CHAPTER III

RESULTS

3.1 CANDIDATE GENE SELECTION

A combination of microarray analysis and candidate-gene approach was employed to identify candidate biomarkers or oncogenes in NPC. First, overexpressed genes in NPC were identified from a previously published expression microarray study that compared 25 primary NPC against 3 non-malignant nasopharyngeal tissues (Bose et al., 2009). Subsequent overlapping of this gene list with genes that are expressed at low level in normal essential organs (microarray data set from bone marrow, brain, lung, liver, heart; Prof Yusuke Nakamura from Human Genome Centre, University of Tokyo, Japan), uncovered high priority candidate genes. 41 genes were identified to be upregulated in NPC but expressed at low level in important normal human organs.

For the purpose of developing monoclonal antibody therapies and serum biomarkers, 18 genes that encode for secreted or membrane-bound proteins were selected. Among these genes, seven of them were chosen on the basis of their involvement in aspects of normal development or in cancer pathophysiology as reported in literature from the period of December 2006 until February 2007. They are: 1) FJX1 (Ashery-Padan et al., 1999; Rock et al., 2005a; Rock et al., 2005b; Snijders et al., 2005; Yamaguchi et al., 2006; Jarvinen et al., 2008), 2) WNT5A (Rodolfo et al., 2004; Blanc et al., 2005a; Blanc et al., 2005b; Katoh et al., 2005; Turashvili et al., 2006), 3) CLCA2 (Gruber & Pauli, 1999; Abdel-Ghany et al., 2001; Bustin et al., 2001; Elble & Pauli et al., 2001; Konopitzky et al., 2002), 4) FZD6 (Katoh, 2005, 2007), 5) FGFR3 (Mhawech-Fauceglia et al., 2006; Schulz et al., 2006; Luis et al., 2007), 6) CLDN1 (Swisshelm et al., 2005; Kominsky, 2006), and 7) RALA (Ehrhardt et al., 2002; Oxford & Theodorescu, 2003; Camonis & White, 2005)

3.2 VALIDATION OF GENE EXPRESSION

3.2.1 Overexpression in primary NPC tissue samples

qPCR was performed to validate the overexpression of the 7 candidate genes in primary NPC tissues. Except RALA, all other 6 genes showed overexpression in virtually all of the NPC samples analyzed when compared to two biopsies of normal nasopharynx (NPC3 and TSE5) and a biopsy of nasopharyngitis (TSE16) that were used as controls. For each gene, the control sample with the highest level of expression was used as the calibrator (Figure 3.1).

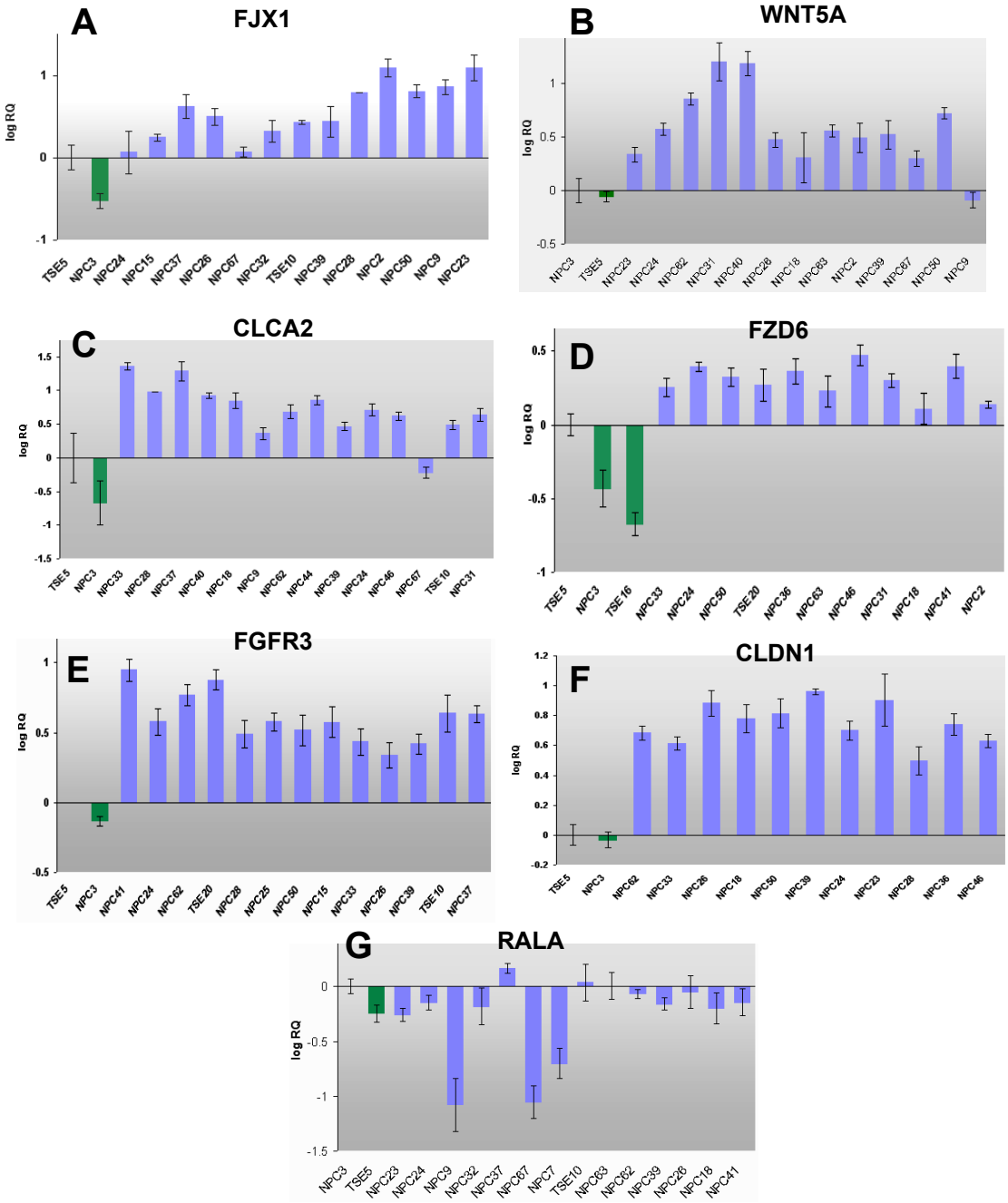

Figure 3.1 | Validation of upregulated expression of candidate genes in primary NPC tissues. (A to F) The overexpression of 6 out of the initial 7 candidate genes which was observed in microarray (Bose et al., 2009) were validated using qPCR. Compared to non-malignant controls (TSE5, NPC3, TSE16), the upregulation of these 6 candidate genes (FJX1, WNT5A, CLCA2, FZD6, FGFR3, and CLDN1) were shown in nearly all of the primary NPC tissue samples examined. (G) RALA, while was observed to be upregulated in the microarray data, failed validation. For each candidate gene, the control sample with the highest level of expression was used as the calibrator. Error bars delineate log RQ Min and Max.

