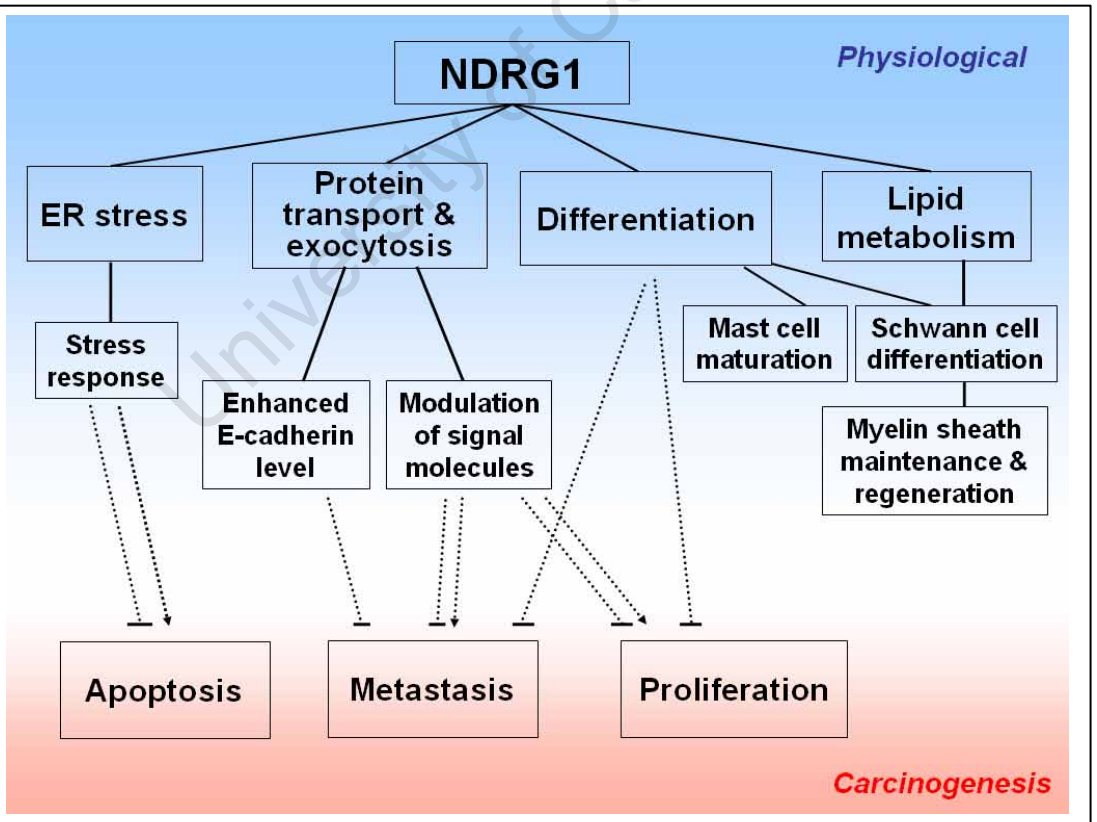


Fig 1-4: Schematic illustration of the biological effects of NDRG1 in various physiological processes, as well as the reflected potential NDRG1 functions during carcinogenesis based on its physiological roles.

subcellular localization, which is tightly regulated by specific protein-protein interaction, further suggests the versatile functions of this protein. Finally, since tumour progression may also be viewed as disorganization of normal physiological processes, in which the function of individual protein may be still retained, the physiological regulation and function of NDRG1 may also provide useful clues for the roles of NDRG1 in neoplastic processes (Fig 1-4). In the next sections, basic information about the molecular events during the progression of oesophageal squamous cell carcinoma (OSCC) will be first discussed. Afterwards, we will review the current data about the NDRG1 regulation and function in cancer, aiming for the clues to elucidate the still unknown roles of NDRG1 in OSCC.

1.3 Molecular biology of squamous cell oesophageal carcinoma

As summarized by Hanahan and Weinberg [97], the molecular biological properties of malignant solid tumour can be traced in the following aspects:

- **Acquisition of unchecked growth**, due to the self-sufficiency in proliferative signals.
- **Loss the ability of apoptosis and senescence**, leading to limitless replicating potential despite internal or external inhibitory signals.
- **Acquisition of sustained angiogenesis**, allowing the tumour to grow beyond the limitations of passive nutrient diffusion.
- **Acquisition of ability of metastasis**, including neighbouring tissue invasion and metastases at distant sites, allowing the tumour to spread for unlimited space and nutrients.

Not surprisingly, validating the potential regulation and function of a gene (such as NDRG1) in these biological events becomes essential in exploring its role in the development of cancer (such as OSCC). Considering the importance of stage background to the whole scenario, in this section, the molecular biological events during the progression of OSCC is reviewed, with the information related to this project highlighted.

1.3.1 Proliferation and apoptosis

1.3.1.1 Proliferation

One of the most striking features differentiating cancer cells from their normal counterparts is the liberation of cancer cells from exogenous growth stimulations. Mitogenic growth factors, through binding their distinctive transmembrane tyrosine kinase receptors (or receptor tyrosine kinases, RTKs), are among the most important category of signalling molecules for triggering proliferation. Such growth factors include EGF (epithelial growth factor), IGF (insulin-like growth factor), VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), and many others. In cancer cells, the growth factor receptors are kept activated through either uncontrolled autocrine loop or mutation of the receptors, thereby transducing mitogenic signals to the cells continuously.

Several signalling pathways can be initiated when mitogenic growth factors bind to their receptors (RTKs). Activated RTK undergoes dimerization, tyrosine autophosphorylation and recruitment of effectors or adaptor proteins which determine the exact pathway to be activated. Typical RTK downstream signalling includes MAPK pathway (via SHC-Grb2-SOS activated Ras), PKC pathway (via PLCγ), PI-3K/Akt pathway, JAK/STAT pathway and FAK pathway [98] (Fig 1-5). However, due to the different affinities and activities of the RTK effectors or adaptor proteins determined by the difference of the RTK C-terminal sequences as well as the type of the cells on which the RTK resides, the particular pathways activated by a certain RTK are under a delicate special and temporary control. For example, EGF can effectively activate both MAPK pathway and PI-3K/Akt pathway. However, compared to the strong and sustained activation of MAPK pathway by EGF, the PI-3K/Akt pathway activated by this ligand is relatively insignificant and temporary [98;99].

Mitogen-activated protein kinases (MAPKs) are a superfamily of protein serine/threonine kinases. MAPKs include extracellular signalregulated kinases (ERKs), stress-activated protein kinases (SAPKs) (also known as c-jun terminal kinases, JNKs), and p38-mitogen-activated protein kinases. When activated, MAP kinases can translocate into the nucleus and phosphorylate

transcription factors to make them active to regulate gene expression. For example, the engagement of the MAPK cascade comprised of Raf, MEK1/2 and ERK1/2 is essential for most RTK mediated proliferation [100]. Activated ERK can be translocated to the nucleus, where it directly or indirectly phosphorylates a number of transcription factors such as Elk, c-myc, CREB,

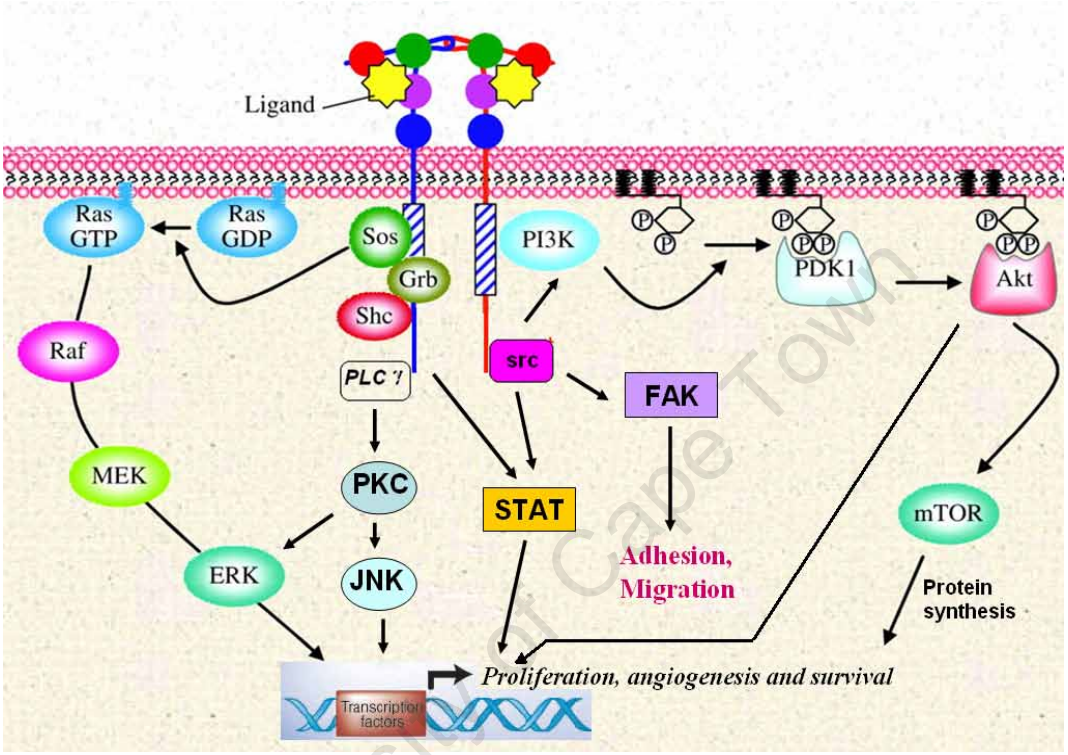


Fig 1-5: A schematic diagram of RTK signalling. Binding of specific ligands to RTK initiates homodimerization/heterodimerization of the receptor and autophosphorylation of specific tyrosine residues for receptor activation. Activated receptor interacts with effectors directly or through adaptor proteins (such as SOC-Grb2-SOS complex, PLC-γ, PI-3K and Src), phosphorylates specific tyrosine residues on the effectors, and activates these effecters to initiate signalling cascades. Typical RTK downstream signalling pathways include Ras/Raf/MAPK pathway, PI-3K pathway, PLCγ/PKC pathway, JAK/STAT pathway, and non-receptor tyrosine kinase src related activation of PI-3K, STAT and FAK. Activation of RTK downstream signalling is responsible for transcription dependent or independent regulation of cell proliferation, survival, adhesion, migration and invasion.

Abbreviations: ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; JNK, c-jun N-terminal kinase; mTOR, mammalian target of rapamycin; PDK1, phosphoinositol dependent kinase 1; PI-3K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase C-γ; PKC, protein kinase C; STAT, signal transducers and activators of transcription protein.

Ets-2 and components of AP-1 such as ATF1, c-jun and c-fos. These transcription factors facilitate the rapid induction of immediate early genes following mitogenic stimulation, leading to increased cellular activities such as proliferation, survival, metastasis, angiogenesis as well as cell cycle progression.

The control of cell cycle primarily ensures the distribution of complete and accurate replicas of the genome to daughter cells. Thus, a series of checkpoints are set up at various stages of the cell cycle (Fig 1-6). The DNA

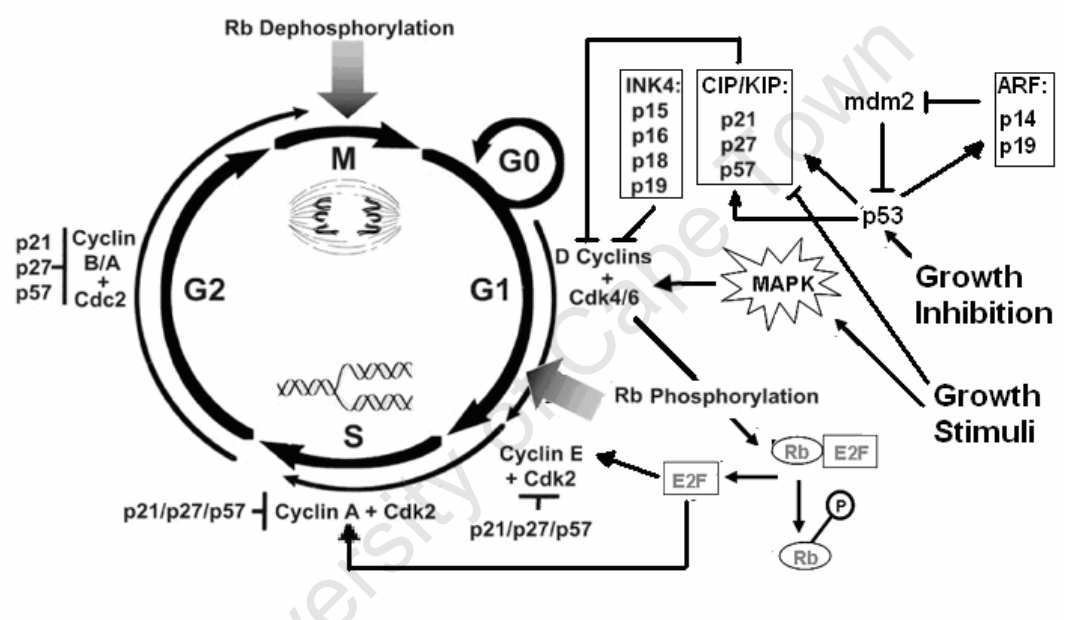


Fig 1-6: A schematic diagram illustrating cell cycle and the different cyclins, cyclin dependent kinases (CDKs) and CDK inhibitory proteins (CKIs) controlling the progression through each phase. In response to growth stimuli, cyclin D is induced and activates CDK4 or CDK6 by direct binding. CyclinD-CDK4/6 phosphorylates and inactivates RB protein, leading to the release of E2F transcriptional factor and the induction of proteins important for cell cycle progression. The cyclin-CDK complex is also under negative regulation from the CKIs, including the proteins of INK4, CIP/KIP and ARF families. Various growth inhibitory stress or signals can cause the upregulation of CKI proteins, via p53 dependent or independent pathways. Members of CIP/KIP families can bind and inhibit all kinds of cyclin-CDK complex, leading to either G1/S or G2/M arrest. In contrast, INK4 proteins mainly target cyclin D-CDK4/6 complex. Proteins of the ARF family are transcriptional variants of the INK4 proteins. The ARF proteins are engaged in the p53 network, functioning as a potent negative regulator of MDM2, stabilizing p53 protein through antagonizing the MDM2 mediated p53 degradation.

damage checkpoint (G1/S checkpoint) enables cells to screen for the potential challenges to DNA integrity. Upon DNA damages, cells activate this checkpoint that arrests cell cycle at late G1 phase, allowing the repair of damaged DNA before DNA replication. When DNA replication is disturbed by exogenous stimuli that interfere or block DNA synthesis, cells activate DNA replication checkpoint that arrests cell cycle at G2/M transition until DNA replication is complete. Finally, the spindle checkpoint is present in mitosis, ensuring the equal distribution of chromosomes into daughter cells. Disruption of spindle checkpoint will lead to aneuploidy [101]. Transition of cell cycle checkpoints are mediated by a network of cyclin/CDK complex and CDK inhibitory proteins (CKIs) under tight transcriptional and post-translational regulations of proliferative and growth inhibitory signals, such as ERK, cellular stress (often mediated by p53) and differentiation stimuli (Fig. 1-6).

Phosphatidylinositol 3-kinase (PI-3K) is another important RTK effector participating in cell growth, metastasis, angiogenesis and apoptotic evasion [102-104]. Of the various PI-3K downstream effecters such as PDK1, Akt, PKC-ζ and Rac/Cdc42, the PDK1 activated Akt (also known as PKB) is the primary mediator of PI3K-initiated signalling [102;103]. Akt is a serine-threonine kinase with a broad spectrum of substrates for its growth promoting and apoptosis suppressing functions. Akt is capable of abolishing the functions of cell cycle inhibitors such as $p21^{CIP1}$ and $p27^{KIP1}$ or apoptosis inducers such as Bad, Bim and procaspase-9 by direct phosphorylation. AKT also regulates transcription activities to carry out its function. Intermediate transcription factors include FoxO (forkhead family proteins), p53, CREB, and NFκB. AKT inactivates FOXO and p53 by either directly phosphorylating FOXO proteins or by phosphorylating and activating MDM2, a negative regulator of p53. In both cases, the expression levels of growth suppressing or pro-apoptotic genes (such as $p21^{CIP1}$, $p27^{KIP1}$ or Bax, Bad, Bid, Bim, Noxa, PUMA) are decreased on the transcriptional level. Conversely, Akt activates transcriptional activities of CREB (via direct phosphorylation) and NFκB (via activating IKKα and IKKβ, which in turn in activate the NF-κb antagonizer Iκb), leading enhanced expression of anti-apoptosis genes such as Mcl-1 and survivin [105].

In addition, PI-3K pathway is also negatively regulated by phosphatases such as PTEN (phosphatase and tensin homolog) [106-108] and SHIP-1/2 (SH2-containing inositol phosphatase 1/2) [109] through the removing of phosphates from PIP3. These genes are categorized as tumour suppressors as mutations, which eliminate their phosphatase activity, can lead to tumour progression.

1.3.1.2 Apoptosis

Apoptosis is one of the main types of programmed cell death that play critical role in tissue development and homeostasis. Apoptosis can be activated when a cell is damaged beyond repair. Typical apoptosis inducing stimuli include DNA damage, endoplasmic reticulum (ER) stress, nutrient starvation, hypoxia and immune attack. The trigger for apoptosis can either come from the cell itself or from the surrounding environment (e.g. immune surveillance) tissue, or from a cell that is part of the immune system. There are three pathways by which a cell commits apoptosis: (1) the intrinsic or mitochondrial pathway, (2) the extrinsic or death receptor pathway, and (3) the ER-stress related pathway. In cancer, apoptosis functions as a negative regulation to eliminate the transformed cells, hence blocking the spread of genetic deficiency and phenotypic malignancy. However, the apoptotic machinery is usually impaired in tumour cells.

In contrast to necrosis, which is a form of traumatic cell death that results from acute organism injury or inflammation, apoptosis is delicately executed by a series of signal cascades, hence avoiding the irritation or other damages to the surrounding tissue. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. Caspases, closely associated with apoptosis, are aspartate-specific cysteine proteases with a broad spectrum of substrates. The caspases and the main related regulating factors in extrinsic, intrinsic and ER stress related apoptotic pathways is summarized in Fig 1-7 (figure adapted from the review by Fan et al. [110]).

The intrinsic apoptosis pathway is initiated by cellular stress (such as DNA

damage and hypoxia) and controlled by the opening of mitochondrion permeability transition pores (MPTPs), which is primarily mediated by proteins of Bcl-2 family. Functionally, Bcl-2 family proteins are divided into three groups: (1) the anti-apoptotic Bcl-2 subfamily such as Bcl-2, Bcl-xL and Mcl-1; (2) the pro-apoptotic Bax subfamily such as Bax and Bak and (3) the pro-apoptotic BH3 subfamily such as Bad, Bid, Bik, Bim, NIP3, Noxa and PUMA. The Bcl-2 proteins, originally localized in the cytoplasm, can translocate to the mitochondrial outer membrane after an apoptotic program starts. Following the

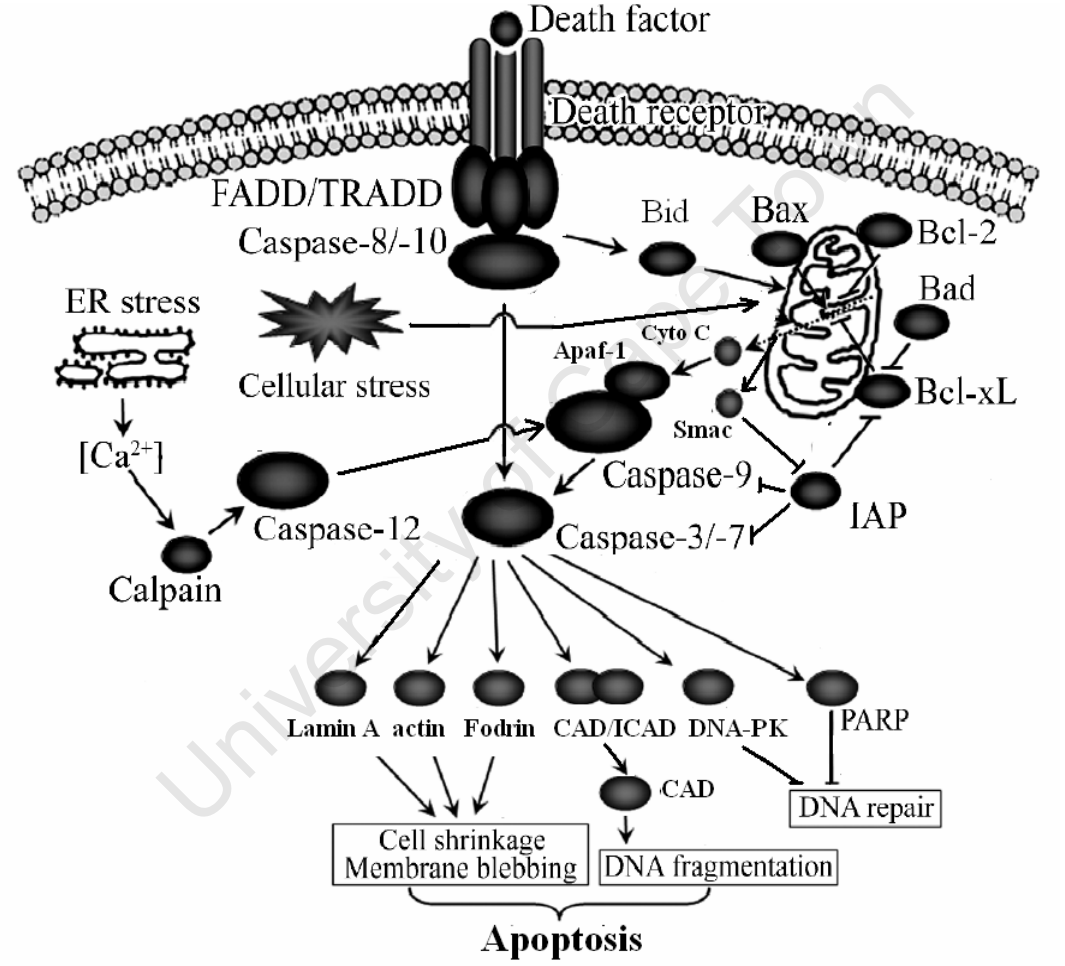


Fig 1-7: Caspases and the main related regulating factors in intrinsic, extrinsic and ER stress related apoptotic pathways.

Abbreviations: Apaf-1, apoptotic protease activation factor-1; CAD, caspase-activated deoxyribonuclease; Cyto C, cytochrome C; DNA-PK: the DNA-activated protein kinase; FADD, Fas-associated death domain; IAP, inhibitor of apoptosis protein; ICAD: inhibitor of caspase-activated deoxyribonuclease; PARP: poly ADP ribose polymerase; Smac, second mitochondrial activators of caspases/direct IAP binding protein with low pI (Smac/DIABLO); TRADD, tumour necrosis factor receptor-associated death domain.

translocation, Bax and Bak form homodimers and insert into the mitochondrial outer membrane to elevate the permeability of MPTPs. This process can be blocked by Bcl-2 subfamily proteins, through direct binding to the activated Bax and Bak. The main function of the BH3 subfamily members is to interact with Bcl-2 subfamily proteins and inactivate them. As a result of elevated mitochondrial permeability, mitochondrial proteins such as cytochrome c, AIF (apoptosis-inducing factor) and Smac/DIABLO (second mitochondrial activators of caspases/direct IAP binding protein with low pI) are released into cytosol. Of these factors, AIF is responsible for caspase-independent chromatin condensation and apoptosis [111]; Smac/DIABLO binds to IAP proteins such as XIAP and survivin, abolishing the inhibitory effects of IAP proteins on caspase-3, -7 and -9 [112;113]; while cytosolic cytochrome c is the most important herald of intrinsic apoptosis.

Once released, cytochrome c binds to Apaf-1 and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. In the apoptosome, caspase-9 is activated, which in turn activates procaspase-3 and procaspase-7. The activated caspase-3 will then activate procaspase-9 and other caspases, forming a positive feedback activation pathway. Caspase-3 and -7, as final apoptosis executors, target and degrade a broad spectrum of substrates including: cytoskeleton components (such as actin, lamin A and fodrin), DNA polymerase and DNA repair proteins (e.g. PARP and DNA-PK). Caspase-3 and -7 also activates the CAD endonuclease (caspase-activated deoxyribonuclease) through degrading its inhibitory counterpart ICAD, and cause DNA fragmentation.

Extrinsic apoptosis pathway is activated by cell death signals originated from immune cells or cytokines. Such signals as the tumour necrosis factor α (TNF-α), TRAIL or CD95/Fas ligand can bind to their specific membrane receptors, resulting in the recruitment of death receptors (such as Fas-associated death domain, FADD or TNFR-associated death domain, TRADD) and the cleavage and activation of procaspase-8 and procaspase-10. Next, caspase-8 and -10 cleave and activate the apoptosis executor caspase-3 and caspase-7. Moreover, caspase-8 and -10 can also cleave Bid,

a pro-apoptotic member of Bcl-2 family proteins, functioning as a positive regulator in the release of cytochrome c and Smac/DIABLO from mitochondria and further facilitating the apoptotic response [114].

Similarly to extrinsic apoptosis, the apoptosis induced by ER-stress is also mitochondrial independent. Caspase-12 is the initial caspase for ER-stress induced apoptosis [115]. In ER-stress, the aggregation of unfolded and misfolded proteins disturbs the calcium homeostasis in the ER lumen and cause calcium release which in turn activate the calcium dependent protease calpain. Next, calpain activates caspase-12 which is capable of activating caspase-9 independent of cytochrome c and link the cascade to the intrinsic apoptotic pathway [116;117].

1.3.1.3 Molecular alteration of proliferation and apoptosis in OSCC

There is a general agreement that aberrant growth-related RTK function and expression are implicated in the progression of oesophageal squamous cell carcinoma (OSCC). Epidermal growth factor (EGF), transforming growth factor α (TGF-α) and insulin growth factor (IGF) have all been demonstrated to function as autocrine growth factors for the proliferation of OSCC in cell culture models [118-120]. In OSCC, elevated EGFR activity due to either EGFR gene amplification or increased transcriptional activity has been demonstrated in 40%-90% of the tumours [121-124]. Elevated EGFR activity is regarded as an independent poor prognosis factor for OSCC, correlating with advanced disease, lymph node metastasis, vascular invasion, resistance to chemo- or radio-therapy and poorer prognosis [121-125]. Similarly, elevated IGF level has been detected in the sera of the OSCC patients [126;127]. The overexpression of IGFR has also been found in OSCC tissue samples [128]. In these cases, the elevation of either IGF or IGFR is associated with increased depth of tumour invasion, metastasis, advanced tumour stage and poor prognosis [126-128]. Other RTK downstream effecters or their regulators involved in the progression of OSCC include Ras [129;130], Akt [131] and PTEN [132-134]. Although discrepancy exists, a general trend observed in OSCC is that Ras and phosphorylated Akt levels are often elevated and associated with poor prognosis or chemo-resistance while loss of the tumour

suppressor PTEN often correlates with advanced disease and decreased survival. Collectively, it is suggested that growth factors together with their receptors and downstream signal transducers play important roles in the progression of OSCC.

Many tumour suppressor genes and oncogenes function as cell cycle regulators. They interact with each other and control the cell cycle checkpoints, determining the fate of the cells to proliferation, differentiation or apoptosis. Loss of the functions of the p53 (ARF–Mdm2–p53–p21) and pRb (INK4–cyclin D–pRb–E2F) pathways play a central role in the development of most human cancers [135]. In OSCC, inactivation of the pRb pathway is caused by the gene amplification of cyclin D1 as well as locus inactivation (including homozygous deletion, genetic mutation, or aberrant DNA methylation) or loss of heterozygosity of $p16^{INK4a}$, $p15^{INK4b}$, and occasionally pRb. Deregulated pRb pathway results in amplified E2F activity and elevated proliferation activity. On the other hand, deactivation of the p53 pathway is caused by p53 mutation itself, overexpression of the MDM2 protein (which causes degradation of wild-type p53), or the genetic inactivation of $p14^{ARF}$ inactivation (which suppresses MDM2 activity), resulting in a disabling of cell cycle arrest and apoptosis. Moreover, the genetic alteration and the subsequent aberrant expression of cyclin D1, pRb, p53, $p16^{INK4a}$, $p15^{INK4b}$, and $p14^{ARF}$ are associated with various clinicopathological parameters of OSCC in numerous studies (reviewed in [136-139]).

Besides genetic alteration, deregulation of the cell cycle surveillance proteins is also frequent in OSCC. $p21^{Cip1}$ and $p27^{Kip1}$ are important genes in this category. Stepwise reduction of $p21^{Cip1}$ and $p27^{Kip1}$ were observed in the multistep progression of OSCC, from dysplasia to distal invasive carcinoma. The decreased expression of $p21^{Cip1}$ and $p27^{Kip1}$ significantly correlate with increased cell proliferation observed with Ki-67 labelling [140]. In addition, it is demonstrated that $p21^{Cip1}$ or $p27^{Kip1}$ expression may correlate with the histologic differentiation of OSCC [141-143]. Since the genes for $p21^{Cip1}$ and $p27^{Kip1}$ are intact in most cases of OSCC, positive regulation of these genes may represent a potential therapeutic approach for this disease.

1.3.2 Metastasis and angiogenesis

Metastasis consists of the spreading of tumour cells from the primary neoplasm to distant sites. It is believed that the metastasis is influenced by intrinsic changes of the tumour cells as well as the tumour cell mediated modulation of the tumour micro-environment in both original and distant host sites (seed and soil theory). Metastasis consists of a series of steps in which cancer cells break through the primary tumour and disseminate to other parts of the body via the blood, lymphatic system or both. Many proteins including proteases, adhesion molecules, angiogenesis, and growth factors are involved in metastasis. As a common event in late stages of cancer, metastases formation is a major factor in cancer progression and accounts for the majority of cancer deaths [144].

As described in previous section, metastasis can be readily triggered or enhanced by mitogenic growth factors in OSCC cells, especially EGF and IGF (refer to Section 1.3.1.3 and Ref. [145]). In response to mitogenic signalling, genes responsible for metastasis such as zymogens and angiogenic factors are activated. In this section, function and significance of these metastatic and angiogenic factors in OSCC progression is discussed.

1.3.2.1 Extracellular matrix degradation

The extracellular matrix (ECM) includes the interstitial matrix and the basement membrane. A defining feature of connective tissue, the interstitial matrix comprises a gel of proteoglycans and glycosaminoglycans (e.g. heparin and hyaluronic acid) and fibrous proteins (e.g. collagen, elastin and fibronectin). The basement membranes are sheet-like depositions of ECM proteins such as laminin and type IV collagen, on which various epithelial cells rest. Destruction of ECM, especially the basement membrane, is a primary event in tumour invasion, which allows the tumour cells to escape the primary site as well as to allow their entry to the distant target (intravasation and extravasation). In addition, ECM also acts as a local depot for growth factors and cytokines. Tumour cells can trigger protease activities that cause local release or activation of these growth factors without *de novo* synthesis, which further

facilitates their rapid growth and epithelial-mesenchymal transition [146;147].

Endopeptidases are known to play important roles in the ECM remodelling during the process of tumour invasion and metastasis. Tumour cells mainly secrete two kinds of endopeptidases: zinc and calcium dependent matrix metalloproteinases (MMPs) and divalent metal ion independent serine proteases (e.g. uPA).

The urokinase plasminogen activator system comprises urokinase-type plasminogen activator (uPA) a serine protease, its specific receptor uPAR and its endogenous inhibitor PAI-1 and PAI-2. uPA catalyzes the conversion of plasminogen into plasmin. Plasmin is a proteolytic enzyme of broad substrate specificity which is involved in direct extracellular matrix (e.g. basement membrane) degradation and zymogen activation. Zymogenic substrates of uPA include but are not limited to the latent form of uPA (positive feedback loop) and several kinds of MMPs including collagenases, gelatinases and stromelysins (Fig 1-8). Binding of uPA to uPAR initiates the conversion of pro-uPA to active uPA on the cell surface, while the uPA-uPAR complex can be blocked by PAI-1 and PAI-2 through lysosome internalization and degradation. Besides ECM degradation, uPA system also participates in the regulation of cAMP levels, inositol phosphorylation and diacylglycerol/PKC pathways [148].

Although uPA system has been associated with invasion and metastasis in various types of cancer including OSCC [148;149], contradictory evidence from immunohistochemical analysis of OSCC clinical samples largely weaken the assumed correlation between uPA system and the progression of OSCC. A report by Torzewski et. al. suggests that the percentage of uPA-positive tumour cells in OSCC has no significant correlation with tumour size, histological grade and blood/lymphatic vessel invasion [150]. In another study by Tang et al., elevations of both uPA/uPAR and PAI-1 are observed in OSCC tumour, but these changes have no correlation with tumour grade or survival [151].

The matrix metalloproteinases (MMPs) family, as a whole, is capable of cleaving all the ECM components. Although some overlaps do exist, based on their structure and substrate specificity, MMPs are roughly divided into four

groups: collagenases (MMP-1, 8, 13 and 18), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10 and 11), and membrane-type MMPs (MT-MMP 1 to 6). The main substrates for the collagenases and stromelysins are interstitial matrix proteins such as collagen, elastin and fibronectin. Besides matrices components, gelatinases mainly target the components of basal membrane including collagen type IV, laminin, fibronectin and gelatin, that makes gelatinises essential for intravasation and extravasation. Except for MMP-11, all the other MMPs are secreted as inactive zymogens and need to be cleaved by other endopeptidases for activation. Plasmin, stromelysins and membrane type MMPs are among the most important activators. All the active forms of MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs). Antagonizing by binding to the catalytic sites of the MMPs, TIMPs are also able to block the MMP activation cascade. Therefore, regulation of the MMPs

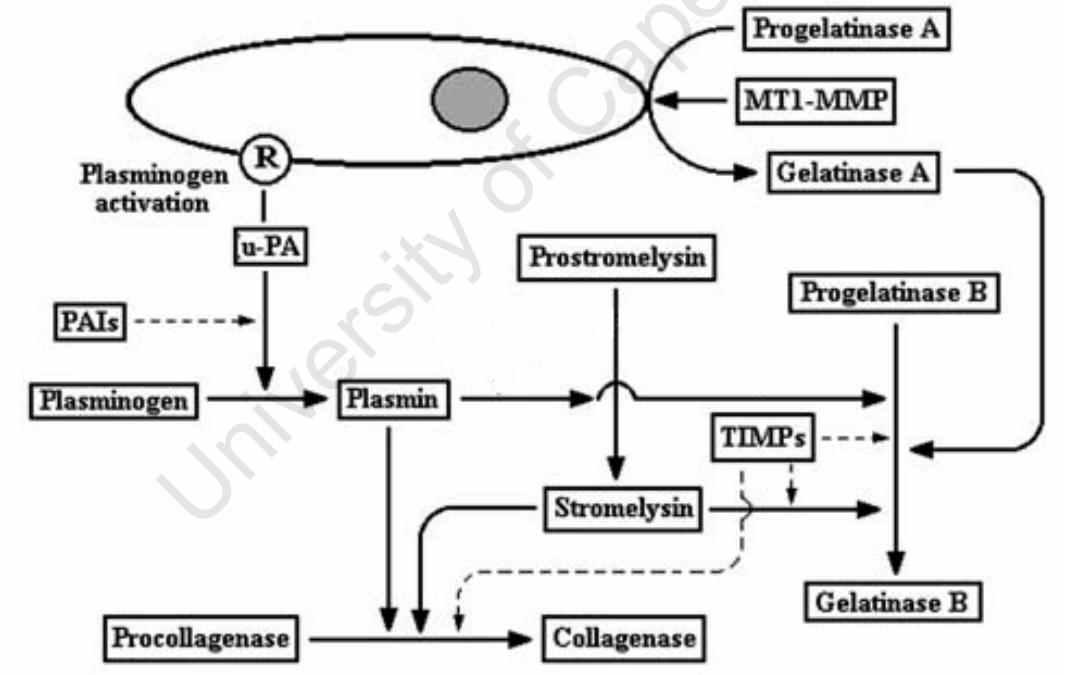


Fig.1-8: The activation cascade of MMPs (→, activation/conversion; ⇢, inhibition). Plasmin, activated by the uPA/PAR system, is the main initiator for MMP activation. Once activated, MMPs are also capable of reciprocal activation, in which the plasmin-mediated activation of stromelysins (MMP-3, and 10) is central to this cascade of processing plasmin-cleaved pro-collagenases and MMP-9 to their active forms. MMP-2, on the other hand, is substrate of neither plasmin nor stromelysin. In fact, membrane-type MMPs, pre-activated by plasmin, are responsible for MMP-2 activation.

occurs on three levels: gene expression alteration, latent zymogen activation and inhibition by TIMPs. The cascade of MMP activation is summarized in Fig 1-8 [146;152;153].

In oesophageal squamous cell carcinoma, upregulation of MMP-1, MMP-2, MMP-3, MMP-9 and MT-MMP-1 have previously been demonstrated by immunohistochemical studies of clinical samples [154-160]. The upregulation of these MMPs also positively correlate with tumour grade, depth of invasion, angiogenesis and poor prognosis, suggesting the oncogenetic roles of MMPs during the carcinogenesis of OSCC. Nevertheless, until now, reports about the expression and function of TIMPs in OSCC still remain controversial [160-162] .

In this study, MMP-2 and MMP-9 are explored due to their broad plethoric function in the modulation of tumour growth, metastasis and angiogenesis [153;156;163;164], as well as the solid correlation of these two gelatinases with the progression of OSCC [154-157].

1.3.2.2 Angiogenesis

The formation of new blood vessels (angiogenesis) and lymph vessels (lymphangiogenesis) are fundamental steps in the transition of solid tumours from a dormant state which corresponds to small lesions of not more than 1–2 mm in diameter, to a malignant and metastatic state [165]. Angiogenesis and lymphangiogenesis are mediated by distinct cytokines (angiogenic factors) and their receptors (RTKs) on the cells of endothelial origin. Activation of the angiogenic RTKs distinctively activate mitogenic signals such as Grb2/SOS/Ras, PLCγ/PKC, MAPK, PI-3K, Src and STAT, leading to the enhancement of endothelial proliferation and permeability as well as protect endothelia from apoptosis [166-169]. In human malignancies, tumour associated blood and lymphatic vessels are well known key accesses for metastatic spread. Moreover, penetration of tumour cells into peritumoral lymphatics and lymph node spreads are the most common initiations of tumour dissemination in some types of cancer, especially OSCC [170;171].

The best characterized and most specific angiogenic cytokines are the vascular endothelial growth factors (VEGF-A, -B, -C and -D). VEGF-A binds to VEGFR-1 and -2 expressed on vascular endothelial cells and is believed to be the main inducer of angiogenesis [166]. VEGF-C and VEGF-D are ligands of VEGFR-2 and VEGFR-3, playing an essential role in lymphangiogenesis since VEGFR-3 is expressed mainly on lymphatic endothelial cells [170-172]. Another major class of angiogenesis/lymphangiogenesis inducers is the angiopoietin (Ang-1 and Ang-2) and their receptor Tie1 and Tie2 [167;173]. In complement with VEGF and angiopoietin, platelet-derived growth factors (PDGFs) and their cognate tyrosine kinase α- and β-receptors are also involved in promoting angiogenesis and lymphangiogenesis [173-175].

Deregulation of angiogenetic cytokines is an important feature of solid tumours. In OSCC, a close relationship is established between the overexpression of angiogenic factors and malignant features such as advanced tumour grade, nodal infiltration, distant metastasis and poor prognosis. Candidate angiogenic factor includes VEGF-A and -C (reviewed in Ref. [176-178]), PDGF-A and -B [179], as well as angiopoietin-1 (Ang-1) [178;180]. Moreover, functional interference of the VEGF, PDGF and angiopoietin system in OSCC cell lines all markedly decreases the tumorigenesity of these cells further indicating the significance of these angiogenic system in the progression of OSCC [181-184].

1.4 NDRG1 in cancer

Although the role of NDRG1 in OSCC still remains largely unexplored, NDRG1 has been demonstrated to be implicated in several types of cancer. In this section, we review the current data about the regulation and function NDRG1 in the context of cancer cells, for clues on the possible role of NDRG1 in OSCC.

1.4.1 NDRG1 expression in cancer

Numerous studies have revealed altered expression levels of NDRG1 in cancer relative to normal tissue, suggesting its involvement in cancer

development. However, huge discrepancies exist in analysing the NDRG1 levels of patient tissue samples through *in situ* hybridization or immunohistochemical analysis.

Most early studies report the downregulation of NDRG1 during neoplastic progression. For example, immunohistochemical analysis of prostate cancer specimens has shown reduced NDRG1 expression in tumour tissues, compared to its prominent expression in counterpart normal tissues [185;186]. In addition, the level of NDRG1 expression has been found to be inversely related with tumour grade, showing that reduced NDRG1 levels are associated with advanced tumour characterized by poor differentiation status, increased distant metastasis and unfavourable prognosis [185]. Similarly, observations from breast [186;187], colon [24;49], pancreas cancers [188;189] and neuroblastoma [190] also revealed decreased mRNA or protein levels of NDRG1, especially in metastatic tumours [49;187]. Consistently, in these studies, high NDRG1 levels in patients are associated with better prognosis. Taken together, these results contribute to the hypothesis that NDRG1 may play the role of a tumour suppressor in the development of cancer.

In contrast, a growing body of evidence support the notion that NDRG1 may play an oncogenic role in cancer development. NDRG1 is reported to be upregulated during human and mouse skin carcinogenesis [191;192]. Elevated NDRG1 expression in cancer tissue was also observed in human oral squamous cell carcinoma [193], cervical adenocarcinoma [194;195], renal [64], colon [196;197], and liver cancers [198;199]. In most of the above cases, increased NDRG1 is associated with advanced tumour grade, metastasis, vascular invasion and poor prognosis. Moreover, Cangul and colleagues has reported that NDRG1 is expressed at higher levels in multiple human cancer types including brain, breast, lung, colon, kidney, liver and prostate cancers when compared with normal tissue [51;200]. Finally, clinical trials of anti-tumour drug irinotecan (or CPT-11, a camptothecin derivative) in colon cancer patients showed that upregulated NDRG1 was positively linked with chemo-resistance, indicating the potential oncogenic anti-apoptotic property of NDRG1 [201;202].

In addition, NDRG1 levels are sometimes observed to be irrelevant to cancer progression. As suggested by Fotovati and co-workers, although a positive trend was observed between NDRG1 expression and advanced tumour grade from immunohistochemical analysis of breast cancer samples, no significant correlation was found between NDRG1 levels and tumour size, lymph node metastasis and five year survival rate [77], resembling the lack in correlation between NDRG1 mRNA expression and breast cancer progression previously reported by Guan et al [49]. Similarly, except for a positive correlation between NDRG1 overexpression and enhanced liver metastasis, no other clinicopathologic correlation was found in a study using colorectal carcinoma samples [202]. Another study examining NDRG1 expression in a large number of prostate cancer specimens also failed to find conclusive NDRG1 upregulation or downregulation in these samples [203]. Even in cases which show NDRG1 involvement in cancer progression, inconsistencies are apparent in some parameters. For example, although NDRG1 was shown to be related to histopathological grade, microvessel density and depth of invasion in pancreatic cancer, no correlation was observed between NDRG1 expression and lymph node tumour metastasis, which is closely related to the above parameters in the same batch of samples [195]. In another study on breast cancer, NDRG1 expression level was observed associated to tumour metastasis but not the size or the histological grade of the primary tumour [187].

Many factors affect the result of immunohistochemical analysis, especially sample selection (such as the sample quality and the relatively smaller sample size) and experimental approach (such as the difference in staining method, antibody and scoring criteria). These deviations make it difficult to predict the tumorigenecity of a certain gene merely through the immunohistochemistry data, especially in the presence of contradictory observations. Since contradictory immunohistochemical observations regarding NDRG1 expression exist even in the same cancer type, immunohistochemistry result alone might not be a good indicator of NDRG1 tumorigenecity in cancer. Regardless of the above deviations, which could account for the inconsistencies in these NDRG1 immunohistochemical studies, the

contradictory observations may at least suggest the complexity of the NDRG1 regulation and function during the carcinogenesis. In fact, it is highly tempting to speculate the regulation and function of this gene to be tissue or even cell type specific [204].

It was recently reported that decreased NDRG1 mRNA level is correlated with tumour progression and poor prognosis in patients with oesophageal squamous cell carcinoma (OSCC) [205]. However, in another study, NDRG1 level was shown to be irrelevant to the OSCC pathological stages, tumour differentiation, lymph node metastasis as well as prognosis [206]. Again, inconsistent immunohistochemical observations are elicited in the same type of cancer. Moreover, the lack of functional studies of NDRG1 in OSCC further questions the values of these two studies.

1.4.2 Regulation of NDRG1 in cancer

Diverse cellular stress response mechanisms have been proposed in NDRG1 regulation. The expression of NDRG1 is strongly induced by various stresses and stimuli, most of which are closely involved in carcinogenesis. Typical NDRG1 regulators include carcinogens (phorbol esters, nickel, cobalt and cadmium) [23;73;83;207-212], differentiation inducers (retinoic acid, steroids, vitamin A and vitamin D) [31;46;73;213], DNA damage [25;214], hypoxia [26;51;198;215], endoplasmic reticulum stress inducers (reducing agents, calcium ionophore and etc.) [22;23;83;84] as well as DNA methylation and histone deacetylation targeting drugs [49;187]. Furthermore, various oncogenes and tumour suppressor genes participate in the regulation of NDRG1 expression. NDRG1 is reported to be upregulated by p53 [25;214;216], HIF-1 [200;217], AP-1 [218;219], Egr-1 [220] and PTEN [186;221], while N-myc, c-myc [21;75] and VHL [222] play suppressive roles in NDRG1 regulation. These observations indicate that NDRG1 is frequently associated with neoplastic processes. Here, the information about NDRG1 regulation is reviewed for clues regarding its function in cancer progression.

1.4.2.1 NDRG1 and hypoxia

Hypoxia is an important and common feature of the microenvironment of solid malignancy, when the rapid growing tumour outstrips the accompanying angiogenesis. All cells must be within 1–2 mm of a blood supply for the efficient permeabilization of oxygen and other nutrients. Therefore, it is not surprising that many parts of a developing solid tumour are hypoxic [223]. Hypoxia triggers many adaptive responses modulating the cellular activity of metabolism, proliferation, apoptosis and angiogenesis, and selects tumour cells with highest adaptability - a more malignant phenotype. It has been demonstrated that hypoxia may compromise the effects of to radio- or chemo-therapy, introduce higher mutation rates, promote epithelial-mesenchymal transition and finally lead to tumour invasion and metastasis [224-226].

Many research groups have demonstrated that hypoxia [26;51;67;189;198;215] and hypoxia mimicking reagents such as Co^{2+}, Ni^{2+} and iron chelators could strongly induce NDRG1 expression [51;67;189;212;217;219]. Although it has been reported in poorly differentiated pancreatic ductal adenocarcinoma cells that hypoxia and its mimics fails to upregulate NDRG1 regardless of HIF-1α stabilization [189], hypoxia still ranks one of the most prominent induction mechanisms of NDRG1 during cancer progression. Here, we survey the available data in an order of the transcription factors modulating the hypoxia response, despite the possibility of hypoxia independent NDRG1 regulation by these transcription factors due to the lack of literature until recently.

HIF-1:

The transcription factor hypoxia-inducible factor 1 (HIF-1) plays an essential role in the adaptive response of cells to reduced low oxygen tension in tumour progression [224;227]. HIF-1 is a heterodimeric transcriptional complex composed of HIF-1α and HIF-1β subunits. HIF-1β, also known as arylhydrocarbon receptor nuclear translocator (ARNT), is a common subunit of multiple transcription complexes. Associated with other transcription factors or co-factors (such as AP-1 or p300), HIF-1 docks with the hypoxia-responsive element (HRE), a five nucleotide DNA sequence (5'-RCTCG-3') in the promoter, and regulates the transcription of target genes.

In contrast to the relatively constant nuclear level of HIF-1β, the expression of HIF-1α varies a lot, and is tightly controlled mainly in the post-translational level. Therefore, HIF-1α becomes the key subunit determining the transcriptional activity of HIF-1. Although synthesized constitutively, HIF-1α is quickly targeted for degradation in the presence of molecular oxygen (normoxia), showing a half life of less than 10 minutes. This process is mediated through the hydroxylation of the proline residues by members of the prolyl-4-hydroxylases (namely PHD1, PHD2 and PHD3), which facilitates interaction between HIF-1α and the von Hippel-Lindau tumour suppressor (VHL), an E3 ubiquitin ligase, for proteasomal degradation of HIF-1α. A second level of HIF-1α regulation is mediated by an asparagine hydroxylase known as Factor Inhibiting HIF (FIH), which inhibits the interaction of HIF-1α with transcriptional coactivators such as CBP/p300 by hydrolyzing a conserved asparagine residue in its C-terminal transactivation domain. In hypoxia, as oxygen-dependent proline and asparagine hydroxylation are inhibited by low intracellular oxygen concentration, HIF-1α accumulates, translocates to the nucleus, heterodimerizes with HIF-1β, and associates with co-activators to regulate transcription [224;227;228]. In addition, since Fe^{2+} is required by both HIF-1α-regulating enzymes (PHDs and FIH), transition metals (such as cobalt and nickel ions, through competitive binding) as well as iron chelators (such as desferrioxamine) can stabilize HIF-1α as well [228].

It has been shown that NDRG1 induction by hypoxia, Co^{2+} and Ni^{2+} is largely diminished, at least in the short-term exposure, in HIF-1α double knock-out mouse embryonic fibroblast cells (HIF-$1\alpha^{-/-}$ MEF), indicating the participation of HIF-1α in the above processes [51;212;217]. Moreover, compared to HIF-$1\alpha^{+/+}$ MEF, HIF-1α double knock-out completely eliminate the induction of the NDRG1 core promoter by deferoxamine and okadaic acid, further supporting the requirement of HIF-1α in NDRG1 upregulation by hypoxia and its mimics. However, NDRG1 accumulation (albeit lower levels) can still be observed in HIF-$1\alpha^{-/-}$ MEF when cells are subjected to long-term hypoxia, suggesting HIF-1α may not be the only route for NDRG1 upregulation under hypoxia. Similarly, NDRG1 induction under iron chelation has also been demonstrated

via both HIF-1α dependent and HIF-1α independent pathways [217].

Nevertheless, the mechanism by which HIF-1α induce NDRG1 is still unknown. Analysing the NDRG1 promoter up to -1122 bp revealed no apparent HIF-1 binding motif (HRE) [222]. Although one less optimal HRE has been suggested by Zhang et al., neither mutation nor deletion of this site has been found to cause any appreciable effects on either basal or hypoxia inducible promoter activities [220]. In addition, even in HIF-1α efficient cells, the intracellular Ca^{2+} chelator BAPTA-AM completely abrogates NDRG1 induction by both hypoxia and nickel. This observation strongly implicates the involvement of intracellular calcium as well as its downstream effecters (such as AP-1) in NDRG1 induction by hypoxic conditions [212]. Hence, it may be proposed that induction of NDRG1 by hypoxia and its mimics may not be mediated by direct HIF-1α binding to its promoter. In contrast, secondary modulation and/or cooperation with other transcription factors or co-factors may be involved in HIF-1α mediated NDRG1 upregulation, with the possibility that HIF-1α itself may even act as a co-factor of other transcription factors.

In addition to HIF-1α, hypoxia-induced gene expression has been shown to be mediated by many other transcription factors such as AP-1, Egr-1, SP-1, NF-κB and p53 [224;227].

AP-1:

The activator protein 1 (AP-1) is a homo- or hetero- dimeric transcription factor composed of proteins belonging to the Fos, Jun and ATF families. AP-1 proteins are known as basic leucine-zipper (bZIP) proteins because they dimerize through a leucine zipper motif and contain a basic domain for interaction with the DNA backbone containing the TPA response element (TRE, TGAG/CTCA) or cAMP response element (CRE, TGACGTCA). In this way, AP-1 exerts its physiologic, oncogenic or anti-oncogenic effects by regulating genes involved in cell proliferation, differentiation, apoptosis, angiogenesis and tumour invasion. The AP-1 activity is primarily defined by the identity of its constituent dimers. Because of the oncogenic or tumour suppressive activity exhibited by distinct Jun, Fos and ATF family members depends on the cell context and the genetic background of the tumour, the AP-1 complex has been

called a "double-edged sword" in tumorigenesis. However, two of the most important components of AP-1, c-Jun and c-Fos, have been linked positively to neoplastic transformation and progression ever since they were cloned as cellular homologues of viral oncogenes, despite their frequent participation in promoting cell apoptosis [229-231].

