CHAPTER FOUR

DISCUSSION

The knowledge of the molecular pathogenesis of NPC has improved significantly over the years; however, the current understanding is still not sufficient to change the clinical practice in the management of NPC. Therefore, a better molecular understanding of the disease is needed, not just to identify which molecular pathways that are dysregulated, but also to have an in-depth understanding on their clinical values. For example, EGFR is overexpressed (Fang et al., 2007) and amplified (Hui et al., 2002) in NPC, but unlike in lung cancer, targeting the EGFR pathway has proved limited clinical significance in NPC (Elser et al., 2007; Ma et al., 2008).

The starting point of this study was to identify genes that are overexpressed in NPC while having negligible expression in normal human organs using microarrays. Seven candidate genes, FJX1, CLCA2, WNT5A, RALA, FZD6, CLDN1 and FGFR3 were initially identified as they code for secreted or membrane-bound proteins, as well as having prior association with neoplasia or normal development. Only FJX1 was found to be expressed at negligible level across normal human organs examined, as well as overexpressed at the protein level in primary NPC tissues. Therefore, FJX1 was chosen for future studies. The effects of elevated FJX1 expression in NPC were subsequently investigated *in vitro*. FJX1 was found to enhance cell proliferation, invasion, and colony-formation in soft agar, indicating a potential role of FJX1 as an oncogene in NPC.

4.1 CANDIDATE GENE SELECTION

The current mainstay methods of treatment for NPC, which are radiotherapy and chemotherapy, have been unable to improve the outlook of the disease due to severe undesirable complications. Targeted therapy, which includes small molecule inhibitors, monoclonal antibodies and cancer vaccination, is a new treatment method which functions by interfering with specific molecules involved in tumor growth and maintenance. A route for developing this type of therapy is prior identification of the target molecule preceding the development of the inhibiting agent.

During validation of expression of the initial seven candidate genes, RALA failed validation for its overexpression in primary NPC tissues (Section 3.2.1). Three other genes FZD6, CLDN1 and FGFR3 failed validation in normal human organs (Section 3.2.2) as they showed broad and significant expression across the organs examined, suggesting their involvement in normal physiological processes in the organs. Targeting these molecules could result in adverse events in patients. As an example, CLDN1 is a well-known component in epithelial tight junction that regulates paracellular permeability in many tissues, as well as is a component in keratinization process (Angelow et al., 2008; Morita et al., 2011). While therapy against CLDN1 has not been developed for any cancer so far, previous experience in targeting the angiogenic VEGF pathway has shown that disruption of this pathway causes impairment of important physiological systems, including cardiovascular and renal systems, with associated serious and potentially fatal consequences (Chen & Cleck, 2009). In addition, the immunohistochemical staining of CLCA2 and WNT5A could not be completed due to the insensitivity and unspecificity of the antibodies.

This study has focused on the human FJX1, the homolog of Drosophila *fj* which is predicted to be a type II transmembrane protein (Villano & Katz, 1995). Like Fj and murine FJX1, human FJX1 protein might also be secreted and function at the extracellular (Probst et al., 2007; Rock et al., 2005; Villano & Katz, 1995). In addition, Fj is a Golgi kinase that phosphorylates specific extracellular cadherin domains of two gigantic transmembrane molecules, Fat and Dachsous (Ishikawa et al., 2008). If FJX1 mainly exerts its function at the extracellular, the development of monoclonal antibody against the protein could be viable. Meanwhile, the amino acid residues crucial for the kinase activity is conserved in human FJX1, suggesting a possibility to develop FJX1-specific small molecule inhibitors.

4.2 FJX1 EXPRESSION IN NPC AND NORMAL ORGANS

During the course of the present study, a meta-analysis (Lan et al., 2010) comparing three independent expression microarray datasets (Fang et al., 2008; Shi et al., 2006; Sriuranpong et al., 2004) also identified FJX1 to be overexpressed in NPC compared to normal controls. Several lines of evidence indicate FJX1 might be involved in carcinogenesis. Endothelial cells isolated from ovarian cancers showed increased FJX1 expression compared to those from healthy ovaries (Buckanovich et al., 2007; Lu et al., 2007). FJX1 was also amplified and overexpressed in oral squamous cell carcinoma (Jarvinen et al., 2008; Snijders et al., 2005). In addition, FJX1 expression is associated with kidney metastasis of non-small cell lung cancer cells in mice (Kakiuchi et al., 2003). However, the protein expression and functional role of FJX1 in NPC has not been studied.

After validation of FJX1 overexpression at the transcript level via qPCR in primary NPC tissues (Section 3.2.1), immunohistochemistry was employed to determine FJX1 protein expression in paraffin-embedded primary NPC tissues (Section 3.3). FJX1 protein was overexpressed in a subset of NPC cases, which however lacked any correlation with clinicopathologic parameters (T category, N category and overall staging). This might be due to the small sample size in certain categories that was unable to offer a statistically significant inference.