3.2.2 Low expression in normal human organs

To validate the low expression of the 6 candidate genes in human organs, the expression of these genes was determined in a panel of 16 normal human organs using commercially available cDNAs, Human Multiple Tissue Panel™ (MTC) I and II, via semi-qPCR. The cDNA amount for each sample was normalized by the manufacturer (Clontech), obviating the need for loading control reactions.

When semi-qPCR was first performed using Panel I cDNAs which consists of cDNAs from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas, the expression of FJX1, WNT5A, and CLCA2 was shown to be generally low in the initial 8 organs examined (Figure 3.2A). These 3 genes were chosen for further validation in Panel II which consists of cDNAs from spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte. Similarly, the expression of the 3 candidate genes was negligible in these 8 organs (Figure 3.2B), confirming the low expression of these genes in a wide range of normal human organs.

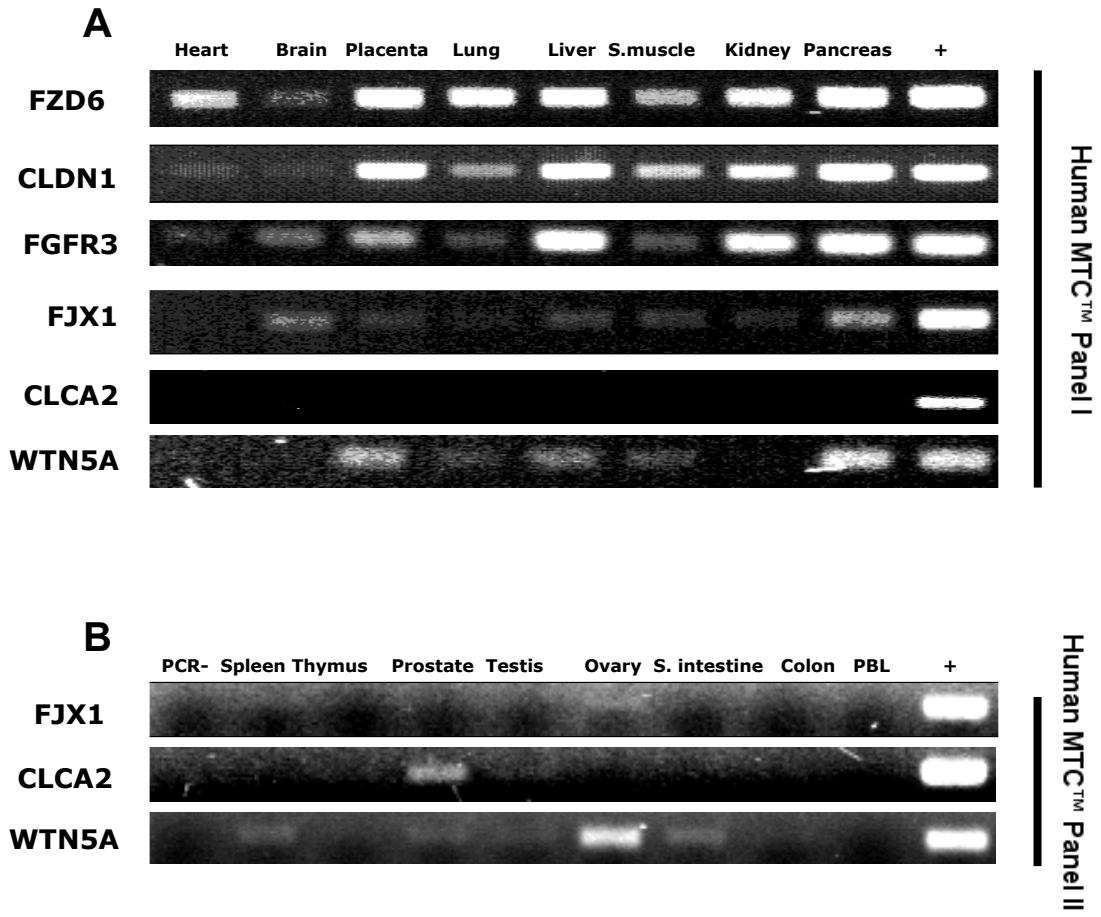

Figure 3.2 | Validation of low expression of candidate genes in normal human organs. Semi-qPCR for 35 cycles waperformed using commercial cDNAs derived from normal human organs (Human MTC™ Panel I and II) as templates. Experiment were done first using Human MTC™ Panel I, where FJX1, CLCA2 and WNT5A were deemed to be expressed at low level across the initial 8 normal human organs tested (A). Further semi-qPCR experiment using Human MTC™ Panel II showed that FJX1, CLCA2 and WNT5A were expressed at low level in another 8 normal human organs (B). "S.muscle" denotes smooth muscle (A), "PBL" denotes peripheral blood leukocyte (B), while "+" denotes positive control cDNA from NPC cell lines.

3.3 PROTEIN EXPRESSION IN PRIMARY NPC TISSUES

To determine whether the protein levels of FJX1, WNT5A and CLCA2 were overexpressed in primary NPC tissues, their protein expression and cellular location were examined in 43 samples of formalin-fixed paraffin-embedded (FFPE) primary NPC and 11 samples of non-malignant nasopharyngeal tissues by immunohistochemistry. The assessment of the staining was performed by a consultant pathologist, Prof Rajadurai Pathmanathan, from Sime Darby Medical Centre Subang Jaya, Subang Jaya.