Independent of HIF-1, hypoxia upregulates and activates AP-1 through MAP kinase, intracellular Ca^{2+} release and non receptor tyrosine kinase such as Src [218;232;233]. During hypoxia, AP-1 functions at least partly as an important facilitator of hypoxia induced gene expression through interaction with HIF-1 [224;227]. Salnikow et al. [218] demonstrated that c-Jun/AP-1 is involved in hypoxia and nickel induced NDRG1 expression. NDRG1 has also been reported to be upregulated by influx of Ca^{2+}, an important event in hypoxia [83;218]. c-Jun/AP-1 dependent, HIF-1 independent transcription is responsible for this induction. Dominant negative AP-1 as well as pan serine/threonine kinase inhibitor K252A (a potent inhibitor of CaMK, PKC, PKA and JNK) partly diminished these inductions, suggesting the involvement of AP-1 in modulating NDRG1 expression under hypoxia. Similarly, c-Jun/AP-1 is also involved, either HIF-1 dependent or independent, in NDRG1 upregulation by the iron chelator minosine and its analogue kojic acid [219]. Therefore, despite the unknown mechanism, AP-1 becomes one of the important regulators of NDRG1, especially under the circumstance of intracellular Ca^{2+} disruption.

Egr-1:

Early growth response-1 (Egr-1) is a zinc finger transcription factor involved in a number of early responses to a variety of stress and stimuli. Egr-1 preferentially binds to the GC-rich sequence GCGGGGGCG or the consensus DNA element TGCGT(G/A)GGCGGT to modulate the expression of genes involved in cell growth and survival [234]. Egr-1 is *de novo* synthesized under hypoxia in an Elk dependent, HIF-1α independent manner [235]. Since Elk is a canonical effecter of MAP kinase and PKC, the involvement of these kinases is thought to be important for Egr-1 activation [236]. According to the work of Zhang et al. [220], hypoxia and treatment of iron chelator deferoxamine leads to the transcriptional upregulation of NDRG1 by Egr-1 via the core promoter

containing the GC-rich sequence GCGGGGGCG. Mutation of this binding site abrogates the promoter activity. Consistently, Egr-1 knock-down or knock-out significantly reduced the NDRG1 induction in response to hypoxia or deferoxamine. Collectively, these results indicated the requirement of Egr-1 in the positive regulation of NDRG1 gene expression in response to hypoxia signaling.

Sp1 and Sp3:

Sp1 and Sp3 are ubiquitous transcription factors that are involved in basal transcription and housekeeping gene expression. There is growing evidence that Sp1 and Sp3 play a critical role in the growth and metastasis of many tumour types by regulating expression of cell cycle genes and angiogenetic cytokines [237]. Although the level of SP1/SP3 is generally unchanged under hypoxia, some evidence suggests that enhanced SP1/SP3 activity is required in hypoxia induced gene expression [224;227]. Interestingly, an overlapping binding motif GCGGGGGCGG(G/A)(G/A)(C/T) for Egr-1 and Sp1/Sp3 (underlined nucleotides) was identified in the NDRG1 promoter. Although *in vitro* electrophoresis mobility shift assay (EMSA) demonstrated binding of Sp1/Sp3 to this motif, mutation of the Sp1 binding site has no affect on promoter activity, either in normal or hypoxia conditions. In another study, Masuda et al. [222] reported a putative Sp1 binding site located between -286 and -62 on the proximal promoter of NDRG1 in VHL null renal cancer cell line 786-O. In comparison with other renal cancer cell lines which have efficient VHL, 786-O cells shows a much higher basal NDRG1 level but loses the capability of NDRG1 induction in response to hypoxia and nickel. Ectopic expression of VHL in 786-O cells down-regulates the NDRG1 expression but restores the NDRG1 induction by hypoxia and nickel. Moreover, mutation of this motif diminished basal transcriptional activity of NDRG1 promoter and partially blocked the decreasing effect of exogenous VHL on NDRG1 transcriptional activity. Considering that the involvement of VHL in regulating the expression of hypoxia related genes through Sp1 modulation is previously reported [238;239], Sp1 is expected to be specifically involved in the VHL-induced downregulation of NDRG1 gene expression as well as its basal gene expression activity.

Other factors:

NF-κB is a homo- or hetero- dimeric transcriptional factor consisting of transcriptional active members p65, c-Rel and RelB and regulating members p50 and p52. An increasing body of evidence suggests that NF-κB is positively involved in tumour progression [240], including OSCC [241]. Although constitutively expressed, NF-κB is bound to the repressor molecule IκB in the absence of stimulus. Upon stimulation, IκB is phosphorylated and inactivated by IκB kinases (IKKα and IKKβ). Consequently, NF-κB is released, translocates to in the nucleus, and initiates the transcription of NF-κB responsive genes. NF-κB activation has also been reported to be involved in hypoxia and hypoxia mimicking nickel insult [224;227;242]. However, it has been shown that NF-κB seems to be irrelevant in nickel induced NDRG1 expression in human bronchial epithelial BEAS-2B cells, since ectopic overexpression of dominant negative IKKβ (a kinase dead form) has no effect on NDRG1 upregulation by nickel [242].

During hypoxia, the cellular levels of reactive oxygen species (ROS) are elevated, leading to various changes in cellular signal transduction and gene expression [243]. Nickel or cobalt exposure also triggers ROS. However, neither the ROS generator hydrogen peroxide, nor ROS scavengers affects NDRG1 induction by hypoxia, Ni^{2+} or Co^{2+} [211]. Similarly, eliminating the ROS generated by doxorubicin also has no effect on its upregulation of NDRG1 [244]. Collectively, NDRG1 expression seems to be independent of ROS generation.

* * *

Taken together, it is tempting to consider NDRG1 as a hypoxia responsive gene based on the tight relationship between hypoxia and NDRG1 upregulation. Besides the hypoxia responsive transcription factors discussed here, activated by JNK during hypoxia [224;227], p53 is another candidate transcription factor accounting for the hypoxia mediated NDRG1 induction. In the next section, the potential effect of p53 on NDRG1 expression is reviewed.

1.4.2.2 NDRG1 and p53

The tumour suppressor protein p53 plays a key role in preventing the progression of cancer. p53 is activated by external and internal stresses including DNA damage, untimely expression of oncogenes, oxidative stress, hypoxia and nucleotide depletion. p53 activation involves its own stabilization, nuclear accumulation and enhancement of DNA binding and transcriptional activity. Once activated, depending on the incoming signals and cellular context, p53 will either induce a cell cycle arrest to allow repair and cell survival or apoptosis to self destruct the damaged cell. Phosphorylation (by MAP kinases such as ERK, JNK and p38 or checkpoint kinases such as ATM, ATR, CHK1 and CHK2) and acetylation (by transcriptional co-activator p300) are believed to be important for regulating p53 stability and transcriptional activity. Deacetylases such as Sirt1, can deacetylate p53, leading to an inhibition of apoptosis [245-247].

Kurdistani et al. first showed that NDRG1 is positively regulated by p53 through ectopic overexpressing p53 in various cell types as well as comparing renal NDRG1 levels in normal and $p53^{-/-}$ mice [25]. They also hypothesized that the induction of NDRG1 mRNA by DNA damage was dependent on the transcriptional activity of p53 because no significant induction is detected in cells with mutated p53 (DLD1 and T47D) as compared with cells with wild-type p53 (HCT116 and MCF7). In agreement, Stein et al. isolated NDRG1 as one of the p53 responsive genes necessary for p53-dependent apoptosis [214]. Furthermore, a p53 binding motif has been identified in the proximal promoter of NDRG1 at -406 upstream of the transcriptional initiation site by *in vivo* luciferase and CHIP assay. Mutation or deletion of this binding motif abrogate the p53 induction of NDRG1, confirming NDRG1 as a direct target of p53.

However, p53 dependent induction of NDRG1 is far from ubiquitous but rather cell-type specific. Ectopic overexpression of p53 in the p53-null lung cancer cell line H1299 failed to increase the NDRG1 level. In contrast, other p53 downstream targets such as $p21^{Cip1}$ and Mdm2 were readily induced after p53 overexpression [214]. Since p53 is accumulated and activated in hypoxia condition, it is conceivable p53 may participate the hypoxia induced NDRG1

upregulation [224;227]. However, deletion of the putative p53 binding site does not affect either the basal or hypoxia-inducible activity of the NDRG1 promoter [220], suggesting the hypoxia induction of NDRG1 is irrelevant to p53. In addition, immunohistochemical analysis of clinical samples of breast and prostate cancer showed no signi?cant correlation of NDRG1 protein expression with p53 status, suggesting that NDRG1 is predominantly regulated *in vivo* by p53 independent pathways [186;187].

The contradiction on the role of p53 on NDRG1 induction is further highlighted by the conflicting reports within the same experimental system. Le et al. [217] and Kurdistani et al. [25] independently treated MCF-7 cells with similar concentrations of DNA damaging agent, actinomycin D (6.3 ng/mL and 10 ng/mL, respectively) and mitomycin C (both 10 μg/mL) to induce the expression of p53 responsive genes. Effective treatment reflected by the marked induction of the canonical p53 effecter $p21^{Cip1}$ was demonstrated by both groups, however, NDRG1 induction was only observed by Kurdistani an co-workers. According to Le et al, DNA damaging agents such as actinomycin D, mitomycin C and cisplatin has no effect on NDRG1 induction in MCF-7 cells. In another report, the same group demonstrated that the induction of NDRG1 by another DNA damaging agent doxorubicin in mouse embryonic fibroblast was probably due to the iron-chelating character of doxorubicin, since this effect could be reversed by replenishing iron to the culture system [244].

Considering the intact p53 response demonstrated in nearly all of these contradictory papers, it is suggested that epigenetic diversity may explain the failure of p53 to induce NDRG1 expression in some cases. Epigenetic events such as DNA methylation and histone deacetylation block the access of transcription factor to the promoter, thereby silencing the gene transcription even with the existence of activated transcription factors [248]. Interestingly, NDRG1 is reportedly to be induced by DNA methylation and histone deacetylation inhibitors in colon [49] and breast [187] cancer cell lines, including MCF-7, indicating the NDRG1 gene is, at least partially, epigenetic silenced in these cells. Hence, it is conceivable that the NDRG1 promoters in H1299 cells and other cases may be epigenetic silenced, which blocks the p53

mediated NDRG1 upregulation. Moreover, since the methylation of promoter DNA is characterized as gradually accumulating during the physiological aging and cancer cell duplication [248], it may be postulated that the epigenetic status of the MCF-7 cells used in Ref. [217] and [25] may not be the same. In fact, as early as 1987, biological differences among MCF-7 cells from different laboratories were reported, despite the similar karyotypes shared by these cells [249]. In summary, although inconsistency does exist, due to the strong evidence from the identification of p53 responsive motif on NDRG1 promoter, we still consider the positive regulation of NDRG1 by p53 as reliable.

1.4.2.3 NDRG1 and Myc

The Myc family of proto-oncogene includes three evolutionarily conserved genes c-myc, N-myc and L-myc. Myc is a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family. The best understood function of the myc proteins is their ability to activate gene transcription. Myc exerts its function through dimeriziation with another bHLH-Zip protein, Max. The heterodimer binds to promoters containing a specific DNA sequence CACGTG, called E-box for transcriptional activation [250-252]. Either Max dependent or independent, Myc also executes its multiple activities through transcriptional suppression. In this case, Myc is recruited to core promoters through protein–protein interactions and may not bind DNA directly. Candidate proteins that have been proposed to target Myc to core promoters are Miz-1, Sp-1 and YY-1 [253;254].

The Myc transcription factors are thought to play a key role in cell differentiation. Myc expression is suppressed by growth-inhibitory signals and differentiation inducers while activated by mitogenic signals [250-252;255]. In embryonic development, Myc is consistently expressed but rapidly down-regulated when tissues become terminally differentiated and growth-arrested [256]. Oncogenic activation of the Myc genes generally results in their constitutive expression, resulting in uncontrolled cell cycle progression and poor differentiation status in a wide range of human and animal tumours [251;252;255;257;258].

NDRG1 was first identified as a Myc target gene by Shimono et al. through direct subtraction of whole mouse embryo cDNAs between wild type and N-myc mutant [21]. *In situ* hybridization results further revealed an inverse relationship between the expression of N-myc and NDRG1 in various developing tissues of wild type embryos. With diminished N-myc expression, NDRG1 expression is activated in the terminal differentiation phase of mouse embryonic tissue. Later, Li et al. and Zhang et al. observed similar induction of NDRG1 with Myc suppression in various human neuroblastoma and leukaemia cell lines when cells were treated with differentiation inducing reagents [74;75]. Furthermore, forced N-myc or c-myc expression consistently suppressed NDRG1 transcription in these studies [21;74;75].

Promoter analysis of both mouse and human NDRG1 revealed binding of c-myc or N-myc to the NDRG1 core promoter may participate in the transcriptional suppression [74;75]. Repression of NDRG1 by myc can be reversed by the histone deacetylases inhibitor trichostatin A, suggesting the recruitment of histone deacetylase to the promoter region is required for Myc mediated NDRG1 transcriptional repression [21;74]. Nevertheless, the necessity of direct DNA binding by the Myc is still obscure, since no canonical E-box sequence is revealed in this region by mutational studies. It is conceivable that Myc may require cooperating factors to repress NDRG1 transcription through indirect DNA binding, since it has been demonstrated that Myc down-regulates the transcription of another NDRG family member, NDRG2 through interaction of with Miz-1 [259]. However, the abrogation of Myc mediated repression of NDRG1 by protein synthesis inhibitor cycloheximide, seems to indicate the participation of other nascent proteins (or transcriptional factors) activated by Myc in the repression process [74].

Taken together, although the mechanism underlying Myc dependent NDRG1 repression still remains obscure, evidence from many different systems independently suggest the existence of this process during the cell differentiation. Intriguingly, the suppression of Myc was also observed in nickel treated neuroblastoma cells [75]. Considering the ability of Myc in interplaying with many other transcription factors, such as HIF-1α [260], this observation

further indicates that the participation of Myc in NDRG1 regulation may not be limited to differentiation, but a broader spectrum of biological processes.

1.4.2.4 NDRG1 and PTEN

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylates PIP_3 phospholipids generated through the activity of PI3K, thereby antagonizing PI3K activity as well as its downstream signal transduction pathway [106-108]. Moreover, independent of PI3K/Akt pathway, PTEN directly regulates the phosphorylation status of p53 and cyclin D through its phosphatase activity, leading to increased p53 stability and transcriptional activity and decreased cyclin D activity [261]. Similarly, directly through its phosphatase activity, PTEN diminishes the activities of focal adhesion kinase (FAK), Shc and MEK/ERK, and thus negatively regulates cell migration [262-264]. Like p53, PTEN deficiency is found is many types of cancer, including OSCC [132-134].

Upregulation of NDRG1 on the transcriptional level by PTEN has been observed in breast, prostate and ovary cancer cells [186;221]. Overexpression of PTEN resulted in increased NDRG1 level, while siRNA mediated PTEN silencing decreases NDRG1 expression in a dose and time dependent manner [186]. Consistently, immunohistochemical analysis revealed significant correlation of PTEN and NDRG1 expression in both prostate and breast cancer samples [186].

However, another immunohistochemical study of endometrial carcinoma by Chen et al. revealed negative correlation between PTEN and NDRG1 [265]. According to this study, NDRG1 expression is gradually increased in the progression from normal endometrium, atypical hyperplasia, to endometrial carcinoma, whereas PTEN expression is gradually diminished in this process. Another contradictory observation comes from the mouse epidermal cells C141 [208]. In this study, PI-3K activation is demonstrated to be necessary for nickel induced NDRG1 upregulation in C141 cells. Dominant negative PI-3K and Akt significantly blocked the nickel mediated HIF-1α and NDRG1 induction. Considering the fact that PI-3K positively regulates HIF-1α, a potent inducer of

NDRG1, either hypoxia dependently or independently [266], the above observations largely compromise the plausible positive correlation between PTEN and NDRG1.

Despite the tissue type specific impact on different immunohistochemical observations about the relationship of NDRG1 and PTEN, it should also be noted that Li et al. (Ref. [208]) examined cells under nickel treatment (hypoxia mimic condition) as opposed to normal condition in Ref. [186] reported by Bandyopadhyay and co-workers. Since PI-3K/PTEN controls a broad spectrum of downstream effectors, many of which may interplay with each other, clarifying the potential transcription factors for direct NDRG1 regulation may also help to elucidate the above contradiction.

1.4.2.5 Patterns of NDRG1 regulation and its potential functions:

It is widely accepted that expression patterns of a gene largely determines its potential functions, either as an executor or antagonist of its upstream

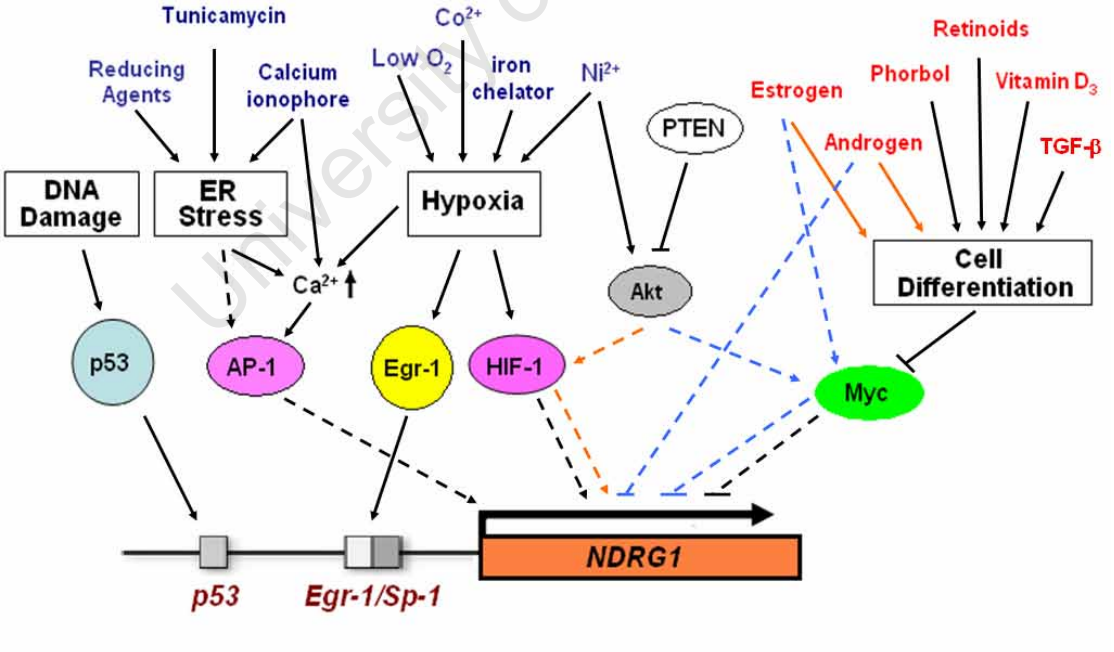


Fig. 1-9: Networks of the transcriptional regulation of NDRG1. Solid arrows indicate confirmed links or transcriptional regulation with direct evidence such as luciferase, EMSA or CHIP assays on wild type or mutated promoter. Broken arrows indicate possible links of regulation. Pairs of orange and blue arrows indicate contradictory information from literature.

regulator. As summarised in Fig 1-9, the expression of NDRG1 is linked to various stresses and stimuli. While the evolutional conservation of NDRG1 attests to its importance in biological function, the diverse regulation patterns of NDRG1 suggest it as a multi-functional protein.

Nevertheless, except for hypoxia and myc, most of the regulating mechanisms of NDRG1 are highly inconsistent and dependent on the cellular context. Considering the causality between regulation and function, it may be difficult to predict the general function of NDRG1 in cancer merely from the above inconsistent data of NDRG1 regulation. Moreover, the complexity of NDRG1 regulation may also provide the possibility to explain the discrepancy in the functional study of NDRG1 in different experimental systems.

1.4.3 The function of NDRG1 in cancer

Ectopic alteration of protein expression level through knock-in (overexpression) and knock-down (RNA interference) is one of the most important approaches for protein function investigation. Through subjecting cancer cells as well as their derivatives (transfectants) with altered NDRG1 levels to various kinds of functional assays, the function of NDRG1 in cancer have been studied extensively. Although it is agreed that NDRG1 is frequently associated with neoplastic development, observations regarding the function of NDRG1 in tumour progression are again, contradictory.

1.4.3.1 NDRG1 and primary tumour growth

Since malignant neoplasms are distinctively and nearly ubiquitously characterized by uncontrolled growth, the ability of a gene to regulate primary tumour growth becomes one of the "gold criteria" in defining a gene as either a tumour promoter (oncogene) or tumour suppressor.

The intrinsic cell proliferation activity reflected by *in vitro* cell growth is the fundamental factor determining the primary tumour growth. To investigate the role of NDRG1 in cell proliferation, various transfectants were established using cell lines derived from prostate [185], bladder [25], breast [25;267], lung [214], colon [49;214;216;221;268] , ovarian [221], endometrial [221],

pancreatic [188] and liver [269] cancers as well as neuroblastoma [75]. These transfectants were then subjected to different *in vitro* cell proliferation assays including cell counting, soft agar colony formation and DNA synthesis rate assay (through H^3-thymidine or BrdU incorporation). As summarised in following tables, these results are highly contradictory. This may suggest a tissue or even cell type specific function for NDRG1 in cancer cell proliferation.

Moreover, NDRG1 overexpression failed to promote soft agar colony formation or serum-independent growth of NIH3T3 cells. Since NIH3T3 cells are widely used to monitor the ability of oncogenic transformation, these results suggest that NDRG1 alone is not sufficient for initiating tumourigenesis [269].

Attempting to explain the discrepancy of the NDRG1 effect on *in vitro* cell growth, Stein and co-workers hypothesized that NDRG1 is involved in reducing cell proliferation in metastatic tumour cells but is ineffective in non-metastatic cells [214]. However, results in Table 1-1 and 1-2 do not support this hypothesis. In fact, the effect of NDRG1 on cell proliferation is specified by tissue or even cell types, regardless of the metastatic status or the presence of functional p53 in the cell line tested.

Recently, Wang et al. reported that NDRG1 overexpression in MDA-MB-231 leads to G1/S cell cycle arrest which may account for the decreased cell proliferation [25]. However, the flow cytometry results from Kim et al. did not show any significant change in DNA content profile in either normal breast epithelial 76N hNMEC or cancerous EJ, HCT-116 (p53 +/+), HCT-116 (p53 -/-) and DLD-1 cells with either NDRG1 knock-in or knock-down [60]. According to the same study, NDRG1 may be important in maintaining G2/M arrest induced by the microtubule spindle disruptor nocodazole. However, another study by Motwani et al. showed that NDRG1 eliminated by antisense RNA did not abrogate the G2/M arrest induced by DNA damaging agent irinotecan (a camptothecan derivative and DNA topoisomerase I inhibitor) [268].

Table 1-1: Effect of ectopic NDRG1 overexpression on *in vitro* cell proliferation:

Cell line	Origin	Description *	Method	Effect	Ref.
ALVA	Human prostate cancer	low-metastatic	cell counting & colony formation	No effect	[185]
AT6.1	Rat prostate cancer	metastatic	cell counting & colony formation	No effect	[185]
EJ	Human bladder cancer	metastatic, p53 null	cell counting & colony formation	Suppression	[25]
MCF-7	Human breast cancer	non-metastatic, p53 functional (wild type)	cell counting & colony formation	Suppression	[25]
MDA-MB-231	Human breast cancer	metastatic, mutant-inactive p53	BrdU incorporation	Suppression	[267]
H1299	Human lung carcinoma	metastatic, p53 null	cell counting & colony formation	Suppression	[214]
DLD-1	Human colon adenocarcinoma	non-metastatic, mutant-inactive p53	cell counting & colony formation	No effect	[214]
SW620	Human colon adenocarcinoma	metastatic, mutant-inactive p53	cell counting	No effect	[49]
SW480	Human colon adenocarcinoma	non-metastatic, mutant-inactive p53	colony formation	No effect	[221]
LoVo	Human colon adenocarcinoma	metastatic p53 functional (wild type)	colony formation	No effect	[221]
Sawano	Human endometrial adenocarcinoma	N/A	colony formation	No effect	[221]
Ishikawa3-H12		N/A	colony formation	No effect	[221]
MDAH2774	Human ovarian cancer	low-metastatic, mutant-inactive p53	colony formation	No effect	[221]

HCT-116 $p53^{-/-}$	Human colon adenocarcinoma	metastatic, double knock-out p53	H^3 thymidine incorporation and cell counting	Suppression	[216]
PANC-1	Human pancreas adenocarcimoma	metastatic, mutant-inactive p53	cell counting	No effect	[188]
MIApaca-2	Human pancreas adenocarcimoma	metastatic, mutant-inactive p53	cell counting	No effect	[188]
SK-N-SH	Human neuroblastoma	metastatic, deficient wt p53 (cytoplasmic sequestered)	colony formation	Suppression	[75]
NIH3T3	Mouse fibroblast	non-metastatic, functional (wild type) p53	colony formation	No effect	[269]

Table 1-2: Effect of ectopic NDRG1 RNAi on *in vitro* cell proliferation:

Cell line	Origin	Description *	Method	Effect	Ref.
IEC-6 **	Normal rat intestine	wild-type functional p53, non-tumorigenic	H3 thymidine incorporation and cell counting	Promotion	[216]
HepG2	Human hepatocellular carcinoma	metastatic, functional (wild type) p53	cell counting	Suppression	[269]
Hep3B	Human hepatocellular carcinoma	metastatic, p53 null	cell counting	Suppression	[269]

***** The metastatic status is determined from either the origin of cell line or more importantly, the ability of spontaneous metastasis when cells are transplanted into nude mice

****** Decreased NDRG1 by the stable transfection with siRNA significantly promoted cell growth in the absence of cellular polyamines which was responsible for physiological NDRG1 induction and growth inhibition in IEC-6 cells

Table 1-3: Effect of ectopic NDRG1 overexpression and knock-down on *in vivo* xenograft growth:

Cell line	Origin	Mice used	Method	Effect	Ref.
AT6.1 †	Mouse prostate cancer	SCID mice, BALB/C stain, 5 weeks	dorsal flank s.c. injection	No effect	[185]
EJ †	Human bladder cancer	nude mice, stain unknown	dorsal flank s.c. injection	Marked growth suppression	[25]
SW620 †	Human colon adenocarcinoma	nude mice, BALB/C stain male, 8 weeks	spleen injection	No effect	[49]
SW620 †	Human colon adenocarcinoma	nude mice, NCr-nu stain, male, 8-10 weeks	dorsal flank s.c. injection	No effect	[268]
HCT116 *	Human colon adenocarcinoma (p53 wild type, functional)	nude mice, NCr-nu stain, male, 8-10 weeks	dorsal flank s.c. injection	No effect	[268]
MIApaca-2 †	Human pancreas adenocarcimoa	nude mice, BALB/C stain, male, 6-10 weeks old	dorsal flank s.c. injection	Marked growth suppression	[188]
HepG2 **	Human hepatocellular carcinoma	nude mice, male, 4-6 weeks	dorsal flank s.c. injection	Marked growth suppression	[269]

† Stable NDRG1 overpression transfectant compared to parent cells

* Stable NDRG1 antisense transfectant compared to parent cells

** Intratumoral injection of NDRG1 siRNA to pre-formed HepG2 xenografts

Spindle checkpoint ensures even segregation of chromosomes in daughter cells and prevents polyploidy (aneuploidy) which a common characteristic in cancer [101]. Kim et al. showed that NDRG1 may compensate for mitotic checkpoint defects resulting from p53 deficiency and rescue polypoidy induced by the mitotic spindle disruption agent nocodazole. Further investigation revealed that NDRG1 co-localizes with the centrosome (γ-tubulin), and binds to microtubules *in vitro* and *in vivo* (in DLD-1 and 76N hNMEC cells). Furthermore, NDRG1 inhibition by siRNA resulted in the loss of astral microtubules and dividing spindle fibre. Reduced levels of α-tubulin, and particularly acetylated α-tubulin, which is important for microtubule dynamics (stabilization) and integrity [270] was supposed to account for this NDRG1 RNAi induced microtubule disturbance. Therefore, it was suggested that NDRG1 may contribute to genomic stability through p53-mediated euploidy maintenance [60].

Similar to the *in vitro* studies, observations of the effect of NDRG1 on *in vivo* primary tumour growth in xenograft models also do not agree with each other (Table 1-3). Unlike *in vitro* cell growth which is exclusively determined by the proliferative machinery of the cell itself, *in vivo* tumour growth is also controlled by the crosstalk between tumour cells and the host environment.

Angiogenesis, generating micro-vessels for oxygen and nutrients supply, which are essential for the rapid growth when the tumour reaches a certain size, is thus highlighted. Maruyama et al. found that NDRG1 overexpression had no effect on cell proliferation of pancreatic ductal adenocarcinoma cell line MIApaca-2 in culture, while in a mouse xenograft model, there was a marked decrease of tumour growth. This observation suggested potential modulation of angiogenesis by NDRG1 [188]. Indeed, further investigation in pancreatic ductal adenocarcinoma showed an inverse correlation between NDRG1 and the expression of angiogenic factors such as VEGF-A, IL-8 and MMP-9 (refer

to Ref. [271] and [272] for the angiogenic properties of IL-8 and MMP-9). In tumours expressing high levels of NDRG1, the *in vitro* and *in vivo* potential for microvessel formation is compromised [188]. Moreover, angiogenesis inhibition mediated by the anti-tumour drug ZD6474 (VEGFR-2 and EGFR inhibitor) and mycophenolic acid (*de novo* guanosine synthesis inhibitor) in gastric cancer and glioblastoma cell lines respectively, was associated with a significant induction of NDRG1 mRNA. The upregulation of NDRG1 by angiogenesis suppressive agents provides further evidence for the plausible involvement of NDRG1 in the modulation of tumour angiogenesis [273;274].

However, the above hypothesis is not consistent with cases from prostate [185] and colon [49;268] cancer, in which ectopic NDRG1 overexpression has no effect on the *in vivo* xenograft growth in mice (Table 1-3). These observations raise a concern that NDRG1 may have no effect on tumour angiogenesis in these two types of cancer. Paradoxically, in another report from cervical adenocarcinoma, high NDRG1 expression was significantly correlated with high VEGF expression and microvessel density. Hence, it was suggested that NDRG1 was positively associated with angiogenic activity in cervical adenocarcinoma, which in turn, made NDRG1 a marker of poor prognosis in this type of cancer [195].

Taken together, results from both *in vitro* and *in vivo* experiments are highly contradictory regarding the role of NDRG1 on primary tumour growth.

1.4.3.2 NDRG1 and apoptosis

A link between NDRG1 and stress response has been established through various studies showing NDRG1 upregulation under a broad spectrum of stress stimuli such as DNA damage, hypoxia and ER stress (as summarised in previous sections). Moreover, NDRG1 has been demonstrated to be transcriptionally regulated by tumour suppressor p53 [214;216], a key

regulator of both intrinsic and extrinsic apoptosis [275-277]. Due to the association of NDRG1 expression with various stress stimuli, numerous studies have explored the potential involvement of NDRG1 in apoptosis.

Using RNA interference and inducible ectopic gene overexpression approaches, Stein and co-workers suggested that NDRG1 was necessary but not sufficient for p53-mediated caspase activation and apoptosis. In this study, DNA damaging agent doxorubicin, a topoisomerase II inhibitor, was used to induce apoptosis in colon and lung cancer cell lines DLD-1 and H1299 (originally p53 deficient, with re-introduced wild type p53 expression when cells were cultured without tetracycline) [214].

In contrast to the pro-apoptotic features of NDRG1 claimed by Stein and colleagues, in another relevant study, NDRG1 induction by DNA damaging agent irinotecan was shown to participate in resistance to this drug [268]. Irinotecan is a topoisomerase I inhibitor, which is able to induce p53 accumulation and activate p53-mediated cell cycle arrest and apoptosis. Two colon cancer cell lines HCT-116 and SW620 with high and low endogenous NDRG1 expression respectively, were used in this study. Decreased NDRG1 expression by stable expression of antisense NDRG1 in HCT116 cells (p53 functional) sensitized cells to irinotecan induced apoptosis, while ectopic overexpression of NDRG1 in SW620 cells (p53 mutated, non-functional) exhibited decreased sensitivity to irinotecan treatment. Intriguingly, although flavopiridol, a broadspectrum kinase inhibitor, has no effect on NDRG1 expression itself, when applied in combination with irinotecan, it strongly abolishes the irinotecan induced NDRG1 expression with the cells quickly undergoing apoptosis. These data strongly suggest the ability of NDRG1 as a suppressor of apoptosis. Moreover, NDRG1 mediated irinotecan resistance also seems to be independent of p53, since it also manifests in p53 non-functional SW620 cells. Consistent with *in vitro* experiment, NDRG1

mediated chemo-resistance was also observed in both *in vivo* mouse xenograft and clinical colon cancer patients subjected to irinotecan [201;202].

It was also observed in primary human placental trophoblasts that NDRG1 attenuates the apoptosis induced by hypoxic injury [67]. Similarly, exposure of hypoxic trophoblasts to the omnipotent kinase inhibitor staurosporine results in marked downregulation of NDRG1 and switches cells to apoptosis. Moreover, trophoblasts with decreased NDRG1 level by siRNA become more susceptible to hypoxia mediated apoptosis as this effect can be antagonized by the caspase-3 inhibitor zVAD.fmk. Exposure to hypoxia leads to the accumulation of p53 which can in turn lead to rapid apoptosis [278]. Interestingly, overexpression of NDRG1 in trophoblast cultured in either standard or hypoxic conditions diminished the expression of p53, possibly through upregulated Mdm2, which sequesters nuclear p53 for proteasome degradation and thus negatively regulates p53 activity [279].

Despite the bulk of information indicating the involvement of NDRG1 in apoptotic modulation, there are also reports suggesting lack of involvement of NDRG1 in apoptosis. According to Zhang and co-workers, overexpression of NDRG1 failed to alter the susceptibility to tumour necrosis factor α /cycloheximide (together or separately) induced apoptosis [216]. Furthermore, several xenograft studies showed no effect of altering NDRG1 expression level on primary tumour growth. Since apoptotic response is inevitably involved as hypoxic ischemia is a common insult during the growth of xenograft (as solid tumour), it seems that NDRG1 does not participate in the modulation of hypoxia induced apoptosis in these cases [49;185;268].

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Although NDRG1 was shown to be potentially involved in cell death when cells are challenged by apoptotic stimulators, in most of studies, both NDRG1 knock-in and knock-down failed to elicit any characteristic sign of apoptosis when cells were cultured under normal condition [60;67;214;216]. However, it

was demonstrated that silencing NDRG1 by siRNA induced apoptosis in two hepatocellular carcinoma cell lines HepG2 (with functional p53) and Hep3B (p53 deficient). Besides the anti-apoptotic (or pro-survival) effect of NDRG1, this observation additionally suggested the involvement of NDRG1 in the homeostasis maintenance in these hepatocellular carcinoma cell lines [269].

To date, the functions of NDRG1 in the regulation of both spontaneous and induced apoptosis are paradoxical. Available results suggest that the role of NDRG1 in apoptotic response is cell type specific and does not seem to be dependent on the p53 status of the host cells [214;269].

1.4.3.3 NDRG1 and tumour metastasis

Metastasis reflects the abilities of a primary tumour to "break out" from the initial site of tumour development (basement membrane rupture, invasion), "spill" through circulation system (initialized by angiogenesis and lymphangiogenesis), and "settle down" to distant normal tissues. As a hallmark of aggressive malignancy and poor prognosis, it is not surprising that the relationship between metastasis and NDRG1 levels in clinical tumour samples has been carefully investigated. However, the results are contradictory.

Various immunohistochemistry results from breast [186;187], prostate [185] and pancreatic [188] cancers showed significant reduction of NDRG1 expression in patients with both primary invasion [186;188] and distant metastasis [185;187]. In cell culture models, lower endogenous NDRG1 level is often shown to be associated with the invasive phenotype in cancerous cell lines. Accordingly, ectopic overexpression of NDRG1 markedly suppresses *in vitro* Matrigel invasion and *in vivo* spontaneous metastasis in nude mice. These phenomena were observed with a broad spectrum of cell lines, including cell derived from breast (MDA-MB-468 and MDA-MB-231) [187;267], prostate (At6.1 and ALVA) [185], pancreatic (PANC-1 and MIApaca-2) [188]

and colon (SW480 and SW620) [49] cancers.

The recruitment of microvessels (angiogenesis) is a key step for tumour dissemination to distant organs. Recently, Maruyama et al. observed a significant effect of NDRG1 on suppressing the mRNA and protein levels of matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) in pancreatic cancer cell line PANC and MIApaca-2 [188]. Previous data indicate that MMP-9 plays a critical role in the angiogenic switch during carcinogenesis [272], while VEGF and IL-8 are two cytokines amongst the most potent angiogenic factors [271]. These data suggest NDRG1 may suppress angiogenic triggering. Moreover, immunohistochemical analysis of mice tumour xenograft showed a significant decrease in the number of neo-microvessel developed in xenograft derived from NDRG1 overexpression transfectants. A reverse correlation of NDRG1 overexpression level and microvessel development in samples from pancreas cancer patient is also detected.

Moreover, comparing to primary lesions, an additional significant decrease of NDRG1 expression was observed in metastatic counterparts from patients with colon cancer [49], indicating that the involvement of NDRG1 in colon cancer metastasis may not be limited to the initiation but late stages as well. However, in another relevant study of 131 colorectal liver metastases, NDRG1 expression was not significantly different between the *in situ* tumour and liver metastasis [202]. This is consistent with a previous report demonstrating no appreciable difference in NDRG1 expression by Northern blot analysis in 36 clinical specimens of primary and metastatic breast cancer lesions [49].

Taken together, the antagonizing role of NDRG1 in the initiation of metastasis and angiogenesis is thus suggested. However, more evidence has been presented to contradict the above idea. Immunohistochemical analysis of patient samples from oral squamous cell carcinoma [193], cervical adenocarcinoma [194;195], and liver cancers [198;199] all suggested positive

relationship between increased NDRG1 expression and tumour metastasis. Moreover, in another immunohistochemical study using colorectal cancer samples, both NDRG1 mRNA and protein levels were found gradually upregulated through the colorectal carcinogenesis with a significant difference between non-metastatic and metastatic tumours. In the same study, high expression of NDRG1 was also observed in the metastatic sites of corresponding regional lymph nodes which showed no obvious difference compared to the original primary tumour [196]. Finally, functional studies using NDRG1 siRNA effectively suppressed the migration and invasion abilities of HepG2 and Hep3B cells, indicating the potential metastatic promoting roles in these hepatocarcinoma cell lines.

1.4.3.4 NDRG1 and ATF-3

Recently, using cDNA microarray analysis, Bandyopadhyay et al. have reported in prostate cancer cells that NDRG1 attenuates the transcriptional level of activating transcription factor 3 (ATF3), through which NDRG1 exerts its inhibitory role on tumour metastasis. Reverse correlations of NDRG1 and ATF3 levels in both *in vitro* cell culture model and clinical prostate cancer samples were also observed in this study, further strengthen this potential relationship [280]. ATF3 is a member of the ATF/cAMP binding protein family of stress related transcription factors, which selectively bind to cAMP–responsive element (CRE) of the promoter and activate or suppress the expression of the target genes. ATF3 represses transcription as a homodimer; however, as a component of AP-1, ATF3 heterodimerized with c-Jun to behave as a transcriptional activator [229;231;281].

Interestingly, like NDRG1, the role of ATF3 in the context of tumour progression is very complex, which makes it difficult to define this gene as an oncogene or tumour suppressor. Depending on the cellular context and type of cancer, the dichotomous role of ATF-3 has been observed in nearly all the aspects of cancer development, including oncogenic transformation,

proliferation, apoptosis and metastasis [280;282-288]. Therefore, the link between NDRG1 and ATF-3 may be useful to explain the inconsistent NDRG1 function in different tissue or cell types, if the existence of this link is not tissue or cell type specific. Nevertheless, the report by Bandyopadhyay et al. (Ref. [280]) is the only information about the mechanism of NDRG1 function at present, and further confirmation on the link between NDRG1 and ATF-3 should be a high priority. Moreover, the mechanism underlies the antagonizing effect of NDRG1 on ATF-3 transcription is also unclear.

1.4.4 Summary and project aim

It is highly possible that NDRG1 is implicated in the cancer progression, from the aspects of both its diverse carcinogenesis related regulatory pathways and its function in modulating the tumour growth, apoptosis and metastasis. However, observation from neither aspect is conssistent, with exceptions or contradictions presented in different tissue types and sometimes even different cells derived from the same type of cancer. Therefore, it is very difficult to category NDRG1 as a friend or foe in neoplastic progression, especially in oesophageal squamous cell carcinoma (OSCC), of which the molecular mechanism still remains quite obscure.

The general aim of this project is to explore the role of NDRG1 in OSCC. This aim will be specifically achieved by addressing the following objectives:

1. Determine phenotypic changes in OSCC cells in response to ectopic alteration of NDRG1 expression using *in vitro* and *in vivo* functional assays.
2. Explore regulation of NDRG1 in cultured OSCC cells in response to various stimuli related to the neoplastic progression of OSCC.

Chapter Two

Functional analysis of NDRG1 in oesophageal squamous cell carcinoma cell lines

2.1 Introduction

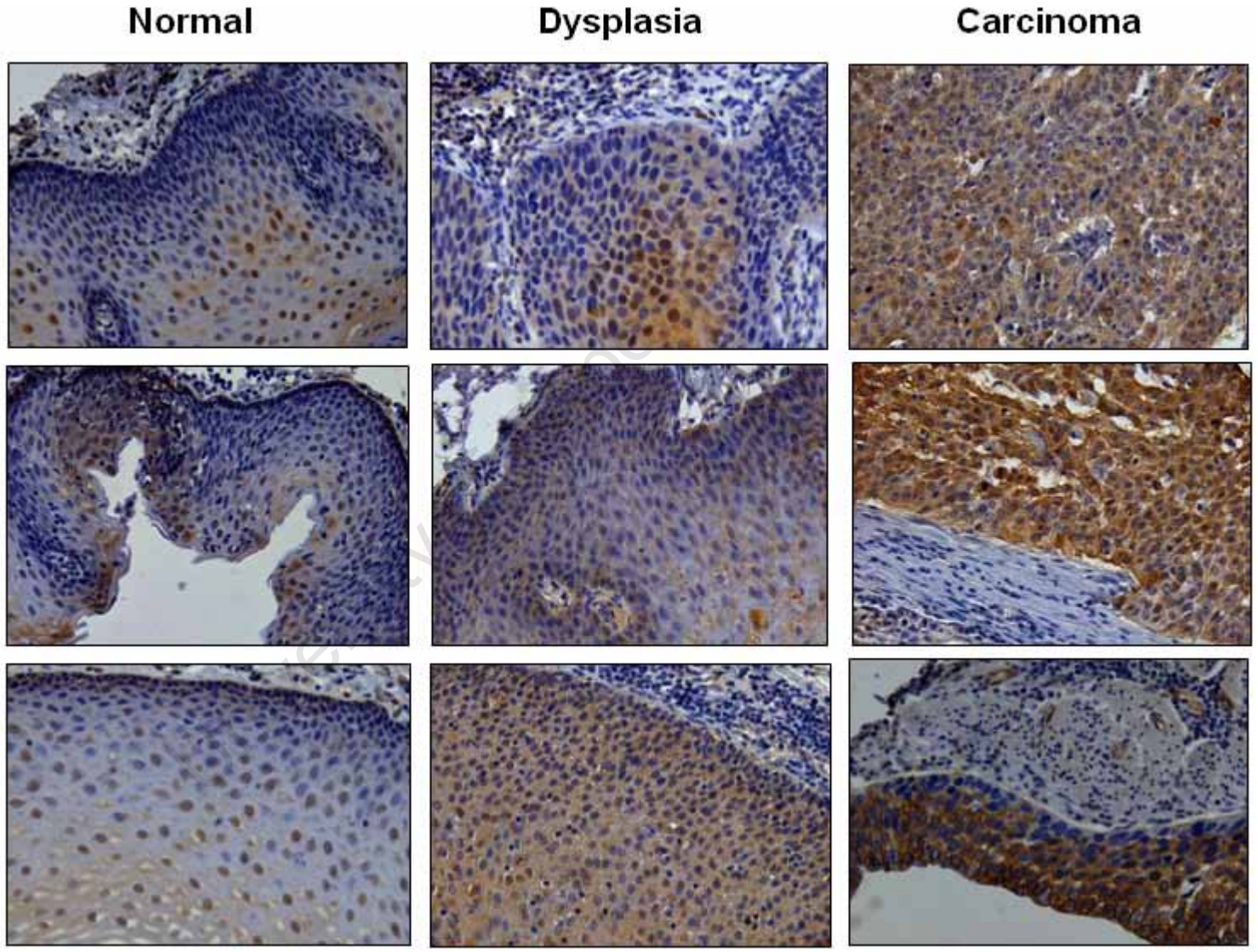
Although N-myc downstream regulated gene 1 (NDRG1/Cap43/Drg-1) was previously implicated in various types of cancer, the exact role of NDRG1 in oesophageal squamous cell carcinoma (OSCC) still remains unclear. As indicated in the previous chapter, OSCC ranks as one of the tumours with poorest prognosis, highlighting the need for knowledge regarding the molecular biology of this deadly disease. As part of the objective in our research group to identify genes potentially involved in the development of OSCC, our previous investigation using differential display techniques demonstrated aberrant expression of NDRG1 in OSCC tissue compared to adjacent normal tissue (unpublished data). This observation makes NDRG1 a potential candidate to contribute the understanding of the molecular events involved in the development and maintenance of OSCC.

Subsequent immunohistochemical analysis of OSCC samples from South African patients (n=70) further revealed a trend of NDRG1 upregulation in OSCC tumour tissue, with well differentiated tumours showing the highest expression level (by Wamunyokoli F, PhD Thesis, 2002). Two independent studies, however, reported decreased levels of NDRG1 in OSCC tissue relative to normal tissue in Japanese [205] and Chinese (He'nan Province) [206] patients. Consequently, another immunohistochemical analysis was performed independently for this project, with 52 clinical OSCC specimens, mostly paired with adjacent normal and dysplasitic tissues, from Chinese patients in Shan'xi Province, using the facility of our collaborator Prof. Xiaohang Zhao in China (Fig 2-1).

Consistent with the previous report [50], regardless of the status of the tumour tissue (pathological stage and histological grade), NDRG1 immunoreactivity was found mainly confined to the oesophageal epithelium. Regarding NDRG1 protein expression, although NDRG1 was detected in all specimens, the level of NDRG1 was found lowest in normal tissue, increased in dysplasia and highest in tumour tissue. Statistical analysis using Fisher's exact test suggested that the gradual upregulation of NDRG1 from normal oesophageal mucosa to squamous carcinoma is unlikely attributable to chance alone ($p<0.001$) (Fig 2-1, A and B). Moreover, in normal oesophageal epithelia, NDRG1 immunoreactivity was mainly detected in the matured, static cells distal to the basal membrane, while proliferating cells near the basal membrane are largely absent of NDRG1 expression. Considering the fact that only these proliferating cells (showing low levels of NDRG1) represent the pool of cells that are thought to develop into cancer cells in response to carcinogenic insults, it is tempting to speculate that increasing NDRG1 expression may be involved or at least reflect the neoplastic development in these cells. Although NDRG1 expression was observed irrelevant to the differentiation level or histological subtype of the tumour tissues ($p=0.292$, from Fisher's exact test, Fig 2-1, C), we still demonstrated a general trend of NDRG1 upregulation in the OSCC carcinogenesis.

Taken together with our previous immunohistochemical analysis of South African samples as well as the two published reports, all the existing immunohistochemistry results commonly indicate that NDRG1 is actively modulated in OSCC, suggesting the potential involvement of NDRG1 in the neoplastic progression of OSCC. However, inconsistent trends of NDRG1 expression during the progression of OSCC have been revealed in these studies. Therefore, further exploration through ectopic gain- and loss-of-function of NDRG1 was performed in OSCC cell lines, since the contradictory immunohistochemistry results presents a rather conflicting picture regarding the role of NDRG1 in this disease.

A



B

NDRG1 expression in different stages of OSCC

Stages	Weak	Moderate	Strong	Total
Normal	14 (45.2%)	15 (48.4%)	2 (6.5%)	31
Dysplasia	9 (22.5%)	19 (47.5%)	12 (30.0%)	40
Tumour	6 (11.5%)	15 (28.8%)	31 (59.6%)	52

C

NDRG1 expression and OSCC differentiation status

Differentiation	Weak	Moderate	Strong	Total
Well	3 (14.3%)	5 (23.8%)	13 (61.9%)	21
Moderate	3 (18.8%)	3 (18.8%)	10 (62.5%)	16
Poor	0 (0.0 %)	7 (46.7%)	8 (53.3%)	15

Fig 2-1: Immunohistochemical analysis of NDRG1 expression in human OSCC and adjacent normal and dysplastic tissues. A, Representative images with a magnification of 100× showing the NDRG1 expression levels in normal mucosa (left), dysplasia (middle) and carcinoma (right). Immuno-stained sections were first developed with DAB substrate chromogen resulting in a brown-coloured precipitate at the antigen site and then lightly counterstained with Mayer's hematoxylin. Samples from three patients are shown. **B,** Comparison of NDRG1 expression level in different stages of OSCC. **C,** Comparison of NDRG1 expression level in different histological subtypes of OSCC (the degree of tumour cell differentiation).

In this chapter, we describe our work of evaluating the NDRG1 function in two OSCC cell lines using the criteria summarized by Hanahan and Weinberg [97]. Stable transfectants developed from KYSE30 and KYSE150 cell lines were generated by lentiviral transduction that altered NDRG1 expression levels (ectopic overexpression or knock-down). Next, both transfectants and parental cells were subjected to *in vitro* functional assays to investigate the potential phenotypic changes in cell proliferation, differentiation, apoptosis, angiogenesis and metastasis in response to ectopic NDRG1 expression modulation. To confirm the *in vitro* observations, studies using a nude mouse model were further performed. Finally, our preliminary data showed suppressive effect of NDRG1 on ATF-3 expression, indicating ATF-3 as a potential downstream effector of NDRG1 in KYSE30 and KYSE150 cells.

2.2 Ectopic NDRG1 knock-in (overexpression) and knock-down

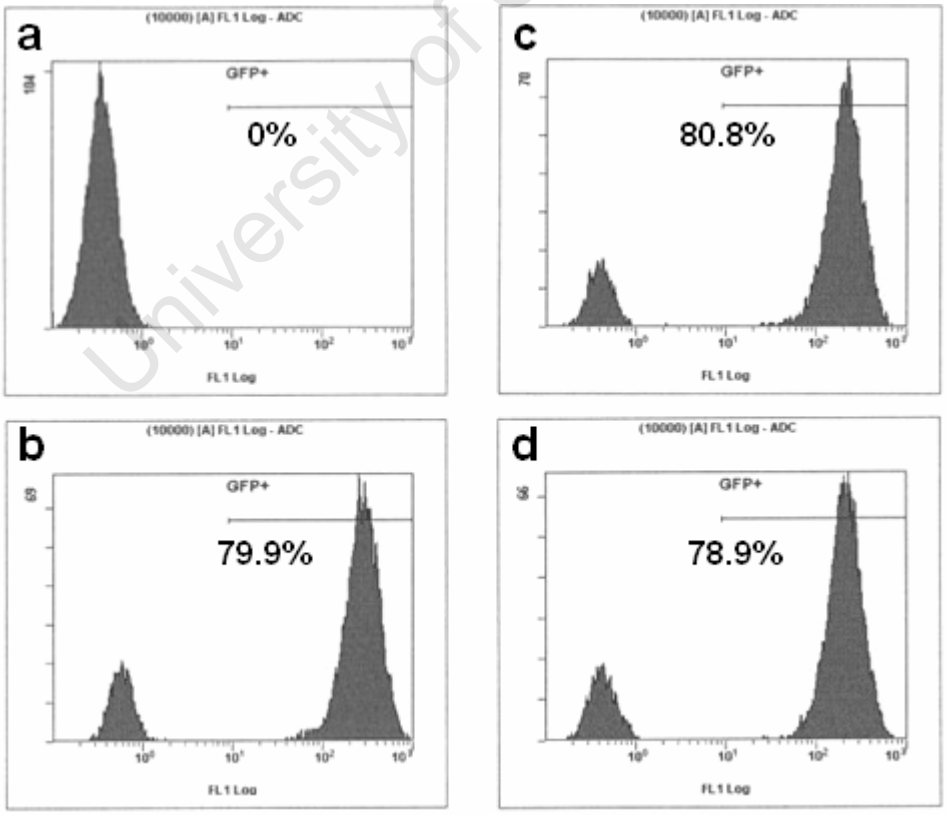
Ectopic NDRG1 knock-in (overexpression) and knock-down (by short-hairpin RNA/shRNA mediated RNA interference or RNAi) in OSCC cell lines was performed using human immunodeficiency virus type 1 (HIV-1) derived lentiviral vectors as delivery vehicles. These lentiviral vectors as well as the packaging system were obtained with the permission of the original developer, Dr. Didier Trono (University of Geneva, Switzerland). The details of the vector information and the methods of applying these vectors for stable NDRG1 knock-in and knock-down are described in the Section 2 of Chapter 5.