In general, FJX1 transcript was detected at low levels in 16 of the normal human organs tested (Figure 3.2). The expression of FJX1 in some of these organs is comparable as previously seen in mouse (Ashery-Padan et al., 1999; Rock et al., 2005). In particular, Rock *et al.* reported that mouse FJX1 expression in the kidneys was lower than in the brain (Rock et al., 2005), an observation that was similar in this study. The low expression of FJX1 in normal organs implies that systemic introduction of FJX1-targeted therapy is likely to result in minimal adverse effect.

4.3 KNOCKDOWN AND OVEREXPRESSION OF FJX1

The effect of introducing FJX1-targeting siRNAs could only be measured at the transcript level via qPCR as there was no reliable antibody that could detect endogenous FJX1 protein. Although the antibody used in the immunohistochemical analysis was specific against FJX1 protein in paraffin-embedded tissue samples, it was not suitable in Western blotting. This might be due to FJX1 protein being easily and irreversibly denatured by the SDS treatment in Western blotting procedure, resulting in loss of epitope conformation that might be required for recognition by the antibody. An attempt to generate a custom-made antibody against an-FJX1

derived peptide was made during the course of this study; however, the antibody also failed to detect FJX1 protein in Western blotting.

Analysis of FJX1 overexpression using V5 antibody revealed the occasional detection of three different molecular weight of FJX1 protein in Western blotting (Figure 3.6A). This could be as a result of differential glycosylation of the recombinant protein. There are two potential *N*-linked glycosylation sites on human FJX1 protein sequence which are also conserved in mouse (Figure 1.3) (Rock et al., 2005). The potential existence of an unglycosylated form of FJX1, plus two other different forms with glycoslyation at a single or both sites, could explain the occurrence of the three bands. However, to resolve whether this is a natural occurrence or merely an artifact of overexpression awaits the development of a reliable antibody to detect endogenous FJX1.

The recombinant FJX:V5 protein was localized at the perinuclear area of the cells (Figure 3.7), an observation that agrees with previous reports showing *Drosophila* Fj is located at the Golgi compartment both *in vivo* and *in vitro* (Ishikawa et al., 2008; Strutt et al., 2004). Double staining of FJX1 with specific intracellular vesicle or compartment marker is warranted to confirm its Golgi localization.

4.4 *IN VITRO* STUDIES OF FJX1

Both knockdown and overexpression experiments showed that FJX1 promoted aggressiveness of NPC cells by enhancing cell proliferation, anchorage-dependent growth and cellular invasion. The oncogenic traits tested are important in determining FJX1 oncogenicity in NPC: sustained proliferation and active tissue invasion have been proposed as cancer hallmark

capabilities (Hanahan & Weinberg, 2011), while anchorage-independent growth in soft agar is considered to be a stringent measurement of oncogenic properties as it is a close approximation to tumerigenicity assays in mouse (Chin & Gray, 2008).

Given that Drosophila *fj* and murine FJX1 are known to be downstream targets of the Notch signaling pathway (Buckles et al., 2001; Rock et al., 2005; Zeidler et al., 1999), it is possible that FJX1 is also a direct target of the pathway in NPC. The Notch signaling pathway, conserved across the metazoans, regulates many fundamental biological processes in a variety of tissues. The dysregulation of this pathway has been frequently reported in human cancers (Kopan & Ilagan, 2009), including NPC (Zhang et al., 2010). In particular, Notch can drive tumorigenesis through the promotion of cell cycle progression by inducing Cyclin D1 and Myc (Ranganathan et al., 2011). Furthermore, the inhibition of the Notch pathway in an NPC cell line CNE1 has been shown to inhibit proliferation and promote apoptosis, accompanied by downregulation of cyclins D and E1 (Chen et al., 2011). In this study, concomitant change of the levels of cyclin D1 and E1 is observed with FJX1 expression level, suggesting that FJX1 might promote proliferation through the Notch signaling.

The dysregulation of the Hippo tumor suppressor pathway is another potential mechanism through which FJX1 acts to modulate cell proliferation. The Hippo pathway regulates animal organ size by controlling cell proliferation and cell death (Dong et al., 2007), and its dysregulation has been increasingly implicated in human cancers (Zhao et al., 2010). The Drosophila Fj modulates the interaction of Fat and Dachsous, the upstream signalling regulators of Hippo pathway, through its kinase activity (Brittle et al., 2010; Ishikawa et al., 2008; Simon et al., 2010). This pathway has been shown to control cell proliferation by regulating Cyclin E in

Drosophila (Bennett & Harvey, 2006; Harvey et al., 2003; Silva et al., 2006; Udan et al., 2003; Willecke et al., 2006). In the present study, the level of Cyclin E1 positively correlated with FJX1 expression in NPC cells (Section 3.5.3.1), implicating FJX1 could be a downstream target of Hippo pathway in human. Currently the only knowledge on the Hippo pathway in NPC is that an important regulator LATS2 was reported to be overexpressed in NPC with its expression correlates with poor prognosis (Zhang et al., 2010).