While all 11 non-malignant nasopharyngeal tissues consistently showed no staining (Figure 3.3), FJX1 expression was significantly higher in 42% (18 out of 43) NPC tissues examined (P=0.010, Fisher's exact test). The staining was cytoplasmic, ranged in score from +1 to +3, and varied in pattern from focal to diffuse. Ductal structures in the tissue samples were always positive, demonstrating the specificity of the antibody (Figures 3.3C, 3.3E and 3.3G). However, no correlation was found between patient TNM status and FJX1 staining (Table 3.1).

Due to the insensitivity and unspecificity of the antibodies against WNT5A and CLCA2, the staining of these two candidate proteins on NPC tissues could not be completed. This study hereafter focused on FJX1 as the candidate biomarker/oncogene.

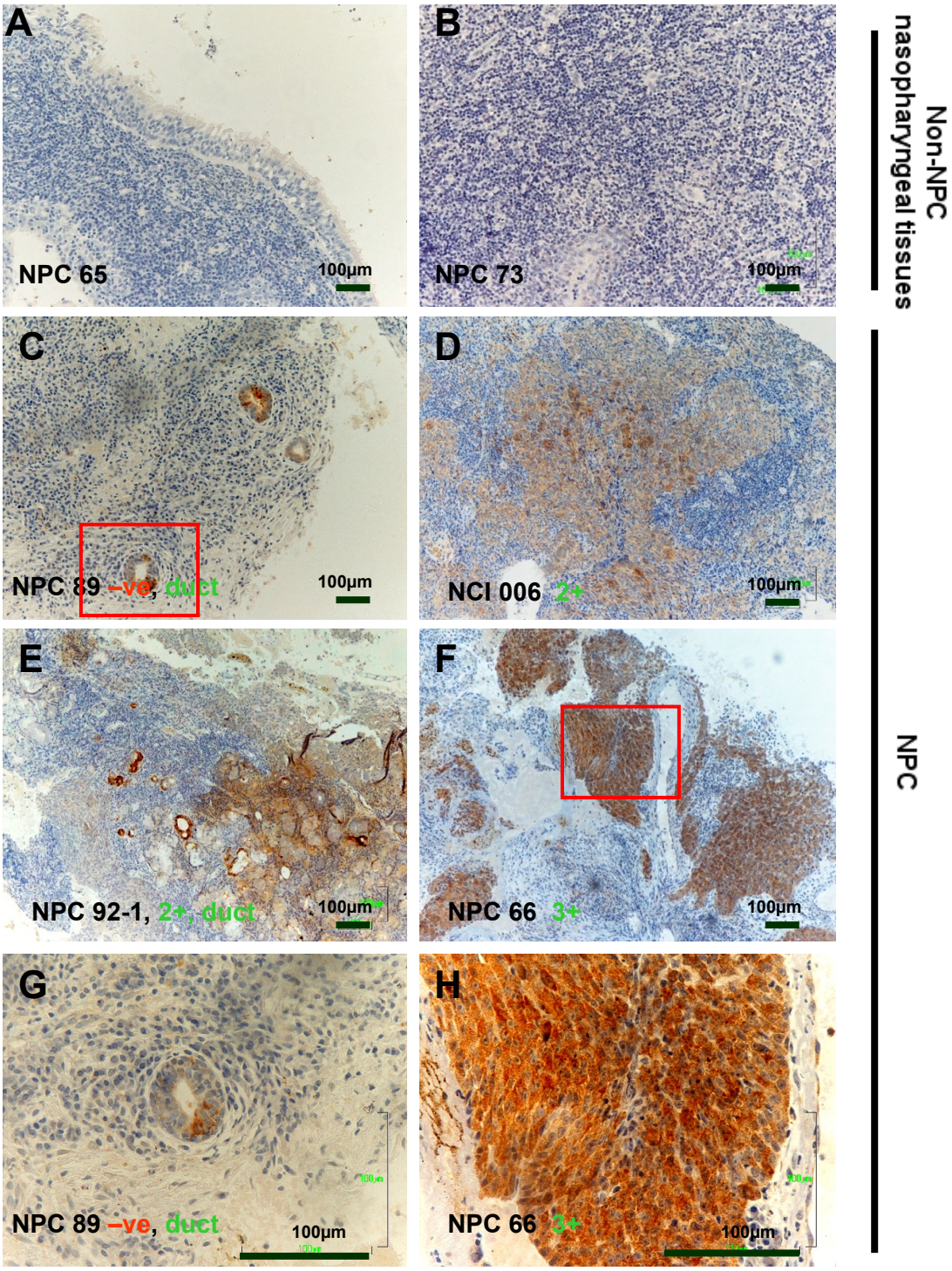

Figure 3.3 | FJX1 was upregulated at the protein level in NPC. Non-malignant nasopharyngeal tissues consistently stained negative for FJX1 (A). (B), a no-primary antibody negative control. (C), a representative NPC tissue void of FJX1 stain in malignant cells, but with staining in endothelial structure. Representative 2+ stain (D and E) and 3+ stain (F) are shown. (G) and (H) are close-up images of (C) and (F), respectively.

Table 3.1 | FJX1 immunohistochemistry analysis. FJX1 was overexpressed at protein level in NPC samples (18/43=42% NPC, all 11 non-NPC negative, *P*=0.010) as determined by immunohistochemistry. However, no correlation between patient TNM status with FJX1 immunohistochemistry stain was observed. *P* values were derived from Fisher's exact tests.

Parameter		FJX1 staining status			
		n	+	-	% +
Disease					
	non-NPC	11	0	11	0
	NPC	43	18	25	42
					P = 0.010
Tumor size					
	T1	6	3	3	50
	T2	19	8	11	42
	T3	12	4	8	33
	T4	6	3	3	50
					P = 0.948
Lymph node metastasis					
	N0	12	5	7	42
	N1	6	4	2	67
	N2	23	9	14	39
	N3	2	0	2	0
					P = 0.841
Stage					
	I	3	2	1	67
	II	7	4	3	57
	III	24	9	15	38
	IV	9	3	6	33
					P = 0.840

3.4 FJX1 expression in NPC and immortalized nasopharyngeal epithelium cell lines

In order to select cell lines that could be used for FJX1 *in vitro* studies, endogenous FJX1 transcript level was determined in a series of NPC and two immortalized nasopharyngeal epithelium cell lines (NP69 and NP460) by qPCR. Due to a lack of reliable commercial antibody against FJX1 for use in Western blotting, the level of endogenous FJX1 protein in the cell lines could not be determined. As determined by qPCR, the NPC cell lines expressed FJX1 at varied levels (Figure 3.4); when nonmalignant epithelial cell line NP69 was used as calibrator, HONE1 and HK1 cells expressed high amount of FJX1 transcript, whereas SUNE1 and TW04 expressed FJX1 transcript at low level. Interestingly, another nonmalignant cell line NP460 expressed high level of FJX1 mRNA.