Compared to the conventional vector systems, lentiviral vectors have a much greater transgene delivery efficiency which may even approach 100% for many cultured cell lines. Besides the high efficiency, lentiviral vector system also mediates the integration of the transgene into the genome of targeted cells, resulting in stable expression of the exogenous fragment. Therefore, no selection marker is necessary, preventing the protracted usage of toxic selection chemicals such as puromycin and G-418 which may inflict unwanted influence on the phenotype of the transfectants. Finally, a green fluorescent protein (GFP) reporter carried on Trono's lentiviral vectors enabled convenient monitoring of transgene delivery (transfection) efficiency as well as transfectant enrichment through flow cytometry.

KYSE30 and KYSE150 cell lines [289] were chosen from our OSCC cell line repository, since both cell lines had been demonstrated capable of growing in nude mice [290;291]. KYSE30 cells, established from a well-differentiated tumour have high levels of endogenous NDRG1, while KYSE150 cells derived from a poorly-differentiated tumour have low NDRG1 levels (Fig. 2-3). Stable transfectants with ectopic NDRG1 knock-in (overexpression) or knock-down (RNAi) were generated using both cell lines. The vector coding a scrambled,

null-targeting sequence for RNA interference was designated as vector control for both NDRG1 knock-in and knock-down, due to the identical nature of the two lentiviral vectors. Considering the relatively high transgene delivery efficiency of lentivirus, a transfectant "pool" was directly used for subsequent functional assays rather than clones, since the "pool" which consists of numerous clones has statistical advantage over individual clones. Moreover, using a transfectant "pool" avoids the potential problem of individual clones in which the vector integration may happen in non-redundant DNA, resulting in unwanted alteration in gene expression profile. Nevertheless, to minimize the potential inconsistency of the "pool" composition as a result of prolonged culture, we managed to perform all the functional assays using the "pool" with a passage number of 6-10 after initial lentiviral transduction.

A



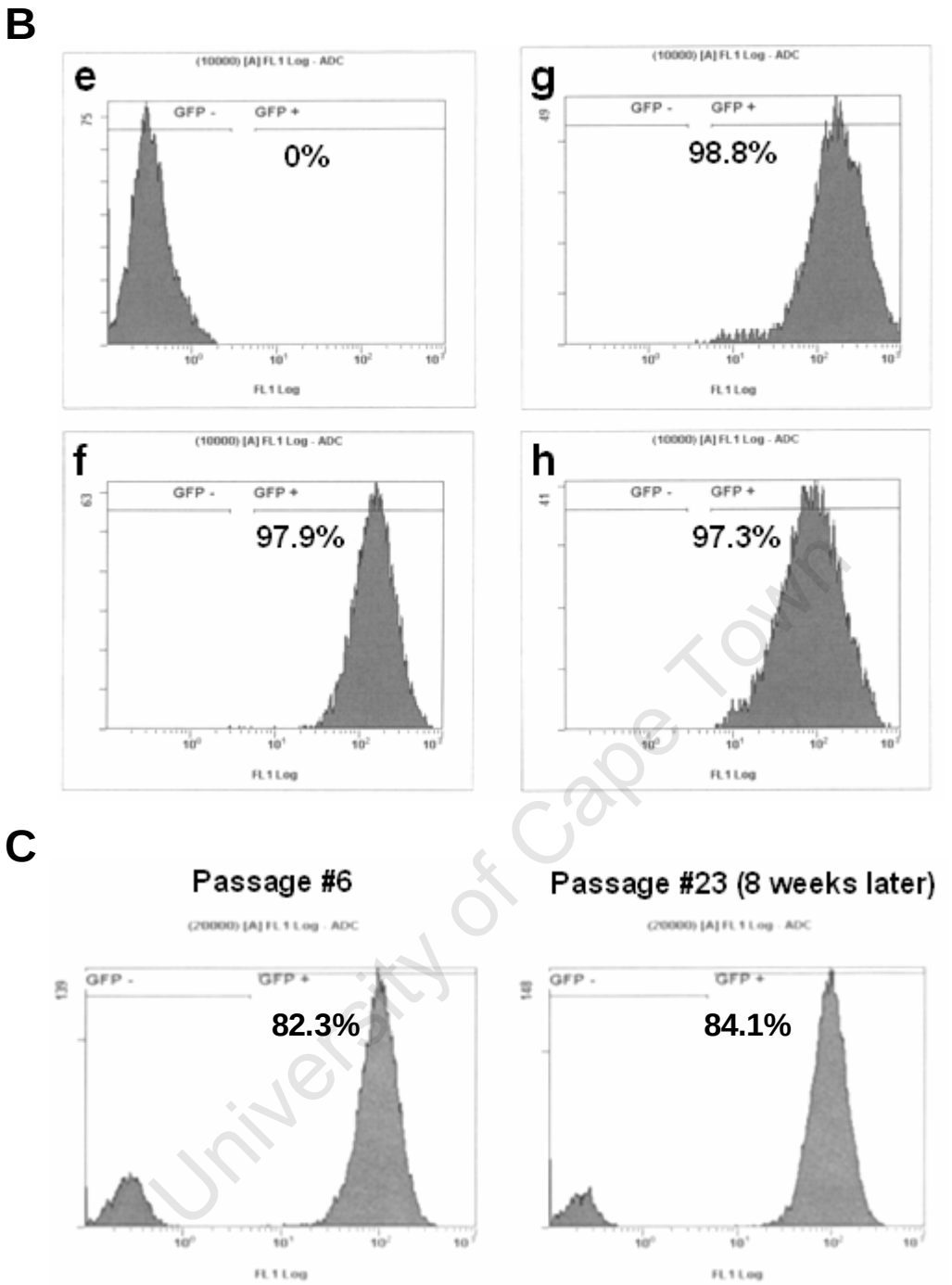


Fig 2-2: Flow cytometry analysis of the lentiviral transfected cells (GFP positive cells) in the transfectant "pool". Subconfluent cells were trypsinized, resuspended in 1 × PBS and directly subjected to flow cytometry analysis. The percentage of GFP positive (GFP+) cells analysed by the subsidiary software is shown within the histogram.
A, KYSE30 transfectants; **B,** KYSE150 transfectants; (**a & e,** wild type cells; **b & f,** NDRG1 overexpression; **c & g,** NDRG1 knock-down; **d & h,** vector control) **C,** Comparison of the percentage of GFP positive cells in the KYSE30 transfectant "pool" with NDRG1 RNAi immediately after transduction (passage #6) and 2 months thereafter (passage #23), showing the stable transgene delivery in this transfectant.

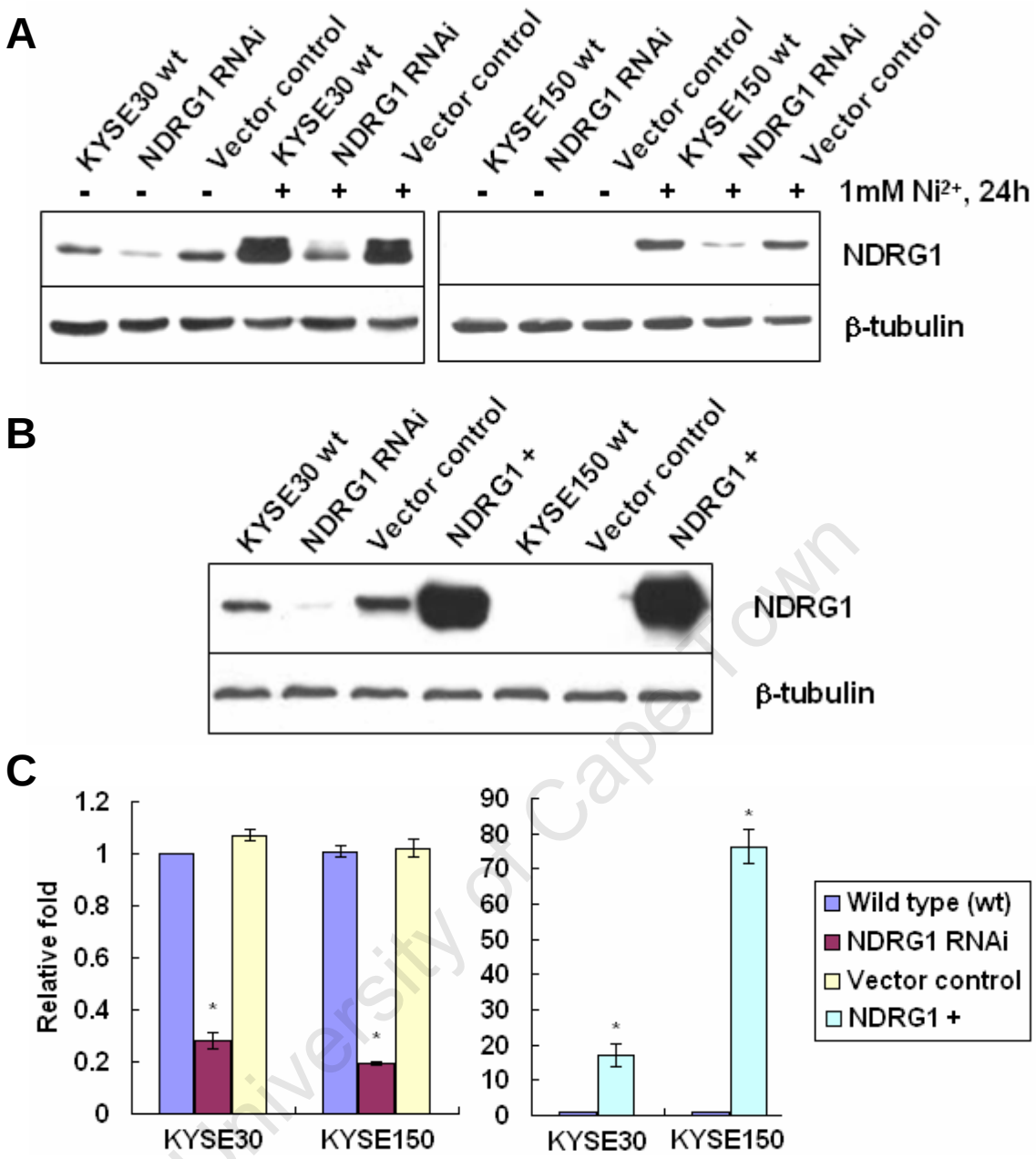


Fig 2-3: Stable KYSE30 and KYSE150 transfectants with ectopic NDRG1 knock-in (overexpression) and knock-down (RNAi). A and B, Whole cell lysates were harvested from subconfluent cells (or transfectants "pools", passage #6 for **A** and passage #23 for **B**) and subjected to Western blot analysis for the protein level of NDRG1. A rabbit polyclonal antibody from Zymed (now part of Invitrogen, San Francisco, CA) was used. β-tubulin was used as loading control. **C,** Quantitative real-time RT-PCR to check the level of NDRG1. Total RNA was extracted from subconfluent wild type (wt) cells and transfectants. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene. *Columns,* mean of the data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type control.

➡In this and following figures in this chapter, **wt** designates untransfected wild type cells; while **NDRG1+** designates NDRG1 overexpression transfectants.

The characteristics of the KYSE30 and KYSE150 transfectants are shown in Fig 2-2 and Fig 2-3. First, the percentage of GFP positive cells in the transfectant "pool" was analysed by flow cytometry to detect the transgene delivery efficiency or the "purity" of the "pool", since only transfected cells express exogenous protein GFP coded on the vector. As shown in Fig 2-2, a GFP positive rate of at least 80% (for KYSE30) or 95% (for KYSE150) was shown immediately after transduction (passage #6). The transfectants can be kept without apparent loss of GFP after 8-week culture (passage #23), indicating that the cells were effectively and stably transfected. Altered NDRG1 expression level was verified using Western blot analysis and quantitative RT-PCR as well, even when the transfectants with high passage number were tested (Fig 2-3). Both approaches showed 70-80% decrease as well as more than 15-fold increase in mRNA and protein levels in NDRG1 knock-down and overexpression transfectants, respectively. Moreover, a high efficiency of NDRG1 knock-down was also observed in those cells treated with nickel compound, a potent NDRG1 inducer due to its ability to mimic hypoxia (refer to Section 1.4.2.1 and 3.2.2 for details). Taken together, successful ectopic NDRG1 overexpression and knock-down were achieved.

2.3 Oncogenic effects of NDRG1 in KYSE30 cells

2.3.1 *In vitro* proliferation and differentiation

Malignant cancer cells are generally characterized by the ability to proliferate in an uncontrolled manner. It is the increase in tumour cell number (tumour burden) and the consequent biological effects that ultimately account for the adverse effects on the host. Dysregulated cell proliferation is an important component of carcinogenesis, since it accelerates the accumulation of mutations and genomic instability, facilitating the deterioration of cells to a more malignant phenotype. Frequently, aberrant cell proliferation can be reflected in defects of cell cycle control [97].

Hence, we compared the proliferation rates of wild type KYSE30 and derivative transfectants with ectopic NDRG1 overexpression and shRNA in the presence of 10% serum by MTS assay. Besides the conventional method of measuring anchorage-dependent cell growth by plating cells in plain 96-well plates, we also investigated the potential effect of NDRG1 on anchorage-independent cell growth using poly-heme coated plates. The application of poly-heme (poly-2-hydroxyethyl methacrylate) to the substratum of tissue culture plates prevents cell adhesion and results in anchorage-independent, aggregational cell growth, which partially mimics the *in vivo* environment for cancer cells [292;293]. Neither NDRG1 overexpression nor NDRG1 knock-down had any effect on the proliferation of KYSE30 cells, measured either in the adherent dependent or independent assays (Fig 2-4, A & B). Further confirmation was obtained through the similar DNA content profiles revealed by FACS analysis of propidium iodide stained cells (Fig 2-4, C) as well as the similar expression levels of several cell cycle regulators such as $p21^{Cip1}$ and $p27^{Kip1}$ (Fig 2-4, D).

In addition to proliferation, differentiation ranks as another useful marker to evaluate the malignancy of tumour cells. In tumour cells, the differentiation programme is frequently severely impaired by either genetic alteration or perturbed signalling networks. Conversely, restoring differentiation by extrinsic stimuli or genetic manipulation to reverse aggressive malignant tumour cells to a static, dormant phenotype has been successfully used as a therapeutic approach against leukaemia as well as some solid tumours [294]. In OSCC, it has been demonstrated that the grade of tumour differentiation (usually assessed by keratinization) is positively associated with reduced proliferative rate as well as increased apoptotic activity. Accordingly, the prognosis of patients with keratinizing OSCC has been reported to be significantly better than that of patients with non-keratinizing tumours [295].

Besides reports implicating NDRG1 in the physiological differentiation of several kinds of cells and tissues, NDRG1 has also been reported to affect the differentiation level of cancer cells. In SW620 colon cancer cells, ectopic overexpression of NDRG1 induces differentiation as reflected by the enhanced expression level of several colonic epithelial cell differentiation markers, including alkaline phosphatase, carcinoembryonic antigen, and E-cadherin [49]. Interestingly, although differentiation is usually linked with decreased proliferation, the growth rate of SW620 remains unaffected during NDRG1 induced differentiation, which makes it worthwhile to examine the putative relationship between NDRG1 and differentiation in the context of KYSE30

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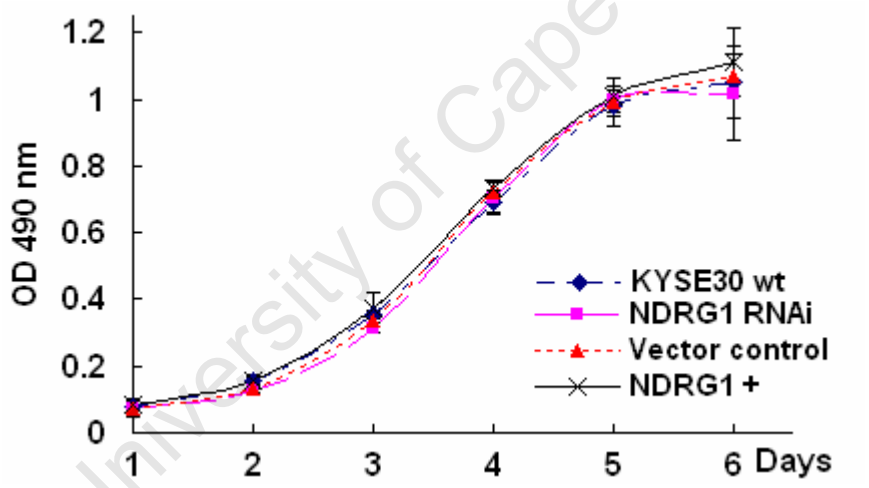

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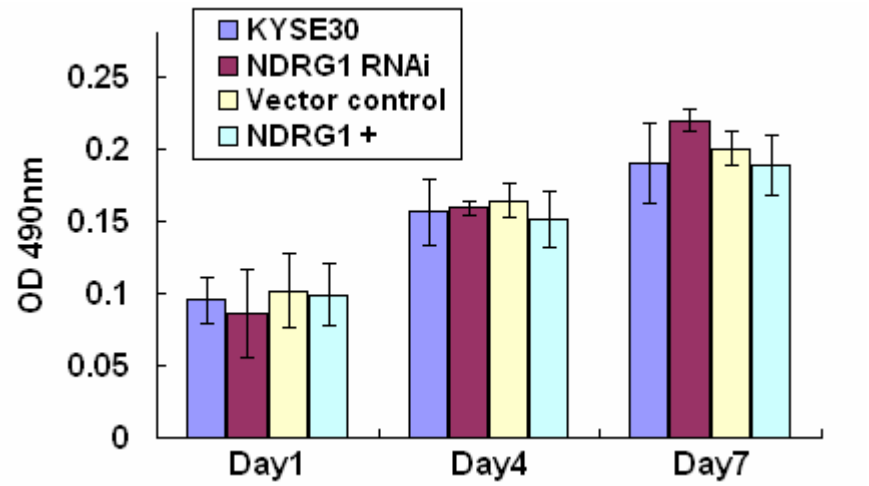


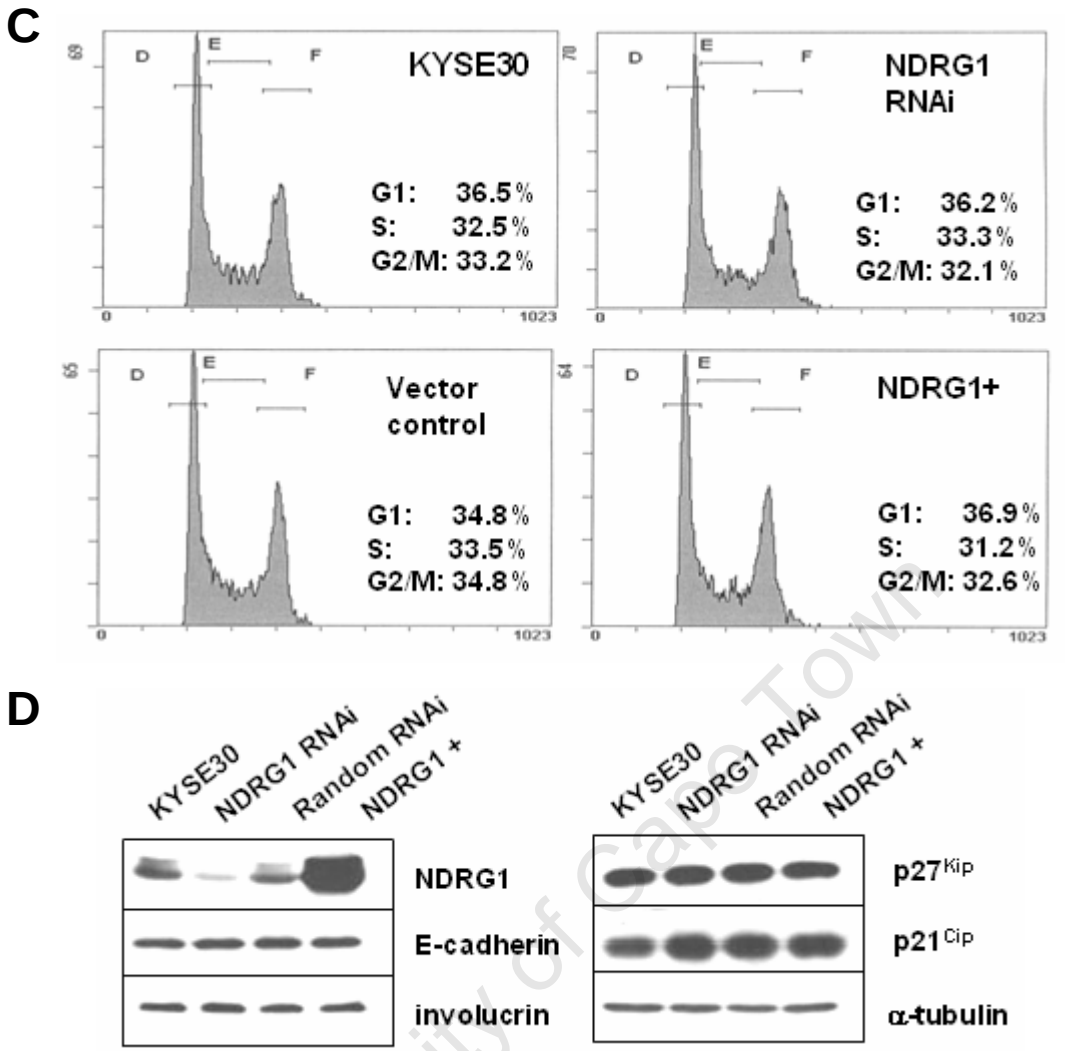


Fig 2-4: Ectopic alteration of NDRG1 expression level in KYSE30 cells and its effect on cell proliferation and differentiation. A & B, MTS assay to measure the proliferative activity in anchorage dependent (**A**) or independent (**B**) growth. Cellular proliferative activity was reflected by the absorbance at 490 nm. Each point (**A**) or column (**B**) represents the mean of four readings; *Bars*, ± SD, *asterisk* (*), $p<0.05$ versus wild type control. One of three independent experiments was shown. **C,** DNA content analysis to detect the effect of ectopic NDRG1 alteration on cell cycle. Subconfluent wild type cells and transfectants were trypsinized, fixed in 70% cold ethanol and stained with propidium iodide before flow cytometry analysis. The distribution of cells in each phase (G1, S, G2/M) is shown along with the histogram. A representative result from three independent experiments is shown. **D,** Western blot analysis of NDRG1 and other proliferation and differentiation related markers. Whole cell lysates were harvested from subconfluent cells. α-tubulin was used as loading control.

➡ A sheep polyclonal anti-NDRG1 antibody from Kinasource (Dundee, Scotland) was used to detect NDRG1. Unless specified, this antibody was always used for NDRG1 immunoblotting, due to its better specificity.

cells. The protein levels of two reliable markers of the OSCC differentiation levels, involucrin and E-cadherin [296;297], were checked by Western blot analysis. However, no significant changes in E-cadherin or involucrin were observed with either NDRG1 knock-in or knock-down despite the substantial change in NDRG1 levels (Fig 2-4, D). Conversely, the protein level of NDRG1 was found unchanged in response to all-trans retinoic acid induced differentiation, although a marked induction of involucrin was detected (refer to Section 3.2.1 for details). Thus, the lack of function for NDRG1 in the differentiation of OSCC cells is further indicated.

In summary, our result indicated that NDRG1 is probably not directly linked to *in vitro* cell proliferation as well as cell differentiation in KYSE30 cells.

2.3.2 Metastasis and angiogenesis

Metastasis represents the most insidious and life-threatening aspects of cancer. In OSCC, once metastasis occurs, the 5-year survival rate dramatically drops to less than 5% [1;3]. The putative effects of NDRG1 on the metastatic ability of KYSE30 cells were investigated by several approaches, since metastasis is a complex process involves cell motility, invasiveness and angiogenesis.

First, significantly enhanced motility of NDRG1 overexpressing KYSE30 cells was demonstrated through scratch/wound-healing assay performed on Matrigel coated surface which mimics, to some extent, the cell migration *in vivo* [298]. During the re-establishment of cell-cell contacts mediated by the cell migration of the cells along the wound edge, enhanced spreading speed was observed associated with ectopic NDRG1 overexpression (Fig 2-5, A). Moreover, the potential interference of cell proliferation to "fill up" the scratch could be neglected due to the similarity of the growth rates of the cells tested

(Fig 2-4, A). Therefore, we assume that NDRG1 may be positively related to endogenous cellular mobility without the stimulation of chemotaxis.

Consistent results were also obtained through evaluating the cell migration/invasion abilities under chemotaxis using transwell filters. Compared to wild type KYSE30 and vector control transfectants, significantly increased migration in response to chemotaxis was shown for cells ectopically overexpressing NDRG1 (Fig 2-5, B). Besides motility, the invasiveness of cells is also determined by the proteolytic ability to degrade the extracellular matrix (ECM). ECM degradation mediated by proteases (zymogens) allows cancer cells to penetrate the basement membrane, a prerequisite step for both intravasation and extravasation. Thus, the penetration of cells through transwell filter coated with a thick layer of Matrigel, which was originally prepared from solubilized basement membrane extraction from the Engelbreth-Holm-Swarm mouse sarcoma, was widely accepted as an effective approach to monitor the cellular invasiveness in the presence of chemotaxis. Based on the significant increased invasiveness observed in transwell assay for ectopic NDRG1 overexpressing cells (Fig 2-5, B), we next checked the probable participation of gelatinases in the modulation of cell invasiveness by NDRG1. The selection of gelatinases is based on their substrate preferentiality of basal membrane component [146;153] as well as their crucial contributions to the aggressive phenotype in the progression of OSCC comparing to other zymogens such as urokinase-type plasminogen activator (uPA) [154]. Our zymography results indicated elevated MMP-2 activity in response to NDRG1 knock-in, with the MMP-9 activity remaining similar (Fig 2-5, C). Comparable observations were shown in quantitative real-time RT-PCR to check the messenger RNA levels of MMP-2 and MMP-9, indicating that MMP-2 but not MMP-9 may be a downstream effector of NDRG1 in modulating the cell invasiveness (Fig 2-5, D).

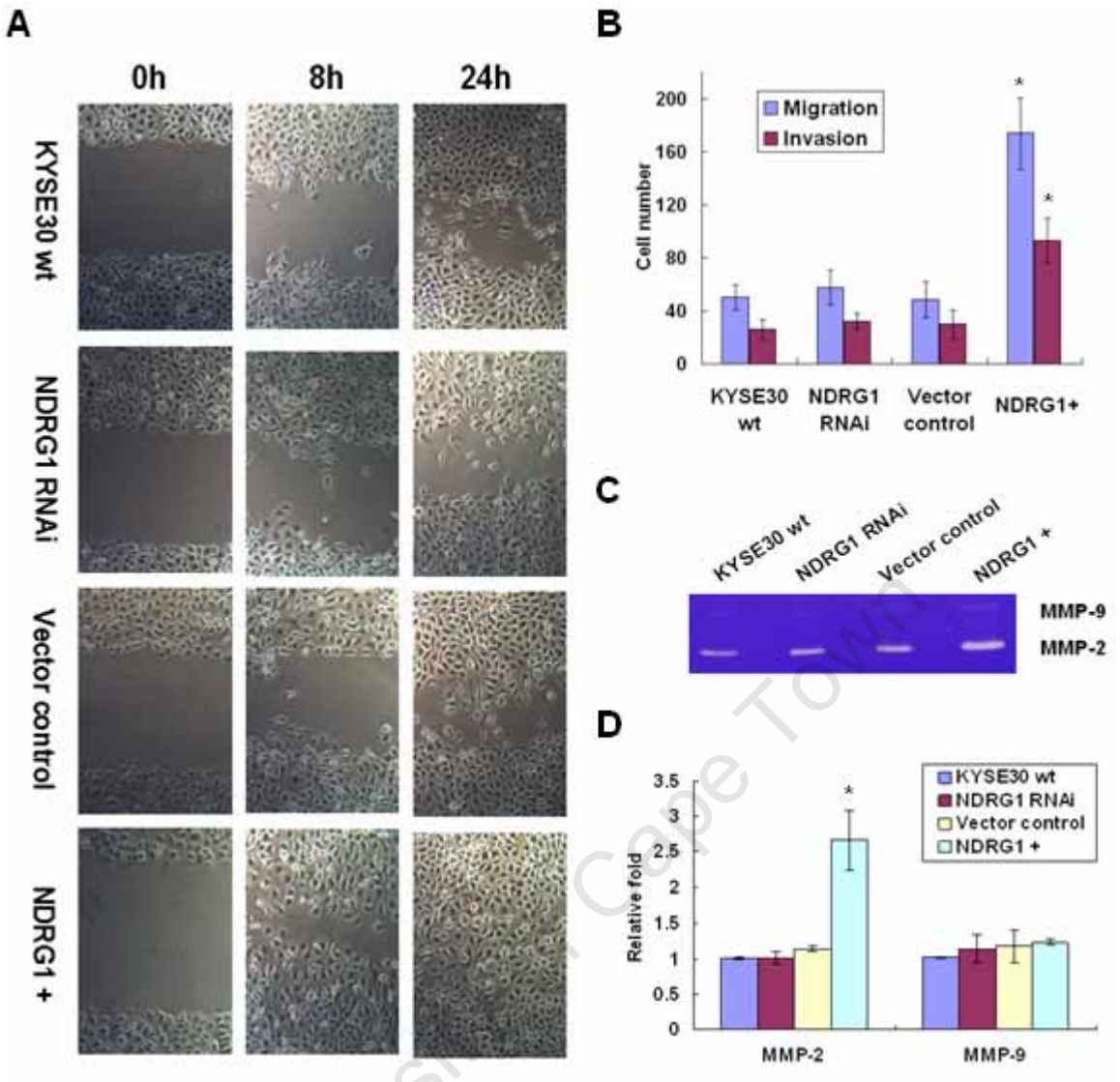


Fig 2-5: Effect of NDRG1 on KYSE30 cell migration and invasion. **A,** Scratch/wound-healing assay to measure cell motility without chemotaxis. Confluent cells were scratched using a P200 tip. Photos of the same spot were taken at indicated time points. **B,** Transwell migration and invasion assay, with EGF (100 ng/mL) and FBS (20%, v/v) used as chemotaxis. Cells that migrated through plain (migration assay) or Matrigel coated (invasion assay) 8.0-μm porous membranes 48 hours after seeding were stained with crystal violet and quantified. The average cell number for 6 randomly chosen 200× fields is shown. **C,** Gelatin zymography to measure the levels of secreted gelatinases (MMP-2 and MMP-9) from the indicated cell lines. Subconfluent cells in 60-mm dishes were incubated in 2 mL RPMI-1640 medium with 0.1% FBS for 24 hours. A portion of concentrated medium representative of 1×10^5 cells was subjected to the zymography assay. **D,** Quantitative real-time RT-PCR for MMP-2 and MMP-9. Total RNA was extracted from subconfluent KYSE30 cells and transfectants. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene.

For **A** & **C**, representative images were shown from three independent experiments. For **B** & **D**, *Columns,* mean of the data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type and vector control.

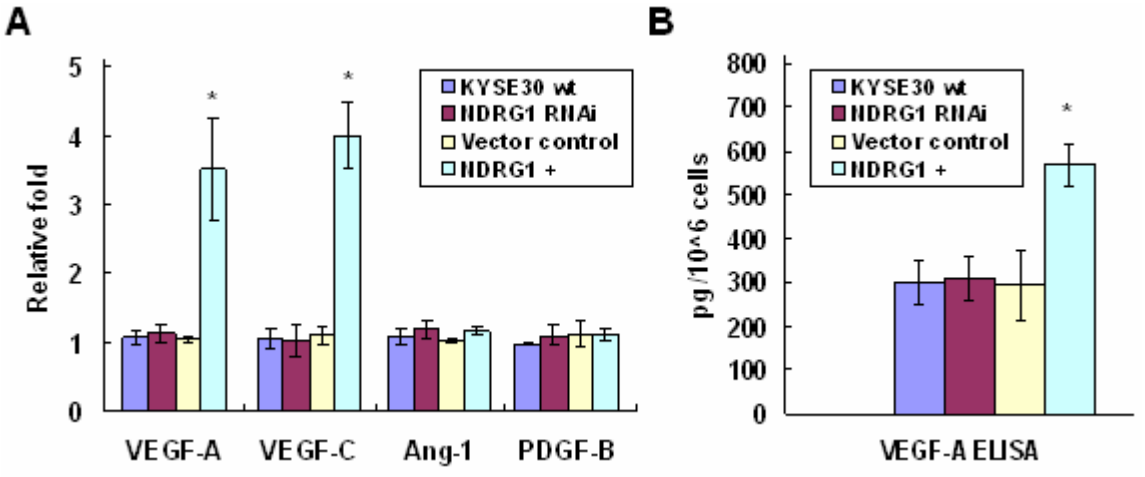


Fig 2-6: Effect of NDRG1 on the expression of angiogenic cytokines in KYSE30 cells. A, Quantitative real-time RT-PCR was performed to determine the mRNA levels of VEGF-A, VEGF-C, angiopiotein-1 (Ang-1) and PDGF-B. Total RNA was extracted from subconfluent KYSE30 cells and transfectants. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene. **B,** Comparison of the VEGF-A secreted into the medium by ELISA. Subconfluent cells in 60-mm dishes were incubated in 2 mL RPMI-1640 medium with 1% FBS for 24 hours. A portion of medium (100 μL) was directly subjected to ELISA analysis. The amount of VEGF-A was normalized with the number of cells at the time of conditioned medium harvest and reported as picograms (pg) per 10^6 cells.
Columns, mean of the normalized data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type and vector control.

Angiogenesis and lymphangiogenesis play important roles in solid tumour growth and metastasis. Besides supplying the necessary oxygen and nutrients for the maintenance of the rapid tumour growth, angiogenesis and lymphangiogenesis also provide access for the distant metastasis of cancer cells. In literature, angiogenic cytokines such as VEGF-A, VEGF-C, PDGF-B and angiopoietin-1 (Ang-1) were all found positively associated with OSCC progression and malignancy [179;180;299]. Through quantitative real-time RT-PCR, elevated mRNA levels of VEGF-A, VEGF-C but not PDGF-B and angiopoietin-1 were detected to accompany NDRG1 overexpression (Fig 2-6, A). Furthermore, similar result was obtained when using ELISA to determine the amount of VEGF-A secreted into the media (Fig 2-6, B). These data suggest the supportive role of NDRG1 in angiogenesis and

lymphangiogenesis, probably through upregulation of VEGF-A and VEGF-C.

In contrast, no significant phenotypic response of NDRG1 knock-down by shRNA was observed in the above assays. Taken together, in KYSE30 cells, we propose NDRG1 as a positive modulator of metastasis but this gene may not be necessary for the maintenance of the metastatic phenotype.

2.3.3 Apoptotic response

Apoptosis is another crucial regulator of carcinogenesis, especially *in vivo*, where cancer cells are continuously challenged by internal stresses (e.g. DNA damage, hypoxic response) as well as external attack from immune system. Considering that the expression of NDRG1 can be modulated by various stress conditions (Ref. [204] and Section 1.4.2 and 3.2.2), we next checked whether NDRG1 was able to modulate the cellular response to apoptotic stimuli. After 24h treatment of DNA damaging agents (2 μM doxorubicin or 5 μg/mL cisplatin) or hypoxia mimics (1 mM Ni^{2+} or Co^{2+}), KYSE30 cells underwent apoptosis which was measured by cleaved PARP protein and elevated caspase 3/7 activity. As shown in Fig 2-7 and Fig 2-8, compared with wild type KYSE30 cells, reduced apoptosis was observed in transfectants with ectopic NDRG1 overexpression, while similar extent of apoptosis was found in both NDRG1 knock-down and vector control transfectants. Our results indicate the protective effect of NDRG1 against intrinsic apoptosis initiated by cellular stresses such as genotoxicity and hypoxia.

In addition to intrinsic stresses, the putative effect of NDRG1 against the stress from immune surveillance was investigated by the administration of TNF-α, alone or with 5 μg/mL of cycloheximide for 24 hours. Although apoptosis is independent of *de novo* protein synthesis, cycloheximide was introduced owing to its inhibitory effect on protein synthesis for TNF-α induced cellular stress adaptation. In fact, the use of cycloheximide significantly

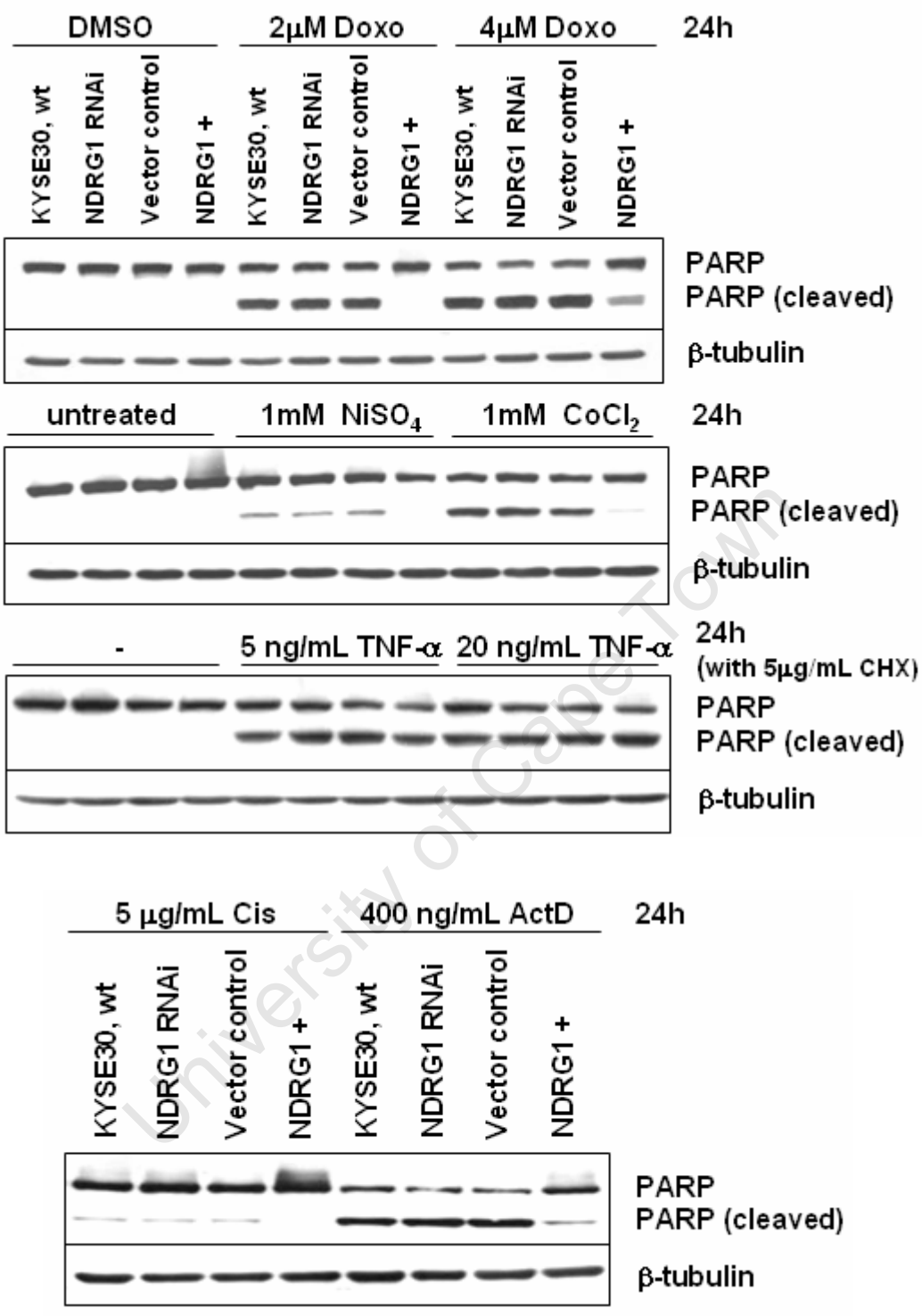


Fig 2-7: The effect of NDRG1 on apoptosis (KYSE30 cells, analyzed by PARP cleavage). Subconfluent cells were challenged with indicated reagents for 24 hours. Total cell lysate (including the floaters) was subjected to Western blot analysis to detect the intact (p116) and cleaved (p85 or p89) forms of PARP. Cleaved PARP serves as marker of apoptosis. β-tubulin was used to confirm equal loading. *Abbreviations:* ***Doxo:*** doxorubicin, ***Cis:*** cisplatin, ***ActD:*** actinomycin D, ***CHX:*** cycloheximide.

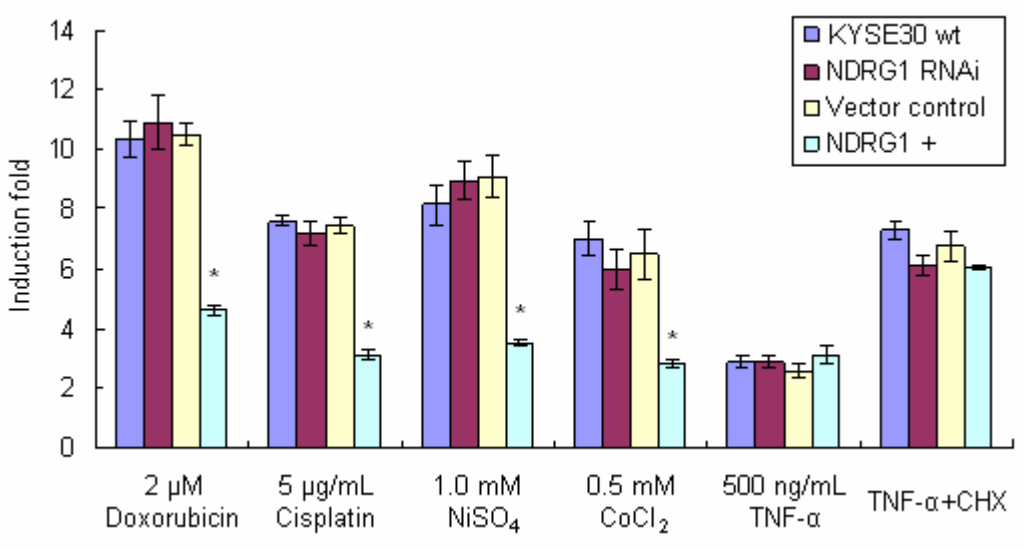


Figure 2-8: The effect of NDRG1 on apoptosis (analyzed by the sum activities of caspase -3 and caspase-7). Subconfluent cells plated in 96-well plate were subjected to apoptosis inducing reagents as indicated (*TNF-a+CHX*: 10 ng/mL TNF-a + 5 μg/mL cycloheximide). Caspase 3/7 activities were determined 24 hours after treatment, and were plotted as induction fold against untreated cells to avoid the variation introduced by cell plating. In one experiment, each treatment was assayed in triplicate. *Columns,* mean of the data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type and vector control.

enhanced the ability of TNF-α to induce massive apoptosis from 500 ng/mL to 5 ng/mL, although 5 μg/mL of cycloheximide alone did not show apparent apoptosis inducing effects within 24 hours. Both PARP cleavage and caspase 3/7 assay failed to show any effect of ectopic NDRG1 knock-in or knock-down on the modulation of TNF-α induced apoptosis (Fig 2-7 and 2-8). Besides, we also noticed that TNF-α treatment (from 5 ng/mL to 500 ng/mL, 24 and 48 hours) was unable to modulate NDRG1 expression (data not shown), further suggesting the lack of involvement of NDRG1 in TNF-α mediated apoptosis during inflammatory or host immune response.

Hence, the possible targeting point of NDRG1 in apoptotic cascade may be addressed through the irrelevance of NDRG1 in TNF-a mediated extrinsic apoptosis. Considering the apoptotic cascade (Fig 1-7), it may be hypothesized that NDRG1 may not impact on IAP proteins (such as survivin) to modulate the caspase activity which is shared by both intrinsic and extrinsic

apoptosis [300], but rather interfere with the apoptotic signalling pathway or mitochondrial permeability. Moreover, the punctate pattern of NDRG1 in cytoplasm revealed by immunofluorescence analysis of KYSE30 cells indirectly indicates the potential association of NDRG1 in mitochondria (Fig 2-9), which is further supported by the identification of several mitochondrial proteins as potential NDRG1 binding partner in a published yeast two-hybrid screen of mast cells [43]. Thus a potential relationship between NDRG1 and mitochondrial permeability is certainly on record. Collectively, although the mechanism still unclear, our results suggest that in OSCC, NDRG1 can function as a negative modulator of intrinsic apoptosis resulted from environmental stress (e.g. hypoxia) rather than extrinsic apoptosis due to immune attack.

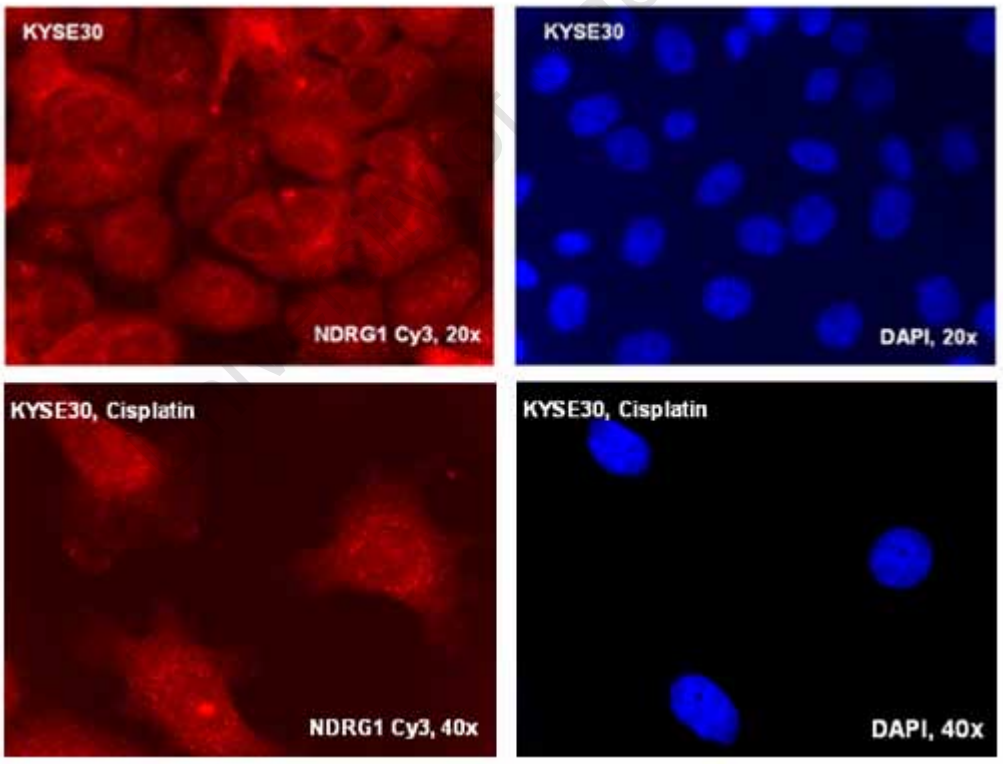


Fig 2-9: Subcellular localization of NDRG1 in OSCC cell lines cultured in normal and genotoxic (by 5 μg/mL cisplatin, 24 hours) conditions. Immunofluorescence analysis of NDRG1 was performed using a rabbit polyclonal antibody from Zymed (San Francisco, CA) which was visualized by Cy3 labelled secondary antibody. Nuclei were visualized using DAPI (blue). Magnification of the objective lens is also shown.

Activation of Akt and JNK pathways are commonly involved in stress response and act as modulator of apoptosis [301;302]. Both pathways can be effectively activated by DNA damaging agents and hypoxia mimics. To test whether the NDRG1 mediated apoptosis alleviation is related to Akt or JNK pathway activation, a preliminary experiment of PARP cleavage assay using 2 μ M doxorubicin as stress inducer was performed with the cells pre-treated with 10 μM Akt Inhibitor VIII trifluoroacetate salt hydrate (Akt inhibitor) and 1 μM SP600125 (JNK inhibitor). As shown in Fig 2-10, both inhibitors enhanced the susceptibility of cells to doxorubicin. In JNK inhibitor treated cells, no significant change was observed in the protective effect of NDRG1, indicating its independence of JNK activation. In contrast, Akt inhibition markedly ablated

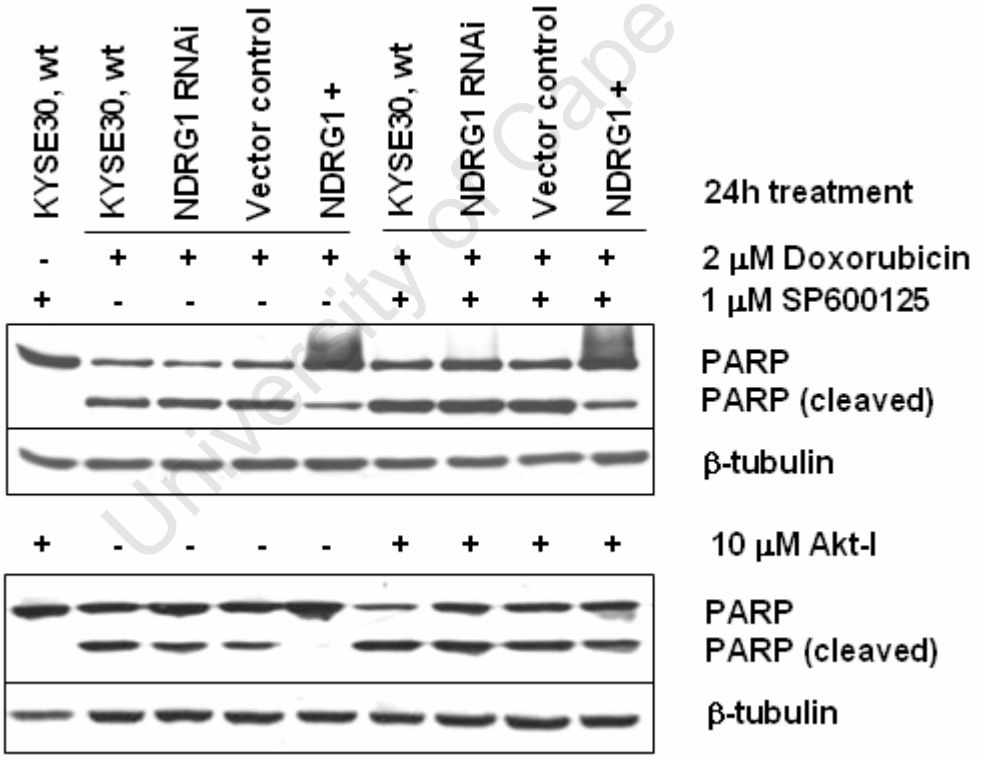


Fig 2-10: Effect of JNK and Akt inhibition on NDRG1 mediated apoptosis evasion in KYSE30 cells (analyzed by PARP cleavage). Subconfluent cells were pretreated with kinase inhibitors for 1 hour to suppress the JNK or Akt activity, before challenged with 2 μM doxorubicin in the presence of kinase inhibitors for 24 hours. Total cell lysate (including the floaters) was subjected to Western blot analysis to detect the intact (p116) and cleaved (p85 or p89) forms of PARP. Cleaved PARP serves as marker of apoptosis. β-tubulin was used to confirm equal loading.

the apoptotic evasion mediated by ectopic NDRG1 overexpression. Therefore, although needed to be further confirmed, NDRG1 may impact on the apoptotic response of cells through modulating the activity of anti-apoptotic Akt pathway.