Cyclins D1 and E1 are responsible in mediating G1-S phase progression of the cell cycle. However, cell cycle analysis using Fluorescence-Activated Cell Sorting (FACS) in the FJX1-knockdown or -overexpressing cells yielded inconsistent results (data not shown). Therefore, additional experiments to determine the role of FJX1 in regulating cell cycle progression in NPC cells are required.

One of the cellular responses to oncogenic transformation is structural rearrangement of the actin cytoskeleton, leading to the formation of specialized structures involved in cell locomotion (Hall, 2009; Nurnberg et al., 2011). To determine whether FJX1 influences the migration of NPC cells, experiments were undertaken to examine the effect of FJX1 knockdown and overexpression using transwell assays. FJX1 did not cause any change in cell migration when FBS was used as a chemoattractant (Figure 3.11). Other chemoattractants such as fibronectin and vitronectin were however not included in this study. In addition, the use of transwells to examine cell migration does not faithfully recapitulate *in vivo* situation as they lack the complex interplay between tumor and the stromal environment that is vital for cell migration. Therefore, the role of FJX1 in regulating cellular migration remains inconclusive.

FJX1 enhanced invasive ability of the NPC cells through Matrigel (Section 3.5.4.2), a gelatinous protein mixture derived from culture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma that is known to resemble extracellular environment in many tissues (Benton et al., 2011). The re-initiation of the developmental regulatory program EMT during tumorigenesis could enable epithelial tumor cells to acquire invasive capability (Kalluri & Weinberg, 2009). However, no alteration in the expression of EMT-associated markers was seen in both knockdown and overexpression experiments (Section 3.5.6). Previously, tumor cells have been found to be able to remodel the actin cytoskeleton via podoplanin, a small mucin-like protein, to acquire invasive phenotype that is independent of EMT. Podoplanin induces actin remodeling to promote filopodia formation, important cellular structure for invasion, via downregulation of small Rho family GTPases (Wicki & Christofori, 2007; Wicki et al., 2006). The acquisition of more invasive phenotype via podoplanin-mediated pathway might offer an explanation on the lack of correlation of invasion with EMT seen in this study.

While de-regulation of the Notch or Hippo pathways might contribute to the up-regulation of FJX1 in NPC, other possible mechanisms also exist, including chromosomal amplification and EBV infection. FJX1 is located at 11p13, a region that has been repeatedly shown to be amplified in many types of human primary cancer tissues and cell lines, including cancers of the breasts (Klingbeil et al., 2010), gastric (Carvalho et al., 2006; Zhang et al., 2011), oral (Snijders et al., 2005), lung (Lockwood et al., 2008; Starczynowski et al., 2011; Sung et al., 2010), esophageous (Chattopadhyay et al., 2010; Miller et al., 2006), prostate (Ma et al., 2009), small bowel (Diosdado et al., 2010) and acute myeloid myeloma (Sarova et al., 2010). In addition, 11p13 is found to be significantly amplified in cancer cell lines derived from various

different origins (Beroukhim et al., 2010). EBV is the viral etiological agent in NPC and it is well recognized that EBV alters many functional properties that are involved in tumor progression (Raab-Traub, 2002). It is a possibility EBV infection could contribute to the overexpression of FJX1 in NPC. Therefore, additional studies investigating the mechanisms that are responsible for overexpression of FJX1 in NPC are warranted.

CONCLUSION

FJX1 is a candidate oncogene in NPC. It is overexpressed at both the transcript and protein levels in NPC, as well as expressed at low level in human organs. It promotes proliferation, invasion and anchorage-independent growth in NPC cells. Furthermore, its expression is accompanied by concomitant change in Cyclins D1 and E1, without change in neither EMT nor differentiation status. In summary, FJX1 represent an attractive therapeutic target for NPC.

In the future, it is necessary to establish the genetic circumstance of FJX1 overexpression in NPC, not only to ascertain the mechanism of FJX1 overexpression, but also to shed light onto the pathways to which it belongs. Furthermore, as FJX1 is also amplified in other cancers as well as potentially has kinase activity, the protein might also be a candidate oncogene in other human neoplasia. *In vitro* assays on knocking down of FJX1 in FJX1-amplified cancer cell lines would verify whether the gene is a driver of the 11p13 amplicon that is observed in various cancers. To conclusively implicate FJX1 as a candidate oncogene in NPC as well as other cancers, *in vivo* tumerigenicity assay would be required. Finally, the identification of an FJX1-specific inhibitor might pave the way for an FJX1-based therapeutic intervention in NPC and potentially other cancers.