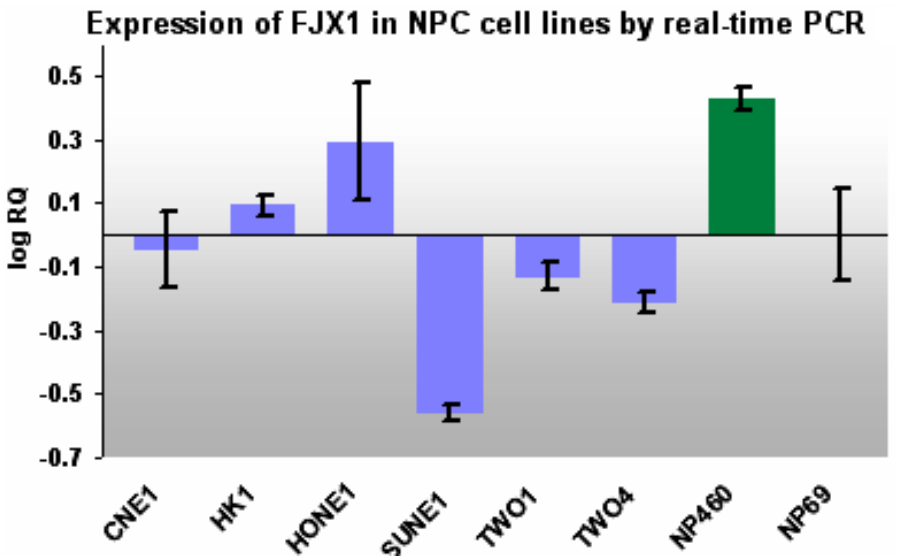

Figure 3.4 | FJX1 expression in NPC cell lines varies. qPCR on NPC and immortalized normal nasopharyngeal epithelium cell lines showed that FJX1 was expressed at varied levels. Calibration was made against immortalized nasopharyngeal epithelium cell line NP69. HK1 was chosen for loss-of-function while SUNE1 and TWO4 were chosen for gain-of-function studies. Error bars delineate log RQ Min and Max for each sample.

3.5 FJX1 *IN VITRO* STUDIES

3.5.1 Endogenous FJX1 knockdown

Depletion of endogenous FJX1 transcript was carried out using pooled small interfering RNAs (siRNAs) that consisted of siRNAs with four different sequences that target the coding region of FJX1 transcript (the FJX1-targeting siRNAs hereafter designated as siFJX) (Figure 2.1). Pooled siRNAs that bear no significant homology to any known human gene sequence was used as negative control (the control 'non-targeting' siRNAs hereafter is referred as siNT). Initially, HK1 and HONE1 cells which expressed high FJX1 transcript levels were chosen for knockdown purpose.

To establish an optimal condition for knockdown experiments, titration experiments were first performed to assess the siRNA concentration that would give the highest knockdown using 10, 30, 50 or 100nM of siRNA after 2 days. Time-course experiments were subsequently conducted to monitor the knockdown effect for a five day period. As previously mentioned (Section 3.4), the lack of a reliable commercial antibody did not permit the examination of FJX1 protein level on siRNA treatment. qPCR analysis was used instead to determine the level of FJX1 transcript.

3.5.1.1 FJX1 knockdown in HONE1 cells

For reasons unknown, two attempts of titration experiments using HONE1 cells failed to determine a consistent optimal siFJX concentration for FJX1 knowdown. Furthermore, a time-course experiment using 50nM siRNA showed that the inhibition of FJX1 transcript could only be sustained up to 2 days post-transfection (data not shown). Therefore, HONE1 cells were not used for any subsequent FJX1 knockdown assays.

3.5.1.2 FJX1 knockdown in HK1 cells

Among the four different siFJX concentrations tested, HK1 cells consistently exhibited the highest FJX1 knockdown when treated with 50nM siFJX (Figure 3.5A), with the inhibitory effect sustained up to 5 days (Figure 3.5B). The knockdown appeared to be FJX1-specific as no noticeable effect on FJX1 level between mock untreated and siNT-treated cells, as well as on the endogenous control gene GAPDH level between mock untreated, siNT- and siFJX-treated, was observed (data not shown). All ensuing loss-of-function *in vitro* assays employed only HK1 cells. Hereafter, HK-siNT is used to denote HK1 cells transfected with siNT, while HK-siFJX designates HK1 cells transfected with siFJX.