Taken together, these results implicate NDRG1 as a non-essential modulator of certain apoptotic pathways in KYSE30 cells. Moreover, the discriminatory effect of NDRG1 on intrinsic (genotoxicity and hypoxia induced) and extrinsic (TNF-α induced) apoptosis suggests mitochondria-mediated apoptosis as a potential NDRG1 target. This hypothesis is further supported by the fact that NDRG1 may associate with the stress related cellular Akt activity a canonical regulator of Bcl-2 family proteins, which function as switches of mitochondria-mediated apoptosis.

2.3.4 Xenograft model in nude mouse

Xenograft studies were performed to check the applicability of our *in vitro* observations *in vivo*. Six nude mice were randomly divided to each group and xenografts were generated by dorsal flank injection of 5×10^6 of wild type KYSE30 cells as well as transfectants. Primary tumours developed in all the nude mice that became palpable within week 1 after inoculation. However, due to the unexpected widespread, massive necrosis which liquefied the xenograft tissues and prevented accurate measurements, all the mice in the NDRG1 knock-down and vector control groups were euthanized in week 3 for ethical issues, and were excluded from this study. Consequently, the wild type KYSE30 cells were used as control. As shown in Fig 2-11, significant bigger and more vascularized xenografts were generated from NDRG1 overexpressing cells.

Immunohistochemical analysis was next performed to explain the difference in xenograft growth, considering that we had previously shown that overexpression of NDRG1 had no significant effect on either anchorage

dependent or independent growth (Fig 2-4). As expected, significant higher levels of NDRG1 were observed in the xenografts derived from NDRG1 overexpressing cells, indicating that overexpression occurred and was maintained throughout the study period (Fig 2-12). In addition, similar levels of E-cadherin were detected in these xenografts (Fig 2-12). Since E-cadherin is widely accepted as a reliable differentiation marker of cells with epithelial origin [297], our observation in xenografts provided more evidence to support the *in vitro* data that NDRG1 might be irrelevant to cell differentiation in OSCC cells. Furthermore, the potential influence from cell differentiation on xenograft growth may also be excluded.

Considering the primary effect of proliferative activity of tumour cells on xenograft growth, the Ki-67 index was first investigated. The Ki-67 antigen is present exclusively in proliferating cells during active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). Thus, Ki-67 index (percentage of Ki-67 positive cells) becomes an excellent marker for determining the proliferating fraction of a given cell population, which is strictly associated with the growth rate of these cells [303]. Our results indicate a Ki-67 index of 29.8 ± 7.5% in wild type xenografts and 36.2 ± 5.1% in NDRG1 overexpressing xenografts (Fig 2-13, A). Although the difference was significant ($p = 0.021$), we suspected that the small difference in absolute value might not fully explain the big change in xenograft tumour volume. Besides the internal cell proliferative activity, other factors like oxygen and nutrient supply as well as stress (e.g. hypoxia or immune attack) related cell death can also impact on the *in vivo* growth of tumour cells. When the tumour volume reaches > 1 mm^3, it becomes vital for tumour cells to develop anti-apoptotic mechanisms and promote angiogenesis for further growth. Therefore, the significant difference of Ki-67 index may also be, at least in part, a reflection of other adaptations driven by NDRG1.

To investigate whether NDRG1 affects angiogenesis in xenografts, microvessels in xenograft sections were visualized with an antibody against mouse CD31, a specific marker of interacted endothelial cells, since

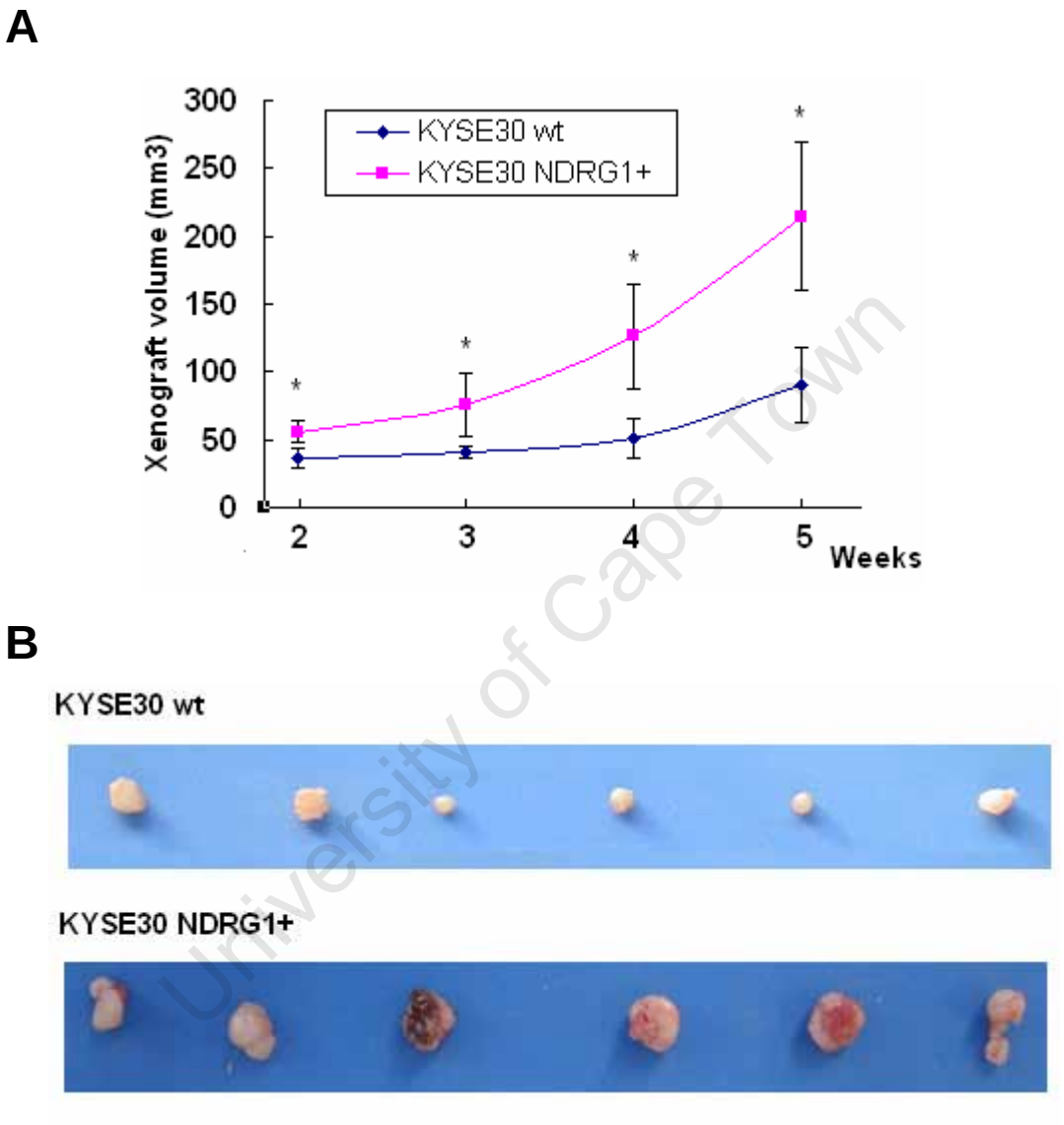


Figure 2-11: Nude mice xenograft model to explore the effect of NDRG1 on *in situ* growth of KYSE30 cells. 5 × 10^6 wild type KYSE30 cells (KYSE30 wt) and transfectants with constitutive NDRG1 overexpression (KYSE30 NDRG1+) were inoculated subcutaneously into the dorsal flank of the nude mice (n=6 in each group). Tumour volumes were measured by calliper every week after the xenograft is measurable (week 2). **A,** Measurements of the tumour volume. *Dots,* mean of the xenograft volume of all the six mice; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type control (KYSE30 wt). **B,** Photograph of the dissected xenografts with the same magnification scale.

microvessels are basically composed of continuous endothelia [304;305]. In xenografts with NDRG1 overexpression, the vascularized area being filled with typical clusters of endothelia, an indication of well developed microvessel network, occupied an average of 26.2% of the total area of the xenograft section. In contrast, microvessel networks could seldom be seen in xenografts derived from wild type cells (vascularization <2% of the total area) (Fig 2-13, B). Therefore, consistent with the *in vitro* observation (Fig 2-6), NDRG1 overexpression significantly correlated with increased angiogenic activity in xenografts (p=0.006). Furthermore, markedly decreased apoptosis activity was also observed in the NDRG1 overexpressing xenografts through *in situ* TUNEL assay (p<0.001) (Fig 2-13, C). Hence, the anti-apoptotic role of NDRG1 as observed in cell culture system (Fig 2-7 and 2-8) is reflected in mice xenografts, although the decreased TUNEL activity can also be traced from the elevated angiogenesis as a crucial stress alleviator of tumour cell growth *in vivo*.

Moreover, we observed that all six mice in the NDRG1 overexpression group developed visible liver metastases, which was detectable in none of the mice in the wild type KYSE30 group, even under the microscope (Fig 2-14). Correlating with the positive modulation by NDRG1 on cell migration and invasion (Fig 2-5), the emergence of liver metastasis may also emphasize the promoting effect of NDRG1 on angiogenesis, since the establishment of distant metastasis is dependent on the penetration of blood or lymph vessel. In contrast, considering the null effect of NDRG1 on TNF-α induced extrinsic apoptosis, it is hard to attribute the enhanced liver metastasis to the escape of immune surveillance (e.g. from macrophages and NK cells).

Taken together, data from the nude mice studies are completely consistent with our results obtained from the *in vitro* experiments. For KYSE30 cells, we propose an oncogenic role for NDRG1, functioning as a metastasis promoter and stress alleviator in the development of OSCC.

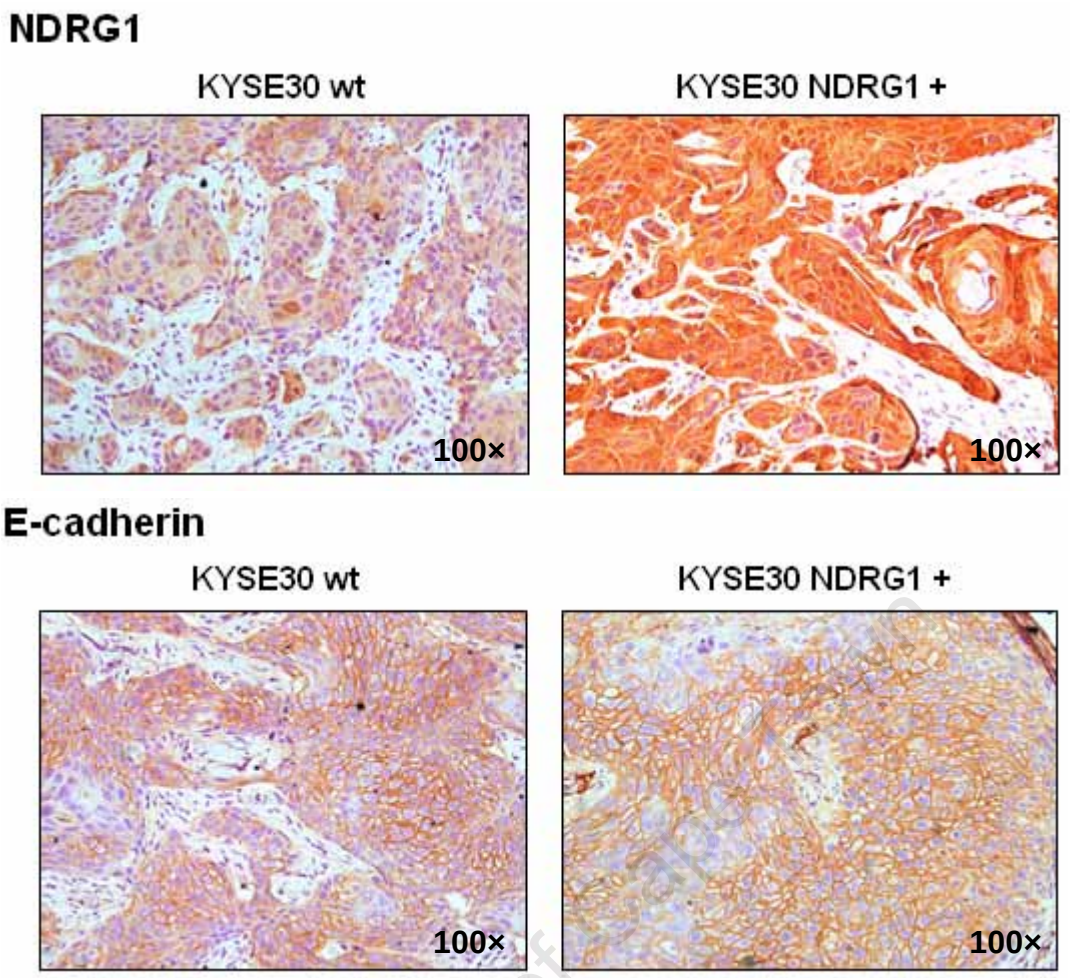
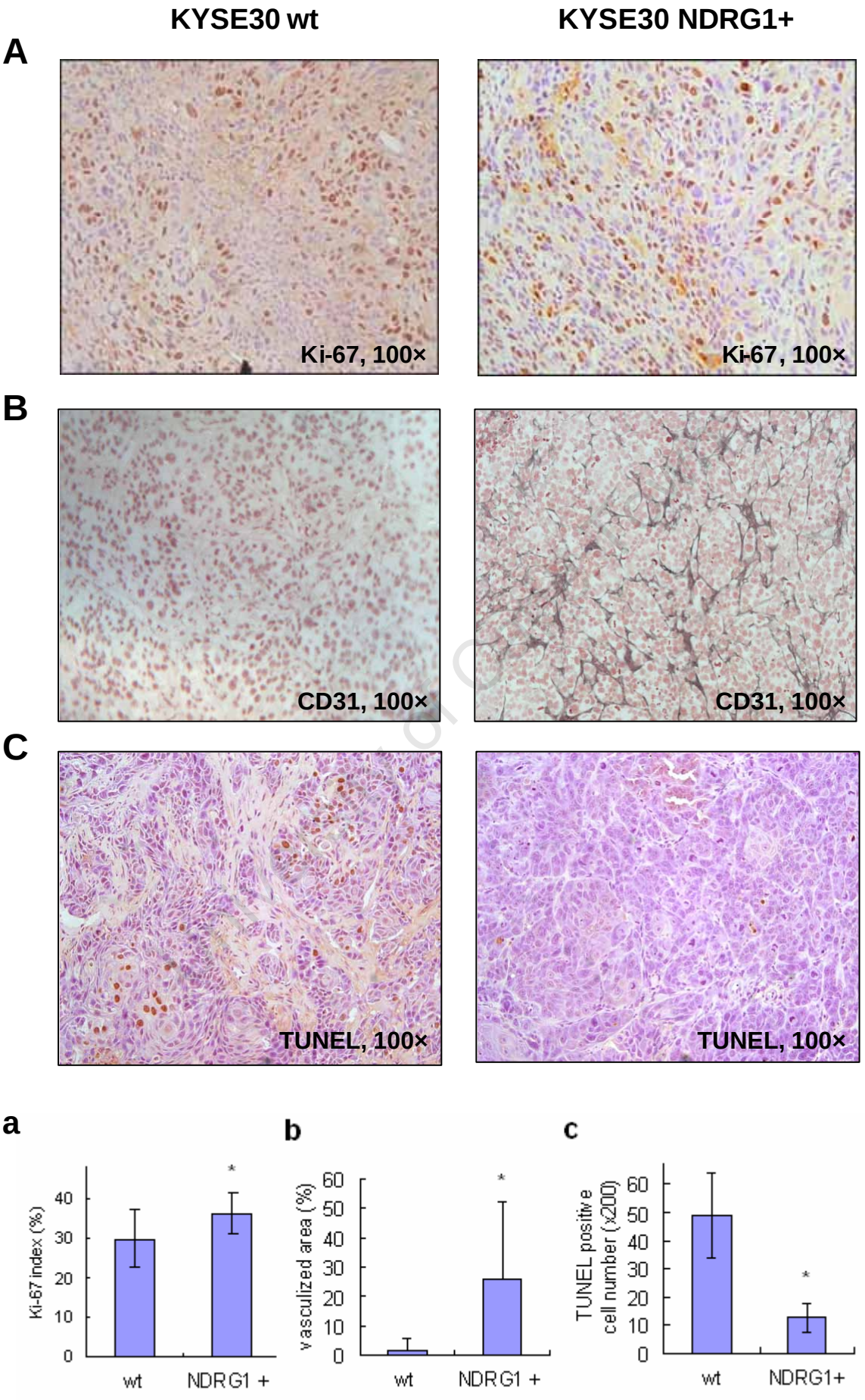


Fig 2-12: Immunohistochemical analysis of the NDRG1 and E-cadherin levels in the KYSE30 xenografts. Sections from wild type KYSE30 cells derived xenograft are shown on the left and sections from NDRG1 overexpressing transfectants derived xenograft are shown on the right. Representative images of 100× magnification are shown.

Fig 2-13 (overleaf): Immunohistochemical analysis of the proliferative activity, vascularization and apoptosis in the KYSE30 xenografts. Both representative images (100× magnification) and the statistical results are shown. *Columns,* mean data of the sections from the six mice in the group; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type control (KYSE30 wt). **A & a,** Proliferative activity was assessed by immunohistochemical staining with antibody against Ki-67 antigen and calculated by the averaged percentage of immunopositive nuclei to the total number of nuclei in three randomly chosen 200× fields with 4000 cells counted in total. **B & b,** Tumour associated neo-vascularization (angiogenesis) was determined by staining the sections with antibody against CD31, and was scored by the percentage of vascularized area showing typical microvessel structure. **C & c,** Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL), and was calculated as the average number of apoptotic cells in three independent 200× fields (~4000 cells) with most intensive staining.


KYSE30 wt
KYSE30 NDRG1+
A
Ki-67, 100×
Ki-67, 100×
B
CD31, 100×
CD31, 100×
C
TUNEL, 100×
TUNEL, 100×
a
Ki-67 index (%)
0
10
20
30
40
wt
NDRG1 +
b
vasculized area (%)
0
10
20
30
40
50
60
wt
NDRG1 +
c
TUNEL positive cell number (x200)
0
10
20
30
40
50
60
wt
NDRG1+

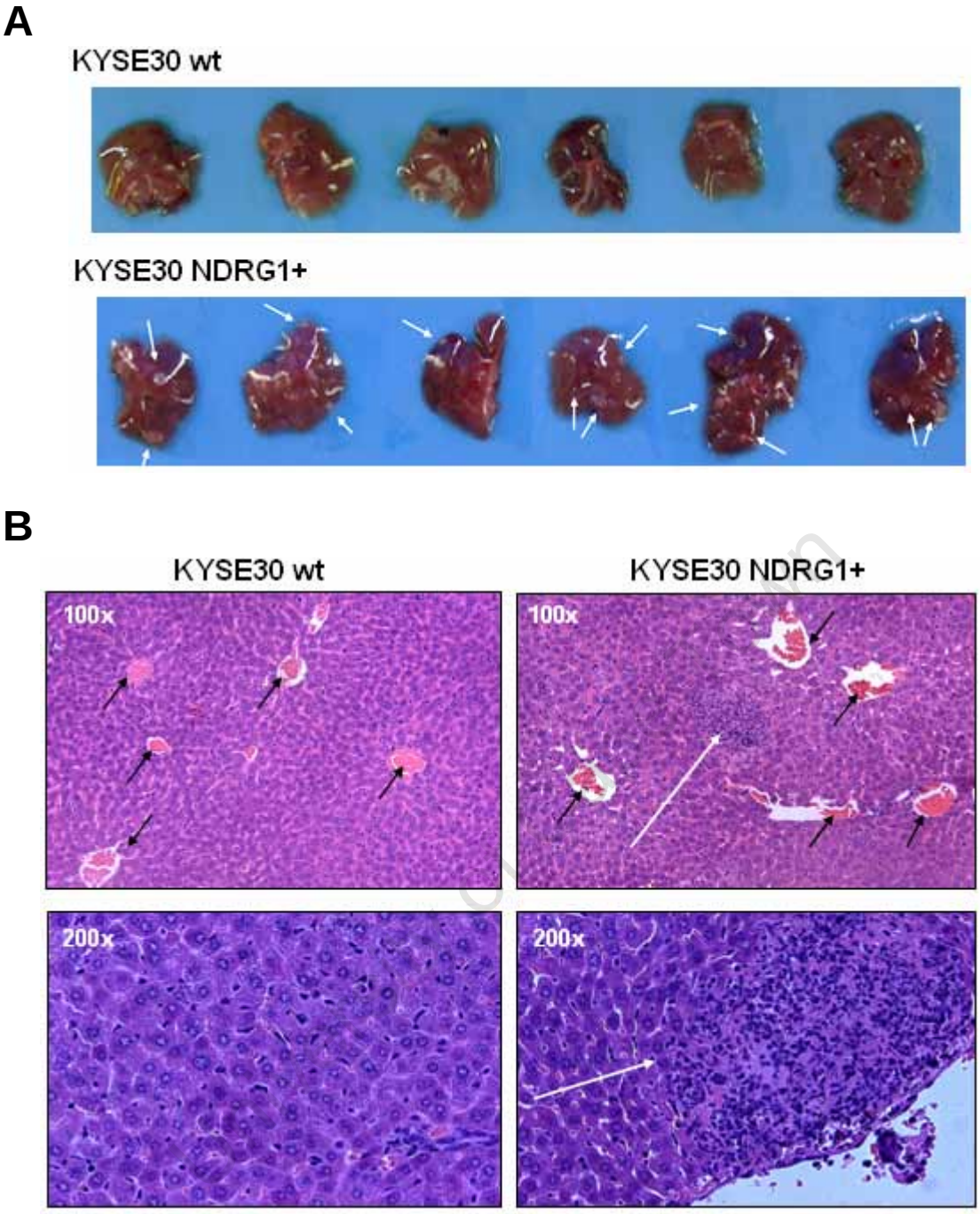
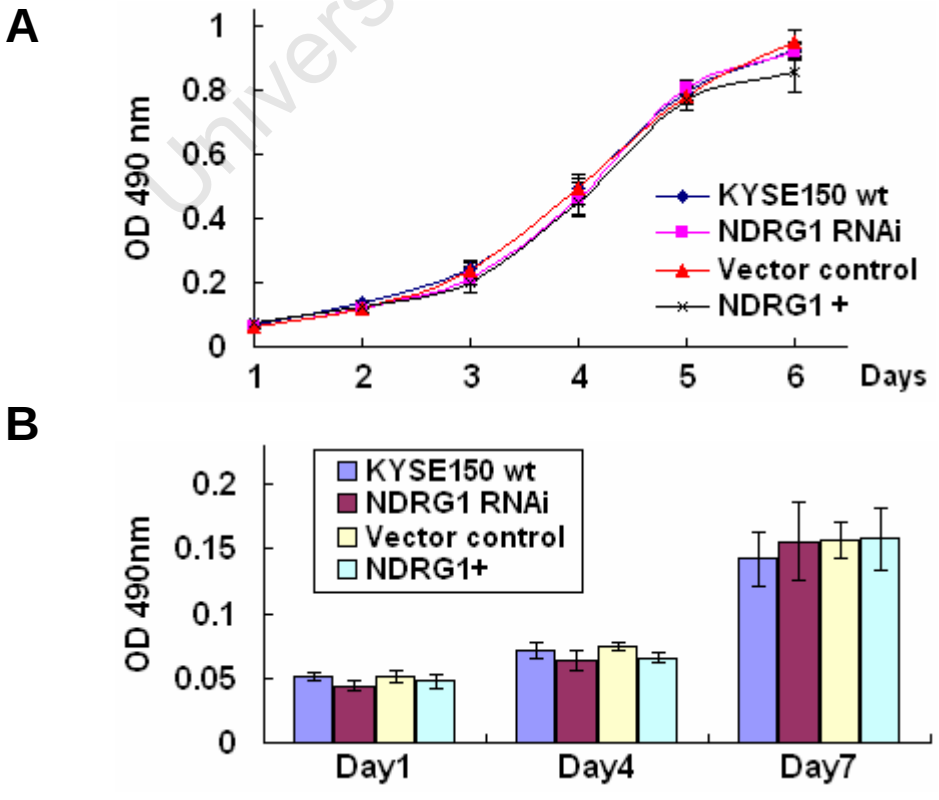


Fig 2-14: Nude mice model to explore the effect of NDRG1 on the liver metastasis of KYSE30 xenograft. Mice were sacrificed 5 weeks after tumour cell inoculation. Liver and lung were dissected to monitor the potential effect of NDRG1 on tumour metastasis. Naked eye visible metastases can only be found in liver but not lung. **A,** Gross examination with the photos taken in the same magnification. Some of the liver metastases (white nodules) are indicated by white arrows. **B,** Histological examination (H+E staining of paraformaldehyde fixed, paraffin embedded sections). The metastatic foci are indicated with white arrows. Black arrows indicate red blood cells in the hepatic vessels, close to micrometastases.

2.4 Null effects of altering NDRG1 expression in KYSE150 cells

Similar functional studies were performed in wild type KYSE150 cells and transfectants with altered NDRG1 expression. As shown in Fig 2-3 and Fig 2-15, effective ectopic modulation of NDRG1 expression was achieved in KYSE150 cells to an extent comparable to (or even better than) that in KYSE30 transfectants. However, in KYSE150 cells, neither ectopic NDRG1 overexpression nor NDRG1 knock-down elicited any phenotypic alteration in proliferation (both adherent and non-adherent, Fig 2-15), differentiation (as reflected by epithelial differentiation marker E-cadherin, Fig 2-15 C), metastasis (Fig 2-16) and the expression of angiogenic cytokines (Fig 2-17). Unlike KYSE30 cells, altering NDRG1 expression level in KYSE150 cells did not affect the sensitivity to apoptotic inducing reagents such as doxorubicin, cisplatin and hypoxia mimics nickel and cobalt (Fig 2-18). Finally, consistent with the *in vitro* observations, nude mice xenograft study comparing the wild type KYSE150 and transfectant with NDRG1 overexpression also showed no significant difference in the growth rate of the *in situ* xenograft as well as the extent of the liver metastasis (Fig 2-19).



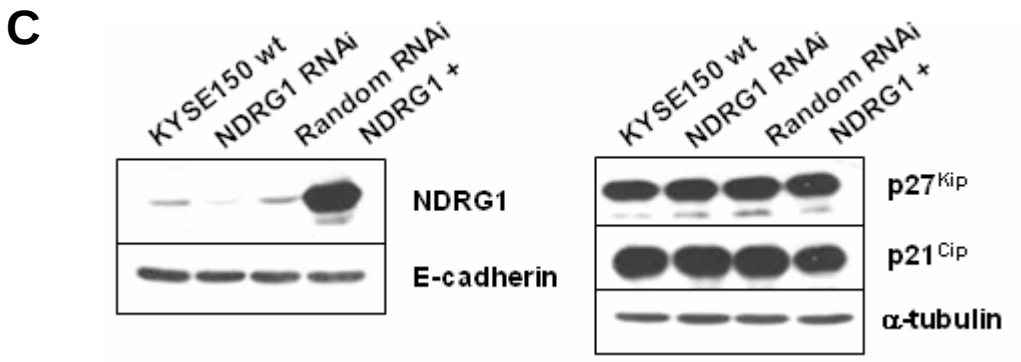


Fig 2-15: Ectopic alteration of NDRG1 expression level in KYSE150 cells and its effect on cell proliferation and differentiation. A & B, MTS assay to measure the proliferative activity in anchorage dependent (**A**) or independent (**B**) growth. Cellular proliferative activity was reflected by the absorbance at 490 nm. Each point (**A**) or column (**B**) represents the mean of four readings; *Bars*, ± SD, *asterisk* (*), $p<0.05$ versus wild type control. One of three independent experiments was shown. **C,** Western blot analysis of NDRG1 and other proliferation and differentiation related markers. Whole cell lysates were harvested from subconfluent cells. α-tubulin was used as loading control.

Note: The involucrin antibody failed to show any positive signal in wild type KYSE150 and transfectants even at a concentration of 10 μg/mL combined with the most sensitive detection reagent available in the lab (data not shown).

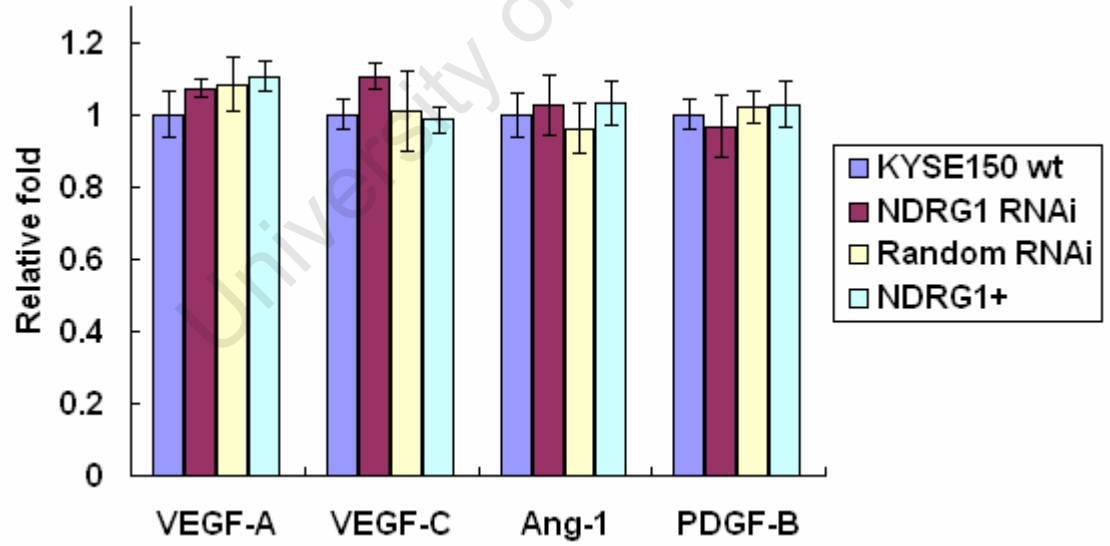


Fig 2-17: Effect of NDRG1 on the expression of angiogenic cytokines in KYSE150 cells. Quantitative real-time RT-PCR was performed to determine the mRNA levels of VEGF-A, VEGF-C, Angiopiotein-1 (Ang-1) and PDGF-B. Total RNA was extracted from subconfluent KYSE150 cells and transfectants. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene. *Columns,* mean of the normalized data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type KYSE150 cells and vector control.

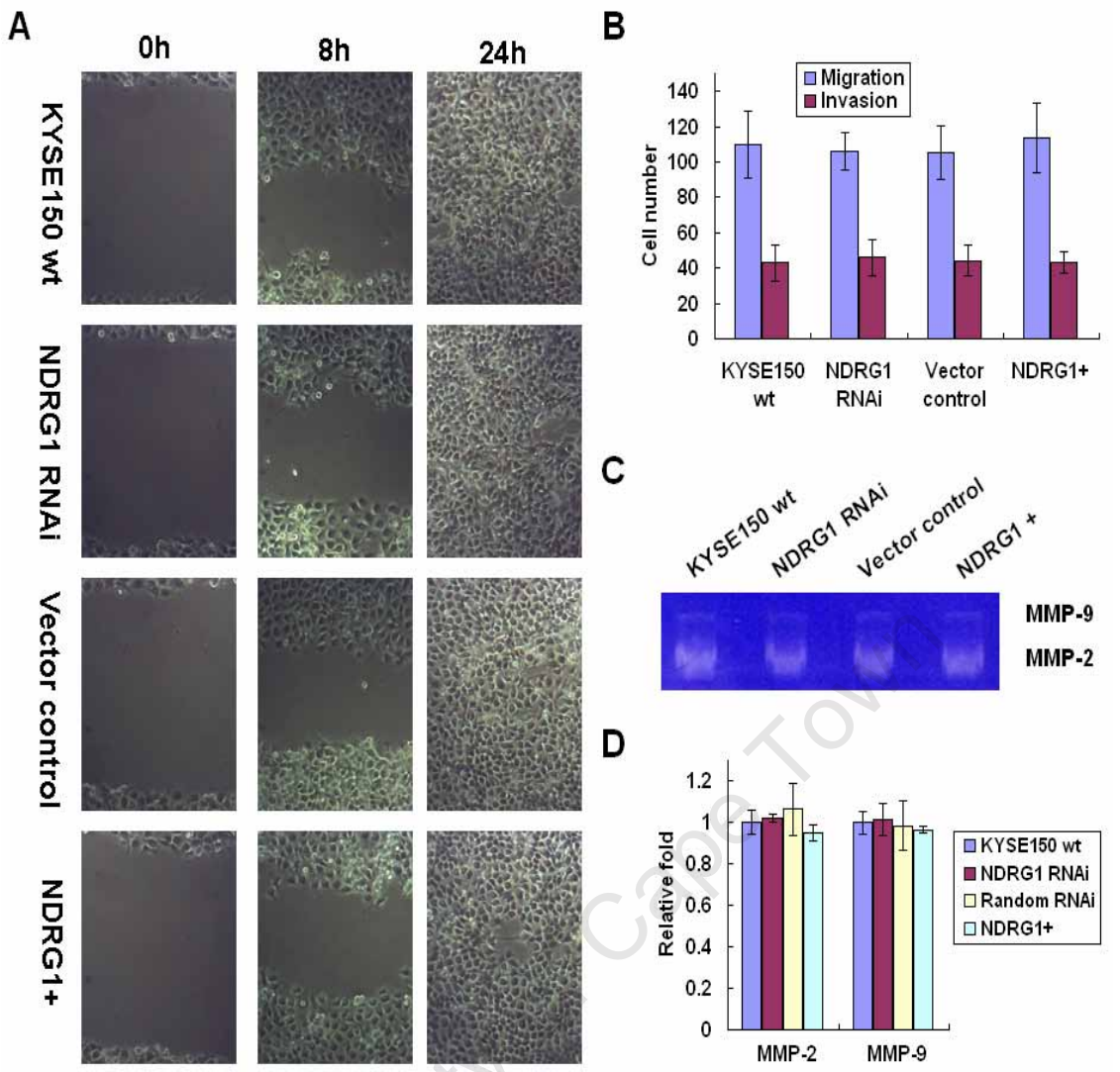


Fig 2-16: Effect of NDRG1 on KYSE150 cell migration and invasion. **A,** Scratch/wound-healing assay to measure cell motility without chemotaxis. Confluent cells were scratched using a P200 tip. Photos of the same spot were taken at indicated time points. **B,** Transwell migration and invasion assay, with EGF (100 ng/mL) and FBS (20%, v/v) used for chemotaxis. Cells that migrated through plain (migration assay) or Matrigel coated (invasion assay) 8.0-μm porous membranes 48 hours after seeding were stained with crystal violet and quantified. The average cell number for 6 randomly chosen 200× fields is shown. **C,** Gelatin zymography to measure the levels of secreted gelatinases (MMP-2 and MMP-9) from the indicated cell lines. Subconfluent cells in 60-mm dishes were incubated in 2 mL RPMI-1640 medium with 0.1% FBS for 24 hours. A portion of concentrated medium representative of 1×10^5 cells was subjected to the zymography assay. **D,** Quantitative real-time RT-PCR for MMP-2 and MMP-9. Total RNA was extracted from subconfluent KYSE150 cells and transfectants. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene.

For **A** & **C**, representative images were shown from three independent experiments. For **B** & **D**, *Columns,* mean of the data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type and vector control.

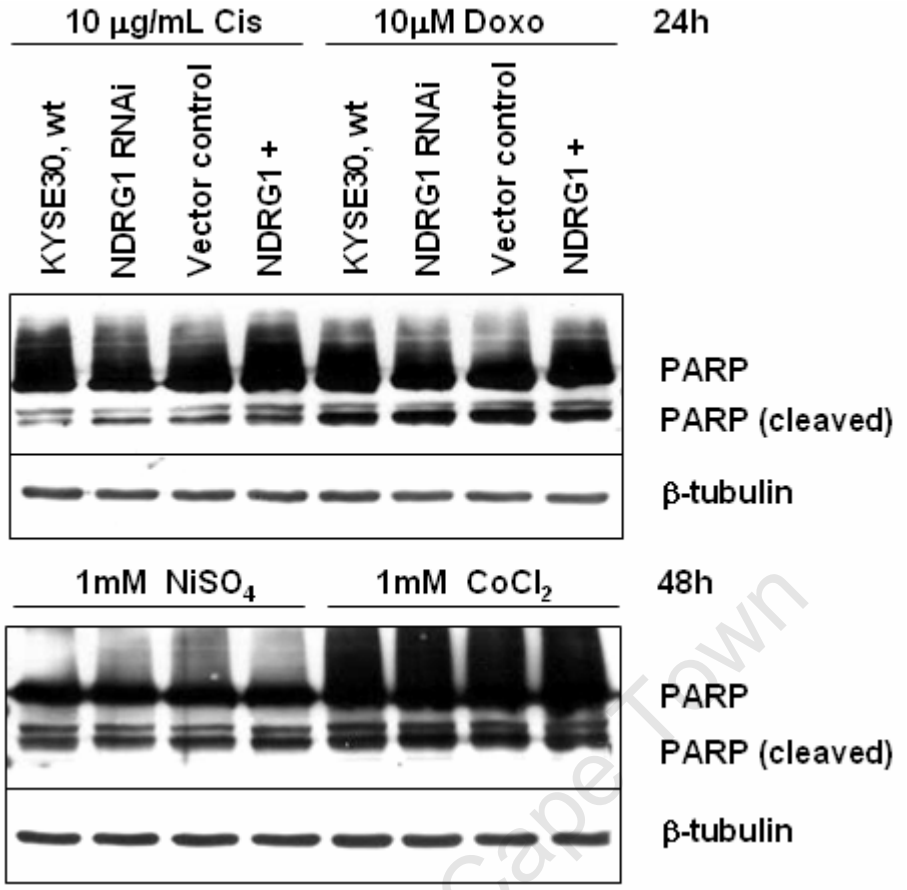
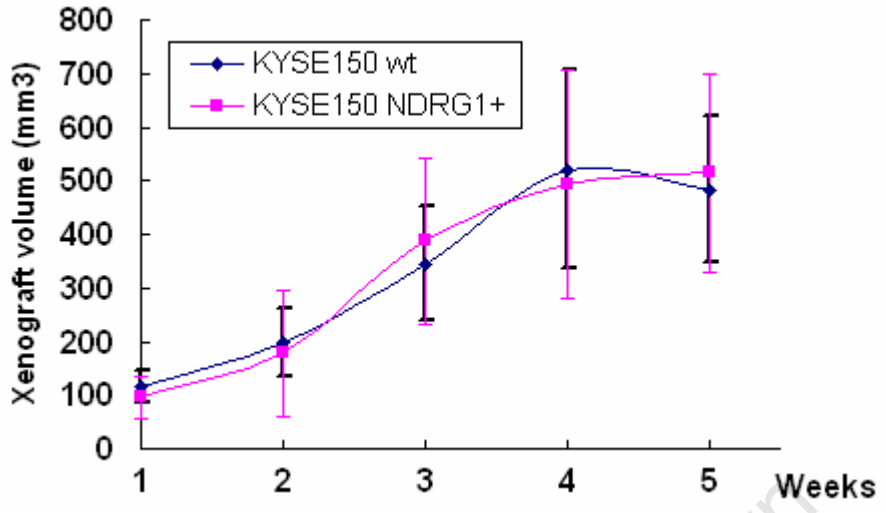


Fig 2-18: The effect of NDRG1 on apoptosis (KYSE150 cells, analyzed by PARP cleavage). Subconfluent cells were challenged with indicated reagents for 24 hours. Total cell lysate (including the floaters) was subjected to Western blot analysis to detect the intact (p116) and cleaved (p85 or p89) forms of PARP. Cleaved PARP serves as marker of apoptosis. β-tubulin was used to confirm equal loading. *Abbreviations:* ***Doxo:*** doxorubicin, ***Cis:*** cisplatin.

Note: KYSE150 cells are resistant to TNF-a (20 ng/mL) induced apoptosis even in the presence of 10 µg/mL cycloheximide (data not shown).

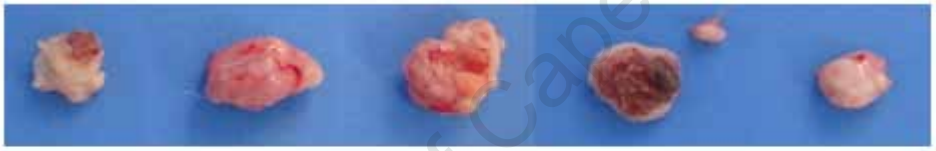
Fig 2-19 (overleaf): Nude mice xenograft model to explore the effect of NDRG1 on *in situ* growth and metastasis of KYSE150 derived xenografts. 5×10^6 wild type KYSE150 cells (KYSE150 wt) and transfectants with constitutive NDRG1 overexpression (KYSE150 NDRG1+) were inoculated subcutaneously into the dorsal flank of the nude mice (n=5 in each group) tumour volumes were measured by calliper every week after the xenograft was measurable (week 1). **A,** Measurements of the tumour volume. Each point represents the mean of the xenograft volume of all the five mice; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type control (KYSE150 wt). **B,** Photograph of the dissected xenografts with the same magnification scale. **C,** Photograph of the dissected liver with the same magnification. Some of the metastases are indicated by either white arrows (tumour nodules) or black arrows (extensive metastases).

A

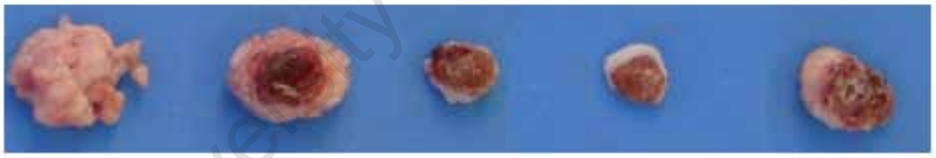


B

KYSE150 wt



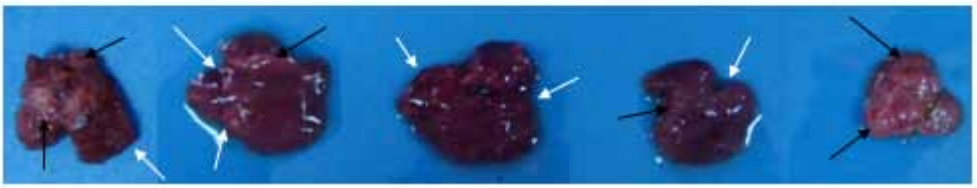
KYSE150 NDRG1+



C

KYSE150 wt

KYSE150 NDRG1+



2.5 NDRG1 and ATF-3

Recently, a microarray analysis in prostate cancer cells has revealed NDRG1 as a transcriptional repressor against activating transcription factor (ATF) 3 [280]. Similar to NDRG1, ATF-3 has been reported to be involved in a variety of neoplastic events such as proliferation, metastasis and apoptosis, despite its cell-type specific, dichotomous role based on the conflicting observations in different model systems [280;282-288]. Interestingly, in both KYSE30 and KYSE150 cells, the mRNA level of ATF-3 was effectively decreased in response to ectopic NDRG1 overexpression, suggesting ATF-3 as a potential NDRG1 downstream effector (Fig 2-20). Although still needed to be confirmed through immunohistochemical, regulatory (through pathway/promoter analysis to clarify the mechanism underlying NDRG1 mediated ATF-3 suppression) and functional (through ectopic knock-in or knock-down in NDRG1 transfectants) studies, the antagonizing effect of NDRG1 on ATF-3 in OSCC cells may be a useful candidate to explain the

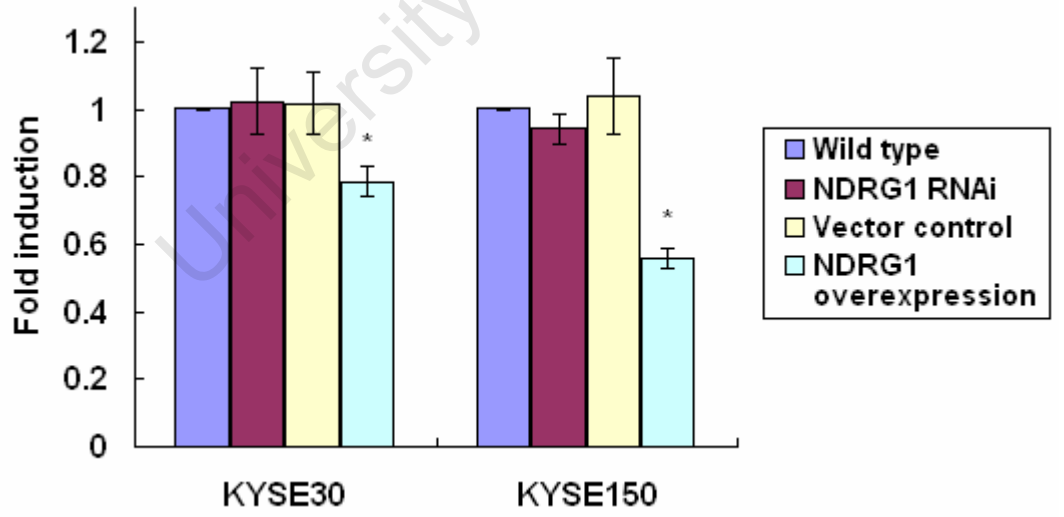


Fig 2-20: Measuring the ATF-3 mRNA level in wild type KYSE30 and KYSE150 cells as well as the transfectants with altered NDRG1 level by quantitative real-time RT-PCR. Total RNA was extracted from subconfluent cells. The relative fold change was determined by $2^{\Delta\Delta Ct}$ method using β-actin as reference gene. *Columns,* mean of the normalized data from three independent experiments; *Bars,* ± SD; *asterisk* (*), $p<0.05$ versus wild type control.

mechanism of NDRG1 function in OSCC. Considering the cell-type specific function of ATF-3, validating ATF-3 as an NDRG1 downstream effector may also help to elucidate the functional difference of NDRG1 in different OSCC cell lines.

2.6 Discussion

2.6.1 NDRG1 as an oncogene

Of the six hallmarks of cancer summarized by Hanahan and Weinberg [97], three were demonstrated to be positively related to NDRG1 in the context of KYSE30 OSCC cell line. These tumour specific capabilities include invasion and metastasis, angiogenesis initiation and apoptotic evasion. Moreover, no significant effect on either cell proliferation or differentiation was observed in response to NDRG1 overexpression or knock-down, indicating the probable irrelevance of NDRG1 to these processes. For each case, at least two independent approaches (*in vitro* functional assays) were employed to ensure the credibility of the data. Besides *in vitro* experiments, the applicability of *in vitro* observation to *in vivo* circumstances has also been confirmed in the nude mice xenograft model. Compared to wild type cells, KYSE30 transfectant with ectopic NDRG1 overexpression elicited significantly elevated tumourigenic and metastatic potential, which could be attributed to the enhanced angiogenic capability and apoptotic evasion as revealed by the immunohistochemical analysis of the xenograft section.

Interestingly, compared to wild type control, a significant higher Ki-67 index was also observed in NDRG1 overexpressing KYSE30 cells derived xenografts. Although the difference in absolute value was not big, it still suggested the potential contribution of enhanced KYSE30 cell growth rate to the bigger xenograft derived from NDRG1 overexpressing cells. In contrast, it was demonstrated through both *in vitro* anchorage dependent and

independent cell proliferation assays that NDRG1 was shown incapable of directly regulating cell proliferative machinery, since the non-stressful culturing condition ensured the cellular growth rate exclusively correlated with the intrinsic proliferative activity. Therefore, the elevated growth rate *in vivo* may indirectly reflect the NDRG1 function *in vivo*. For example, considering tumour cells growing *in vivo* are continuously facing various intrinsic and extrinsic insults, adaptive response mediated by NDRG1 is postulated to indirectly contribute the growth of xenograft. Angiogenesis may lead to a more favourable environment for cell growth, while apoptotic evasion protects cells from existing insults. Both responses thereby desensitize cells to growth-inhibitory stress signalling. Taken together, our results from both *in vitro* and *in vivo* functional assays suggested oncogenic roles of NDRG1 in KYSE30 cells.

2.6.2 Dispensable role of NDRG1

Although convincing oncogenic effects was detected with ectopic NDRG1 overexpression, no significant phenotypic alteration in proliferation, metastasis, angiogenesis and apoptosis was observed in response to NDRG1 knock-down in both KYSE30 and KYSE150 cells, although the efficiency of knock-down reached to 70%-80%, which is accepted as a criteria of effective RNA interference by several top commercial RNAi providers such as Ambion and QIAGEN [†]. On the premise that inefficiency of knock-down can be excluded, the dispensable role of NDRG1 in the neoplastic progression of OSCC may be suggested.

Besides our observation, the non-essential function of NDRG1 has also

[†] Refer to the following websites: http://www.ambion.com/catalog/supp/pd_guarantee.html for Ambion (now part of Applied Biosystems, Austin, TX); http://www1.qiagen.com/Products/GeneSilencing/PredesignedsiRNA/FlexiTubesiRNA.aspx for QIAGEN GmbH (Hilden, Germany)

been revealed through the double knock-out, NDRG1-deficient (NDRG1$^{-/-}$) mice [38;61]. It has been demonstrated in mouse models that NDRG1 is actively regulated during the foetal and postnatal development of brain and kidney, and is kept at a high level in these organs [21;37;70]. These observations implicate the involvement of NDRG1 in the development as well as the maintenance of physiological function of murine brain and kidney. However, no apparent abnormalities were observed in the brain and kidney of NDRG1-deficient mice, at least morphologically. Moreover, although the NDRG1-deficient mice exhibited muscle weakness caused by peripheral nerve degeneration, but their complicated motor abilities were relatively retained. These phenotypes suggest that central nervous system can withstand the loss of NDRG1 [38], which further supports the hypothesis that NDRG1 may be involved but not necessary in the development of brain.

Other evidence for the dispensable role of NDRG1 can be traced from the differentiation in trophoblasts. Although functional studies have demonstrated that NDRG1 participates in trophoblast differentiation, a prerequisite event for the implantation of embryo into endometrium [67], NDRG1 deficient mice are fertile [61], suggesting NDRG1 may not be necessary for functional trophoblasts. Moreover, despite the observation that NDRG1 positively controls the expression of trophoblast differentiation markers (i.e. hCG and hPL), in EGF mediated trophoblast differentiation, NDRG1 is significantly downregulated [67]. In other words, downregulation of NDRG1 failed to eliminate the EGF induced trophoblast differentiation which is marked by the upregulation of hCG and hPL [306].

Considering the high homology between the NDRG family members [29;37], it is tempting to anticipate compensatory effects from NDRG2, 3, or 4 in response to ectopic knock-out or knock-down of NDRG1. However, in NDRG1-deficient mice, no upregulation of NDRG2, NDRG3 and NDRG4 was

observed in brain and kidney [38;61]. Moreover, distinctive expression pattern of NDRG family proteins was revealed in adult murine brain and many organs in murine embryo (including kidney, gut, liver, heart, brain and spinal code) [37;38]. The non-overlapping tissue distribution of NDRG family proteins further argues against the potential mutual compensation for the maintenance of normal phenotype in the brain and kidney of NDRG1-deficient mice. Nevertheless, due to the lack of information of NDRG2, 3 and 4 in OSCC, we still cannot exclude the possibility of compensation by these proteins, which may abolish the effect of NDRG1 knock-down in KYSE30 and KYSE150 cells. Taken together, it is highly possible that NDRG1 is not required for the maintenance of the neoplastic phenotype in the progression of OSCC, such as sustained proliferation, apoptotic evasion, and enhanced metastasis and angiogenesis, at least in the two OSCC cell lines investigated.

2.6.3 Cell-type specific function of NDRG1

Another striking feature we found in our study of the NDRG1 function is the discrepancy observed in the two OSCC cell lines investigated. In KYSE30 cells, NDRG1 manifested as an effective but dispensable promoter in a variety of neoplastic events such as metastasis, angiogenesis and apoptotic evasion. However, null effect was observed in KYSE150 cells from its irresponsiveness to either ectopic NDRG1 overexpression or knock-down. Similar phenomena can also be found in colon cancer derived cell lines, since NDRG1 was demonstrated pro-apoptotic in DLD-1 cells while functioning as an apoptosis suppressor in HCT-116 and SW620 cells [216;268]. Therefore, it is conceivable that the function of NDRG1 in OSCC cells may rely on a particular cellular context. Identification of the proteins determining the function of NDRG1, such as upstream regulators (e.g. kinase, transporter), binding partners or downstream effectors will definitely shed light to the mechanism of NDRG1 functioning. Moreover, the variation in the proteins determining or

reflecting NDRG1 function in different cellular context will be useful to explain the cell-type specific function of NDRG1.

For example, in both KYSE30 and KYSE150 cells, ectopic NDRG1 overexpression effectively decreases the mRNA level of ATF-3. Considering the pleiotropic role of ATF-3 in neoplastic progression, it may be postulated as an executor of NDRG1 function. Interestingly, depending on the type of cell investigated, the role of ATF-3 has been reported to be oncogenic, tumour suppressive or even non-functional [280;282-288]. Hence, the cell-type specific function of NDRG1 can be attributed to the cell-type specific function of its downstream effector ATF-3. Furthermore, considering ATF-3 is effectively downregulated by ectopic NDRG1 overexpression in both KYSE30 and KYSE150 cells, the null effect of NDRG1 in KYSE150 cells may be attributed to cell-type specific defects in ATF-3 downstream effectors but not proteins required for effective NDRG1 function.

Considering the dynamic property of neoplastic development in which genes and signalling pathways are sequentially activated, inactivated or modulated, it is also conceivable that in KYSE150, NDRG1 (or its downstream effectors) may be either inactivated or overwhelmed by other oncogenic signalling. Compared to KYSE30 cells, KYSE150 cell line shows a much more malignant phenotype. According to literature, KYSE150 shows higher proliferative activity but lower differentiation level [289]. As shown in Fig 2-5 and Fig 2-16, higher metastatic potential was shown in both cell motility and invasiveness. Moreover, KYSE150 seems more resistant to apoptosis inducing agents including genotoxicity, hypoxia and TNF-a (Fig 2-7 and Fig 2-18, also refer to Fig 3-3 and Fig 3-4). Finally, in the nude mice xenograft study, from the same number of cells inoculated, KYSE150 cells elicited much larger *in situ* tumours as well as more severe liver metastasis. Not surprisingly, the greater tumorigenecity of KYSE150 *in vivo* can be attributed to its higher degree of

malignancy manifested by its greater activities in proliferation, metastasis and apoptotic evasion. Based on our observation through variety of functional assays, it is suggested that KYSE150 might represent a more advanced stage in the neoplastic progression of OSCC. Therefore, it is highly possible in KYSE150 cells but not in KYSE30 cells that genes (upstream modulators, binding partners or downstream effectors) for functional NDRG1 may have been already inactivated, probably by the genetic mutation or functional modulation due to activation of other oncogenic pathway.

In addition, it is also reasonable to postulate some potent oncogenic pathways, which are only switched on in certain advanced stages of neoplastic progression, but are potent enough to exceed (bypass) the impact of NDRG1 on the malignant phenotype and thus overwhelm the effect of NDRG1 in the cells. In fact, KYSE150 cells show lower endogenous NDRG1 level than KYSE30 cells (Fig 2-3, B), indicating the existence of different oncogenic signalling pathways in their cellular contexts, since the effects of different oncogenic stimuli on NDRG1 expression have been shown dichotomous (refer to Section 1.4.2 and Section 3.3 to 3.4). In summary, we suspect that the cell-type specific effect of NDRG1 in OSCC cells may reflect its role in neoplastic progression -- effective as an oncogene only in the early stage of the OSCC progression. Beyond that, the function of NDRG1 may tend to be either inactivated or overwhelmed by the further disturbed cellular context of the OSCC cells with higher malignancy.

2.6.4 Evaluating the immunohistochemistry on clinical OSCC samples

It is commonly accepted that tumour is a mixture of numerous individual clones, which may lead to huge difference in the cellular context of cancer cell lines even derived from the same type of cancer. To circumvent this problem, immunohistochemical analysis of clinical tumour samples consisting of

"various cell lines" has been widely utilized, although inconsistent sample origin of different studies still becomes a concern. In this case, Ref. [203] as well as the four immunohistochemical studies described in this thesis are good examples for NDRG1. Despite the contradictory function of NDRG1 in different types of cancer, a general trend can be summarized in the immunohistochemical studies, that is, decreasing level of NDRG1 in cancerous tissue often indicates it as a tumour suppressor (e.g. in breast and prostate cancer) while NDRG1 upregulation in cancer always points to its oncogenic effect (e.g. in liver cancer). In both of our immunohistochemical analysis using the OSCC samples from South Africa and China, NDRG1 was shown upregulated in the carcinogenesis, potentially suggesting an oncogenic effect of NDRG1 which also corresponds to the results of the functional analysis of NDRG1 in OSCC cells. Taken together, we consider NDRG1 as an oncogene in the neoplastic progression of OSCC.

Nevertheless, we still cannot neglect the two published works claiming the downregulation of NDRG1 in the carcinogenesis of OSCC [205;206]. In one of these articles, decreased NDRG1 was even reported to be associated with poor prognosis [205]. However, immunohistochemical study can only provide the expression level of the gene investigated in a certain stage of tumour progression, but not the direct causality between the changes of this gene and the resulted tumour stage. Detecting the decreasing NDRG1 level in metastatic stage cannot firmly indicate the functional involvement of NDRG1 downregulation in the development of metastasis, especially in case of lacking the functional studies. Considering the totally disturbed cellular signalling network in cancer, it is also appropriate to assume the downregulation of NDRG1 in the advanced stages of OSCC is a secondary by-product or reflection of other oncogenic changes which in fact execute the neoplastic progression. In fact, null effects of ectopic NDRG1 knock-down observed in the functional studies in both KYSE30 and KYSE150 cells strongly supports the

above hypothesis that downregulation of NDRG1 may not be directly linked to the progression of OSCC. Moreover, as indicated in the previous section (2.6.3), downregulation of NDRG1 in advanced stage of OSCC may reflect the activation of potent oncogenic pathways with overwhelming effect on NDRG1, which further makes NDRG1 irrelevant to neoplastic progression in and beyond this stage.

Chapter Three

The expression of NDRG1 in oesophageal squamous cell carcinoma

3.1 Introduction

One of the concerns regarding the functional assays described in Chapter 2 arises from the "ectopic" gain-of-function or loss-of-function, which is after all an artificial intervention. Thus, it would be useful to determine whether the phenotypic changes observed in the ectopic interventions have parallels in physiological processes. In this regard, to confirm the relationship between NDRG1 and a certain phenotype (e.g. apoptosis) revealed in the ectopic functional studies in physiological level, it would be valuable to obtain evidence that NDRG1 can be regulated by stimuli related to this phenotype (e.g. cellular stress).

Presently, most clues regarding the physiological function of NDRG1 came from studies exploring the regulatory pattern of its expression. In cultured cell lines, NDRG1 expression has been reported to be regulated by a variety of signals, including differentiating stimuli, stress stimuli (such as hypoxic condition, genotoxic agents and ER disturbance) and stimuli from growth factors. Incidentally, many of these stimuli are actively involved in the development of cancer. Considering that the expression profile and probable function of NDRG1 in tumour progression appears to be cell type specific, several questions remain unanswered regarding the role of NDRG1 in oesophageal squamous cell carcinoma (OSCC):

- What factors alter NDRG1 expression, and what is the mechanism?
- In the progression of OSCC, what is the significance of altered NDRG1 expression by a certain regulatory pathway? Is it able to reflect its consequent biological functions?

In this chapter, we describe our work investigating the putative regulatory pathways of NDRG1 in several cell lines developed from OSCC. Through this study, we aim to provide independent clues for putative NDRG1 functions in this disease.

3.2 Differentiation stimuli

In Chapter 2, we described the null effect of NDRG1 on differentiation in OSCC cells based on evidence from ectopic NDRG1 manipulation studies. Considering that NDRG1 has been reported to be upregulated by several kinds of differentiating stimuli (such as all-trans retinoic acid) in both physiological and neoplastic backgrounds, here, we examined the effect of various differentiation stimuli on NDRG1 expression in oesophageal epithelial cells. Our results suggest that NDRG1 may be involved in the differentiating process in normal oesophageal cells but not OSCC cells.

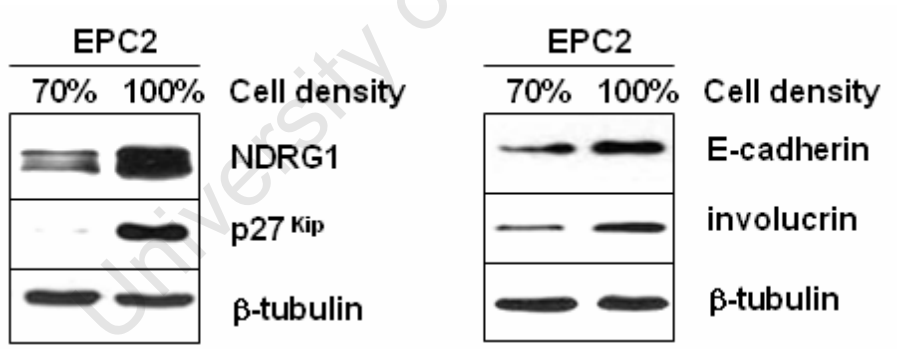


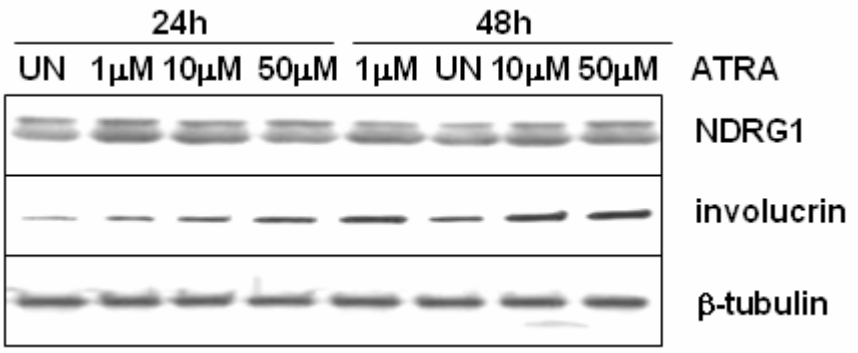
Fig 3-1: Upregulation of NDRG1 and other differentiation markers during the cell density induced differentiation in immortalized normal oesophageal cell line EPC2-hTERT. Subconfluent cells (70%) were left in culture for another 48h to reach post-confluent state (100%) for cell differentiation. Whole cell lysates were harvested and subjected to Western blot analysis for NDRG1 as well as oesophageal epithelial differentiation markers involucrin, E-cadherin and $p27^{Kip}$. β-tubulin was used as loading control.

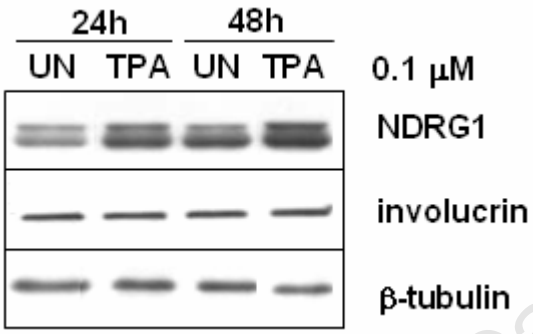
➡ The "double-band" pattern of NDRG1 in the blot was due to the polyclonal NDRG1 antibody (from Kinasource), which recognizes both phosphorylated (upper band) and unphosphorylated (lower band) forms of NDRG1 protein.

Molecular markers such as involucrin, E-cadherin and $p27^{Kip}$ which had been previously identified through immunohistochemical studies of OSCC samples [296;307], as well as in differentiating process of normal oesophageal cells [308-310], were used to monitor the differentiation status of OSCC cell lines. First, these markers were verified in a model of differentiating oesophageal keratinocytes EPC2-hTERT. Despite immortalization by ectopic constitutive expression of the catalytic subunit of telomerase (hTERT) [311], due to its origin from normal oesophageal epithelium, EPC2-hTERT cells retain the ability to undergo differentiation in the post-confluent state [312], a common feature of cultured keratinocytes mediated by cell-cell contact [309;310]. Compared to subconfluent EPC2-hTERT cells, marked inductions of all three differentiation markers, involucrin, E-cadherin and $p27^{Kip}$ were observed in differentiated, post-confluent cultures of EPC-hTERT (Fig 3-1). Interestingly, a marked upregulation of NDRG1 was also observed accompanying the cell density mediated differentiation of EPC2-hTERT cells, indicating the possibility of NDRG1 as a differentiation associated gene in the normal physiological background.

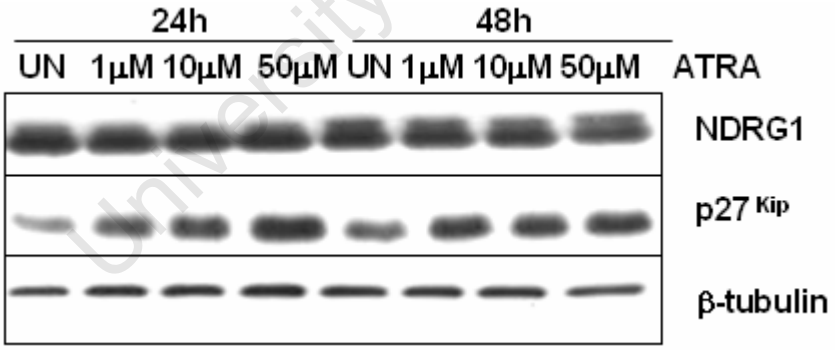
We next subjected OSCC cell lines to reagents capable of inducing differentiation such as 12-o-tetradecanoylophorbol-13-acetate (TPA) and all trans retinoic acid (ATRA) [294] to check whether NDRG1 expression could be modulated in oesophageal cells with cancerous origin (Fig 3-2). Only TPA was able to induce NDRG1 expression in OSCC cells. However, TPA failed to induce differentiation as reflected by the unchanged levels of differentiation markers in all three cell lines tested, similar to an earlier report that TPA was incapable of inducing differentiation in normal oesophageal epithelial cells [313]. In this context, it would be difficult to associate TPA-mediated NDRG1 induction to differentiation. On the other hand, NDRG1 expression was found to be insensitive to ATRA administration, although ATRA effectively induced differentiation in these cell lines (based on the upregulation of differentiation

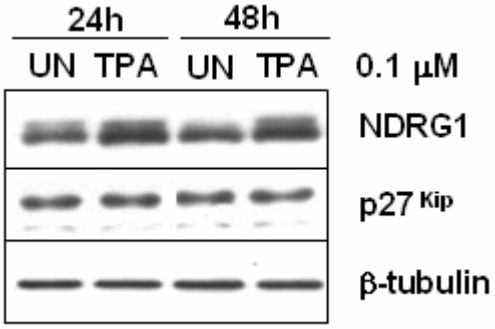
KYSE30





KYSE150





KYSE180

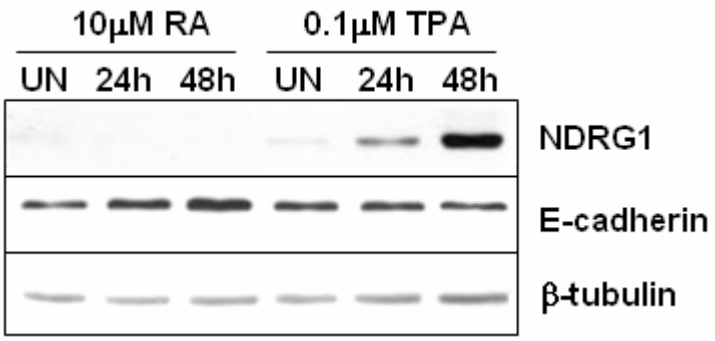


Fig 3-2: Determination of the putative regulatory effects of differentiation stimuli on NDRG1 expression in OSCC cell lines. Subconfluent cells were treated with reagents capable of inducing differentiation. Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis for NDRG1 protein level. The level of a differentiation marker was also determined simultaneously to indicate the cellular differentiation status after treatment. The choice of differentiation marker was mainly based on its basal expression in the OSCC cells tested. β-tubulin was used as loading control. UN represents "untreated" For KYSE180, the "UN" represents untreated cells harvested 24h after treatment.

➡ The "double-band" or smear pattern of NDRG1 in the blot was due to the polyclonal NDRG1 antibody (from Kinasource), which recognizes both phosphorylated (upper band or smear) and unphosphorylated (lower band) forms of NDRG1 protein.

markers). Collectively, our data from OSCC cell lines suggest that NDRG1 expression does not associate with differentiation in these cells. Considering the upregulation of NDRG1 during the differentiation of normal oesophageal epithelial cells (Fig 3-1), it would appear that the association between NDRG1 expression and cell differentiation may be largely dependent on cellular context.

The disparity of NDRG1 regulation in response to differentiation stimuli between normal oesophageal epithelial cells and OSCC cells can also be observed in the immunohistochemical studies. In OSCC samples, the expression of NDRG1 was found to be irrelevant to the differentiation level (Fig 2-1, C). In contrast, it seemed that differentiation in normal oesophageal epithelia was associated with NDRG1 upregulation. In about 50% of the

sections, NDRG1 protein level was elevated in mature or differentiated epithelial cells distal to the basement membrane, compared to the faint or absent NDRG1 immunoreactivity in cells near the basement membrane, which are generally considered undifferentiated and proliferating (Fig 2-1, A). Furthermore, the observed NDRG1 expression associated with epithelial maturation was exclusively accompanied with the characteristic absolute nuclear localization which could not be observed in OSCC cells. This redistribution of NDRG1 further indicates its potential participation in the differentiation of normal oesophageal epithelium.

Taken together, it may be proposed that the role of NDRG1 in differentiation of normal oesophageal squamous epithelial cells is either lost or masked in OSCC cells, which is probably due to the disturbance of cellular context during the cancer development. Therefore, NDRG1 may be considered insignificant in the differentiation process associated with OSCC cells.

3.3 Stress conditions

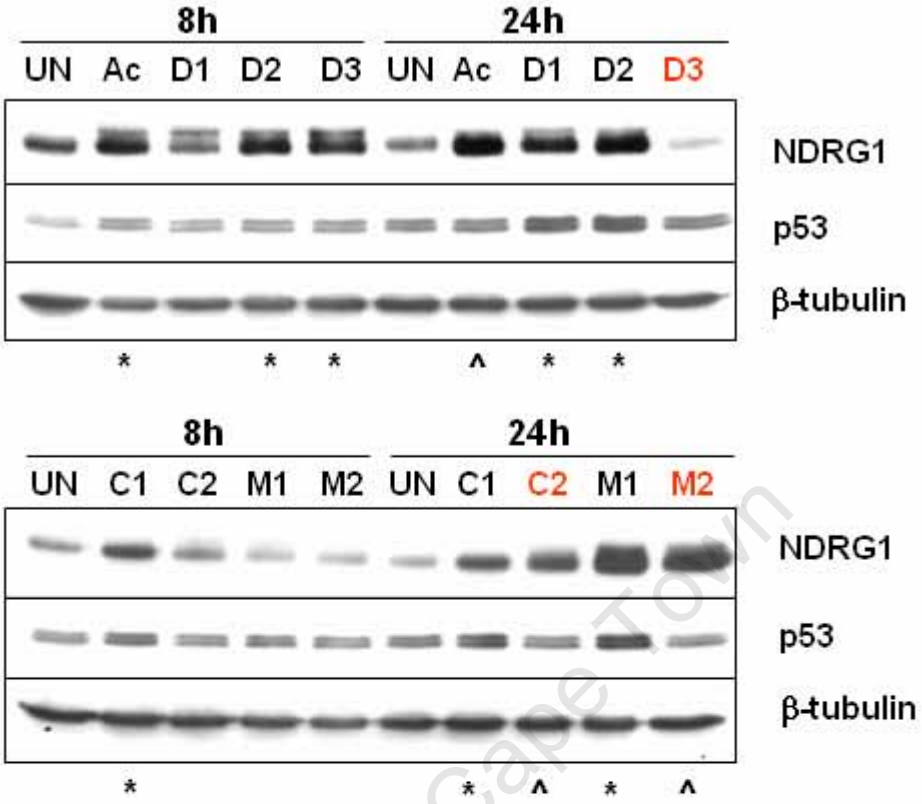
It is well established that OSCC carcinogenesis is both initiated and driven by various intrinsic and extrinsic stress conditions such as DNA damage, hypoxia, oxidative stress and endoplasmic reticulum (ER) stress. Meanwhile, exposure to any of these stresses also has detrimental outcomes characterized by growth arrest or even programmed cell death, which makes it vital for cancer cells to develop certain adaptive response for survival and further proliferation. In the functional studies in Chapter 2, despite being irrelevant to cell proliferation, NDRG1 was found to impact on apoptotic evasion, a typical adaptive response to cellular stress. Here, we explored the regulation of NDRG1 in response to stress signals in OSCC cells, aiming to determine the physiological involvement of NDRG1 in processes that allow cells to cope with these insults.

3.3.1 Genotoxic stress (DNA damage)

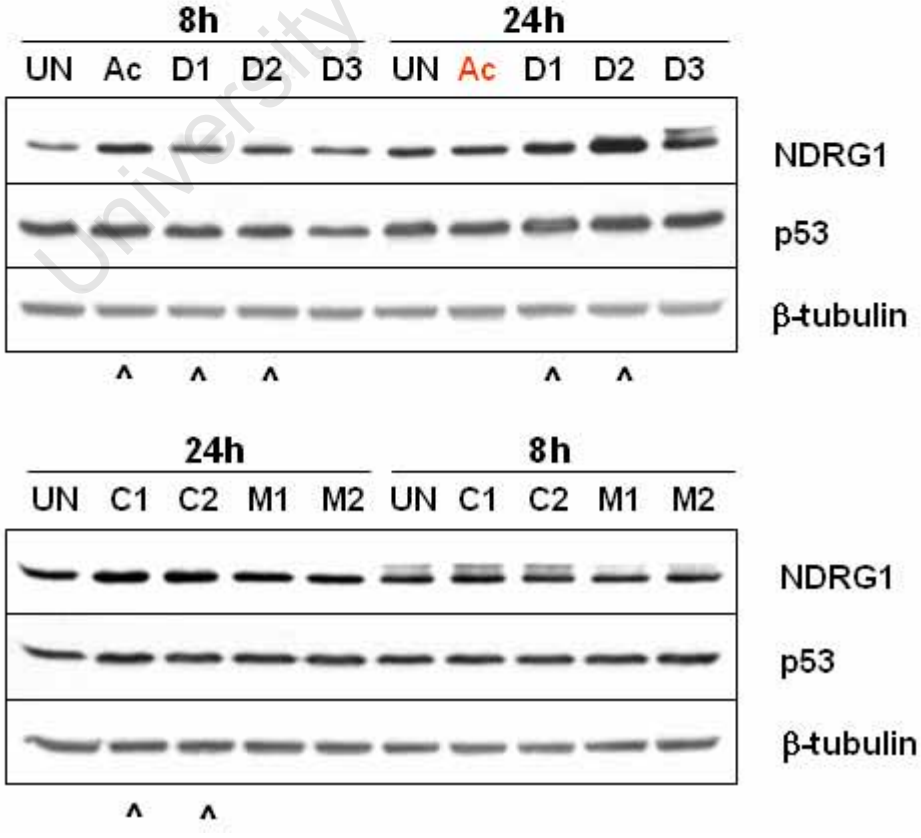
Many carcinogens also elicit genotoxic effects, leading to the mutation and inactivation of tumour suppressive genes or the activation of oncogenes resulting in accelerated malignant progression of cancer cells. On the other hand, excessive DNA damage also exhibits growth inhibitory or even apoptotic effects, which is widely used as a chemotherapeutic approach against neoplasms. p53 is one of the most important transcription factors involved in DNA damage, and it has also been shown capable of activating NDRG1 transcription [25;214;216]. Here we explored the possibility in OSCC cells, that DNA damage may induce NDRG1 expression in a p53-dependent manner. OSCC cell lines KYSE30, KYSE150 and KYSE180 were subjected to several DNA damaging agents with different mechanisms of action, including DNA intercalators actinomycin D and doxorubicin (also an topoisomerase II inhibitor causing double-strand breaks in DNA) as well as DNA alkylating and crosslinking agents cisplatin and mitomycin C. Preliminary experiments demonstrated that all four agents were capable of inducing extensive apoptosis in KYSE30, KYSE150 and KYSE180 cells when used at high dosage. However, the effects of these genotoxic agents on both NDRG1 and p53 were inconsistent in different cell lines (Fig 3-3).

NDRG1 was markedly induced by DNA crosslinking agents in KYSE30 cells, while in KYSE150 and KYSE180 cells, only marginal effects were elicited by these chemicals. On the other hand, NDRG1 upregulation was shown pretty sensitive to the DNA intercalators, doxorubicin and actinomycin D in all cell lines tested. Interestingly, both up- and down-regulation of NDRG1 expression were found to be associated with genotoxicity, especially DNA intercalation. Attenuation of NDRG1 induction or even NDRG1 downregulation was found with excessive treatment from either high dosage or extensive time periods, especially in cases when cells became apoptotic. Although it may reflect the possible inhibitory effect on transcription by these DNA damaging agents,

KYSE30



KYSE150



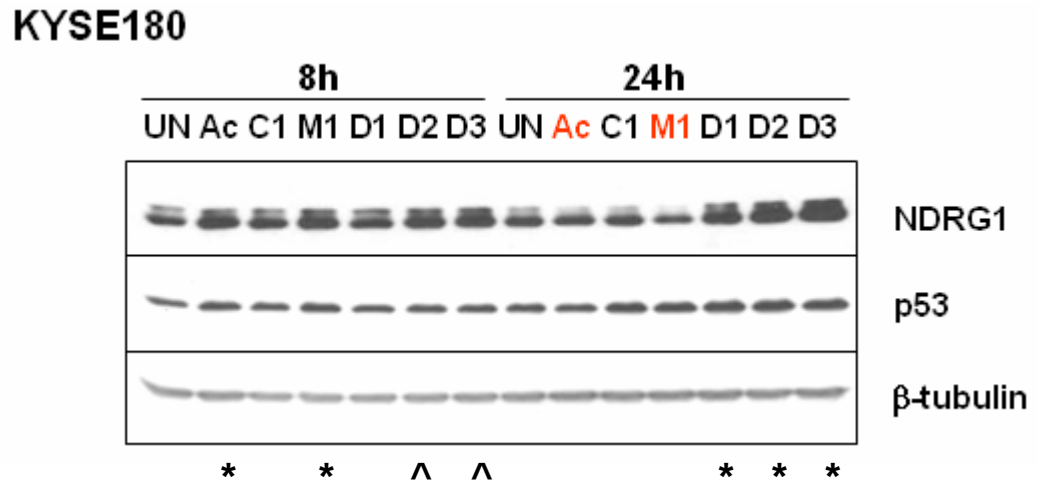


Fig 3-3: Determination of the putative regulatory effects of genotoxic agents on NDRG1 expression in OSCC cell lines. Subconfluent cells were treated with DNA damaging reagents such as actinomycin D (40 ng/mL, **Ac**), cisplatin (2 μg/mL and 5 μg/mL, **C1** and **C2**), mitomycin C (2 μg/mL and 5 μg/mL, **M1** and **M2**) and doxorubicin (0.5 μM, 1 μM and 2 μM, **D1**, **D2** and **D3**) for 8h and 24h. Whole cell lysates were subjected to Western blot analysis for protein levels of NDRG1 and p53. β-tubulin was used as loading control. **UN** represents "untreated".

➡ Apparent apoptosis (cell blebbing and floaters) was observed in conditions marked with red.

➡ For blot quantification, more than 2-fold change is defined as upregulation; *asterisk* (*) indicates upregulation of both NDRG1 and p53 while the *caret* (^) indicates upregulation of NDRG1 without significant change of p53.

since structurally distorted DNA is not an effective template for transcription [314], the concentration used here, at least theoretically, may be too low for the effective inhibition of transcription. Therefore, the inhibitory effect on NDRG1 expression can also be attributed to the complexity of the signalling triggered by these chemicals. Thirdly, in all the three OSCC cell lines, regulation of NDRG1 by DNA damage is clearly not overlapping with the level of p53, especially in KYSE150 cells, in which NDRG1 induction was always accompanied with an unchanged p53 level. Considering the major contribution of p53 accumulation to p53-mediated gene expression in response to DNA damage [315;316], we propose a p53-independent mechanism of NDRG1 regulation in response to DNA damage, regardless of the p53 status

(functional or non-functional) in these OSCC cells.

Collectively, our data suggest the ability of DNA damage to induce NDRG1 in OSCC cell lines, although the extent of the response may vary depending on the genotoxic agents used and the cell types examined. These phenomena indicate that DNA damage, as the common effect shared by these genotoxic agents, may not serve as the only regulatory factor affecting NDRG1 expression during the treatment. Considering the dissimilar regulatory patterns of p53 and NDRG1 in most cases, the participation of p53-independent regulatory mechanisms is suggested. In fact, p53 mediated NDRG1 induction has also been reported to be cell-type specific in the literature (Section 1.4.2.2).

3.3.2 Hypoxia

Hypoxia is a prevalent phenomenon in solid tumours including OSCC [317-319]. Adaptive responses to hypoxia such as angiogenesis and apoptotic evasion, eventually contribute to the malignant phenotype as well as aggressive tumour behaviour [320]. To investigate the potential regulatory effect of hypoxic signalling on NDRG1 expression, OSCC cell lines were subjected to the treatment of nickel and cobalt compounds as hypoxia mimics, owing to the lack of a special cell incubator in our laboratory to achieve low oxygen tension. As shown in Fig 3-4, activation of hypoxic signalling by Ni^{2+} and Co^{2+} can be reflected by the significant upregulation of HIF-1α, a genuine hypoxia marker as well as the most important transcription regulator of the hypoxic response in cells. In all three OSCC cell lines tested, NDRG1 was drastically induced 8 hours after nickel or cobalt administration and was further increased or sustained at the 24h time point, suggesting that it is a bona fide hypoxia responsive gene. The only exception of the time- and dose-dependent induction of NDRG1 by nickel or cobalt appears in 24h treatment of 0.5 mM Co^{2+} in KYSE30 cells, in which the NDRG1 upregulation was

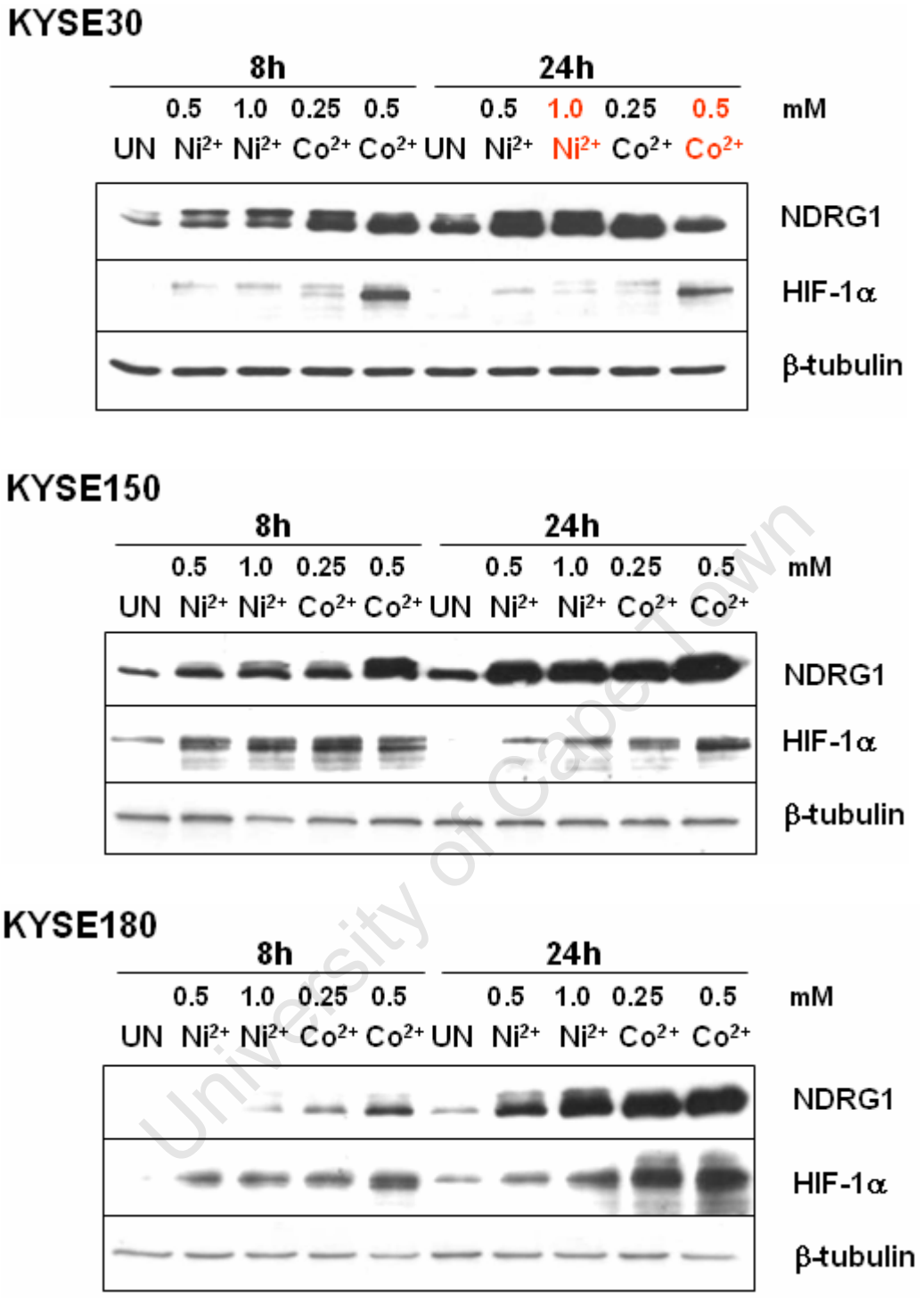


Fig 3-4: Determination of the effects of hypoxia mimics (soluble nickel or cobalt compounds) on NDRG1 induction in OSCC cell lines. Subconfluent cells were treated with nickel sulphate ($NiSO_4$) or cobalt chloride ($CoCl_2$) at indicated concentrations. Whole cell lysates were harvested at 8h or 24h afterwards and subjected to Western blot analysis for the protein levels of NDRG1 and HIF-1a. β-tubulin was used as loading control. **UN** represents "untreated". Apparent apoptosis (cell blebbing and floaters) was observed in conditions marked with red.

diminished along with massive cell death, similar to the observation in genotoxic challenge induced cell apoptosis.

3.3.3 Endoplasmic reticulum (ER) stress

Besides hypoxia, ER stress, due to the excessive glycolysis and the consequent acidic microenvironment in the tumour tissue [321], raises another physiological challenge against the rapid growing solid tumour. Another source of ER stress comes from hypoxia, which disturbs the intracellular calcium signalling and consequently activating the ER resident kinase PERK [322;323]. Since ER stress eventually lead to the accumulation of incorrectly folded, dysfunctional proteins, tumour cells develop adaptive reactions termed "unfolded protein response", which largely contributes to hypoxia tolerance and promotes tumour growth *in vivo* [321;323;324]. The upregulation of GRP94, an ER chaperone responsible for unfolded protein processing and regulating the degradation of abnormal proteins, is one of the reliable markers of the "unfolded protein response" [325]. Two ER stress inducing reagents calcium ionophore A23187 (by calcium homeostasis disturbance) and tunicamycin (by glycosylation suppression) were tested to determine their effects on NDRG1 expression. As shown in Fig 3-5, both reagents were capable of inducing GRP94 as a marker of ER stress.

Like genotoxic challenge, the regulatory effect of ER stress on NDRG1 expression also manifested as a cell type specific response. Both A23187 and tunicamycin actively induced NDRG1 expression in KYSE180 cells. While in KYSE30 and KYSE150 cells, only A23187 acted as an effective NDRG1 inducer, although marked induction of GRP94 was detected, indicating the introduction of ER stress by both reagents. Furthermore, the null effect of tunicamycin on NDRG1 upregulation in KYSE30 and KYSE150 cells suggests that the A23187 mediated NDRG1 induction may not be merely attributed to ER stress. Actually, it is tempting to associate NDRG1 to intracellular calcium,

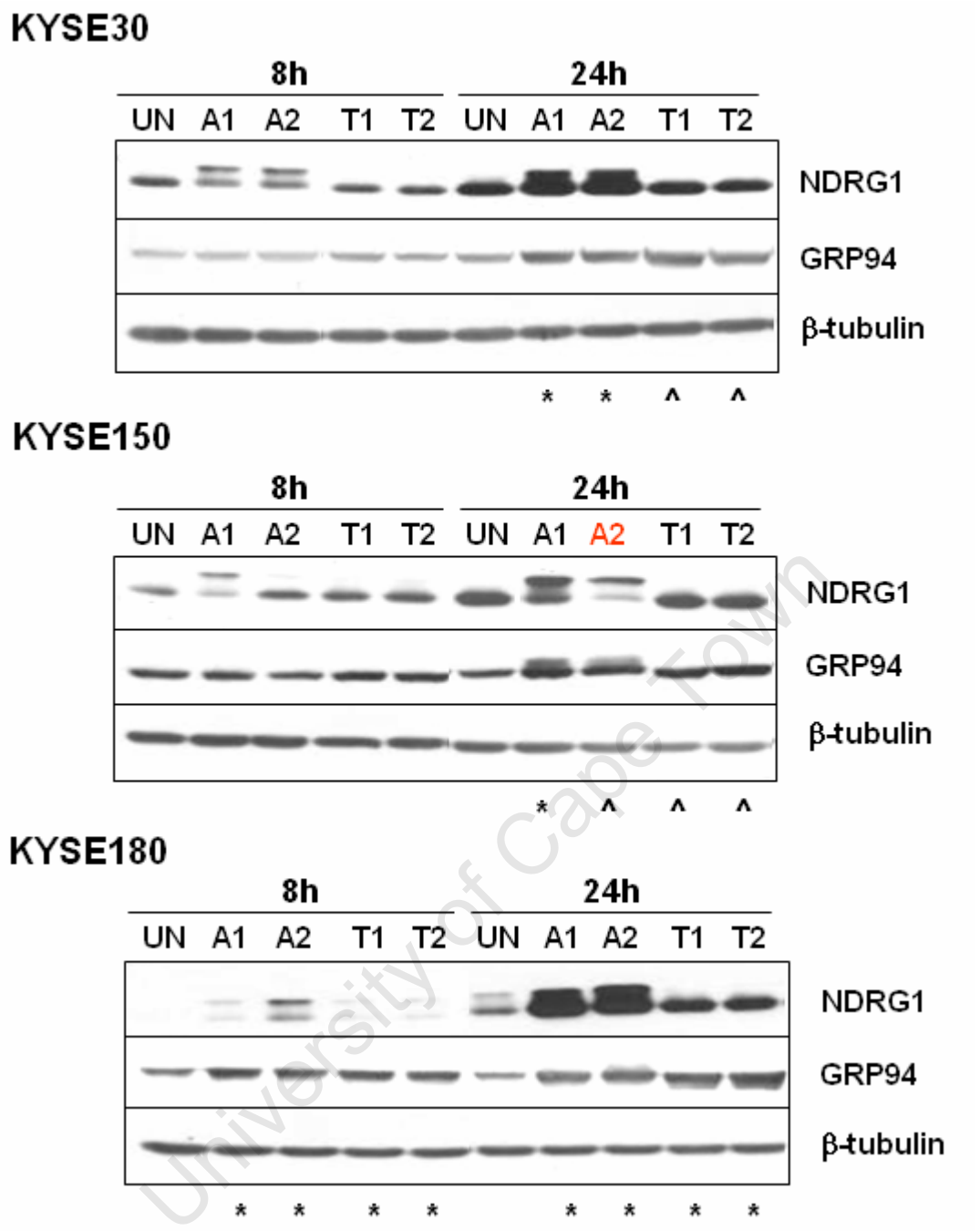


Fig 3-5: Determination of the putative regulatory effects of endoplasmic reticulum stress (ER stress) on NDRG1 expression in OSCC cell lines. Subconfluent cells were treated with reagents capable of inducing ER stress such as calcium ionophore A23187 (5 μM and 10 μM, **A1** and **A2**) and tunicamycin (5 μg/mL and 10 μg/mL, **T1** and **T2**) for 8h and 24h. Whole cell lysates were subjected to Western blot analysis for protein levels of NDRG1 and GRP94 (as a marker of ER stress). β-tubulin was used as loading control. **UN** represents "untreated". Apparent apoptosis (cell blebbing and floaters) was observed in conditions marked with red.

➡ For blot quantification, more than 2-fold change is defined as upregulation; *asterisk* (*) indicates upregulation of both NDRG1 and GRP94 while the *caret* (^) indicates upregulation of GRP94 without significant change of NDRG1.

which is recognized as an important second messenger dealing with variety of cellular signalling such as PKC pathway, and thereby control cell proliferation, differentiation and apoptosis [326]. Interestingly, we again observed the downregulation of NDRG1 with apoptotic KYSE150 cells resulting from A23187 treatment. Taken together, our data suggested that ER stress might serve as an additional factor for NDRG1 upregulation in OSCC cells, despite the complexity associated with the cellular context, which has also been observed in other cancer cell lines [23].

3.3.4 Summary

In summary, a link between NDRG1 and stress-response has been identified in OSCC cells. During the progression of OSCC, challenges such as DNA damage, hypoxia and ER stress can all be candidates that participate in the regulation of NDRG1, although it may be highly dependent on the cellular context. Therefore, a potential role for NDRG1 in the stress response is suggested. Interestingly, regardless of genotoxic, hypoxic or ER stress, we have always observed that when damage becomes harsh enough to induce cell death, the previously triggered NDRG1 upregulation may be largely abolished or even reversed to a downregulated state. Considering the similar observation in colon cancer cells [268], we could speculate that NDRG1 may participate in death signalling, which further suggests the potential function of NDRG1 as an antagonist of apoptosis, as revealed in the functional studies in Chapter 2.

Of the three kinds of stresses investigated, only hypoxia signalling was demonstrated as a comprehensive and reliable NDRG1 inducer with the least exceptions. Therefore, similar as in other types of cancer [51], NDRG1 may be considered as a hypoxia responsive gene in OSCC. In contrast, the discrepancies elicited by different genotoxic or ER stress inducing agents in OSCC and other tumour cells (Section 1.4.2.2 and Ref. [23]) largely motivate

against the hypothesis that considers NDRG1 as a genotoxicity or ER stress related gene. On the other hand, as a gene actively regulated by hypoxia, it is highly plausible to link NDRG1 to hypoxia related phenotypic changes. In fact, phenotypic targets of NDRG1 functions revealed in Chapter 2, such as metastasis, angiogenesis and apoptotic evasion are all typical consequence of hypoxia adaptation.

3.4 Signalling from growth factors

It is generally accepted that OSCC frequently demonstrates uncontrolled amplification of mitogenic signalling triggered by the increased expression of cellular growth factors and corresponding receptors (Section 1.3.1). However, until recently, no direct evidence has been revealed regarding the regulatory effect of growth factors on NDRG1 expression in OSCC or even in the context of cancer cells. Therefore, clarifying the putative relationship between NDRG1 and growth factor signalling may provide useful hints on the participation of NDRG1 in the phenotypic modulation by mitogenic signalling pathways. Moreover, considering the fact that cancer cells frequently display disturbed networks of signalling pathways, some of the regulatory controls which are required for normal and physiological processes may be masked or overwhelmed in cancer cells. Hence, based on the results from our functional studies in Chapter 2, we can also evaluate the significance of NDRG1 regulation resultant from different signalling pathways in the neoplastic progression of OSCC.

Current data suggest that MAPK and PI3K pathways are pivotal to the malignant transformation of OSCC, through the modulation of proliferation, metastasis and apoptosis [121-128]. In this section, we describe our work to elucidate the putative NDRG1 regulation mediated by MAPK or PI-3K.

3.4.1 Signalling from EGF and IGF

Epidermal growth factor (EGF), along with its receptors, represents the most prominent aberration of mitogenic signalling in the progression of OSCC [121-125]. When stimulated by EGF, NDRG1 induction was observed in all the four OSCC cell lines tested (Fig 3-6). In KYSE450 cells, significant (more than 2-fold) NDRG1 induction was observed as early as 6 hours after EGF administration, while about 12 hours was needed by other three cell lines (KYSE30, KYSE150 and KYSE180). The difference in induction dynamics could be attributed to the complexity of EGF signalling.

Further investigation with kinase inhibitors such as GF109293X (1 μM, PKC inhibitor), PD98059 (50 μM, MEK inhibitor), SP600125 (1 μM, JNK inhibitor) and LY294002 (50 μM, PI-3K inhibitor) was performed to elucidate the putative signalling pathway responsible for EGF-mediated NDRG1 induction. Our data indicated the pivotal role of ERK1/2 (p44 and p42), since inhibition of MEK or PKC, both of which act as upstream activators of ERK, significantly abolished the upregulation of NDRG1 by EGF. Furthermore, the participation of JNK was also suggested in KYSE30 cells, due to the antagonizing effect of SP600125, although less effective compared to PD98059 or GF109293X. Moreover, treating cells with GF109293X, PD98059 or SP600125 alone failed to modulate the level of NDRG1 in KYSE30 cells, indicating the probable irrelevance of PKC, ERK or JNK signalling in the maintenance of basal NDRG1 expression (Fig 3-7).

In contrast, the PI-3K inhibitor LY294002 was found to induce NDRG1 expression markedly, regardless of the presence of EGF. Therefore, a negative modulation of basal NDRG1 expression via PI-3K signalling is suggested. To further verify the negative effect of PI-3K signalling on NDRG1 expression, OSCC cell lines were subjected to insulin-like growth factor (IGF), which, as a potent inducer of PI-3K signalling, is also considered an important

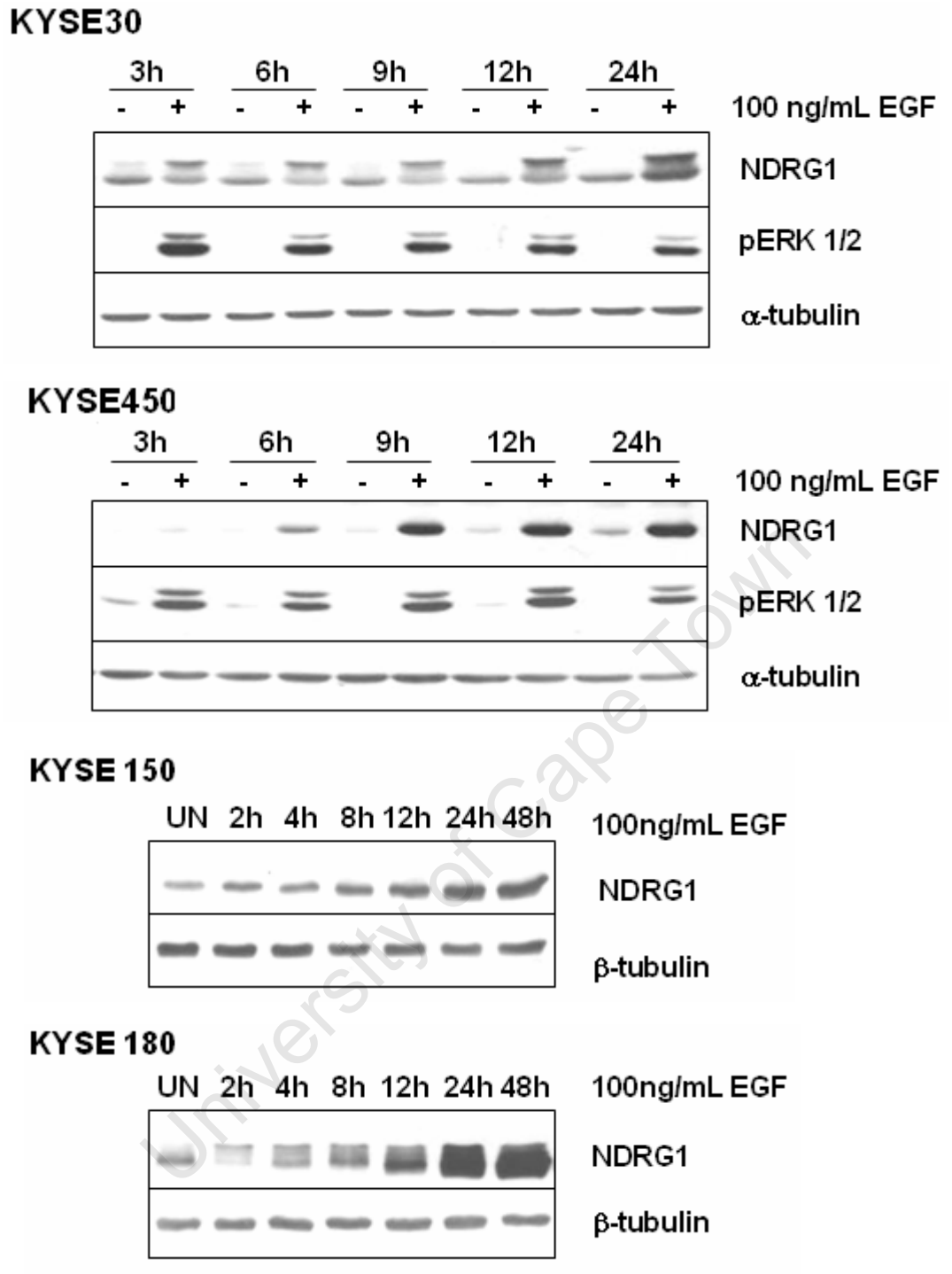


Fig 3-6: Epidermal growth factor (EGF) induces NDRG1 in OSCC cell lines. Before treatment, complete medium was replaced with serum free medium overnight to allow cells to get subconfluent before adding EGF (100 ng/mL, diluted in serum free medium). Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis to check NDRG1 level. Phosphorylated ERK1/2 (pERK 1/2, Thr202/Tyr204 of ERK1 and Thr185/Tyr187 of ERK2) was used to indicate the validity of EGF treatment. α- or β-tubulin was used as loading control. UN represents "untreated", which was harvested at 24h time point for the experiment of KYSE150 and KYSE180.

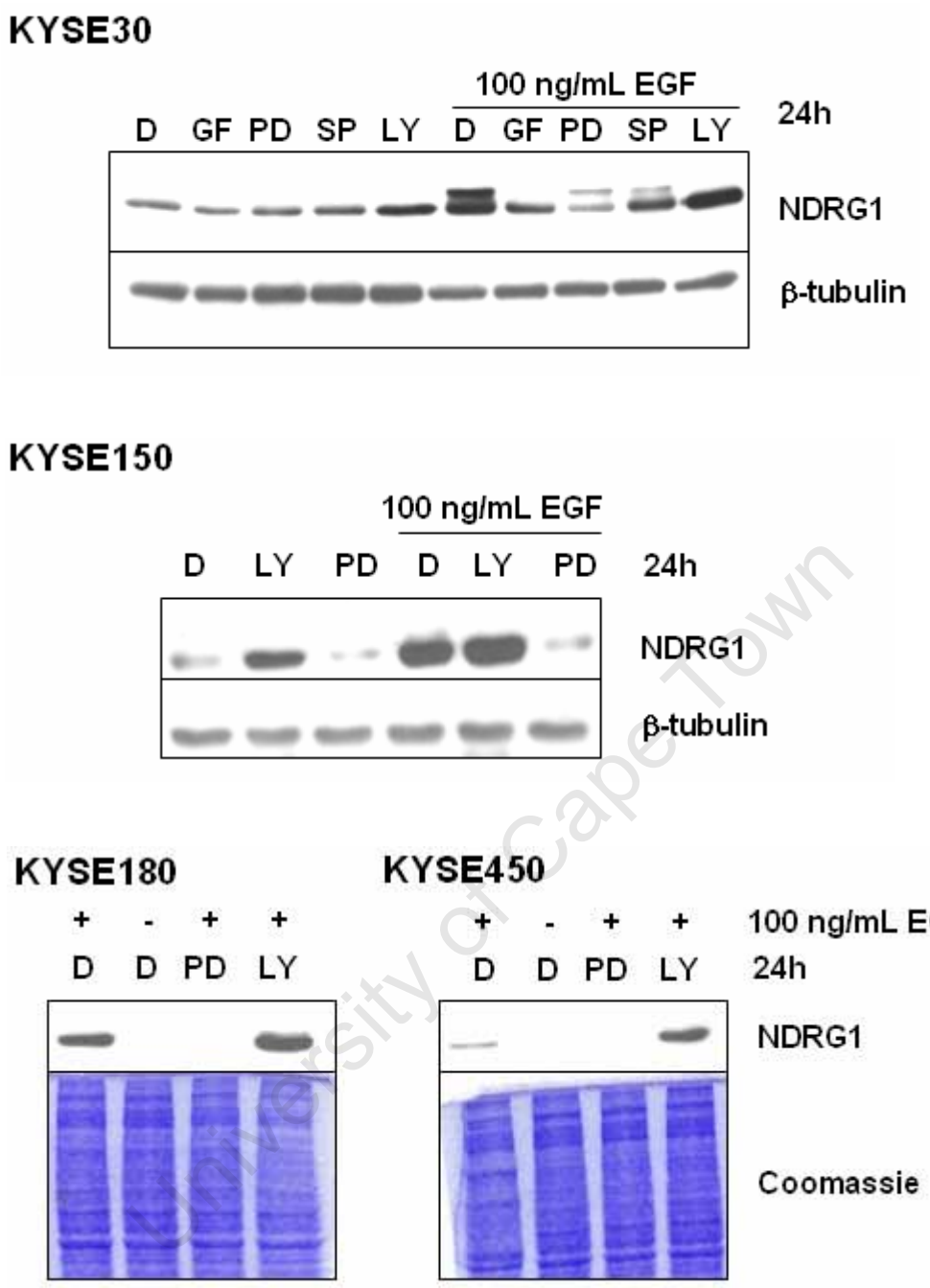


Fig 3-7: Determination of the pathways responsible for EGF mediated NDRG1 induction. Same serum starvation protocol as Fig 3-5 for EGF treatment (100 ng/mL) was used. All kinase inhibitors were added 1 hour before EGF treatment. Whole cell lysates were harvested 24 hours afterwards and subjected to Western blot analysis to check NDRG1 level. β-tubulin or Coomassie brilliant blue R250 staining of the transferred gel was used as loading control. Kinase inhibitor used are listed as GF: 1 μM GF109293X (PKC inhibitor); PD: 50 μM PD98059 (MEK inhibitor); SP: 1 μM SP600125 (JNK inhibitor); and LY: 50 μM LY294002 (PI-3K inhibitor). D represents DMSO as solvent vehicle.

promoter of OSCC [126-128]. As shown in Fig 3-8, IGF dramatically downregulated the expression of NDRG1 in KYSE30 and KYSE150 cells, with the effect reversible by additional treatment of the PI-3K inhibitor LY294002. Conversely, treating OSCC cells with IGF receptor inhibitor PQ-401 (10 μM) in the presence of serum resulted in a significant induction of NDRG1 (Fig 3-9). Taken together, we postulate IGF as a negative regulator, probably through PI-3K activation, on NDRG1 expression in OSCC cells.