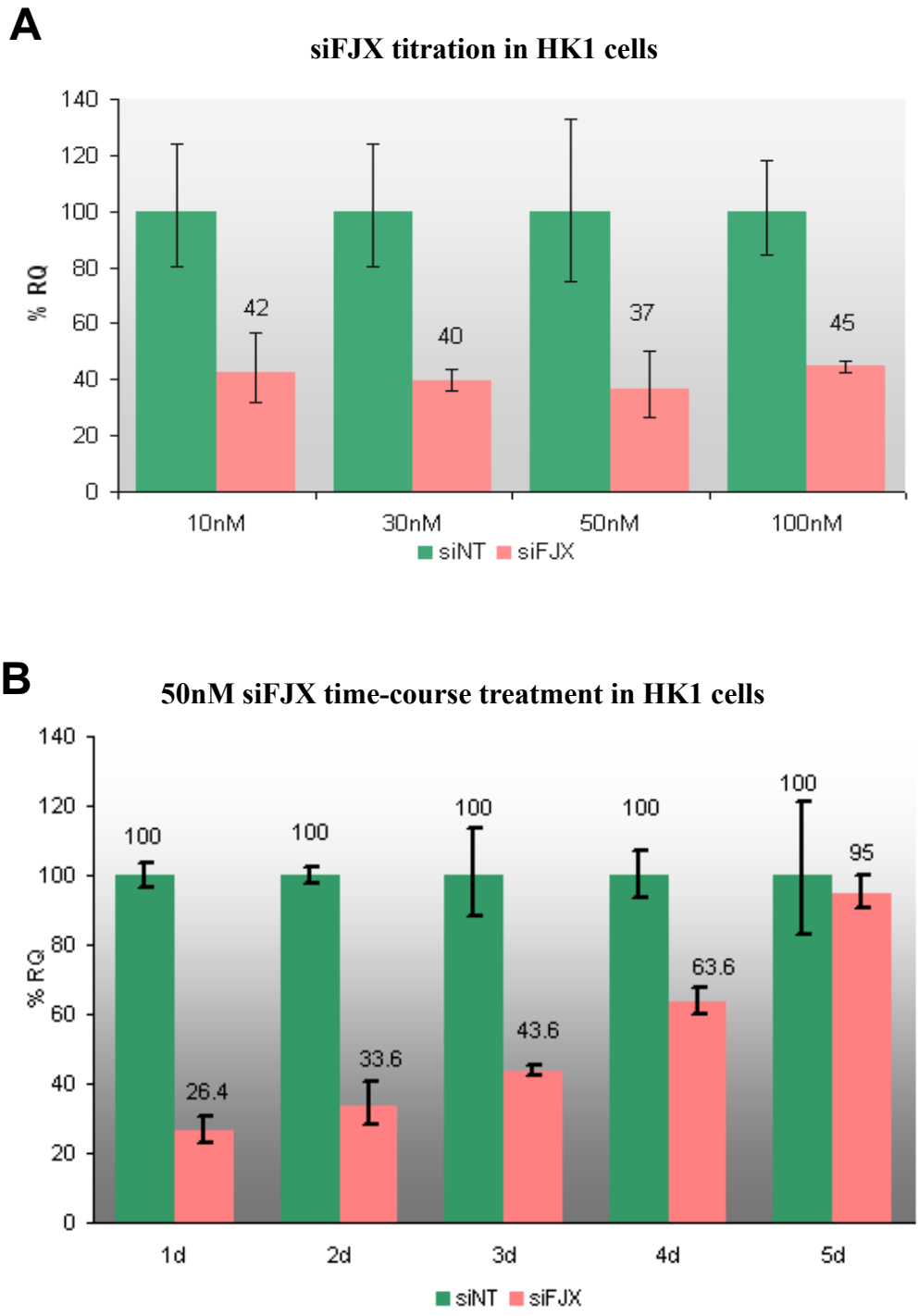

Figure 3.5 | Endogenous FJX1 knockdown in HK1 cells. siFJX titration experiments, performed twice, showed that optimal FJX1 knockdown after 2 days was achieved using 50nM siFJX. (A), Representative FJX1 expression on siFJX titration. Time-course FJX1 expression showed that knockdown of FJX1 was sustained until day 4 post-transfection (B).

3.5.2 Recombinant FJX1 protein overexpression

To perform gain-of-function studies, FJX1 coding region was cloned into pcDNA3.1-V5/His-B, a mammalian expression plasmid. The introduction of this construct in mammalian cells would result in a high expression of recombinant FJX1 protein with a C-terminal tag encoding the V5 epitope followed by a polyhistidine sequence (the recombinant protein hereafter designated as FJX:V5) (Figure 2.2). The expression of the FJX:V5 protein was detected using anti-V5 antibody in Western blotting and immunocytochemistry. Two NPC cell lines, TWO4 and SUNE1 that expressed low level of endogenous FJX1 transcript were chosen for overexpression purpose.

To determine the expression of FJX1 protein, Western blot analysis was performed on pcDNA3.1-FJX1 transfected (designated TW04-FJX and SUNE-FJX) and vector control transfected (designated TW04-pcDNA and SUNE-pcDNA) cells. The recombinant FJX1 protein was not detected in TW04-pcDNA and SUNE-pcDNA, while TW04-FJX and SUNE-FJX showed a high level of recombinant FJX:V5 protein with a molecular mass close to the predicted size of 48kDa. Time course experiment showed that the transient FJX:V5 overexpression was sustained up until fourth day post-transfection (Figure 3.6). The expression of FJX1 protein was further confirmed in SUNE1- and TW04-FJX1 transfectants by immunocytochemistry. While vector-transfected cells were void of signal, intense brown staining was observed in SUNE- and TWO4-FJX cells at the perinuclear area (Figure 3.7).

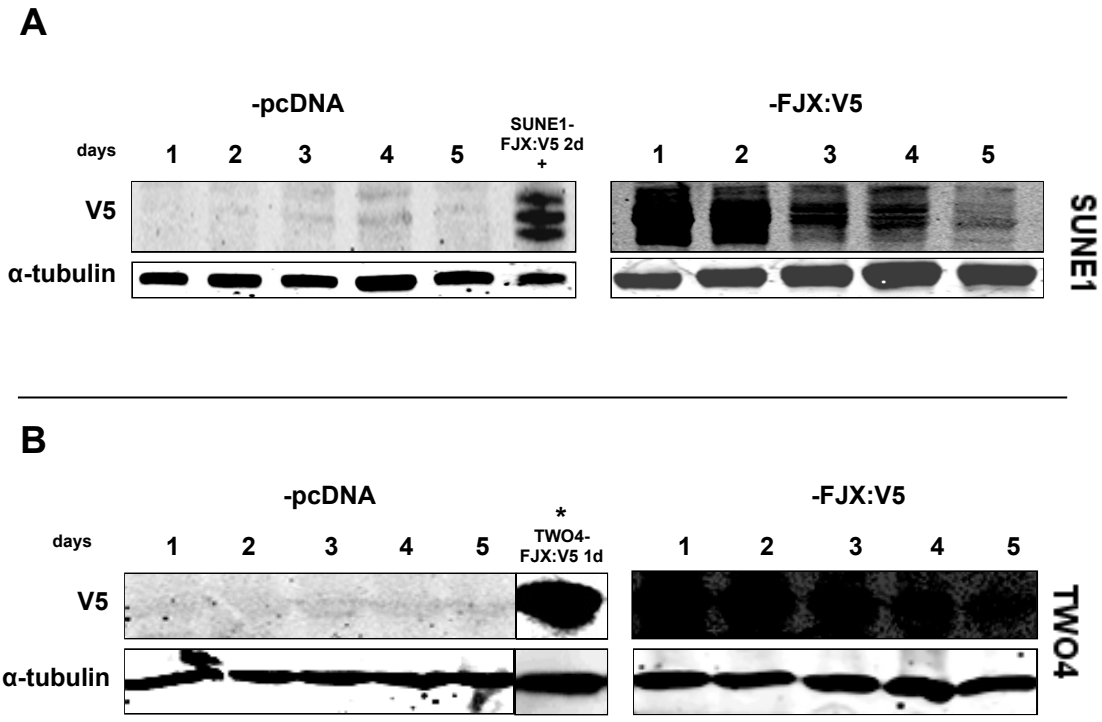

Figure 3.6 | Overexpression of FJX:V5 in SUNE1 and TWO4 cells as detected by Western. Transient overexpression of recombinant FJX:V5 protein was sustained in SUNE1 (A) and TWO4 (B) cells until 4 days post-transfection. Asterisk (*) denotes sample lysate from TWO4-FJX:V5 day 1 that was used as positive control in the same blot for TWO4-pcDNA samples (B).