* * *

In summary, our data suggest the diverse regulatory effect of mitogenic stimuli on NDRG1 expression, with the EGF signalling (probably through ERK and JNK) showing a positive effect and the IGF signalling (probably through PI-3K) showing a negative effect. Considering that EGF also activates PI-3K [98;99], the synergistic effects of EGF and LY294002 on NDRG1 induction also suggested the involvement of PI-3K signalling as a negative regulator during this process. Therefore, the negative effect of PI-3K signalling may also provide a possibility to explain the difference of the dynamics in different cell lines in EGF-mediated NDRG1 induction.

In OSCC, elevated EGF signalling has been shown related to excessive proliferation, lymph node or vascular metastasis as well as resistance to chemo- or radio-therapy [121-125]. Except proliferation, many of the EGF signalling mediated phenotypic changes such as enhanced activities of metastasis, angiogenesis and apoptotic evasion can be also elicited by ectopic NDRG1 overexpression in OSCC cells (Chapter 2). In this aspect, our work may suggest the potential participation of the EGF signalling induced NDRG1 in the EGF-mediated neoplastic progression of OSCC.

Although the exact mechanism of EGF/MAP kinase mediated NDRG1 upregulation in OSCC cells is still under investigation in our lab, through

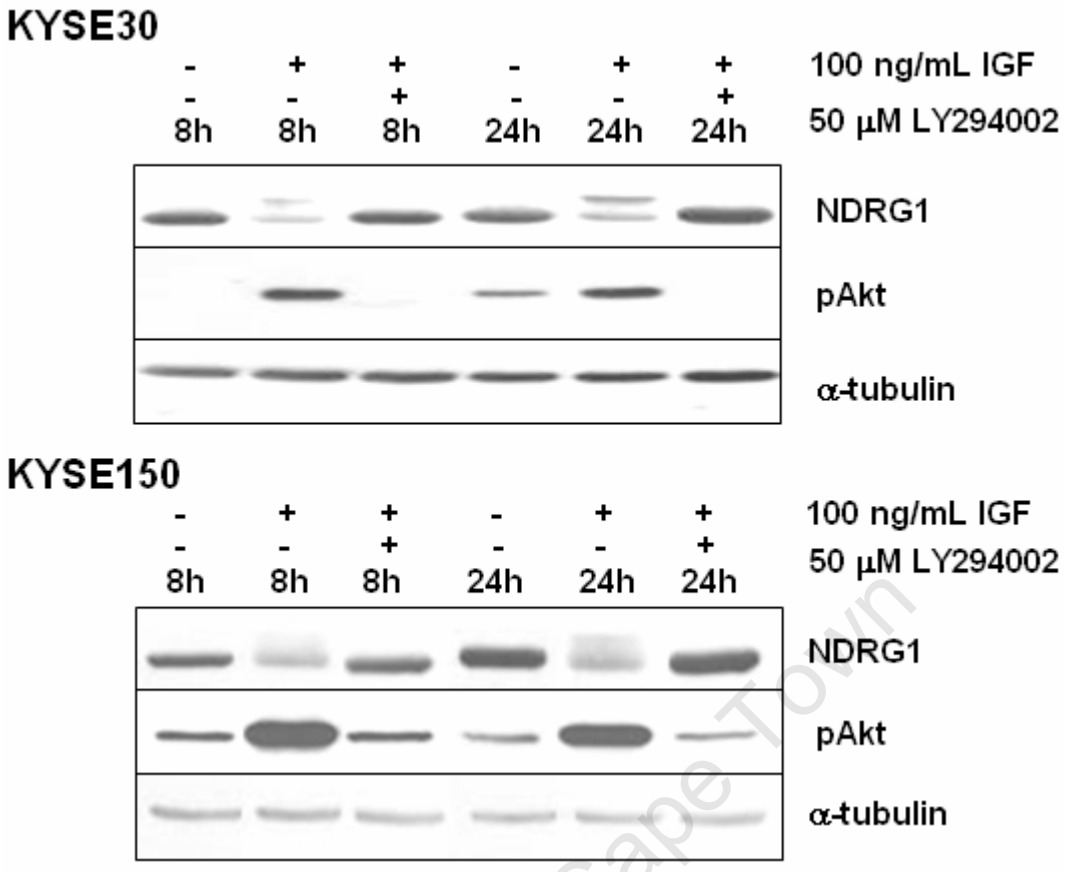


Fig 3-8: Insulin-like growth factor (IGF) suppresses NDRG1 expression in OSCC cell lines. A serum starvation and kinase inhibition protocol similar to Fig 3-7 was used. Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis. Phosphorylated Akt (pAkt, Ser473) was used to reflect the validity of IGF treatment. α-tubulin was used as loading control.

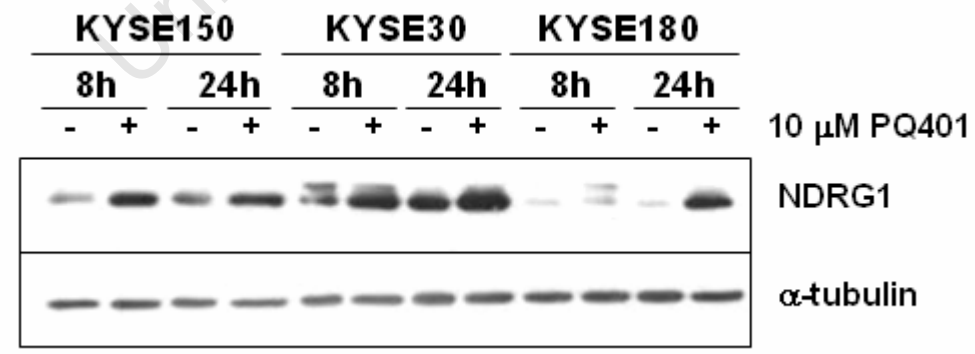


Fig 3-9: Inhibition of insulin-like growth factor receptor (IGFR) upregulates NDRG1 expression in OSCC cell lines. Subconfluent cells kept in complete medium were treated with 10 µM of IGFR inhibitor PQ401. Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis. α-tubulin was used as loading control.

analysing the literature, two transcription factors Egr-1 (as a downstream effector of ERK/Elk) and AP-1 (as a downstream effector of JNK) captured our attention as potential executors of EGF mediated NDRG1 upregulation [218;220]. It has been shown that both Egr-1 and AP-1 play important roles in tumour progression. Moreover, both transcription factors have been shown to participate in the hypoxia mediated NDRG1 induction [218;220]. Therefore, once a direct link (e.g. evidence from promoter analysis or CHIP assay) is established, it is conceivable that NDRG1 may act as one of the executors for the phenotypic changes related to Egr-1 or AP-1.

In addition, if only considering the regulatory data, our results may also suggest the plausible participation of NDRG1 in IGF signalling, which actively promotes the progression of OSCC [126-128]. However, NDRG1, with oncogenic effect shown in OSCC cells (Chapter 2), was found to be downregulated by IGF/PI-3K signalling. A question is thus raised about the importance of IGF induced NDRG1 downregulation in the progression of OSCC. Unfortunately, information from literature is lacking in this regard. Since PI-3K signalling was found to be largely contributory to IGF mediated NDRG1 downregulation, in the next section, we explored the possible mechanism underlying the suppressive effect of PI-3K on NDRG1 expression, aiming to find putative explanation to the above question.

3.4.2 Negative effect of PI-3K signalling on NDRG1 expression

A further step was taken to investigate the mechanism underlying the suppressive effect of PI-3K on NDRG1 regulation, due to the lack of information in literature. Considering the fact that the effect of either IGF administration or ectopic overexpression of PTEN is not limited to modulating PI-3K signalling [98;106], specific PI-3K inhibitor LY294002 was used instead for this approach. For the study using LY294002 and other inhibitors, 10%

foetal bovine serum was always incorporated in the culturing system, as a source of cytokines as PI-3K activator.

Consistent with previous results, it was shown that LY294002 treatment alone significantly upregulated NDRG1 protein level in a time and dose dependent manner (Fig 3-10). Moreover, quantitative real-time RT-PCR

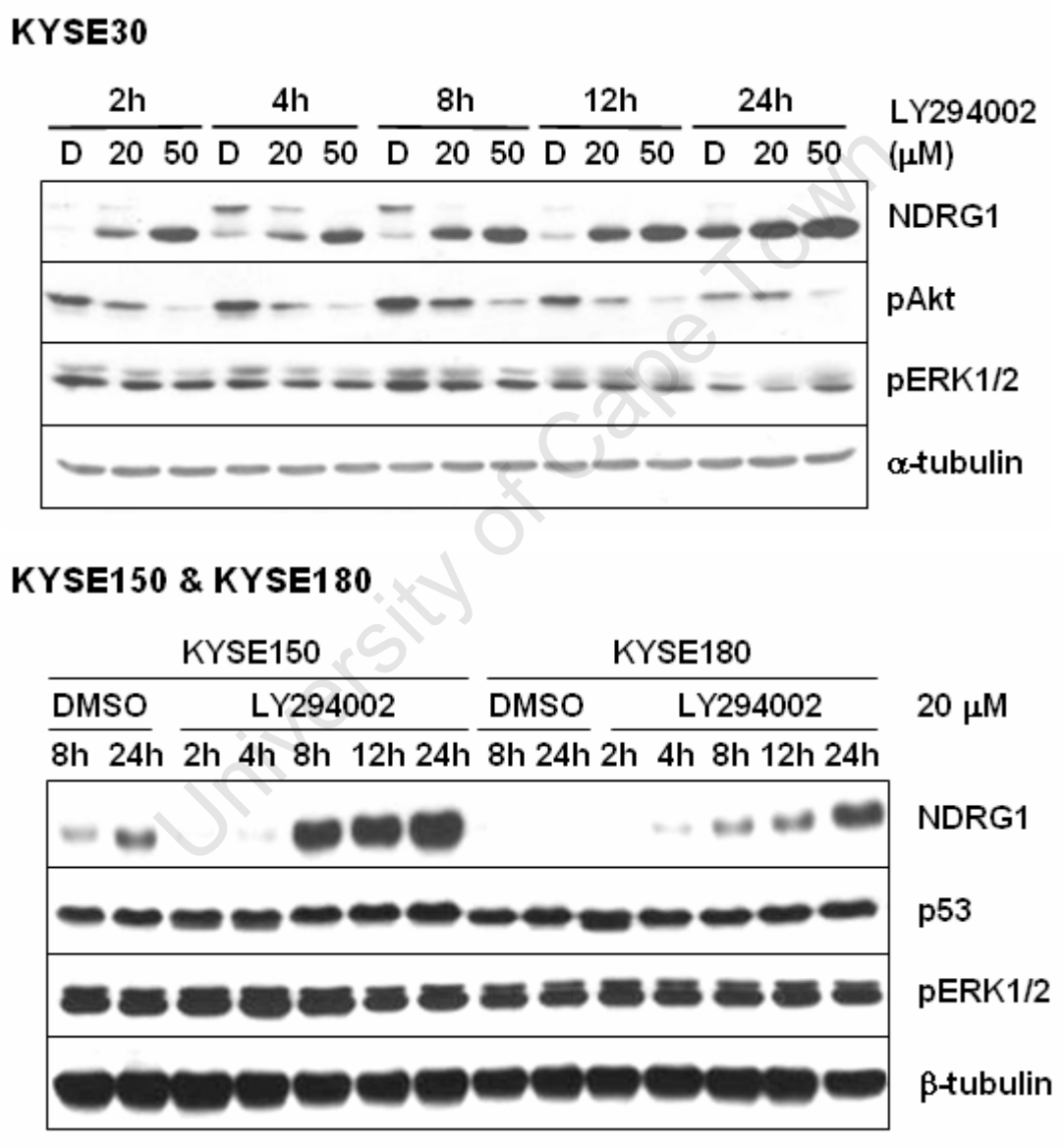


Fig 3-10: Inducing effect of LY294002, a specific PI-3K inhibitor, on NDRG1 expression in OSCC cells. Subconfluent cells kept in complete medium were treated with different concentrations of LY294002. Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis. "D" represents DMSO as solvent vehicle.

revealed marked induction of NDRG1 mRNA level in response to LY294002, especially in the early time points of treatment, suggesting the transcriptional dependence of PI-3K signalling mediated NDRG1 suppression (Fig 3-11). Although significant decrease of phosphorylated Akt (pAkt), a canonical downstream effector of PI-3K, was observed with PI-3K inhibition (Fig 3-10

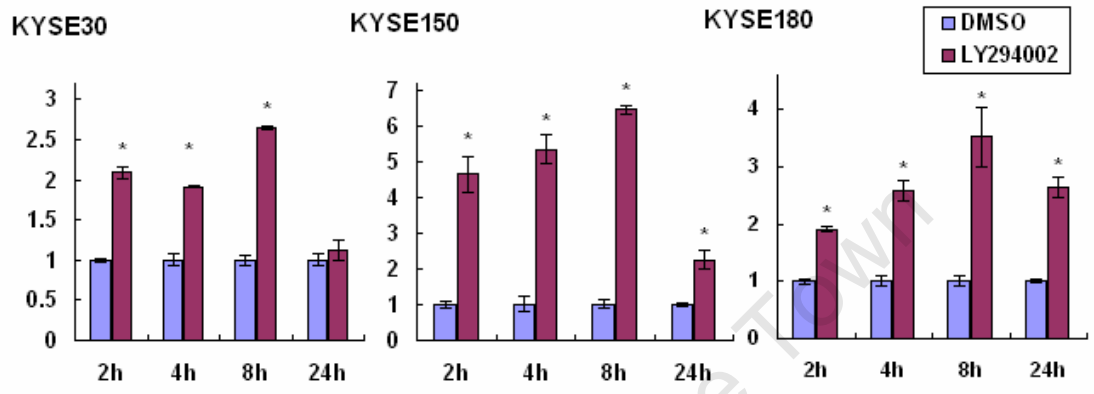


Fig 3-11: LY294002 upregulates the mRNA level of NDRG1 in OSCC cells. Subconfluent cells kept in complete medium were treated with 50 μM of LY294002 and DMSO as solvent vehicle. Total RNA was isolated at indicated time points and subjected to quantitative real-time RT-PCR to analyse the mRNA level of NDRG1. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene. *Columns*, mean of the data from three independent experiments; *Bars*, ± SD; *asterisk* (*), $p<0.05$ versus DMSO treated control.

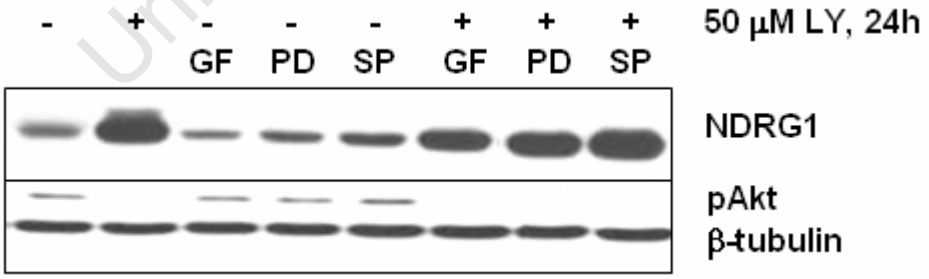


Fig 3-12: Negative effect of PKC, ERK or JNK inhibition on LY294002 mediated NDRG1 upregulation in KYSE150 cells. Subconfluent KYSE150 cells were treated with indicated kinase inhibitors for 24h. Total cell lysates were subjected to Western blot analysis. β-tubulin was used as loading control. Kinase inhibitor used are listed as GF: 1 μM GF109293X (PKC inhibitor); PD: 50 μM PD98059 (MEK inhibitor); SP: 1 μM SP600125 (JNK inhibitor); and LY: 50 μM LY294002 (PI-3K inhibitor).

and data not shown), the level of phosphorylated ERK1/2 was generally left unaffected. Since it is common that blocking one signalling pathway usually leads to compensatory enhancement of other related pathways in the signalling transduction network, to further exclude possible crosstalk (e.g. with MAPK or PKC) in the PI-3K inhibition mediated NDRG1 upregulation, multiple kinase inhibitor treatment with LY294002 was performed in KYSE150 cells. As shown in Fig 3-12, inhibition of PKC, ERK or JNK all failed to impose any significant effects on LY294002 mediated NDRG1 upregulation. Therefore, the negative effect of PI-3K on NDRG1 expression should be attributed to the PI-3K pathway itself.

Akt (also known as protein kinase B, PKB) is one of the most important mediator of PI-3K signalling. Through direct phosphorylation, Akt contributes to tumour progression by modulating the activities of a wide range of targets. Many of the Akt targets controls cellular transcription and translation, such as p53, FoxO (or forkhead transcription factors, FKHR), NF-κB and mTOR [102;103]. To test the role of Akt in PI-3K mediated NDRG1 downregulation, we specifically blocked Akt signalling using Akt Inhibitor VIII trifluoroacetate salt hydrate. In contrast to PI-3K inhibitor LY294002, cell type specific responses were observed (Fig 3-13). Although the Akt inhibitor effectively upregulated NDRG1 protein level in KYSE180 cells, it only showed a marginal effect on NDRG1 expression in KYSE30 and KYSE150 cells. Consistently, ectopic overexpression of dominant negative Akt (activation incompetent and kinase domain mutated) in KYSE150 cells failed to modulate the NDRG1 level (Fig 3-14). Moreover, in KYSE30 cells, Akt inhibition was demonstrated insufficient to reverse the suppressive effect of IGF on NDRG1 expression (Fig 3-15). Inhibiting another two canonical Akt downstream signalling transmitters mTOR (by 100 nM rapamycin, reflected by reduced phosphorylation of $p70^{S6K}$) and IKK (by 5 mM and 10 mM sodium salicylate) in KYSE30 cells both failed to affect NDRG1 expression (data not shown). Taken together, our data

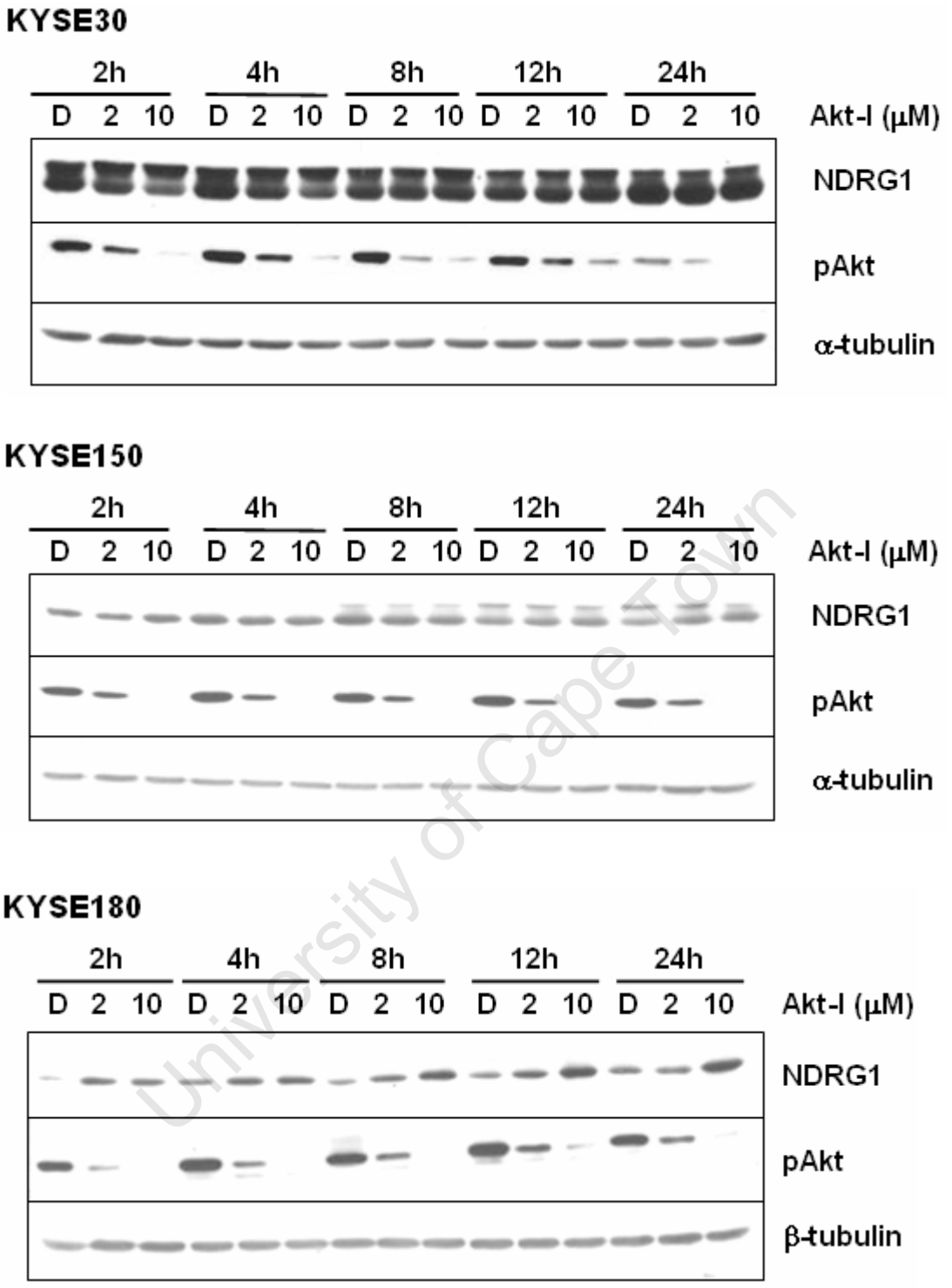


Fig 3-13: Effect of Akt inhibition on NDRG1 expression in OSCC cells. Subconfluent cells kept in complete medium were treated with different concentrations of Akt Inhibitor VIII trifluoroacetate salt hydrate. Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis. The level of phosphorylated Akt (pAkt, Ser473) monitors the efficiency of Akt inhibition. α- or β- tubulin was used as loading control. D represents DMSO as solvent vehicle.

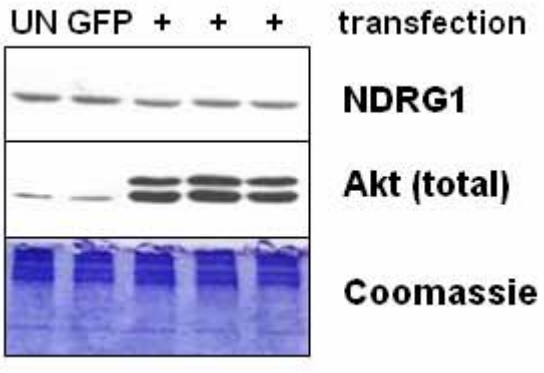


Fig 3-14: Null effect of ectopic overexpression of dominant negative Akt on NDRG1 expression in KYSE150 cells. Subconfluent KYSE150 cells were transfected using Fugene HT reagents (Roche). Total cell lysate was harvest 24h afterwards and subjected to Western blot analysis for the protein levels of NDRG1 and total Akt. The Akt transfected is triple mutated (K179M T308A S473A) resulting in dead kinase and activation deficiency, which can block the endogenous Akt activity through competing the Akt activators. The three "+" represent three independent transfections of mutated Akt. Both mock (UN) and GFP expression plasmids (GFP) were used as negative control. Coomassie brilliant blue R250 staining of the transferred gel was used as loading control.

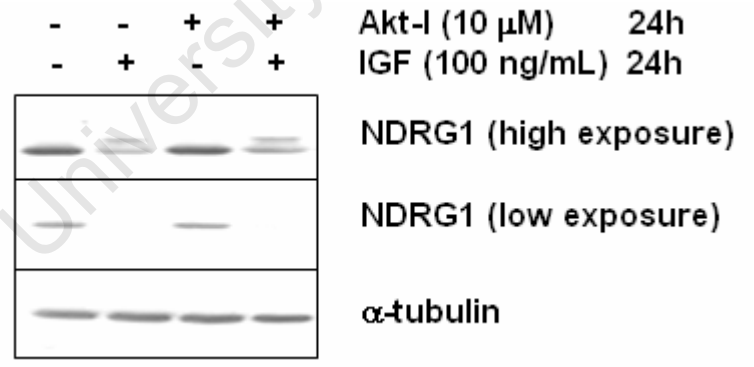
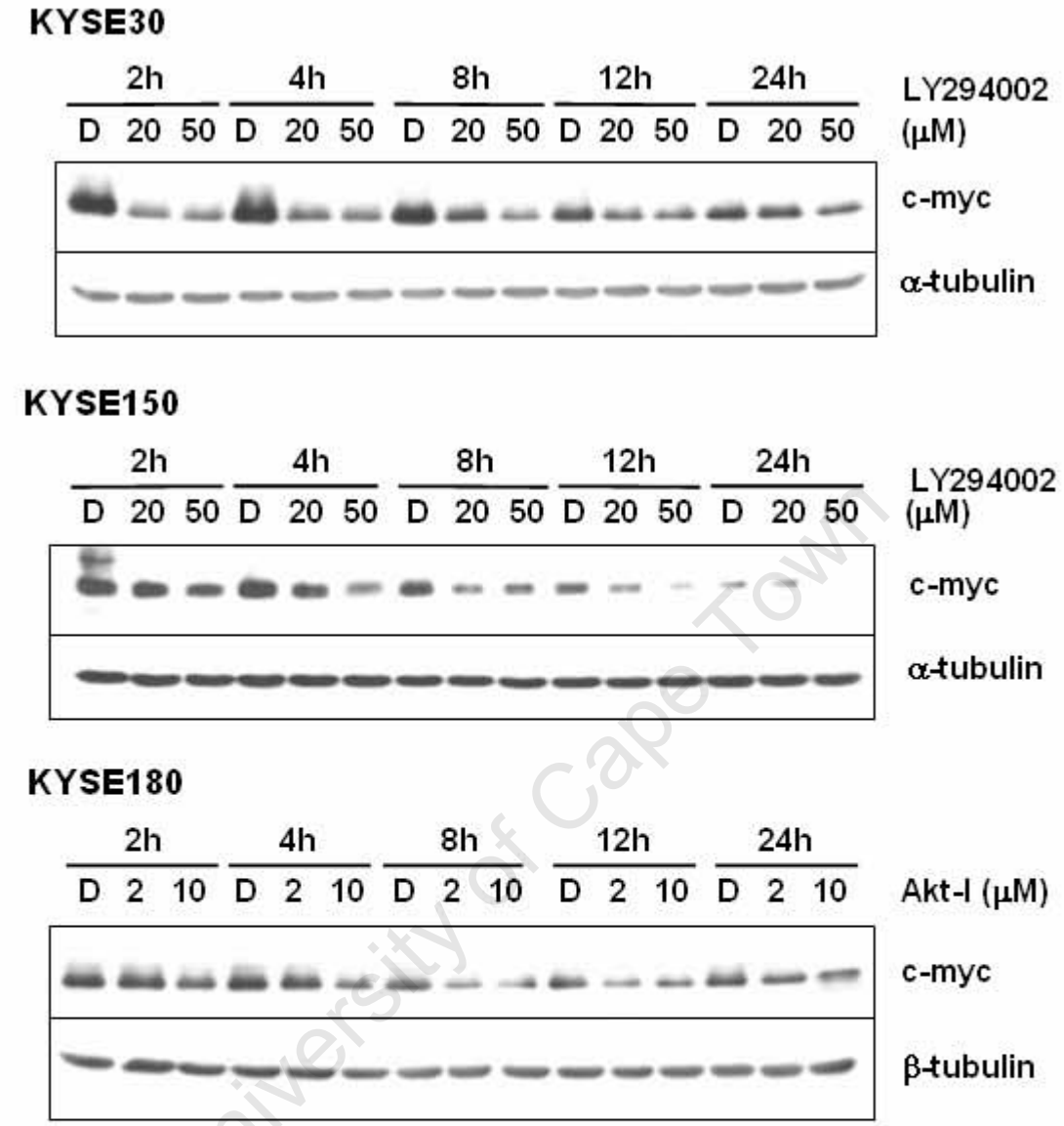


Fig 3-15: Null effect of Akt inhibition on IGF mediated NDRG1 downregulation in KYSE30 cells. Prior to treatment, complete medium was replaced with serum free medium overnight to allow cells to reach subconfluent before adding IGF (100 ng/mL, diluted in serum free medium) and Akt Inhibitor VIII trifluoroacetate salt hydrate (Akt-I, 10 μM, 1 hour prior to IGF treatment). After incubating 24h with IGF, whole cell lysates were harvested and subjected to Western blot analysis to check NDRG1 level. The validity of IGF was reflected by the enhanced phosphorylation of NDRG1. α-tubulin was used as loading control.

suggested that Akt might not be necessary for PI-3K mediated NDRG1 downregulation in OSCC cells, although it might sometimes participate in this process, depending on the cellular context. Finally, the level of p53, either viewed as an Akt downstream effector or not, was found to be constant during the treatment of LY294002, suggesting p53 may not be involved in the upregulation of NDRG1 by PI-3K inhibition, despite the existence of putative p53 binding site on the NDRG1 promoter (Fig 3-10).

Recently, upregulation of myc transcription factors has been demonstrated as a downstream event of PI-3K activation in both Akt dependent and independent manner, although the mechanism has not been fully identified [327-329]. In parallel with NDRG1 induction by PI-3K inhibition, a decreased level of c-myc was also observed (Fig 3-16). Since myc negatively targets the expression of NDRG1, this observation may suggest that the upregulation of NDRG1 by LY294002 (or Akt inhibitor in KYSE180 cells) is potentially mediated through the suppression of c-myc. Unlike the commonly suggested post-translationally stabilization of c-myc by PI-3K signalling [327], in the OSCC cells tested, c-myc is found to be transcriptionally suppressed by LY294002, at least in the early time points of reagent administration. Since Akt independent c-myc activation in transcription level was previously reported to be controlled by Wnt/β-catenin signalling [328;329] which is negatively regulated by GSK3-β [330;331], the potential effect of Wnt signalling was investigated by treating KYSE30 and KYSE150 cells with lithium chloride, a specific GSK-3β inhibitor via phosphorylation (Ser9) for proteasome targeting. Accordingly, significant downregulation of NDRG1 was observed in respond to lithium chloride, being accompanied by the gradual increase of phosphorylated GSK-3β (Fig 3-17). Considering that the level of phosphorylated GSK-3β was also observed inversely correlated with PI-3K, an axis of PI-3K/GSK-3β/c-myc is suggested for the suppressive role of PI-3K on NDRG1 expression, although further study is still required to determine how c-myc is involved in this process.

A



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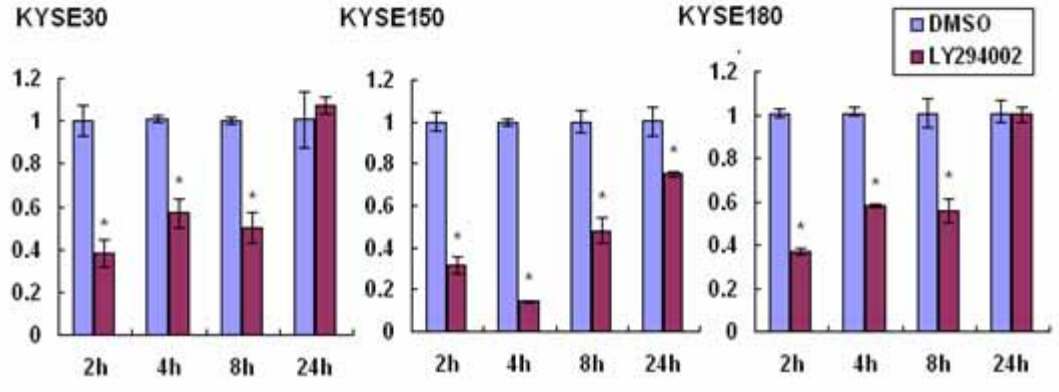


Fig 3-16 (overleaf): PI-3K or Akt inhibition decreases the level of c-myc. A, Subconfluent cells kept in complete medium were treated with different concentrations of LY294002 (KYSE30 and KYSE150) or Akt Inhibitor VIII trifluoroacetate salt hydrate (KYSE180). Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis to check the level of c-myc. α- or β-tubulin was used as loading control. "D" represents DMSO as solvent vehicle. **B**, LY294002 transcriptionally represses the level of c-myc in OSCC cells. Subconfluent cells kept in complete medium were treated with 50 μM of LY294002. Total RNA was isolated at indicated time points and subjected to quantitative real-time RT-PCR to analyse the mRNA level of c-myc. The relative fold change was determined by $2^{-\Delta\Delta Ct}$ method using β-actin as reference gene. *Columns*, mean of the data from three independent experiments; *Bars*, ± SD; *asterisk* (*), $p<0.05$ versus DMSO treated control.

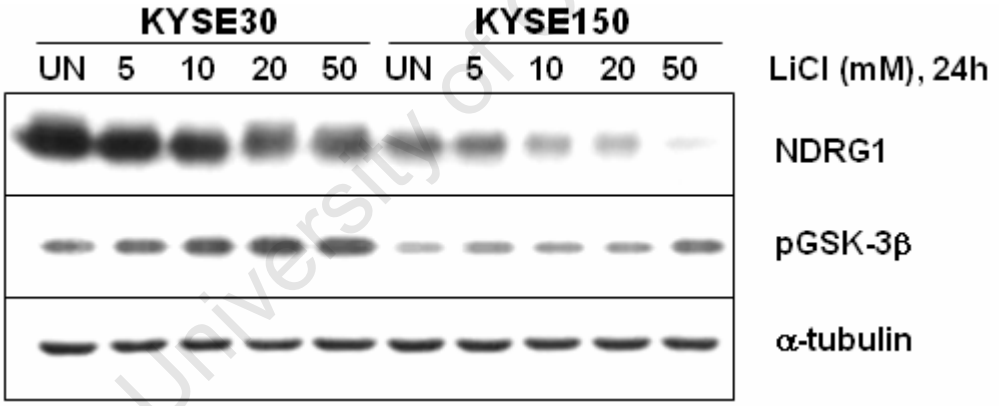


Fig 3-17: Inhibition of GSK-3b by lithium chloride decreases the expression of NDRG1. Subconfluent KYSE30 and KYSE150 cells kept in complete medium were treated with different concentrations of lithium chloride (LiCl). Whole cell lysates were harvested at indicated time points and subjected to Western blot analysis. The inhibition of GSK-3β was reflected by its phosphorylation, which inactivates the kinase and targets it to proteasome for degradation. α-tubulin was used as loading control. UN represents untreated cells.

* * *

Taken together, although the mechanism still needs to be further studied, our observation clearly confirmed the suppressive effect of PI-3K signalling on NDRG1 expression in OSCC cells. Although the intermediate signalling transmitter may differ according to the cellular context, we identified c-myc as a potential mediator in the NDRG1 suppression by PI-3K signalling.

Through literature search, an interesting trend was found that myc mediated NDRG1 repression is always associated with differentiation process [21;73-75]. In these reports, NDRG1 has been reported to be induced by myc suppression resulted from differentiating stimuli. Until recently, no report has been published to describe the myc mediated NDRG1 downregulation in neoplastic development. Considering the importance of myc in the terminal differentiation of many cell types [78;79], myc may be pivotal for the NDRG1 function related to cell differentiation. However, through functional analysis, we demonstrated that NDRG1 might be irrelevant to function as a differentiation modulator in OSCC cells although it is capable of doing so in the leukaemia cell line U937 (from both regulatory and functional studies, Ref. [74] and [332]). Therefore, downregulation of NDRG1 by IGF may be a redundant event with little effect in the progression of OSCC, which is probably determined by the cell context, if the NDRG1 suppression effect of IGF signalling is entirely mediated by myc in OSCC cells. Furthermore, we noticed that the suppressive effect of PI-3K on NDRG1 expression itself is cell type specific, as PI-3K activation has also been reported to be positively linked to NDRG1 expression in human endometrial carcinoma and mouse epidermal cells C141 [208;265]. Therefore, it is highly possible that the IGF/PI-3K/myc mediated NDRG1 suppression may be just a by-product without functional significance of a disturbed signalling network.

3.5 Discussion

In this chapter, we present evidence that NDRG1 protein expression is actively regulated in OSCC, suggesting potential implication of NDRG1 in this disease. Although non-responsive to differentiation stimuli, which further indicates the probable irrelevance between NDRG1 and differentiation in OSCC cells, NDRG1 still has a wide range of regulatory response to growth factor mediated mitogenic signals as well as various cellular stresses. As discussed previously, all of the NDRG1 regulators can contribute to the tumour progression of OSCC.

Nevertheless, similar as in other types of cancer, not many of the NDRG1 regulators demonstrated an identical response in all the OSCC cell lines tested, with the exception of only hypoxia, MAP kinase (ERK), and PI-3 kinase, suggesting the complexity of NDRG1 regulation. Moreover, the regulatory effects of oncogenic stimuli on NDRG1 expression in OSCC also seem to be dichotomous. Both stress response (low extent versus high extent) and mitogenic stimuli (EGF versus IGF) are good examples for this. Therefore, clarifying the significance of a certain NDRG1 regulatory pathway in the development of OSCC based on the information from gain-of-function or loss-of-function studies will greatly contribute the understanding of the role or position of NDRG1 in this deadly disease.

Despite the highly conflicting observations regarding NDRG1 regulation, it is generally agreed to consider NDRG1 as a hypoxia responsive gene [51]. Similar observation has also been demonstrated in OSCC, considering the common involvement of hypoxia in this disease [317-319]. In our study, NDRG1 has been found to be consistently upregulated by hypoxia mimicking agents nickel and cobalt in several OSCC cell lines. Moreover, enhanced invasive and angiogenic abilities, two of the most typical outcomes of hypoxic

adaptation, have been positively linked to ectopic NDRG1 overexpression in KYSE30 cells (Chapter 2).

Besides, hypoxia is also found to be associated with apoptosis [320;333]. Interestingly, an anti-apoptotic (pro-survival) function of NDRG1 is observed in KYSE30 cells against the insults from hypoxia mimics and DNA damage. Consistently, in OSCC cells, NDRG1 can be upregulated by a variety of stress signals, with an intriguing feature that when stress signal become harsh enough to induce cell apoptosis, the previously upregulated NDRG1 is largely diminished or even downregulated. In agreement with our observation in OSCC cell lines, it has been shown that NDRG1 upregulation, either physiologically or ectopically, attenuates the apoptotic response to hypoxia or camptothecin derivatives (as DNA damaging agents) in trophoblast or colon cancer cell lines respectively [67;268], while physiological and ectopic NDRG1 downregulation sensitizes these cells to apoptosis. Hence, evidence from the regulation pattern also suggests the participation of NDRG1 in the apoptotic response. Taken together, both regulatory and functional studies suggest NDRG1 as a stress-adaptive gene, which may alleviate insults and consequently modify the cellular context in response to several kinds of cellular stress, especially hypoxia.

It is well accepted that the function of a gene in the neoplastic development may always be the reflection of its role in normal physiological process. As discussed previously, our work indicated NDRG1 as a hypoxia responsive gene. Interestingly, a similar role of NDRG1 has been observed in the trophoblasts during the early development of placenta which occurs in an environment of hypoxia [67]. Simultaneous with elevating invasive and angiogenic activities, NDRG1 is upregulated in these trophoblasts. Moreover, functional studies directly demonstrated that the upregulation of NDRG1 was crucial for the apoptotic evasion against hypoxia, which ensured the

maturation of trophoblasts. Thus, NDRG1 may be postulated as a hypoxic effector which systematically mediates the hypoxia associated phenotypic changes in both normal physiological (e.g. in trophoblasts) and neoplastic (e.g. in OSCC) developments.

Constitutive activation of mitogenic signalling is a common phenomenon in OSCC cells. In this project, we for the first time described the regulatory effect of oncogenic growth factors such as EGF and IGF on NDRG1 expression in the context of neoplasm. Surprisingly, in OSCC cells, diverse effects of EGF and IGF were observed on NDRG1 regulation, with EGF showing an inductive effect and IGF showing a suppressive effect. Thus, a question is raised that, either up regulation or downregulation of NDRG1 is physiologically important in the progression of OSCC. As previously explained in the result sections, ectopic overexpression of NDRG1 may elicit oncogenic effects to promote cellular metastasis, angiogenesis and apoptotic evasion, which partially overlap the oncogenic outcomes of EGF signalling. In contrast, ectopic NDRG1 downregulation (shRNA mediated knock-down) was demonstrated insignificant in modulating the cellular phenotypes of neoplastic development. Therefore, it is conceivable to suspect the significance of IGF/PI-3K/c-myc mediated NDRG1 downregulation revealed in Section 3.4.2. Moreover, considering the close relationship between myc mediated NDRG1 downregulation and cell differentiation [21;73-75], it may be appropriate to associate the IGF/PI-3K/c-myc mediated NDRG1 downregulation to cell differentiation, probably in normal oesophageal epithelial cells. However, the relationship between NDRG1 and differentiation has been shown masked in OSCC cells (Section 2.3.1 and 3.2), thus making the IGF/PI-3K/c-myc mediated NDRG1 downregulation as a redundant regulatory event in the neoplastic progression of OSCC.

Taken together, our work highlighted NDRG1 as a hypoxia or EGF responsive gene mediating the phenotypic changes when cells were under the stimuli from hypoxia or EGF signalling.

* * *

Besides its wide ranging regulatory responses, NDRG1 is also capable of being phosphorylated physiologically by several kinases (Section 1.2.1). As a result of the antibody used in this study (from Kinasource, Scotland), we were able to detect the phosphorylated form of NDRG1 in the Western immunoblots. Two major patterns of phosphorylated NDRG1 could be observed in the blots. Although potentially affected by the inconsistency of the SDS-PAGE gels that were cast in-house, and the electrophoresis conditions, in most of the cases, a distinctive "double-band" pattern was observed in EPC2 and KYSE30 cells for NDRG1, while in KYSE150 and KYSE180 cells, NDRG1 blots displayed a pattern of a single band together with a faint smear. According to the antibody manufacturer, both the "upper band" and the "smear" can be viewed as phosphorylated NDRG1. In KYSE30 cells, the "upper band" could be readily abolished by serum deprivation and kinase inhibitors such as LY294002 (PI-3K inhibitor) and GF109293X (pan PKC inhibitor). Conversely, elevated levels (or an increased proportion) of the "upper band" could be observed under various kinase activating stimuli including genotoxic stress, hypoxia, calcium ionophore A23187, EGF and IGF. Therefore, we confirmed the "upper band" as a form of phosphorylated NDRG1. Regarding the "smear", due to the low resolution of 1D normal (not gradient) SDS-PAGE gels, hypothesizing the smears (in KYSE150 and KYSE180 cells) as a form of phosphorylated NDRG1 may not be as convincing, although supportive evidence could be also drawn through kinase activating treatment such as genotoxic stress, hypoxia, EGF (KYSE180 only) and IGF. Taken together, our data suggest that part of NDRG1 can be phosphorylated in normal oesophageal and OSCC cells, and the phosphorylation pattern of NDRG1 may be cell-type specific. Interestingly,

the “double-band” pattern could also be effectively manifested in KYSE150 and KYSE180 cells under the treatment of A23187, while JNK inhibition by SP600125 in EGF-stimulated KYSE30 cells significantly reduced the distinctive “upper band” to a “smear”. These observations further substantiate the notion that the different patterns of NDRG1 phosphorylation in different OSCC cells may be a reflection of the diverse patterns (profiles) of the activated kinases in these cells.

Considering the importance of phosphorylation as a modulator of protein function, it is hence postulated that the difference in NDRG1 phosphorylation patterns in KYSE30 and KYSE150 cells may, in part, account for the functional relevance of NDRG1 in KYSE30 cells but not in KYSE150 cells. This proposal is underscored by the observation that the NDRG1 phosphorylation pattern in KYSE30 cells is similar to that in EPC2 cell line, a representation of the normal oesophageal cell context. However, the exact phosphorylation patterns of NDRG1 in KYSE30 and KYSE150 cells still remain unknown, although the available data from specific kinase inhibitors indicate that NDRG1 phosphorylation may relate to PI-3K and PKC pathways, but not Akt and ERK in KYSE30 cells. Moreover, we also do not know the impact of phosphorylation of specific residues (or by a certain kinase) on the function of NDRG1. A research project clarifying the pathways/kinases responsible for NDRG1 phosphorylation as well as the consequent impacts on the function of NDRG1 in KYSE30 cells is currently underway.

Chapter Four

Conclusion

Although the biological function of NDRG1 has been explored in several types of cancer, very little is known about the function of NDRG1 in oesophageal squamous cell carcinoma (OSCC). Here, we provide the first evidence, through both *in vitro* and *in vivo* gain-of-function (ectopic overexpression) studies, that NDRG1 may perform positive but non-essential roles in the progression of OSCC by promoting metastasis, angiogenesis and apoptotic evasion. Consistently, the upregulation of NDRG1 during OSCC progression revealed by our immunohistochemical study of clinical samples, as well as the upregulation of NDRG1 by various oncogenic stimuli, may also serve as predictors of its oncogenic role in the neoplastic development of OSCC.

Although some studies claim NDRG1 as a potential proliferation inhibitor and differentiation inducer, these results could not be reproduced in many other experimental platforms, including KYSE30 and KYSE150 cells in our studies. Moreover, the insensitivity of NDRG1 to differentiation stimuli as well as the insignificant correlation between NDRG1 level and histological subtype in OSCC patient samples by previous [205;206] and our studies, further indicates the probable irrelevance of NDRG1 to differentiation in OSCC.

In addition, no significant phenotypic alteration was observed through loss-of-function studies, although effective knock-down efficiency (70%-80%) elicited in the transfectants investigated. As discussed in section 2.6.2, the dispensable (non-essential) role of NDRG1 in the progression of OSCC may also be inferred from the null effect of NDRG1 knock-down. Despite the lack of direct evidence in OSCC, we noticed that the dispensable role of NDRG1 was

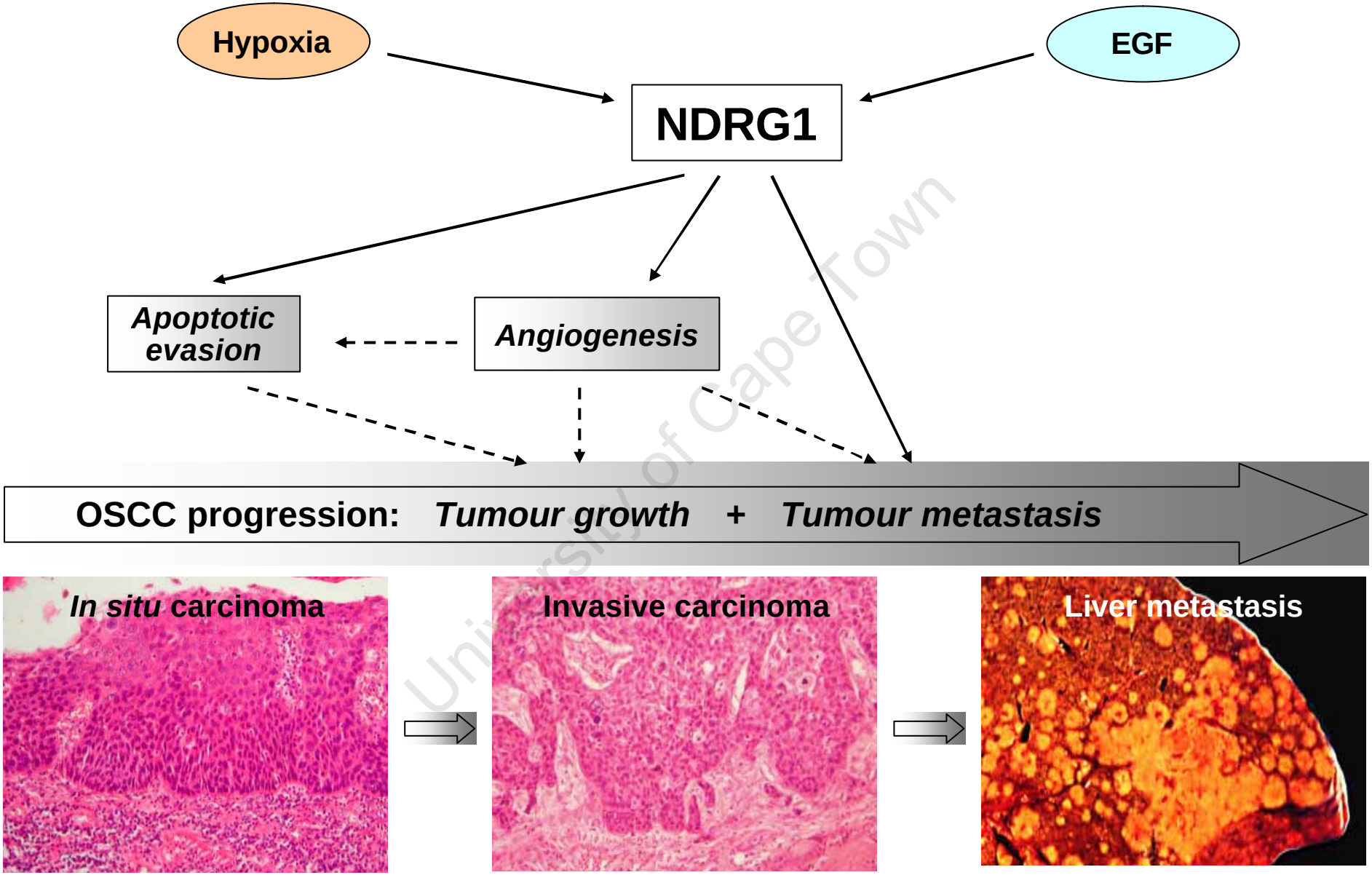
also observed in the foetal and postnatal development of brain and kidney [38;61], as well as in the differentiation of trophoblast [67]. Hence, the existence of compensatory processes for NDRG1 function is proposed, which clearly requires further exploration.

Besides the direct ectopic gain-of-function and loss-of-function studies, many valuable clues regarding NDRG1 function in OSCC cells can also be inferred from our investigation of its regulatory pattern. As suggested by our functional studies, NDRG1 may perform oncogenic roles in the progression of OSCC. Interestingly, a general agreement is found between the regulatory pattern of NDRG1 and its function, suggesting that the findings in the ectopic functional studies were not artifactual. As a potential hypoxia responsive gene in OSCC, NDRG1 facilitates the adaptive response via the enhancement of angiogenic activity and apoptotic evasion. Furthermore, the pro-metastatic property of NDRG1 can also be viewed as a reflection of the outcome from hypoxia adaptation. Similarly, evidence suggesting NDRG1 as an apoptosis alleviator can also be gathered from the response of this gene to stress stimuli, along with its downregulation when cells become apoptotic, since it is prerequisite for cells to eliminate or inactivate anti-apoptotic molecules before executing apoptotic programme. Moreover, partially sharing some of the oncogenic properties of EGF signalling, NDRG1, which can be readily upregulated by EGF/MAPK signalling, is also expected to carry out some of the EGF related phenotypic changes, such as promotion of metastasis, angiogenesis and apoptotic evasion. Finally, although another oncogenic axis of IGF/PI-3K/c-myc was identified in OSCC cells as a suppressive pathway of NDRG1 expression, the functional significance of this pathway is severely suspected due to the null phenotypic alteration in the NDRG1 loss-of-function study using shRNA.

Taken together, all of the above observations may imply an oncogenic role for NDRG1 in OSCC progression as an effector of hypoxia or EGF signalling, through positively modulating the cellular activities of metastasis, angiogenesis and apoptotic evasion. In addition, although NDRG1 has been demonstrated irrelevant to the intrinsic proliferation machinery in OSCC cells, its promotional effects on angiogenesis and apoptotic evasion may indirectly promote the tumour growth *in vivo* as demonstrated in our xenograft study. Considering the mutual relationship between these neoplastic events (phenotypes), it is conceivable that NDRG1 may mediate a series of systematic changes, either directly or indirectly, and eventually facilitate progression of the tumour cell to a more malignant phenotype (Fig 4-1).

Nevertheless, as observed in other types of cancer, the function of NDRG1 in OSCC may be largely dependent on the context of OSCC cells. As shown in this study, NDRG1 only elicits oncogenic effect in KYSE30 cells with a lower malignant grade, but tends to be functionally insignificant in highly malignant KYSE150 cells. As discussed in section 2.6.3, although still needed to be further investigated, the cell-type specific function of NDRG1 may reflect the fact that NDRG1 is only functional as an oncogene at a certain stage of OSCC progression, in which the neoplastic changes still retain the upstream modulators or downstream effectors required for effective NDRG1 function, or the neoplastic changes are still not strong enough to mediate malignant progression independent of NDRG1.

Fig 4-1 (overleaf): Schematic model of NDRG1 function in OSCC. Upregulation of NDRG1 by oncogenic stimuli such as hypoxia and EGF signalling directly promotes metastasis, angiogenesis and apoptotic evasion. Moreover, enhanced tumour growth can be indirectly attributed to angiogenesis and apoptotic evasion. All these phenotypic changes reflect the oncogenic role of NDRG1 in the progression of OSCC.
Arrow (→), direct effects; *broken arrow* (⇢), indirect effects; pictures were taken from http://pathology.tmu.edu.tw/microscopy/showall.asp?no=1931



Although evidence from both functional and regulatory studies indicates the potential oncogenic role of NDRG1 in the progression of OSCC, the molecular mechanism of how NDRG1 exerts its function still largely remains unclear. Similar to the recent microarray analysis in prostate cancer cells [280], we observed in OSCC cells that NDRG1 may act as a transcriptional repressor against activating transcription factor 3 (ATF-3). Like NDRG1, ATF-3 also shows pleiotropic but cell type specific role in the neoplastic development. Thus, ATF-3 could act as a downstream effector of NDRG1 in OSCC cells. Besides ATF-3, NDRG1 was observed capable of modulating the mRNA levels of many other genes (e.g. MMP-2, VEGF-A and VEGF-C), suggesting the ability of NDRG1 to influence the cellular transcriptome. Considering the tight connection between the cellular transcriptome and phenotype, a high-throughput cDNA microarray analysis is now being planned to explore the NDRG1 downstream effectors in the OSCC cells, through which we aim to clarify the mechanism of the oncogenic effects of NDRG1 in the progression of OSCC. In addition, due to the limited number of functional assays we can perform, microarray analysis combined with comprehensive *in silico* analysis may also be useful to discover novel NDRG1 related genes responsible for the currently unknown function of NDRG1 in the progression of OSCC. Finally, identification of NDRG1 downstream effectors will definitely shed light on the explanation of the cell-type specificity of NDRG1 function in OSCC cells.

In conclusion, our study suggests a pro-oncogenic but dispensable role of NDRG1 in the development of OSCC by modulating the metastatic, angiogenic and anti-apoptotic activities. The potential participation of NDRG1 in the response to hypoxia and EGF signalling in OSCC is also suggested, although the exact mechanism of the NDRG1 function still needs to be further investigated.

Chapter Five

Materials and Methods

5.1 Cell culture and reagents

The human oesophageal squamous cell carcinoma (OSCC) cell lines KYSE30, KYSE150, KYSE180 and KYSE450, previously established by Shimada and co-workers [289], were purchased from the German Resource Centre for Biological Material (http://www.dsmz.de). The cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS). EPC2 cell line, characterized as human telomerase (hTERT) immortalized oesophageal keratinocytes, was a gift from Dr. Rustgi [311;312]. EPC2 cells were maintained in keratinocyte serum-free medium containing 40 μg/mL bovine pituitary extract and 1 ng/mL EGF. To avoid terminal growth arrest, EPC2 cells were always subcultured before reaching confluence. All the cells were grown in a humidified incubator containing 5% CO_2 at 37 °C and were routinely tested every 3 months for mycoplasm contamination (by Hoechst 33258 staining). Materials for cell culture are all from Gibco (Invitrogen, Carlsbad, CA).

Antibody purchasing will be described in the sections of Western immunoblotting and immunohistochemistry (section 5.4.3 and 5.7.2). Oligos for shRNA vector construction and PCR primers were obtained from the custom oligo synthesis service of the Integrated DNA Technologies (San Diego, CA). Enzymes for molecular cloning such as restriction endonucleases, T4 ligase and Antarctic phosphatase are all from New England Biolabs (Ipswich, MA). Matrigel was purchased from BD Biosciences (San Jose, CA) and was diluted in serum free RPMI-1640 medium before use. Kinase inhibitors PD98059, GF109293X, SP600125 and rapamycin are purchased from Calbiochem (now part of Merck Ltd., Modderfontein, South Africa). All the other reagents, unless specified, were purchased from Sigma-Aldrich (St-Louise, MO).

5.2 Establishment of transfectants with altered NDRG1 expression

5.2.1 Lentiviral vectors

The bi-cistronic lentiviral vectors pWPI and pLVTHM developed in the lab of Dr. Trono (Ref. [334] and http://tronolab.epfl.ch, Fig 5-1), as well as the second-generation lentivirus packaging plasmid psPAX2 and envelope plasmid pMD2.G, were obtained from a non-profit plasmid repository Addgene (http://www.aggdene.org). pWPI, with a human EF1 promoter and a green fluorescent protein (GFP) reporter, was used to express the NDRG1 gene ectopically. Sharing the same backbone as pWPI vector, the pLVTHM vector carries an additional H1 RNA polymerase III promoter to permit the expression of a short hairpin RNA (shRNA) for RNA interference (RNAi). Due to the similarity of pWPI and pLVTHM vectors, the RNAi sister control vector bearing a "scrambled" non-targeting RNAi sequence was used as vector control for both ectopic NDRG1 overexpression and knock-down by RNAi.

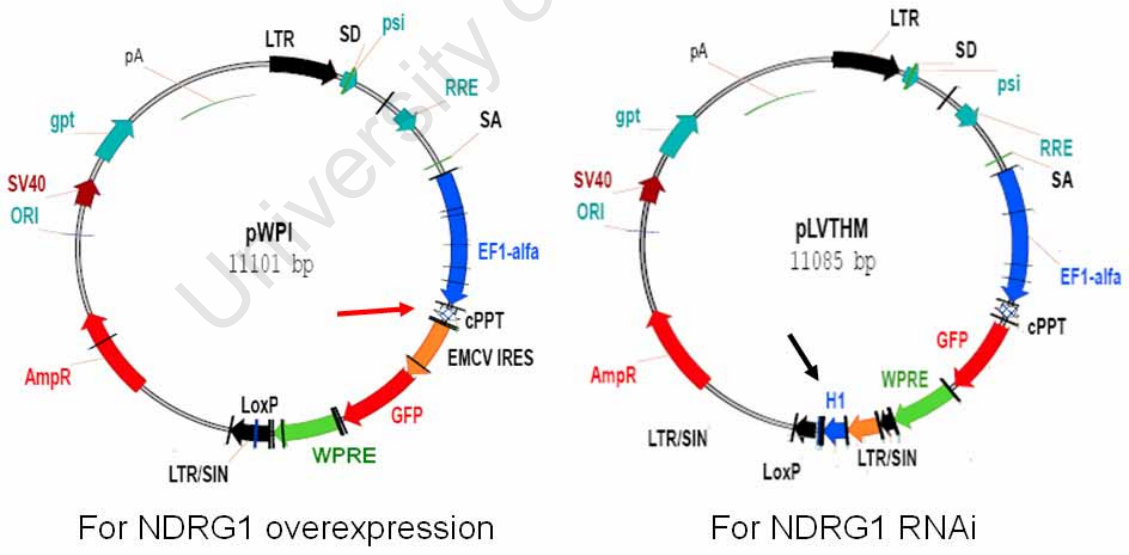


Fig 5-1: Lentiviral vectors for ectopic NDRG1 overexpression (pWPI) and knock-down (pLVTHM). Arrows indicate where exogenous sequences are cloned in for NDRG1 overexpression (under EF1-α promoter) and RNAi (5'-GGAGTCCTTCAACAGTTTG -3'; under H1 promoter). The sequence 5'-GGGTCTTAGAACTAGTTCC -3' was used to generate control shRNA. Vector maps are adapted from http://www.aggdene.org.

5.2.2 Competent cell preparation and transformation

Fresh competent *E. coli* cells (DH5α strain) were prepared using a method described by Inoue et al. [335]. Compared to the traditional $CaCl_2$ method, this method generates competent cells with much higher transformation efficiency (~100 times) and is thus more suitable for the transformation of big sized lentiviral vectors. Briefly, one colony of *E. coli* was inoculated into 100 mL SOB broth and cultured overnight at 18 °C with vigorous shaking (~250 rpm) until the OD_{600} reached 0.6. The culture was then transferred into sterile polypropylene tubes, cooled on ice for 10 minutes and centrifuged at 2500 × g for 5 min at 4 ºC. The bacteria pellet was resuspend in ice-cold Inoue buffer (10mM Pipes-KOH, pH = 6.7, 15 mM $CaCl_2$, 250 mM KCl, and 55 mM $MnCl_2$), incubated on ice for 10 min and centrifuge again at 1000 × g for 5 min at 4 ºC. Finally, the bacterial pellet was resuspended in ice-cold Inoue buffer with 7% (v/v) DMSO; aliquoted of 100 ? L into each pre-chilled Eppendorf tube; and snap frozen in liquid nitrogen. The competent cells can be stored in -80 ºC freezer for at least 3 months without decreasing transformation efficiency.

Transformation was performed as follows. Quickly thawed competent cells was mixed with plasmid DNA (or ligation products), heat-shocked at 42 ºC for 90 seconds and chilled to 4 °C (in a PCR machine). Cells were then incubated at 37 °C with gentle shaking to recover in 0.5 mL of pre-warmed SOC broth before plating onto LB agar plates with 100 μg/mL ampicillin as selector. Plates were incubated at 30 °C to minimize the potential recombination of lentiviral vectors. For the same reason, to expand the lentiviral vectors, plasmid containing *E. coli* was also cultured at 30 °C in LB broth with gentle shake.

5.2.3 DNA preparation

Wizard® Plus Minipreps DNA Purification System (Promega, Madison, WI) was used for small-scale plasmid preparation from 10mL overnight *E. coli*

culture. Products prepared from this kit were used for sub-cloning and sequencing.

The Endofree Maxiprep Kit (QIAGEN GmbH, Hilden, Germany) was used for large-scale plasmid preparation from 250 mL overnight *E. coli* culture. Products prepared from this kit were used for transfection.

QIAquick Gel extraction kit (QIAGEN GmbH, Hilden, Germany) was used to recover separated DNA fragments from agarose gel (TBE buffer based). Products prepared from this kit were used for sub-cloning.

5.2.4 Vector construction

The coding sequence of human NDRG1 gene was previously cloned into the *Xho* I and *Not* I sites of pcDNA 3.1(+) vector (Invitrogen, Carlsbad, CA). To construct pWPI-NDRG1 for ectopic NDRG1 overexpression, the NDRG1 fragment in pcDNA 3.1(+) vector was cut out with *Pme* I and *EcoR* V and sub-cloned into the *Pme* I site of the pWPI vector using T4 ligase (1:3 molar ratio of vector and insert, 22 °C for 2 hours in a PCR machine). The direction of the NDRG1 insert was confirmed by *Xho* I digestion when screening the colonies (Fig 5-3 A, empty vector: 0.4kb, 1.4kb + backbone; pWPI-NDRG1 correct direction: 0.4kb, 2.6kb + backbone; pWPI-NDRG1 incorrect direction: 0.4kb, 1,2kb, 1.4kb + backbone).

For NDRG1 RNAi, two complementary DNA oligonucleotides as shown in Fig 5-2 were synthesized with 5'-phosphorylation (to facilitate ligation) and PAGE purification. The sequences for NDRG1 RNAi and scrambled sister control were

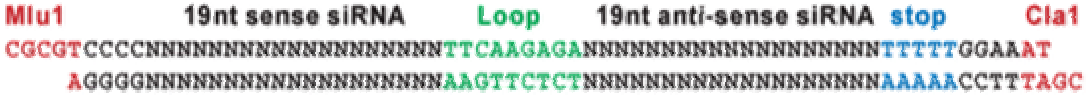


Fig 5-2: Oligo designing for shRNA expression using pLVTHM vector. Annealed fragment is cloned in to the Mlu I and Cla I sites of the pLVTHM vector (http://tronolab.epfl.ch).

5'-GGAGTCCTTCAACGATTTG-3' and 5'-GGGTCTTAGAACTAGTTCC-3' respectively [67]. Oligos were dissolved in TE buffer (pH = 8.0) at a concentration of 1 mg/mL. 2 μL of each oligos were mixed with 46 μL annealing buffer (containing 100mM potassium acetate, 2mM magnesium acetate and 30mM HEPES-KOH, pH = 7.4) and annealing was performed in a PCR machine using the following protocol: 95 ° C for 4 min; 85 ° C for 2 minutes; 82 ° C for 2 min; 80 ° C for 4 min; 78 ° C for 2 min; 75 ° C for 4 min; 72 ° C for 4 min; 70 ° C for 15 min; 65 ° C for 4 min; 60 ° C for 4 min; 50 ° C for 4 min; 40 ° C for 4 min; 37 ° C for 20 min and 4 ° C for ∞. The pLVTHM vector was first digested with *Mlu* I, ethanol precipitated with 3M sodium acetate (pH = 5.2) and digested again with *Cla* I. To decrease the potential of "self-ligation", the digested vector was de-phosphorylated with Antarctic phosphatase (CIP enzyme) before subjected to ligation at 22°C for 2 hours (1:15 molar ratio of vector and insert). Positive colonies were screened by *EcoR* I and *Xba* I digestion (Fig 5-3 B, empty vector: 492bp + backbone; with shRNA construct: 557bp + backbone).

All the three constructions were confirmed by automatic sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). DNA amplification was done according to the manufacturer's instruction. Sample cleanup and automatic DNA sequencing (capillary electrophoresis) were performed by the Core DNA Sequencing Facility in Stellenbosch University using 3130 Genetic Analyser (Applied Biosystems). Sequencing results can be referred to Appendix (Fig S-1 to S-5). Sequencing primers used in this study are listed as below:

- Primers for pWPI-NDRG1, the numbers in the brackets are based on the NDRG1 coding sequence from the mRNA sequence NM_006096.2 (NCBI, http://www.ncbi.nlm.nih.gov) used for the BLAST analysis (in Appendix):

  Forward 1 (SN-F1): 5'- GTCATCCTCACCTACCAT -3' (172-189)

  Forward 2 (SN-F2): 5'- CCTGCTCTGTTGGTGGTT -3' (760-777)

  Reverse 1 (SN-R1): 5'- ACTGTTGAAGGACTCCAG -3' (385-368)

- Primer corresponding to the H1 promoter of pLVTHM vector, for validating the sequences of NDRG1 RNAi and scrambled sister control,:

  H1: 5'- GCATGTCGCTATGTGTTCT -3'

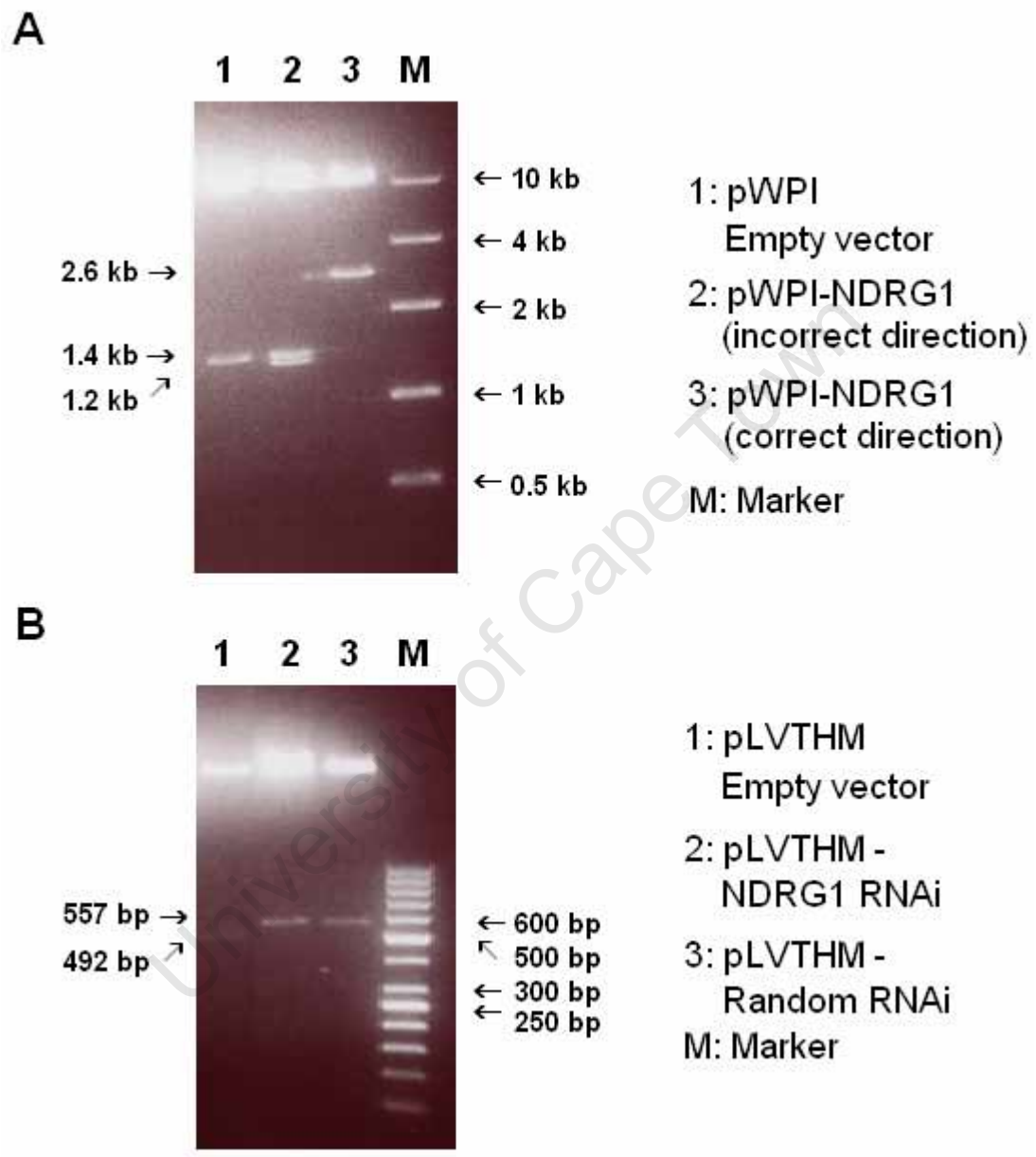


Fig 5-3: Restriction enzyme digestion to verify the constructs. Samples were separated by electrophoresis in 1% agarose gels containing 0.5 µg/mL of ethidium bromide. Results were visualized and photographed under UV light. **A,** pWPI vectors digested by *Xho* I. FastRuler™ DNA Ladder, High Range (Fermentas Life Sciences, Glen Burnie, MD) was used to monitor the size of the bands. The theoretical 400bp band was invisible due to the low amount of plasmid checked. **B,** pLVTHM vectors digested by *EcoR* I and *Xba* I, GeneRuler™ 100bp DNA Ladder (Fermentas) was used to monitor the size of the bands.

5.2.5 Lentivirus production and transduction

Lentiviral particles were produced by transient transfection of human embryonic kidney 293FT cells (Invitrogen). 293FT cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% FBS. Subconfluent, 293FT cells in one 75 cm^2 flask were co-transfected with a cocktail containing 8.5 µg of lentiviral vector, 3.9 µg of the packaging plasmid psPAX2, and 1.3 µg of the envelope plasmid pMD2G diluted in 105 µL of OPTI-MEM (Invitrogen) plus 33.5 µL of FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany) diluted in 386.5 µL of OPTI-MEM. Fresh culturing medium (DMEM with 10% FBS) was changed 12 hours later for virus production. Medium containing lentiviral particles was collected 36 hours post-transfection; filtered through Millex-HV 0.45-µm PVDF filter (Millipore, Billerica, MA) and stored in a -80 °C freezer. In our experience, the lentiviral particles can be stored for at least 1 month without losing infection efficiency.

Before transduction, an aliquot of virus containing medium was titrated using serial dilution method [336]. When targeted cells reached subconfluent, cells were incubated with lentiviral particles with an MOI (multiplicity of infection) of 2-3, along with 8 µg/mL hexadimethrine bromide for 16 hours before fresh culturing medium was changed. Consequently, a transduction “pool” with exotic fragment being expressed in most of the cells was obtained, due to the theoretically high transduction efficiency of lentiviral vector mediated gene delivery. The transduction efficiency and the stability of the transfectants were monitored by FACS analysis through the percentage of GFP positive cells.

5.3 Quantitative Real-time RT-PCR

5.3.1 RNA isolation

Total RNA for quantitative real-time RT-PCR (qRT-PCR) analysis was prepared from subconfluent cells grown in monolayer using TRIzol LS Reagent

(Invitrogen) according to the manufacturer's instructions. TRIzol LS Reagents is a ready-to-use monophasic solution of phenol and guanidine isothiocyanate suitable for total RNA isolation. As recommended by the instruction, 0.75 mL of TRIzol LS reagent was added to a 60-mm culture dish for homogenization.

RNA product was dissolved in nuclease free water and stored at -80 °C. For quality control, the concentration was calculated from the absorption at 260 nm in TE buffer (pH=7.5) and the purity/integrity of the RNA preparations was checked by electrophoresis of a 0.5 µg aliquot on a 1% MOPS/formaldehyde denaturing agarose gel. Only the samples showing ① $A_{280}/A_{260} > 2.0$; ② clear 28S, 18S (with a ratio near 2) and 5S rRNA bands; and ③ faint or invisible genomic DNA band; was used for the further analysis.

5.3.2 Quantitative Real-time RT-PCR

For quantitative real-time RT-PCR, 1 µg of total RNA and 0.5 µg of oligo-dT_{15} were used for cDNA synthesis with ImProm-II™ Reverse Transcriptase system (Promega, Madison, WI). Reverse transcription was performed on GeneAmp PCR System 2700 (Applied Biosystems) under the conditions of 25 °C for 5 minutes, 42 °C for 1 hour, 72 °C for 15 minutes and 4 ° C for ∞. Quantitative real-time RT-PCR was done with KAPA SYBR® qPCR Kit (KAPABiosystems, Boston, MA) on a StepOne Real-time RT-PCR machine (Applied Biosystems) under the conditions of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds, annealing for 20 seconds and 72 °C for 45 seconds. Transcript quantification was performed in triplicate for each sample and three independent experiments were done for each gene. The relative fold change was determined by $2^{-\Delta\Delta cT}$ method using β-actin as reference gene. All the PCR products were checked by melting curve analysis and electrophoresed on 2% agarose gel to ensure PCR specificity (Fig 5-4). Primer sequence and condition for qRT-PCR are listed in Table 5-1. Primers for VEGF-A, VEGF-C and PDGF-B were designed to detect all the transcript variants.

Table 5-1: Primers used for quantitative real-time RT-PCR

Transcript *	Primer	Sequence (5'-3')	Annealing temperature
NDRG1 [67]	Forward	CCGCCAGCACATTGTGAAT	60 °C
	Reverse	GGCTGTTGTAGGCATTGATGAA	
VEGF-A [337]	Forward	TTCATGGATGTCTATCAGCG	60 °C
	Reverse	GCTCATCTCTCCTATGTGCT	
VEGF-C [299]	Forward	GCCAACCTCAACTCAAGGAC	60 °C
	Reverse	CCCACATCTGTAGACGGACA	
Angiopoitein-1 (Ang-1) [299]	Forward	TTCCTTTCCTTTGCTTTCCTC	55 °C
	Reverse	CTGCAGAGCGTTTGTGTTGT	
PDGF-B	Forward	TTATGAGATGCTGAGTGACCAC	55 °C
	Reverse	CCTTCTTCCACGAGCCAAG	
MMP-2 [338]	Forward	TGGCGATGGATACCCCTTT	57.5 °C
	Reverse	TTCTCCCAAGGTCCATAGCTCAT	
MMP-9 [338]	Forward	CCTGGGCAGATTCCAAACCT	53 °C
	Reverse	GCAAGTCTTCCGAGTAGTTTTGGAT	
ATF-3 [280]	Forward	AGTCACTGTCAGCGACAGAC	57.5 °C
	Reverse	TGCTTCTCGTTCTTGAGCTC	
c-myc	Forward	CGCTTCTCTGAAAGGCTCTC	57.5 °C
	Reverse	CTCCTCCTCGTCGCAGTAG	
β-actin **	Forward	ATCGTGCGTGACATTAAGGA	53-60 °C
	Reverse	AGGAAGGAAGGCTGGAAGAG	

* The sizes of the qRT-PCR products are list as follows: *NDRG1*: 67 bp; *VEGF-A*: 236 bp; *VEGF-C*: 200 bp; *Ang-1*: 184 bp; *PDGF-B*: 156bp; *MMP-2*: 117 bp; *MMP-9*: 88 bp; *ATF-3*: 227 bp; *c-myc*: 169 bp; *b-actin*: 178 bp.

** The qRT-PCR of β-actin as reference gene was performed simultaneously with the gene to be tested. Thus, same annealing temperature of the gene to be tested was used for β-actin in the experiments.

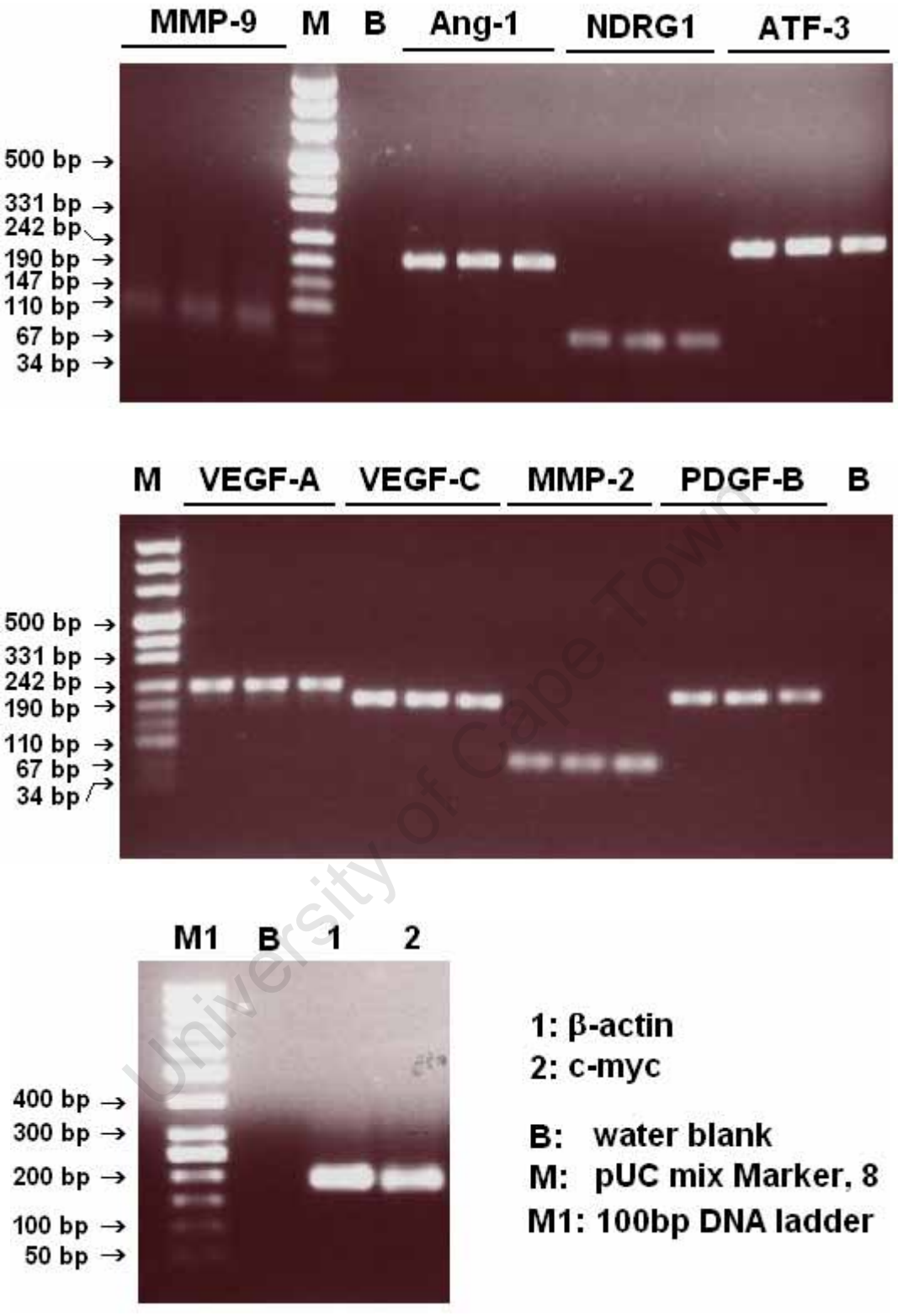


Fig 5-4: Electrophoresis of the qRT-PCR products. The PCR products were separated in 2% agarose gels containing 0.5 µg/mL of ethidium bromide. Results were visualized and photographed under UV light. pUC mix Marker, 8 (Fermentas, **M**) or GeneRuler™ 100bp DNA Ladder (Fermentas, **M1**) was used to monitor the size of the bands. "**B**" represents the water blank (null template) control for qRT-PCR.

5.4 Western immunoblotting

5.4.1 Cell lysate preparation

Cell lysates for Western immunoblotting analysis were, unless specified, always prepared from subconfluent cells. Briefly, cells were rinsed with cold PBS and lysed using RIPA buffer containing 150mM NaCl, 50 mM Tris-HCl (pH=7.5), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EGTA, 1 mM EDTA, phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate) and Complete™ protease inhibitor cocktail (Roche Diagnostics). Cell lysates were cleared by being passed through 25-gauge insulin syringe and centrifuged at 13,000 × g at 4 °C for 15 minutes.

Protein samples were stored in a -80 °C freezer. The protein concentration was determined by the bicinchoninic acid (BCA) assay using a kit from Pierce (Rockford, IL) before electrophoresis (SDS-PAGE).

5.4.2 SDS-PAGE and membrane transfer

SDS-PAGE and membrane transfer are performed using Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Hercules, CA). Gels (0.75-mm thick) were cast according to the recipe shown in Table 5-2. Protein sample (20 µg per well for 10-well gel or 15 µg per well for 15-well gel) was mixed with 5 × loading buffer (containing ß-mercaptoethanol as reducing agent), heated at 95 °C for 5 minutes and rapidly cooled on ice before loading. Electrophoresis was performed under constant current (12 or 15 mA per gel in stacking or resolving gels) until the bromophenol blue was eluted (about 1.5 hours). The size of the protein band is monitored by the Precision Plus Protein Kaleidoscope Standards (250 kDa to 10 kDa) purchased from Bio-Rad.

Table 5-2: Recipe for the SDS-PAGE gel

10% Resolving gel (8 mL):		5% Stacking gel (1.8mL):	
30% Acr + Bis (30:0.8)	2.66 mL	30% Acr + Bis (30:0.8)	300 µL
4× Resolving buffer	2.0 mL	4× Stacking buffer	450 µL
dH_2O	3.33 mL	dH_2O	1.05 mL
10% AP	80 µL	10% AP	30 µL
TEMED	8 µL	TEMED	3 µL

Separated protein was next transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences) at 100 V for 1.5 hours (wet method, done in 4 °C fridge). Transferred gel was stained for 1 hour with Coomassie brilliant blue R-250 staining solution, and de-stained overnight with the aid of a piece of sponge. De-stained gels were dried under vacuum on a piece of filter paper and stored as another reference for protein loading.

5.4.3 Blot processing

All the following steps were performed on an orbit shaker at the speed of 75 rpm. Blots were first blocked with 5% skimmed milk powder or 5% bovine serum albumin (BSA, fraction V, from Roche Diagnostics) dissolved in Tris-buffered saline (pH=7.4) with 0.1% (v/v) Tween-20 (TBST) for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4 °C. Antibody dilution factors were summarised in Table 5-3. Next, three 10-minute washes at room temperature in TBST were performed before blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 for anti-goat/sheep, 1:2500 for anti-mouse and anti-rabbit, diluted in blocking solution) at room temperature for 1 hour. The donkey anti-goat/sheep secondary antibody was obtained from The Binding Site Ltd. (Birmingham, UK), while both of the goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Bio-Rad. After another three 10-minute washes in TBST,

antibody detection was performed with chemiluminescence reagents from SuperSignal West Pico kit or SuperSignal West Dura kit (Pierce, Rockford, IL) according to the manufacturer's instructions. To ensure reproducibility, three independent repeats (from sample preparation) were done for each experiment, and typical blot was shown in the thesis.

In cases that the density (grey scale) of the blot is difficult for direct comparison through naked eye, densitometry was performed using QuantiScan Demonstration 3.0 software (Biosoft Inc, Cambridge, UK). Blots were first scanned to get high resolution images (600 dpi) and then converted to grey-scale images using Microsoft PowerPoint 2003 for quantification. To ensure the linear correspondence between blot density and the relative amount of the antigen, only blots without saturated bands (overexposure) were subjected to quantification. The density of each blot was normalized with the density of corresponding loading control (α- or β- tubulin). More than 2-fold change in the normalized density was considered significant.

5.4.4 Stripping the blot and re-probe

For re-probing, blots were stripped with 1 M glycine (pH=2.5) for 15 minutes at room temperature on an orbit shaker (60 rpm). Compared to the stringent SDS/ß-mercaptoethanol based protocols, the mild acidic glycine based stripping inflicted little damage on the antigens. Next, blots were extensively washed three times for 10 minutes each in TBST and blocked with 5% skimmed milk powder or 5% BSA in TBST for additional 30 minutes (done at 75 rpm) before subjected to another primary antibody.

Table 5-3: Antibody details for Western blot analysis *

Antigen	Description	Source ***	Blocking solution	Dilution Factor
NDRG1 **	sheep polyclonal	Kinasource, AB-160	5% skimmed milk	1:1000 in 1% skimmed milk
NDRG1 **	rabbit polyclonal	Zymed, Anti-Cap43	5% BSA	1:1000 in 1% BSA
c-myc	rabbit polyclonal	SCBT, sc-764	5% skimmed milk	1:500 in 2% skimmed milk
PARP	rabbit polyclonal	SCBT, sc-7150	5% skimmed milk	1:1000 in 5% skimmed milk
E-cadherin	rabbit polyclonal	CST, #4065	5% BSA	1:1000 in 1% BSA
involucrin	mouse monoclonal	Novocastra, NCL-INV	5% skimmed milk	1:200 in 1% skimmed milk
pErk1/2	mouse monoclonal	CST, #9106	5% BSA	1:1000 in 1% BSA
Akt (total)	rabbit polyclonal	CST, #9272	5% BSA	1:1000 in 1% BSA
pAkt	rabbit polyclonal	CST, #9271	5% BSA	1:1000 in 1% BSA
pGSK-3ß	rabbit polyclonal	CST, #9336	5% BSA	1:1000 in 1% BSA
p53	mouse monoclonal	DAKO, DO-7	5% skimmed milk	1:500 in plain TBST
$p21^{Cip/Kip}$	rabbit polyclonal	SCBT, sc-756	5% skimmed milk	1:1000 in plain TBST
$p27^{Cip/Kip}$	rabbit polyclonal	SCBT, sc-528	5% skimmed milk	1:1000 in 4% skimmed milk
GRP94	goat polyclonal	SCBT, sc-1794	5% skimmed milk	1:500 in 2% skimmed milk
HIF-1α	mouse monoclonal	SCBT, sc-53546	5% skimmed milk	1:500 in 0.5% skimmed milk
α-tubulin	mouse monoclonal	SCBT, sc-8035	5% skimmed milk	1:1000 in 1% skimmed milk
ß-tubulin	rabbit polyclonal	SCBT, sc-9104	5% skimmed milk	1:500 in 1% skimmed milk

* Tris-buffered saline (pH=7.4) with 0.1% (v/v) Tween-20 (TBST) is used as diluent for all reagents.

** Unless specified, the Kinasource antibody was used to detect NDRG1 in Western blot analysis due to its better specificity.

*** Both the company for antibody production and the catalogue number are shown. Kinasource is a company from Dundee, Scotland. Zymed is a subsidiary company of Invitrogen from San Francisco, CA. Novocastra is a company from Newcastle, England. SCBT stands for Santa Cruz Biotechnology, a company in Santa Cruz, CA. CST stands for Cell Signaling Technology, a company in Beverly, MA. DAKO stands for DakoCytomation, a company from Glostrup, Denmark.

5.5 Immunostaining

5.5.1 Immunofluorescence

For immunofluorescence, cells were grown to subconfluency on poly-lysine (MW = 150-300 kDa) coated round coverslips in 24-well plate. Treatment (if any) was done 24h prior to the immunofluorescence staining. Regarding the staining, cells were first fixed in 4% paraformaldehyde (diluted in phosphate buffer saline pH=7.4 or PBS) for 10 minutes; briefly rinsed with PBS and then incubated in PBS with 0.1% Triton X-100 and 100 mM glycine for permeabilization as well as quenching the trace of paraformaldehyde. Fixed cells were blocked with 1.2% BSA diluted in PBS with 0.1% Triton X-100 (PBST) for 30 minutes before subjected to primary antibodies for 1 hour with occasional shake at room temperature. Another rabbit polyclonal antibody named anti-Cap43 from Zymed (now part of Invitrogen, San Francisco, CA) was used to detect NDRG1 (1:50 dilution in PBST with 1% BSA). After five 5-minute washes with PBS (with occasional shake), the coverslip was immersed in PBST with 1:1000 diluted Cy3 conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hour with occasional shake at room temperature. From the addition of secondary antibody, the coverslip was kept in dark. After another four 5-minute washes with PBS, cells were counterstained with DAPI (4',6-diamidino-2-phenylindole, 0.5 µg/mL) for nuclear visualization. The coverslip was finally mounted with Mowiol-488 mounting solution (Calbiochem, now part of Merck Ltd.) containing 2.5 mg/mL of n-propyl gallate as anti-fading reagent. After the setting of the mounting medium (needed about overnight at room temperature, in dark), the slides were observed using 20× or 40× objective lens of an inverted fluorescent microscope (model: Axiovert 200M, ZEISS, Germany).

5.5.2 Immunohistochemical analysis of NDRG1 in OSCC samples

OSCC sections for immunochemical analysis were obtained from our collaborator Prof. Xiaohang Zhao in the State Key Laboratory for Molecular Oncology, Peking Union University, Beijing, China. OSCC biopsies (surgical resections) were ethically collected from 52 Chinese patients with primary OSCC prior to radio- or chemo-therapy in Shan'xi Province. In most of the cases, normal mucosa, paracarcinoma tissue (adjacent mucosa with dysplasia) and carcinoma tissue were collected. The OSCC biopsies were paraformaldehyde fixed, paraffin embedded and cut to 4 µm sections. Pathological classification was histologically confirmed by the staff in Prof. Zhao's lab.

Immunohistochemical staining using antibodies against NDRG1 (Kinasource) was performed by the S-P (peroxidase labeled streptavidin) method using the UltraSensitive™ SP Staining kit from Maixin_Bio (Fuzhou, China). After routine xylene deparaffinization, gradient ethanol rehydration, and 10 minutes block of endogenous peroxidase activity by 3% H_2O_2, antigen retrieval was done by boiling slides in 0.01M citrate buffer (pH=6.0) for 15 min in a stream cooker. Sections were then blocked by normal sera provided in the kits and incubated with 1:50 PBS diluted primary antibodies for 1 hour at room temperature. Detection was done according to the manufacturer's instruction using 3,3'-diaminobenzidine (DAB) as chromogen. Slides were finally lightly counterstained with Mayer's hematoxylin before dehydration through gradient ethanol, cleared by xylene, and mounted with neutral balsam.

The immunohistochemical stained sections for NDRG1 was assessed on an arbitrary scale based on the relative staining intensity score multiplied with the positive area of stained cells in the section. Staining intensity was scored as: blank 0, light yellow 1, light brown 2, and dark brown 3. And the positive rates were designated as: <25% of the tissue area: 1, 25%-50%: 2, 51%-75%: 3, and >75%: 4. The multiplied score of 0-4, 5-8 and 9-12 were considered light, moderate and strong staining respectively.

5.6 *In Vitro* functional assays

5.6.1 *In vitro* cell growth assays

96-Well plate based colorimetric assay using MTS tetrazolium compound was employed for this approach. The MTS tetrazolium compound can be metabolized, presumably through dehydrogenases (such as NADPH or NADH) by cells into a soluble coloured formazan product, which has a maximum absorbance at 490 nm. To determine the anchorage dependent cell growth, cells (1,500 per well for KYSE30 and 1,000 per well for KYSE150) were seeded into 96-well plates and cultured routinely in RPMI-1640 medium supplemented with 10% FBS. For anchorage-independent cell growth, cells (15,000 per well for KYSE30 and 10,000 per well for KYSE150) were seeded into poly-heme coated 96-well plates and cultured in RPMI-1640 medium containing 10% FBS and 1.5% methylcellulose. The usage of poly-heme (poly-2-hydroxyethyl methacrylate) prevents cell adhesion and results in anchorage-independent, aggregational cell growth [292;293]. All cell lines tested (wild-type and transfectants) were plated in quadruplicate, and cell growth were measured through CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Briefly, 1/10 volume of the ready-to-use MTS reagent was added to the cells and incubated for 4 hours at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm, reflecting the number of viable cells, was recorded with a microplate reader (BioTek, Winooski, VT). Result from one of the three independent experiments was shown.

5.6.2 DNA content analysis by flow cytometry

Protocol for analyzing the DNA contents of GFP positive (transfected) cells in the transfectant "pool" is adapted from Ref. [339] with small modification. Briefly, subconfluent cells from 60-mm dishes were trypsinized into single cell suspension, washed with PBS, and first fixed in 1% formaldehyde (diluted in

PBS, pH = 7.4) to insolubilize GFP protein for 1 hour before the second fixation in 70% ethanol for 18 hours. The cells were then resuspended in 1 mL staining solution containing 1 mg/mL RNase A, 33 µg/mL propidium iodide, 0.1% (v/v) Triton X-100 in PBS for 30 minutes. Samples were analyzed on a Cytomics FC 500 flow cytometry (Beckman Coulter, Fullerton, CA) to generate DNA histograms. The percentage of each phase (G1, S and G2/M) was determined using the CXP software provided with the machine. Since the percentage of GFP positive cells was more than 80%, similar results were obtained when analyzing the DNA contents of the whole transfectant "pool" by fixing cells directly in 70% ethanol (data not shown).

5.6.3 Scratch/wound-healing assay

To measure the cell mobility without chemotaxis, scratch assays were performed as described previously [298]. Briefly, cells were plated in Matrigel (100 µg/mL) coated 60-mm dishes overnight to reach ~90-100% confluence. A sterilized P200 (yellow) pipette tip was used to scratch lines with the same width on cell monolayer. One hour before scratching, low serum containing medium (with 0.1% FBS) was changed to minimize the cell growth during the assay. Photos of the same spot were taken at 0-hour, 8-hour and 24-hour after the wounding. Representative images were shown from one of the three independent experiments.

5.6.4 Migration and invasion under chemotaxis (transwell assay)

Transwell filters (6.5-mm diameter, 8.0-µm pore size, Costar, Corning, NY) were used. For invasion assays, the top chambers were covered with 50 µL Matrigel (1.5 mg/mL, diluted in RPMI-1640 medium) for 2 hours at 37°C. Cells (4.0×10^4) resuspended in RPMI-1640 medium with 0.1% FBS were plated on the top chambers while chemoattractant (20% FBS and 200 ng/mL EGF) was added to bottom chambers. After 48-hour incubation, non-migrated cells as

well as Matrigel in the upper surface of the filters were gently removed by a cotton swab. The cells migrated to the lower side of the membrane were fixed in 100% methanol (for 20 minutes, at room temperature), stained with crystal violet (0.2% w/v in 2% methanol, 30 minutes, at room temperature) and counted (averaged from 6 random chosen low-power fields for each transwell). To ensure equal plating, an MTS assay was done in parallel (with 10% of the volume of the cell suspension for transwell assay plated in 96-well plate and checked at the time point of staining, data not shown). Each sample was assayed in duplicate and three independent experiments were done for quantification.

5.6.5 Gelatin zymography

Gelatinases secreted into the medium by wild type cells and transfectants were measured by zymography. To prepare conditional medium, subconfluent cells in 60-mm dishes were incubated in 2 mL RPMI-1640 medium with 0.1% FBS for 24 hours. Cell counting (after trypsinization) for normalization was performed immediately after conditioned media samples were harvested for lyophilization. A portion of reconstituted sample resembling 1×10^5 cells was loaded directly on a 10% polyacrylamide gel containing 0.1% SDS and 0.4 mg/mL gelatin. After electrophoresis under non-reducing conditions (10 or 12 mA per gel in stacking or resolving gels, in 4 °C fridge), the gel was washed (on a orbit shaker of 75 rpm) with 50 mM Tris-HCl (pH=7.5) containing 2.5% Triton X-100 (three changes, 20 minutes each) and zymography buffer (50 mM Tris-HCl, pH=7.5 with 0.002% Brij-35, 10 mM $CaCl_2$ and 5 μM $ZnCl_2$) for 20 minutes, then incubated in the zymography buffer at room temperature for 16 hours with gentle shaking (30 rpm). Proteolytic regions indicating gelatinolytic activities were negatively displayed by Coomassie brilliant blue R-250 staining. Representative images were shown from one of the three independent experiments.

5.6.6 Determination of VEGF-A by ELISA

The concentration of VEGF-A secreted into the medium was measured by DuoSet ELISA Development kit (Catalogue Number: DY293, R&D Systems, Minneapolis, MN). Conditioned media (with 1% FBS) from subconfluent wild type KYSE30 cells and transfectants were obtained similarly as gelatin zymography with the number of cells counted immediately after harvest. Without lyophilization and further dilution, conditioned media were directly subjected to ELISA according to the manufacturer's instruction. Results were normalized with the number of cells at the time of harvest and reported as picograms (pg) per 10^6 cells. Three independent experiments were done for quantification.

5.6.7 Caspase 3/7 assay

Caspase-3/7 activity was determined using the Caspase-Glo® 3/7 assay kit (Promega) according to the manufacturer's instructions with a small modification. Briefly, 8,000 cells/well were seeded into 96-well plated in 150 μL medium overnight before the treatment of apoptosis inducing reagents (dissolved in 50 μL culture medium). Prior to the assay, drug-containing medium was carefully removed from each well and replaced with 40 μL of fresh medium (especially essential for Ni^{2+} and Co^{2+} treatment, due to the interference with the assay buffer). Next, 40 μL of Caspase-Glo® 3/7 Reagent was added and 1 hour incubation in dark was allowed for reaction. Afterwards, Caspase 3/7 activity was determined by the relative luminescence units (RLU), which was quantified using a Luminoskan Ascent Luminometer (Thermo LabSystems, Franklin, MA). Cells are plated in triplicate and an MTS assay was introduced in parallel to ensure the equal plating (performed before the drug administration, data not shown). The average induction fold (against untreated cells) of the sum activities of caspase 3/7 from three independent experiments were shown.

5.7 *In vivo* experiments on nude mice

5.7.1 Experimental animals and nude mice xenograft model

Athymic nude mice aged 4-5 weeks were obtained with an approval from the animal centre in Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). Animals were ethically used in accordance with the NIH Guide for the Care and Use of Laboratory Animals for xenograft studies.

To examine the growth rates and metastatic abilities of the transfectants with altered NDRG1 expression in nude mice, mice were randomly divided into each group (six for KYSE30 cells and five for KYSE150 cells) and 5×10^6 cells resuspended in 0.2 mL PBS were subcutaneously injected into the dorsal flank. During the injection, care was taken to maintain uniform cell suspensions and to avoid injecting clumped cells. Tumour dimensions were measured by calliper every week and the tumour volume was calculated using the following formula: V= 0.5 × length × width × width (length=larger diameter and width=smaller diameter). Growth curves were plotted using average tumour volume within each experimental group at the set time points. Mice were sacrificed 5 weeks after tumour cell inoculation. Liver and lung were dissected and the metastatic lesions were counted macroscopically to monitor the potential effect of NDRG1 on tumour metastasis. All the xenografts and tissues were fixed in 4% paraformaldehyde, paraffin embedded and sliced into 4 μm sections for further pathological examination (H-E staining) and immunohistochemical analysis.

5.7.2 Analysing the xenograft sections by IHC staining and TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay, which allows *in situ* labelling of DNA breaks, was performed to assess the *in situ* apoptotic activity. The DeadEnd™ Colorimetric TUNEL System from Promega using 3,3'-diaminobenzidine (DAB) as chromogen was applied for this approach.

Same protocol (S-P method using the UltraSensitive™ SP Staining kit) as described in section 5.5.2 was used for immunohistochemical staining of the xenograft sections using antibodies against NDRG1 (Kinasource), E-cadherin and Ki-67 (both are mouse monoclonal, Zymed). These immunohistochemical stained slides as well as slides for TUNEL staining were finally lightly counterstained with Mayer's hematoxylin before dehydration through gradient ethanol, cleared by xylene, and mounted with neutral balsam.

Another approach with much higher sensitivity was used for detection of blood vessels using antibody against endothelial surface marker CD31 (PECAM-1). Antigen retrieval was carried out with a Proteinase K (DAKO, Glostrup, Denmark) solution at 37 °C for 10 minutes and sections were then incubated for 1 hour with a 1:50 dilution of rat anti-mouse CD31 primary antibody (Research Diagnostics Inc., Flanders, NJ) in 1% BSA in PBS. The primary antibody was detected with a biotin-streptavidin-horseradish peroxidase system (Histomark®, from KPL, Gaithersburg, MD) using 3,3',5,5'-tetramethylbenzidine (TMB) as chromogen, which provides a brilliant blue specific stain with red nuclear counterstain. Sections were further counterstained with a 0.25% neutral red (Saarchem, Midrand, South Africa) solution before mounting.

For each staining, exact steps without adding primary antibody (immunohistochemical staining) or deoxynucleotidyl transferase (TUNEL assay) were also carried out for negative controls. No interfering background staining was observed in both approaches (data not shown).

5.7.3 Staining evaluation

Images were obtained using a Nikon 90i light microscope. All counts and scoring were performed by the author with the surveillance of a pathologist who was blind to this study. Average number and standard deviation (SD) were calculated from all the sections for each group.

Ki-67 labelling index as a marker of proliferation was determined by the percentage of immunoreactive nuclei to the total number of nuclei in three high power fields (200×). An average of 4000 nuclei was selected in each section randomly. Apoptosis activity was determined by the average number of TUNEL positive nuclei in three independent microscopic fields with most intensive staining at 200× magnification (~4000 cells).