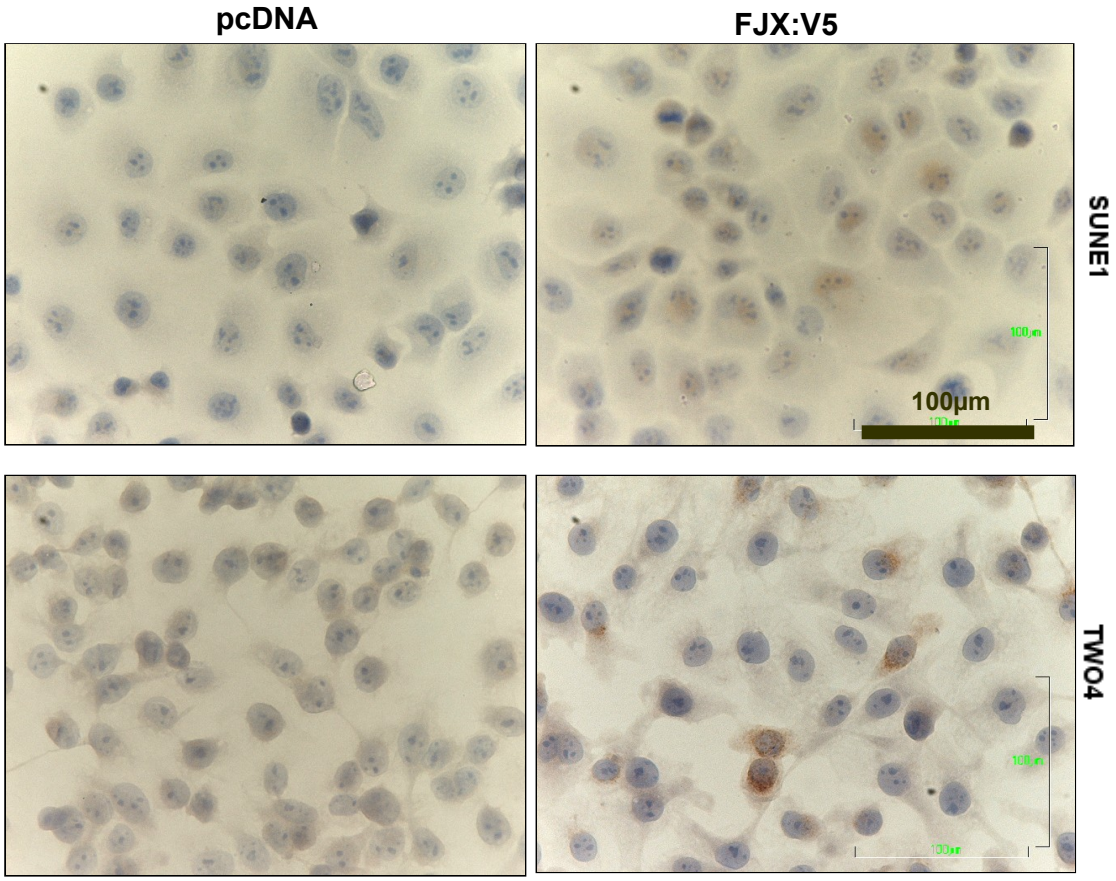

Figure 3.7 | Overexpression of FJX:V5 in SUNE1 and TWO4 cells as detected by immunocytochemistry. SUNE1 and TWO4 cells transfected with pcDNA3.1-FJX produced recombinant FJX1:V5 protein that was localized around the perinuclear area as revealed by immunocytochemistry using V5 antibody (brown stain, right panels). Cells transfected with vector plasmid meanwhile were void of the signal (left panels). The nucleus was stained blue with haemotoxylin. All images were taken at the same magnification.

3.5.3 FJX1 promoted cell proliferation

To determine the contribution of FJX1 in cell growth, proliferation assay was done on HK1 and TWO4 cells following FJX1 knockdown and overexpression, respectively. Endogenous FJX1 depletion in HK1 cells impaired (Figure 3.8A), while enforced FJX:V5 expression in TWO4 enhanced cell proliferation (Figure 3.8B).