The immunohistochemical stained sections for NDRG1 and E-cadherin (as a marker of tumour differentiation level) were assessed on an arbitrary scale based on the relative immunoreactivity (staining intensity) score multiplied with the positive rate (area) of stained cells in the section. Details are described in Section 5.5.2.

The angiogenic activity was determined by the percentage of vascularized area showing endothelial cell clusters versus the total area of the section. Microvessels were stained by the endothelial marker CD31. Images of a complete cross-section were acquired at 32× magnification. All of the ensuing analysis for angiogenetic activity was performed with Visiopharm Integrated Systems (Visiopharm A/S, Hørsholm, Denmark).

5.8 Statistics

Quantitative data was obtained from three independent experiments. Unless specified, statistical significance was determined by Student's t-test using Microsoft Excel 2003, in comparison with samples from the wild type control. Fisher's exact test was performed using SPSS 17.0 statistical package (SPSS Inc., Chicago, IL), which was used to compare the NDRG1 levels in the different stages of OSCC carcinogenesis (immunohistochemical analysis). Of both analysis, p value less than 0.05 was considered statistically significant.

5.9 Solution formula

All chemicals for solution were AnalR grade. Unless specified, double distilled water or dH_2O was used as solvent for solutions.

30% Acr + Bis (30:0.8) (w/w)

Dissolve 30 g acrylamide and 0.8 g bisacrylamide with dH_2O. Add 1 mL 10% SDS (w/w, made from reagent with >99% purity) and make up to 100 mL. Filter through 0.45-μm filter and store at 4 °C with light protection.

10% Ammonium persulfate (10% AP)

Dissolve 100 mg ammonium persulfate in 1 mL dH_2O, store at 4 ºC for less than 1 week.

0.01 M Citrate buffer, pH = 6.0

Dissolve 2.1 g of citric acid monohydrate in 970 mL of dH_2O; adjust pH to 6.0 with NaOH; make up to 1 litre with dH_2O and autoclave.

Coomassie brilliant blue R-250 staining/de-staining solutions:

Add 0.125 g Coomassie brilliant blue R-250 in 250 mL of gel fixing solution containing 50% menthol (v/v), 10% glacial acetic acid (v/v) and 40% dH_2O (v/v). Stir vigorously overnight for maximum solubility, and filter with Whatman No.1 filter paper before use.

De-staining solution was made up with 10% menthol (v/v) and 10% glacial acetic acid (v/v) in dH_2O.

0.5 M EDTA, pH = 8.0

Add 180 mL dH_2O to 37.22 g $Na_2EDTA{\bullet}2H_2O$; adjust pH to 8.0 with 10 M NaOH (~ 10 mL) to dissolve; make up to 200 mL with dH_2O and autoclave.

Inoue buffer (for making competent *E. coli* Cells)

Milli-Q grade H_2O should be used for this approach. Weigh 0.151 g PIPES (1,4-piperazinediethanesulfonic acid), 0.22 g $CaCl_2 \cdot 2H_2O$ and 1.87 g KCl and add about 70 mL H_2O; adjust pH to 6.7 with 5M KOH until everything is dissolved. In another beaker, dissolve 1.09 g $MnCl_2 \cdot 4H_2O$ in about 20 mL H_2O. Slowly combine the two solutions with vigorous stirring to avoid precipitation. Adjust the final volume to 100 mL with H_2O and filter with 0.22-μM filter. Aliquot and store the solution at -20 ºC.

LB (Luria-Bertani) broth and plates

Add 10 g Bacto®-tryptone, 5 g Bacto®-yeast extract and 10 g NaCl to 1 litre dH_2O, adjust pH with 2 mL of 10 M NaOH (to ~7.0) and autoclave.

15 g agar was added per 1 litre LB broth for plate preparation. Agar will dissolve during the autoclave. Ampicillin (100 μg/mL) was added when the broth was cooled to about 50 ºC. The broth was then aliquoted of 30 mL per 100-mm dish. After the setting of agar, plates sealed with parafilm can be stored at 4°C for at least 1 month.

5× Loading buffer (for SDS-PAGE)

Dissolve 5 g SDS (with >99% purity) in 30 mL stacking gel buffer (pH=6.8); add 12 mL glycerol and 1 mL 1% bromophenol blue. Mix well and make up to 50 mL with stacking gel buffer (pH=6.8). Make 0.9 mL aliquots and store at 4 ºC. Before use, add 0.1 mL β-mercaptoethanol per aliquot.

Mowiol-488 mounting solution (for immunofluorescence)

Mix 2.4 g Mowiol-488, 6 g glycerol and 6 mL dH_2O in a 50 mL Falcon conical tube, stir vigorously for several hours at room temperature. Add 12 mL of 0.2 M Tris-HCl (pH = 8.5), heat to 60 °C with stirring for 10 minutes. Remove any undissolved particles by centrifugation at 5000 × g for 15 minutes at room temperature. Aliquot and store at -20 °C. Before use, dissolve 2.5 mg of n-propyl gallate as anti-fade reagent in 1 mL defrosted Mowiol by vortexing.

10 × PBS (phosphate-buffered saline)

Dissolve 2.0 g KCl, 80 g NaCl, 2.4 g KH_2PO_4, and 14.2 g Na_2HPO_4 (anhydrous) in 1 litre dH_2O and autoclave. The pH value of 1 × solution should be around 7.2-7.4 (depends on the water used).

1× Protein transfer buffer

Dissolve 14.4 g Tris base and 3.8 g glycine in dH_2O and mix with 200 mL methanol. Adjust the final volume to 1000 mL and cool to 4 ºC before use.

Resolving gel buffer (for SDS-PAGE)

Dissolve 36.2 g Tris base and 0.8 g SDS (with >99% purity) in dH_2O. Adjust pH to 8.9 and make up to 200 mL with dH_2O. Filter through 0.45-µm filter and store at 4 °C.

10 × Running buffer (for SDS-PAGE)

Dissolve 63.2 g Tris base and 40.0 g glycine in dH_2O. Add 100 mL 10% SDS (w/w, made from reagent with >99% purity) and adjust the final volume to 1 litre (the pH of the solution should be about 8.3). Store at room temperature.

SOB and SOC broth

Dissolve 20 g Bacto®-tryptone, 5 g Bacto®-yeast extract and 0.5 g NaCl in 950 mL dH_2O, add 10 mL of 250 mM KCl, adjust pH to 7.0 with 10M NaOH, make up to 1 litre with dH_2O and autoclave. Before use, add 1/100 volume of a solution containing 1 M $MgSO_4$ and 1 M $MgCl_2$.

SOC broth was prepared by adding 1/50 volume of 1 M glucose to SOB broth.

3 M sodium acetate, pH = 5.2 (for ethanol DNA precipitation)

Dissolve 24.61 g sodium acetate (anhydrous) in 70 mL of dH_2O, adjust pH to 5.2 with glacial acetic acid. Make up to 100 mL with dH_2O and autoclave.

Stacking gel buffer (for SDS-PAGE)

Dissolve 5.9 g Tris base and 0.4 g SDS (with >99% purity) in dH_2O. Adjust pH to 6.8 and make up to 200 mL with dH_2O. Filter through 0.45-µm filter and store at 4 °C.

10 × TBE (for agarose gel)

Dissolve 108 g Tris, 55 g boric acid in dH_2O; mix with 40 mL 0.5 M EDTA (pH=8.0); make up to 1 litre with dH_2O and autoclave.

10× TBS (Tris-buffered saline)

Dissolve 2.0 g KCl, 80 g NaCl, and 30 g Tris base in dH_2O; adjust pH to 7.3 with concentrated HCl; make up with dH_2O to 1 litre and autoclave. The pH of 1 × solution should be around 7.2-7.4 (depends on the water used).

TE buffer, pH = 8.0

Mix 1 mL 1 M Tris-HCl (pH=8.0), 200 µL 0.5 M EDTA (pH=8.0) with dH_2O; make up to 100 ml with dH_2O and autoclave.

1 M Tris-HCl, pH = 8.0

Dissolve 24.22 g of Tris base in 160 mL of dH_2O, adjust pH to 8.0 with concentrated HCl; make up to 200 mL with dH_2O and autoclave.

Appendix: Sequencing results for plasmid constructs

Both of the original reading and sequence alignment (BLAST analysis, http://blast.ncbi.nlm.nih.gov, only for pWPI-NDRG1, with the NDRG1 sequence NM_006096.2 used as template) were shown. Important markers are also manually indicated (in handwriting), including the start and ending codon of the translation as well as the cloning sites of exogenous fragments.

Fig S-1: pWPI-NDRG1, SN-R1 primer:

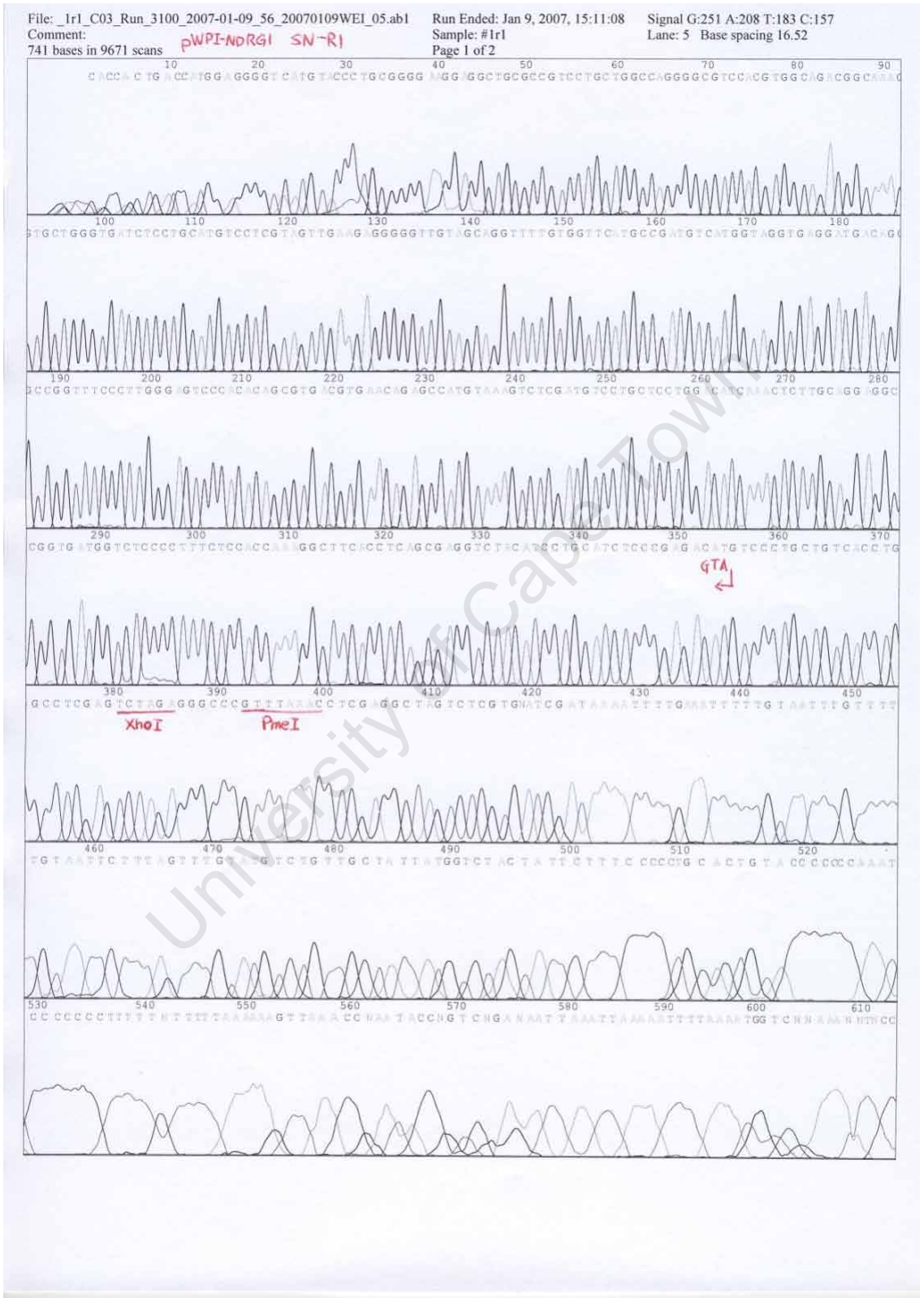


Fig S-1: pWPI-NDRG1, SN-R1 primer (BLAST):

Blast Result Page 1 of 2

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

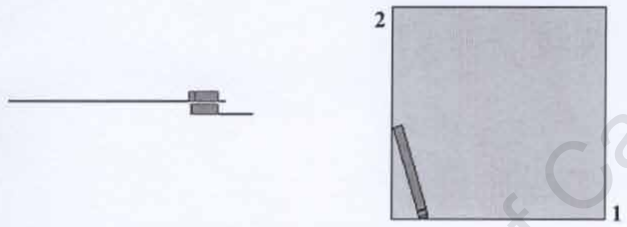
BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.15 [Oct-15-2006]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 50 expect: 10.0000 wordsize: 11 Filter ☑ View option Standard
Masking character option X for protein, n for nucleotide Masking color option Black
☐ Show CDS translation Align

Sequence 1: lcl|1_seq_1
Length = 3022 (1 .. 3022)

pWPI-NDRG1 #7
SN-R1

Sequence 2: lcl|2_seq_2
Length = 854 (1 .. 854)



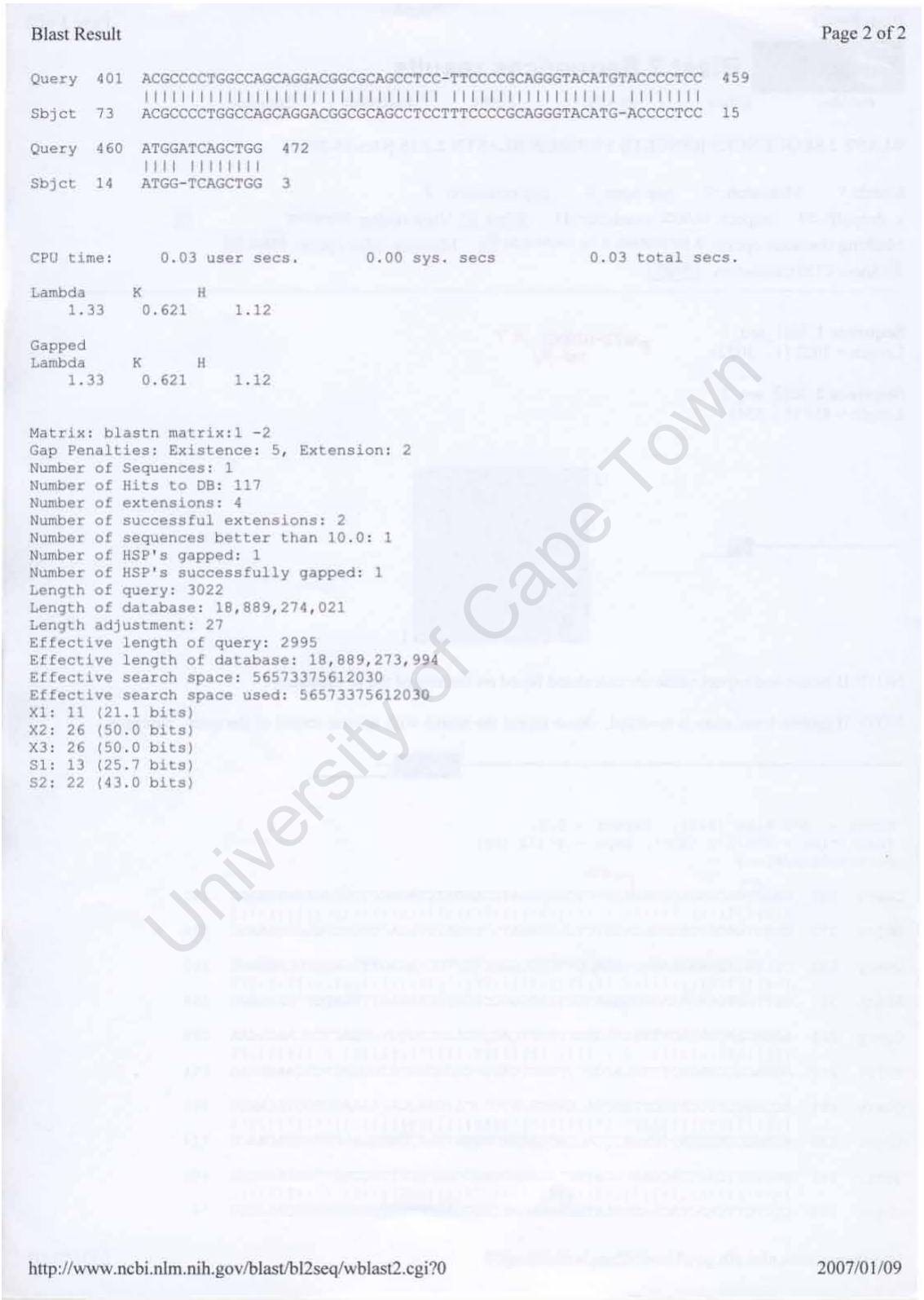
NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score =  671 bits (349),  Expect = 0.0
 Identities = 370/373 (99%), Gaps = 3/373 (0%)
 Strand=Plus/Minus
                                  ATG
Query  101  CAGGTGACAGCAGGGACATGTCTCGGGAGATGCAGGATGTAGACCTCGCTGAGGTGAAGC  160
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  373  CAGGTGACAGCAGGGACATGTCTCGGGAGATGCAGGATGTAGACCTCGCTGAGGTGAAGC  314

Query  161  CTTTGGTGGAGAAAGGGGAGACCATCACCGGCCTCCTGCAAGAGTTTGATGTCCAGGAGC  220
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  CTTTGGTGGAGAAAGGGGAGACCATCACCGGCCTCCTGCAAGAGTTTGATGTCCAGGAGC  254

Query  221  AGGACATCGAGACTTTACATGGCTCTGTTCACGTCACGCTGTGTGGGACTCCCAAGGGAA  280
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  AGGACATCGAGACTTTACATGGCTCTGTTCACGTCACGCTGTGTGGGACTCCCAAGGGAA  194

Query  281  ACCGGCCTGTCATCCTCACCTACCATGACATCGGCATGAACCACAAAACCTGCTACAACC  340
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  ACCGGCCTGTCATCCTCACCTACCATGACATCGGCATGAACCACAAAACCTGCTACAACC  134

Query  341  CCCTCTTCAACTACGAGGACATGCAGGAGATCACCCAGCACTTTGCCGTCTGCCACGTGG  400
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  133  CCCTCTTCAACTACGAGGACATGCAGGAGATCACCCAGCACTTTGCCGTCTGCCACGTGG  74
```

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0 2007/01/09

Fig S-1: pWPI-NDRG1, SN-R1 primer (BLAST):



Blast Result Page 2 of 2

```
Query  401  ACGCCCCTGGCCAGCAGGACGGCGCAGCCTCC-TTCCCCGCAGGGTACATGTACCCCTCC  459
            ||||||||||||||||||||||||||||||| |||||||||||||||| ||||||||
Sbjct  73   ACGCCCCTGGCCAGCAGGACGGCGCAGCCTCCTTTCCCCGCAGGGTACATG-ACCCCTCC  15

Query  460  ATGGATCAGCTGG  472
            |||| ||||||||
Sbjct  14   ATGG-TCAGCTGG  3


CPU time:     0.03 user secs.       0.00 sys. secs          0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 1
Number of Hits to DB: 117
Number of extensions: 4
Number of successful extensions: 2
Number of sequences better than 10.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 3022
Length of database: 18,889,274,021
Length adjustment: 27
Effective length of query: 2995
Effective length of database: 18,889,273,994
Effective search space: 56573375612030
Effective search space used: 56573375612030
X1: 11 (21.1 bits)
X2: 26 (50.0 bits)
X3: 26 (50.0 bits)
S1: 13 (25.7 bits)
S2: 22 (43.0 bits)
```

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0 2007/01/09

Fig S-2: pWPI-NDRG1, SN-F1 primer:

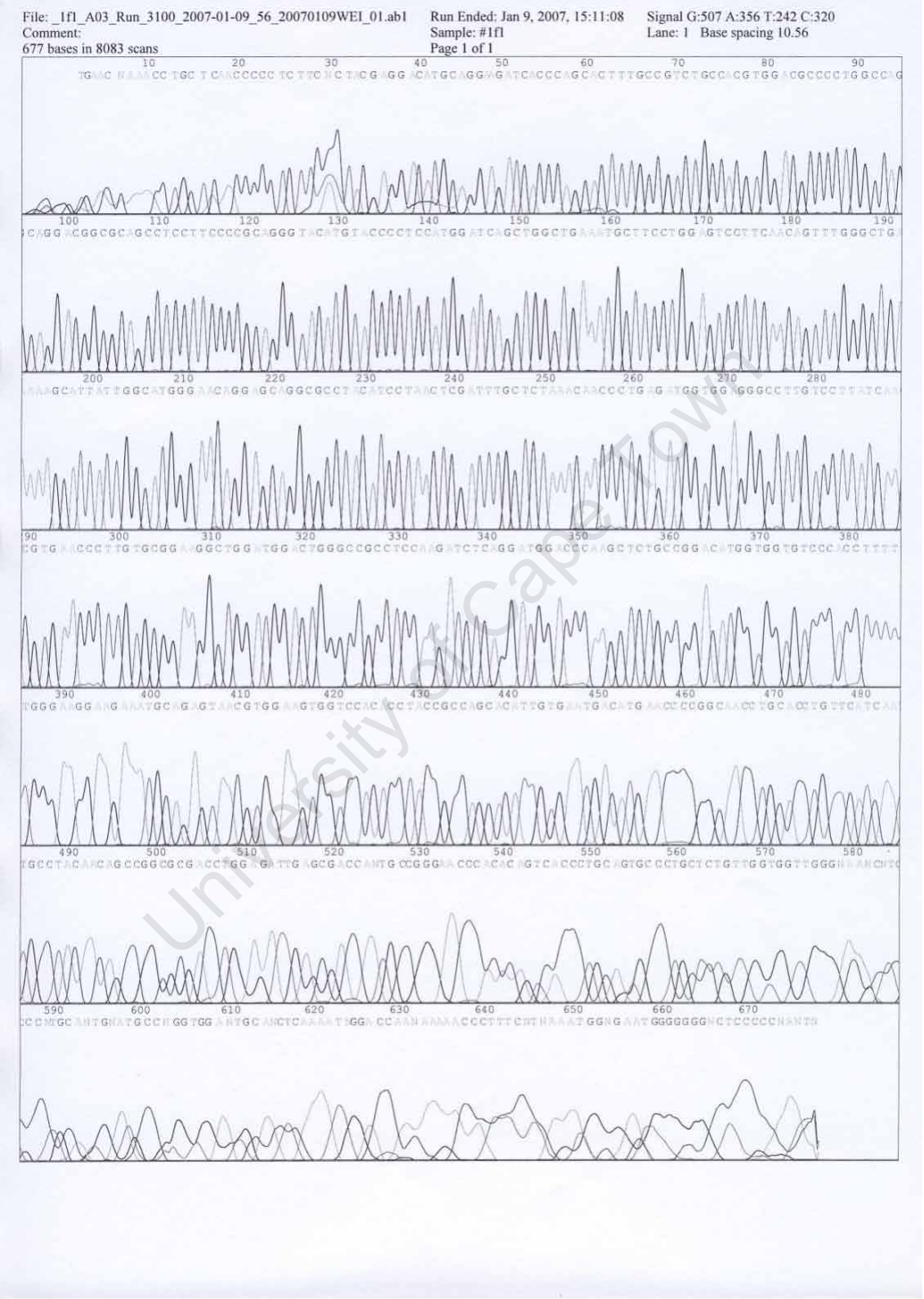


Fig S-2: pWPI-NDRG1, SN-F1 primer (BLAST):

NCBI **Blast 2 Sequences results**

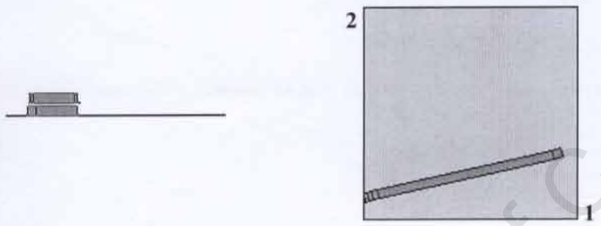
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.15 [Oct-15-2006]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 50 expect: 10.000(wordsize: 11 Filter ☑ View option Standard
Masking character option X for protein, n for nucleotide Masking color option Black
☐ Show CDS translation Align

Sequence 1: lcl|1_seq_1 pWPI-NDRG1 SN-F1
Length = 677 (1 .. 677)

Sequence 2: lcl|2_seq_2
Length = 3022 (1 .. 3022)

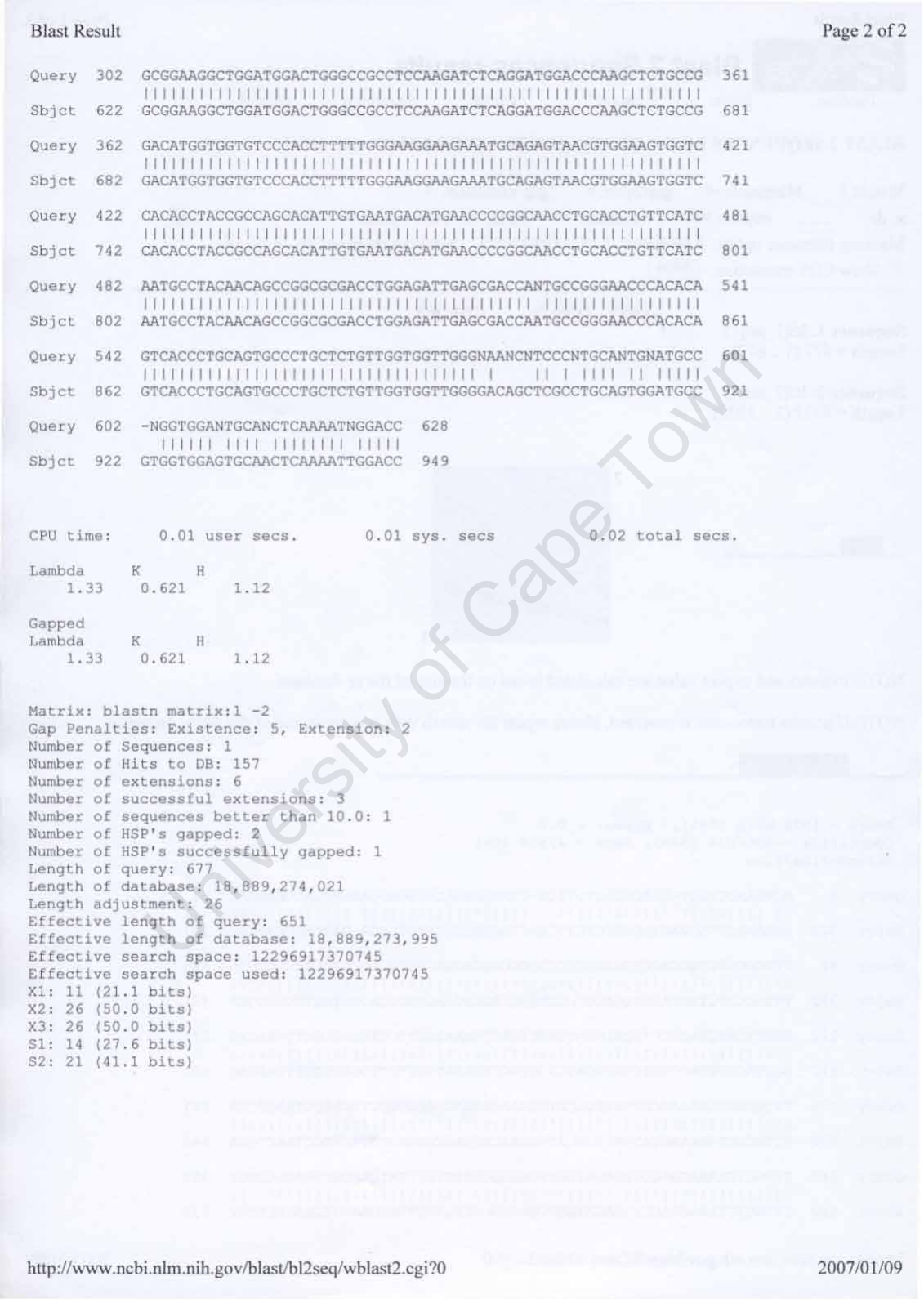


NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score = 1079 bits (561),  Expect = 0.0
 Identities = 608/628 (96%), Gaps = 4/628 (0%)
 Strand=Plus/Plus

Query  4    ACNAAACCTGCT-CAACCCCCTCTTCN-CTACGAGGACATGCAGGAAGATCACCCAGCAC  61
            || ||||||||| |||||||||||||  ||||||||||||||||||| ||||||||||||
Sbjct  323  ACAAAACCTGCTACAACCCCCTCTTCAACTACGAGGACATGCAGGA-GATCACCCAGCAC  381

Query  62   TTTGCCGTCTGCCACGTGGACGCCCCTGGCCAGCAGGACGGCGCAGCCTCCTTCCCCGCA  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382  TTTGCCGTCTGCCACGTGGACGCCCCTGGCCAGCAGGACGGCGCAGCCTCCTTCCCCGCA  441

Query  122  GGGTACATGTACCCCTCCATGGATCAGCTGGCTGAAATGCTTCCTGGAGTCCTTCAACAG  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442  GGGTACATGTACCCCTCCATGGATCAGCTGGCTGAAATGCTTCCTGGAGTCCTTCAACAG  501

Query  182  TTTGGGCTGAAAAGCATTATTGGCATGGGAACAGGAGCAGGCGCCTACATCCTAACTCGA  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  502  TTTGGGCTGAAAAGCATTATTGGCATGGGAACAGGAGCAGGCGCCTACATCCTAACTCGA  561

Query  242  TTTGCTCTAAACAACCCTGAGATGGTGGAGGGCCTTGTCCTTATCAACGTGAACCCTTGT  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  562  TTTGCTCTAAACAACCCTGAGATGGTGGAGGGCCTTGTCCTTATCAACGTGAACCCTTGT  621
```

Fig S-2: pWPI-NDRG1, SN-F1 primer (BLAST):



Blast Result Page 2 of 2

```
Query  302  GCGGAAGGCTGGATGGACTGGGCCGCCTCCAAGATCTCAGGATGGACCCAAGCTCTGCCG  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  622  GCGGAAGGCTGGATGGACTGGGCCGCCTCCAAGATCTCAGGATGGACCCAAGCTCTGCCG  681

Query  362  GACATGGTGGTGTCCCACCTTTTTGGGAAGGAAGAAATGCAGAGTAACGTGGAAGTGGTC  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  682  GACATGGTGGTGTCCCACCTTTTTGGGAAGGAAGAAATGCAGAGTAACGTGGAAGTGGTC  741

Query  422  CACACCTACCGCCAGCACATTGTGAATGACATGAACCCCGGCAACCTGCACCTGTTCATC  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  742  CACACCTACCGCCAGCACATTGTGAATGACATGAACCCCGGCAACCTGCACCTGTTCATC  801

Query  482  AATGCCTACAACAGCCGGCGCGACCTGGAGATTGAGCGACCANTGCCGGGAACCCACACA  541
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  802  AATGCCTACAACAGCCGGCGCGACCTGGAGATTGAGCGACCAATGCCGGGAACCCACACA  861

Query  542  GTCACCCTGCAGTGCCCTGCTCTGTTGGTGGTTGGGNAANCNTCCCNTGCANTGNATGCC  601
            |||||||||||||||||||||||||||||||||||| |    || | |||| || |||||
Sbjct  862  GTCACCCTGCAGTGCCCTGCTCTGTTGGTGGTTGGGGACAGCTCGCCTGCAGTGGATGCC  921

Query  602  -NGGTGGANTGCANCTCAAAATNGGACC  628
             |||||| |||| |||||||| |||||
Sbjct  922  GTGGTGGAGTGCAACTCAAAATTGGACC  949


CPU time:     0.01 user secs.       0.01 sys. secs        0.02 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 1
Number of Hits to DB: 157
Number of extensions: 6
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's gapped: 2
Number of HSP's successfully gapped: 1
Length of query: 677
Length of database: 18,889,274,021
Length adjustment: 26
Effective length of query: 651
Effective length of database: 18,889,273,995
Effective search space: 12296917370745
Effective search space used: 12296917370745
X1: 11 (21.1 bits)
X2: 26 (50.0 bits)
X3: 26 (50.0 bits)
S1: 14 (27.6 bits)
S2: 21 (41.1 bits)
```

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0 2007/01/09

Fig S-3: pWPI-NDRG1, SN-F2 primer:

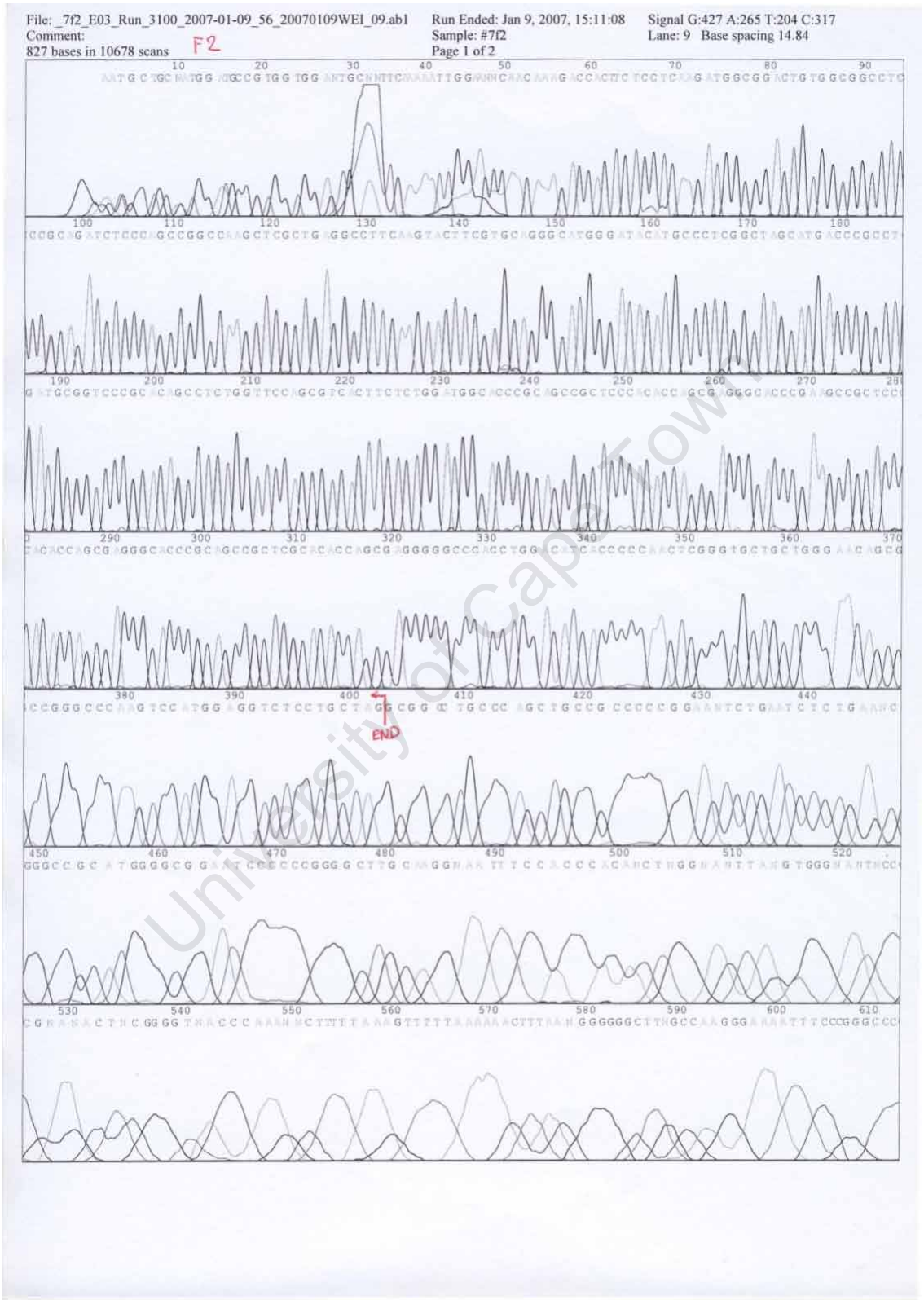


Fig S-3: pWPI-NDRG1, SN-F2 primer (BLAST):

Blast Result Page 1 of 2

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

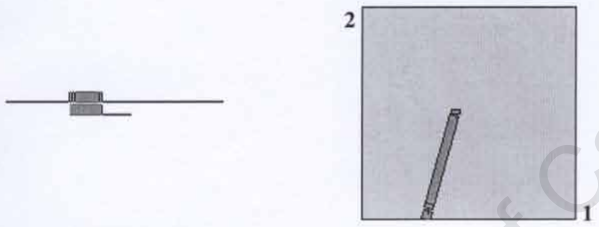
BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.15 [Oct-15-2006]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 50 expect: 10.000(wordsize: 11 Filter ☑ View option Standard
Masking character option X for protein, n for nucleotide Masking color option Black
☐ Show CDS translation Align

Sequence 1: lcl|1_seq_1
Length = 3022 (1 .. 3022)

pWPI-NDRG1 #7 SN-F2

Sequence 2: lcl|2_seq_2
Length = 830 (1 .. 830)



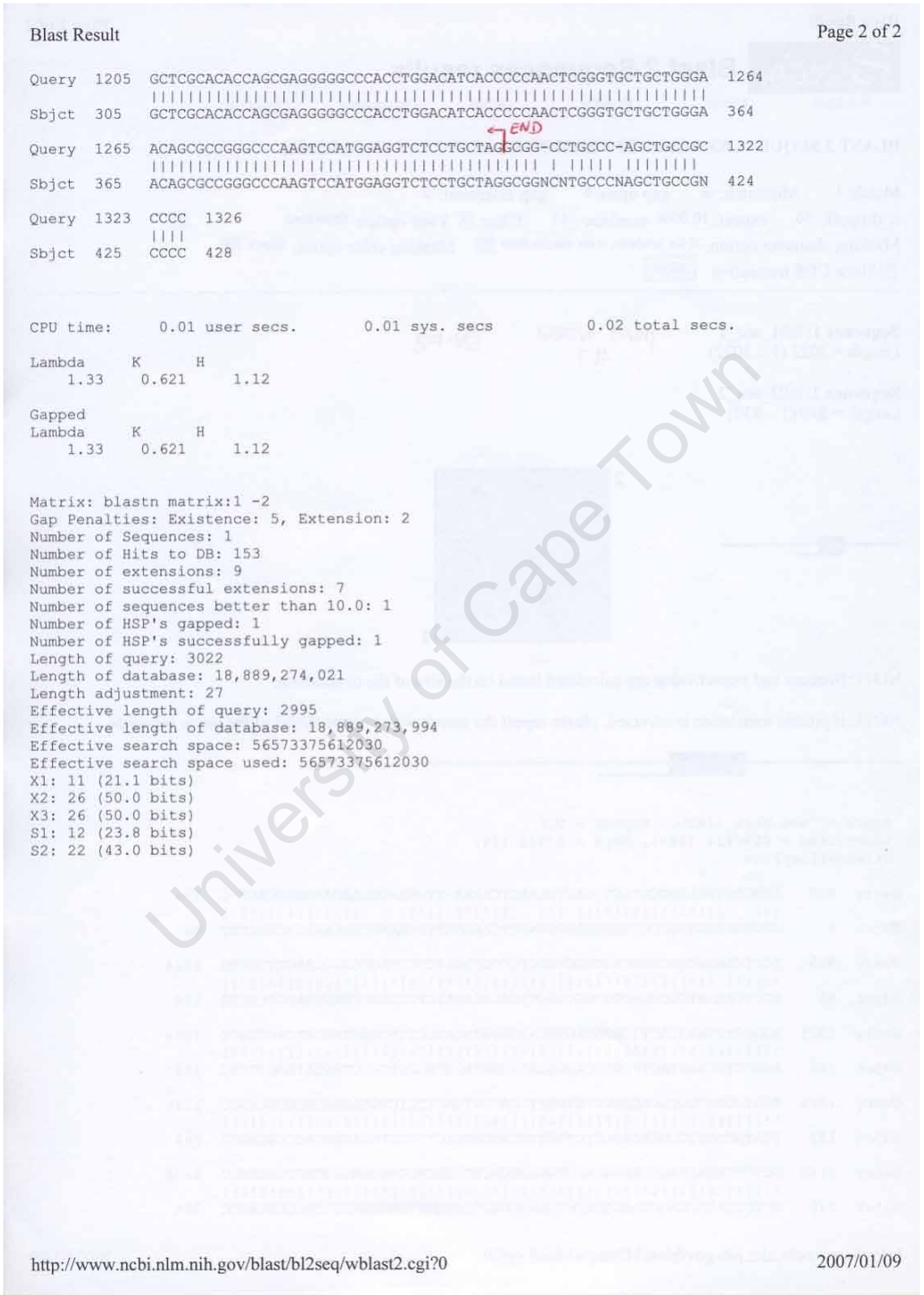
NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score =  696 bits (362),  Expect = 0.0
 Identities = 409/424 (96%), Gaps = 5/424 (1%)
 Strand=Plus/Plus

Query  908   CTGCAGTGGATGCCGTGGTGGAGTGCAACTCAAAA-TTGGA-CCCAACAAAGACCACT-C  964
             ||||  ||||||||||||||||  |||   ||||||  |||||   ||||||||||||| |
Sbjct  5     CTGCNATGGATGCCGTGGTGGANTGCNNTTCAAAAATTGGAANNCAACAAAGACCACTTC  64

Query  965   TCCTCAAGATGGCGGACTGTGGCGGCCTCCCGCAGATCTCCCAGCCGGCCAAGCTCGCTG  1024
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65    TCCTCAAGATGGCGGACTGTGGCGGCCTCCCGCAGATCTCCCAGCCGGCCAAGCTCGCTG  124

Query  1025  AGGCCTTCAAGTACTTCGTGCAGGGCATGGGATACATGCCCTCGGCTAGCATGACCCGCC  1084
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125   AGGCCTTCAAGTACTTCGTGCAGGGCATGGGATACATGCCCTCGGCTAGCATGACCCGCC  184

Query  1085  TGATGCGGTCCCGCACAGCCTCTGGTTCCAGCGTCACTTCTCTGGATGGCACCCGCAGCC  1144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185   TGATGCGGTCCCGCACAGCCTCTGGTTCCAGCGTCACTTCTCTGGATGGCACCCGCAGCC  244

Query  1145  GCTCCCACACCAGCGAGGGCACCCGAAGCCGCTCCCACACCAGCGAGGGCACCCGCAGCC  1204
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245   GCTCCCACACCAGCGAGGGCACCCGAAGCCGCTCCCACACCAGCGAGGGCACCCGCAGCC  304
```

http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0 2007/01/09

Fig S-3: pWPI-NDRG1, SN-F2 primer (BLAST):



Blast Result


```
Query  1205  GCTCGCACACCAGCGAGGGGGCCCACCTGGACATCACCCCCAACTCGGGTGCTGCTGGGA  1264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305   GCTCGCACACCAGCGAGGGGGCCCACCTGGACATCACCCCCAACTCGGGTGCTGCTGGGA  364

                                                    END
Query  1265  ACAGCGCCGGGCCCAAGTCCATGGAGGTCTCCTGCTAGGCGG-CCTGCCC-AGCTGCCGC  1322
             ||||||||||||||||||||||||||||||||||||||||| | ||||| ||||||||
Sbjct  365   ACAGCGCCGGGCCCAAGTCCATGGAGGTCTCCTGCTAGGCGGNCNTGCCCNAGCTGCCGN  424

Query  1323  CCCC  1326
              ||||
Sbjct  425   CCCC  428


CPU time:     0.01 user secs.       0.01 sys. secs          0.02 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 1
Number of Hits to DB: 153
Number of extensions: 9
Number of successful extensions: 7
Number of sequences better than 10.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 3022
Length of database: 18,889,274,021
Length adjustment: 27
Effective length of query: 2995
Effective length of database: 18,889,273,994
Effective search space: 56573375612030
Effective search space used: 56573375612030
X1: 11 (21.1 bits)
X2: 26 (50.0 bits)
X3: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 22 (43.0 bits)
```

Fig S-4: pLVTHM-NDRG1 shRNA:

Reading (refer to Fig 5-2 for comparison):

5’-ACGCGTCCCCGGAGTCCTTCAACAGTTTGTTCAAGAGACA
AACTGTTGAAGGACTCCTTTTTGGAAATCGAT-3’

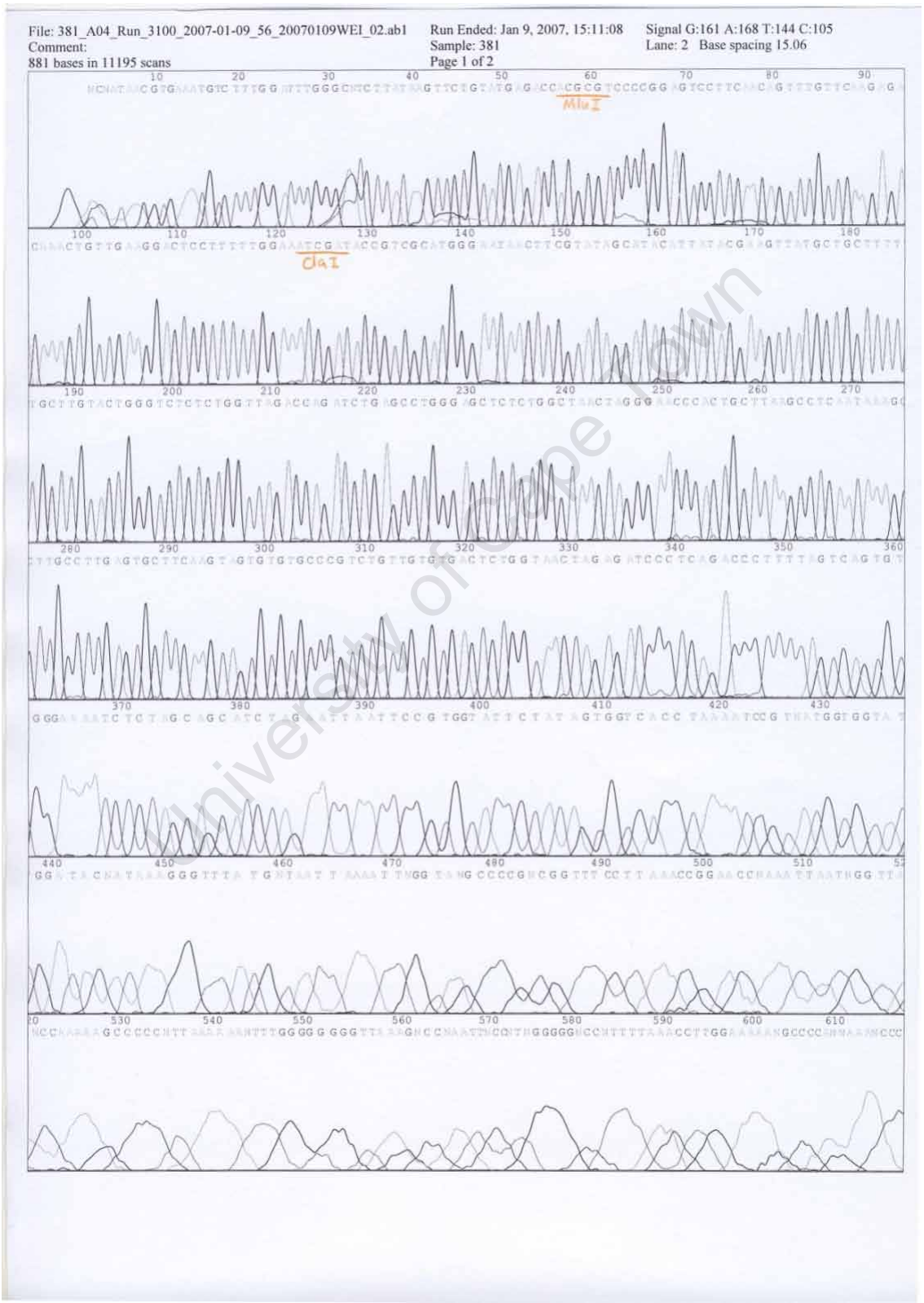
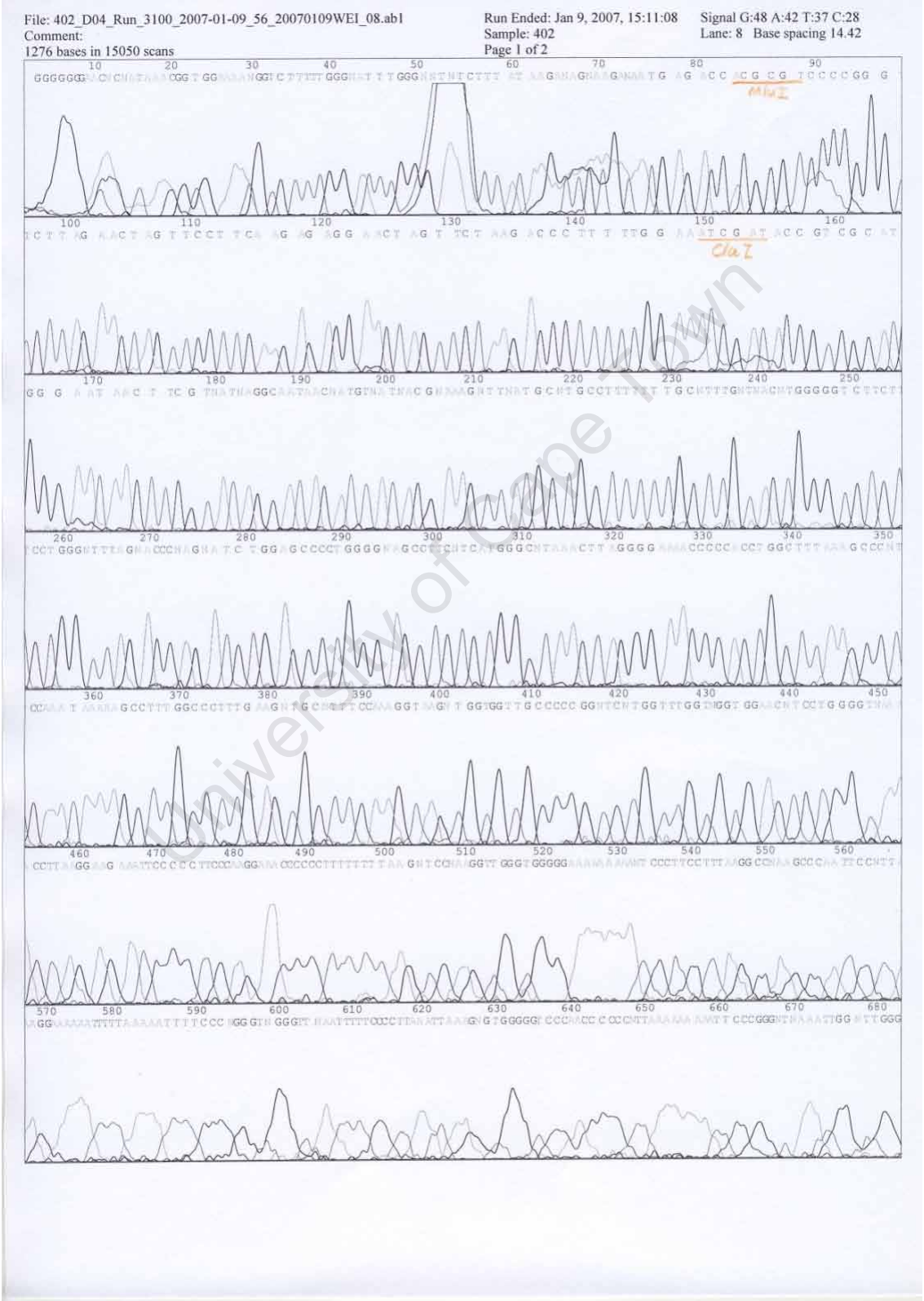


Fig S-5: pLVTHM-Random shRNA (RNAi sister control):

Reading (refer to Fig 5-2 for comparison):

5’-ACGCGTCCCCGGGTCTTAGAACTAGTTCCTTCAAGAGAGG
AACTAGTTCTAAGACCCTTTTTGGAAATCGAT-3’



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