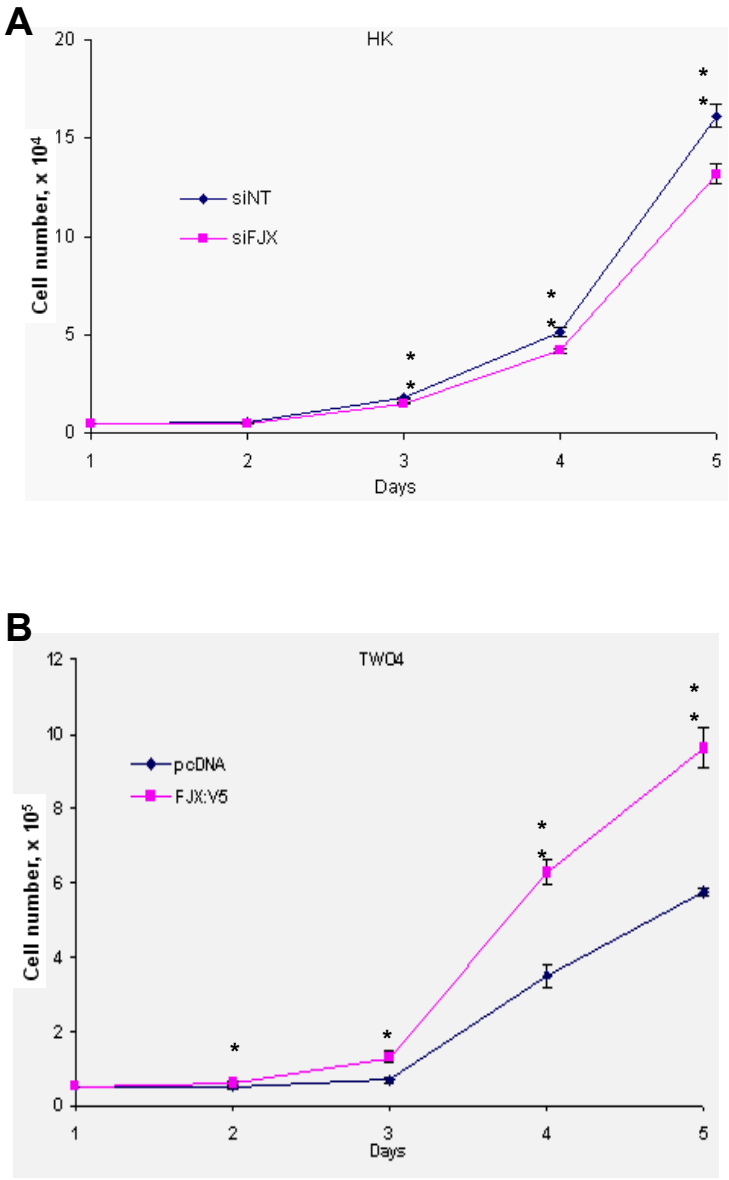

Figure 3.8 | FJX1 enhanced cell proliferation. Knockdown of endogenous FJX1 in HK1 cells decreased (A), while overexpression of FJX1 in TWO4 cells increased (B) cell proliferation as monitored over 5 days period. **P*<0.01, ***P*<0.001 (*P*-value of unpaired two tailed *t*-test).

3.5.3.1 FJX1 enhanced cell proliferation possibly through regulation of Cyclins D1 and E1

To unravel the mechanism by which FJX1 affects proliferation, the transcript levels of cyclins D1 and E1 were determined in cells following overexpression and knockdown of FJX1. Knockdown of FJX1 in HK1 cells and overexpression of FJX:V5 in TWO4 respectively resulted in decrease (Figure 3.9A) and increase (Figure 3.9B) of cyclins D1 and E1 transcript levels.

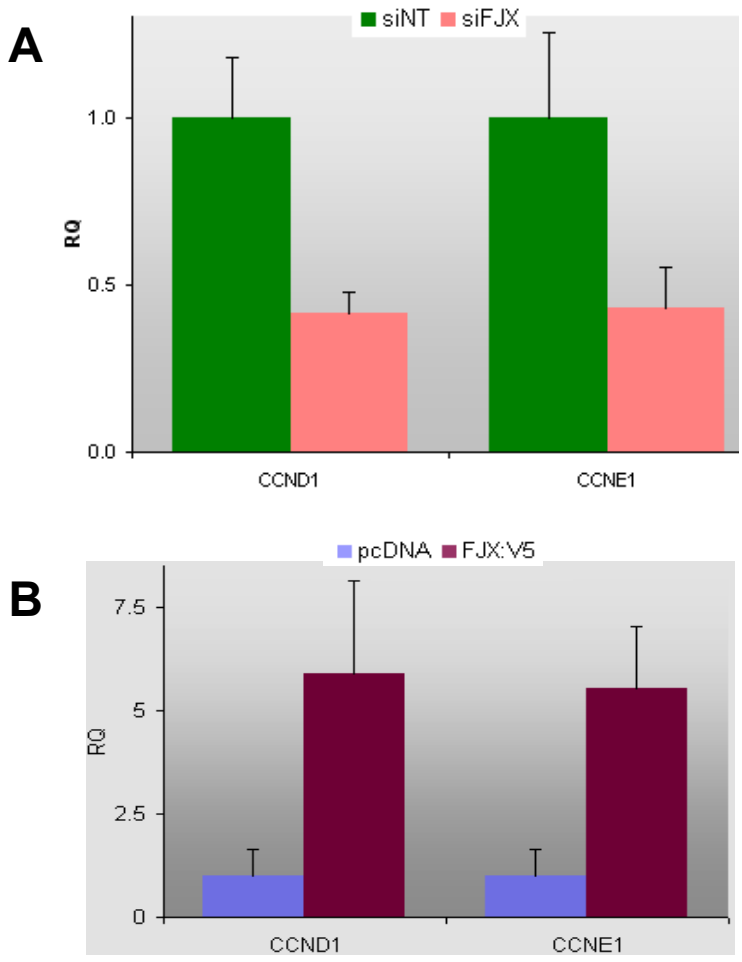

Figure 3.9 | Alteration in FJX1 level was accompanied by change in cyclins D1 and E1 transcript. Knockdown of FJX1 in HK1 cells decreased (A), while overexpression of FJX1 increased transcript level of cyclins D1 and E1 (B).

3.5.4 Mitomycin C arrested cell proliferation without altering the effects of FJX1 knockdown or overexpression

To ensure the results of migration and invasion assays were not swayed by alteration of cell proliferation on FJX1 knockdown or overexpression, cells were treated with Mitomycin C, which inhibits cell mitosis via interfering with thymidine incorporation into DNA during replication (Schwartz et al., 1963), prior to the assays. Herein after, “mito C” suffix denotes cells with prior Mitomycin C treatment.

To assess whether the drug treatment would affect FJX1 expression following knockdown or overexpression, the FJX1 level was measured in the siRNA- or plasmid-transfected cells after mitomycin C treatment. The drug treatment did not alter the effectiveness of FJX1 knockdown or overexpression in HK1 or TW04 cells respectively, as well as did not cause any discernible change in proliferation (Figure 3.10).

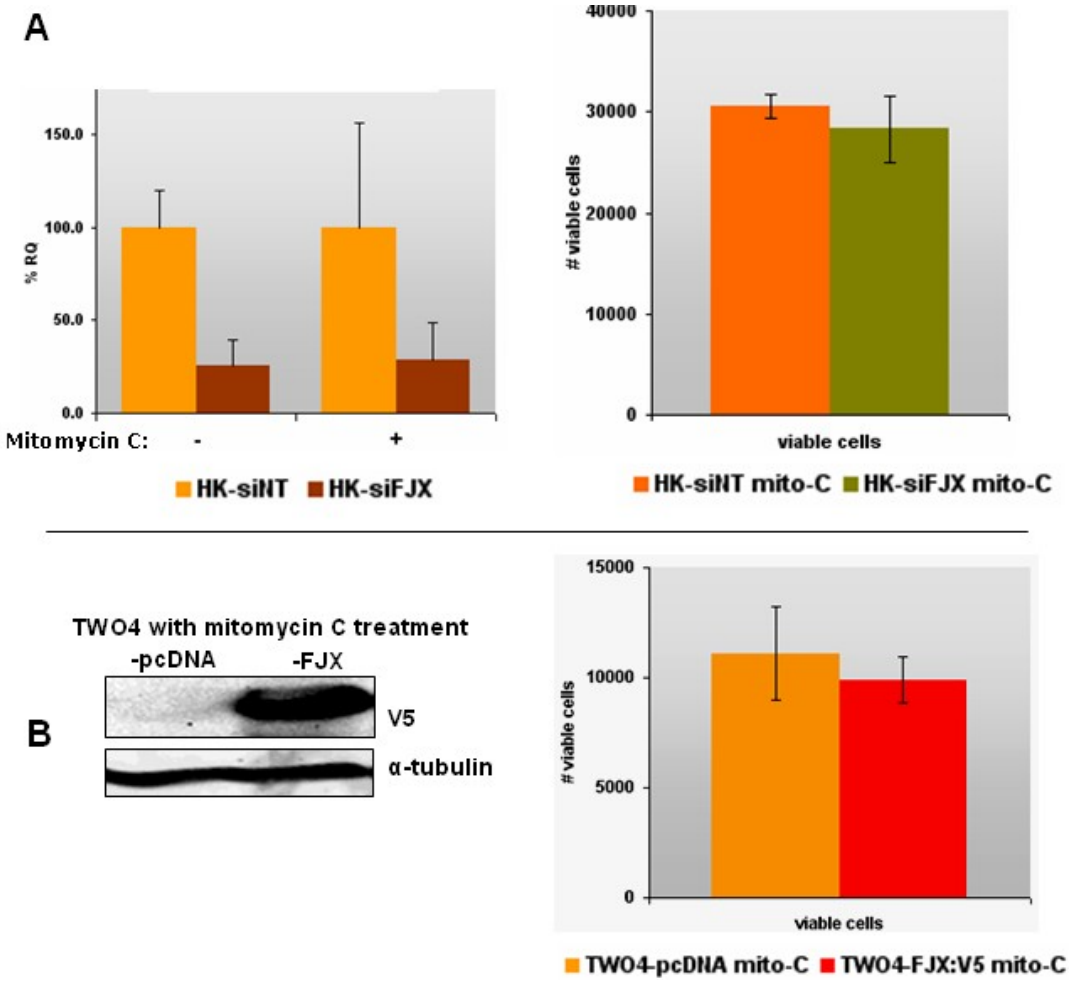

Figure 3.10 | Mitomycin C treatment did not affect FJX1 knockdown or overexpression, but was effective in restricting cell proliferation. The knockdown effect of siFJX on endogenous FJX1 transcript in HK1 cells (A), and the overexpression of FJX:V5 in TWO4 cells (B), were not compromised by mitomycin C treatment. In both cases, the number of cells after a 2-day-period was not statistically different.

3.5.4.1 FJX1 did not change cell migratory ability towards FBS

To assess whether FJX1 is involved in cell migration, transwell migration assay was performed using HK1 and TWO4 cells following knockdown or overexpression of FJX1, respectively. When FBS was used as chemoattractant, neither knockdown nor overexpression of FJX1 resulted in a change of the migratory ability of the cells (Figure 3.11).

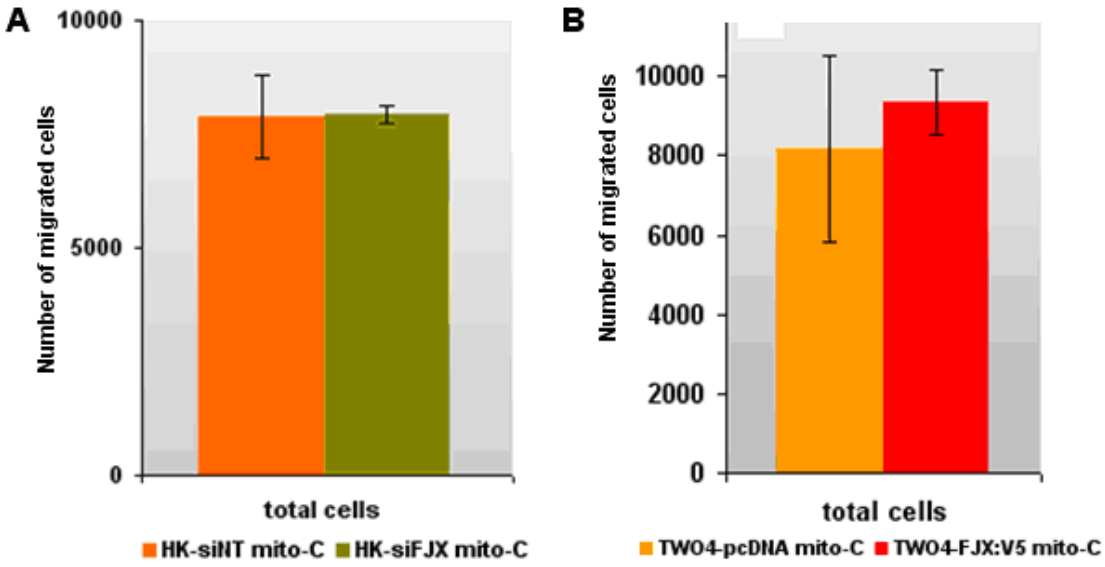

Figure 3.11 | FJX1 did not change migratory ability of cells towards FBS. Neither overexpression (A) nor knockdown (B) of FJX1 caused statistically significant change in the number of cells migrated towards FBS.

3.5.4.2 FJX1 enhanced cell invasion

To investigate the effect of FJX1 on the cell invasive ability, Matrigel invasion assay was performed using HK1 and TWO4 cells following overexpression and knockdown of FJX1, respectively. While knockdown of FJX1 caused reduced invasive ability of HK1 cells (Figure 3.12A), overexpression of FJX1 resulted in increased invasive ability of TWO4 cells (Figure 3.12B).

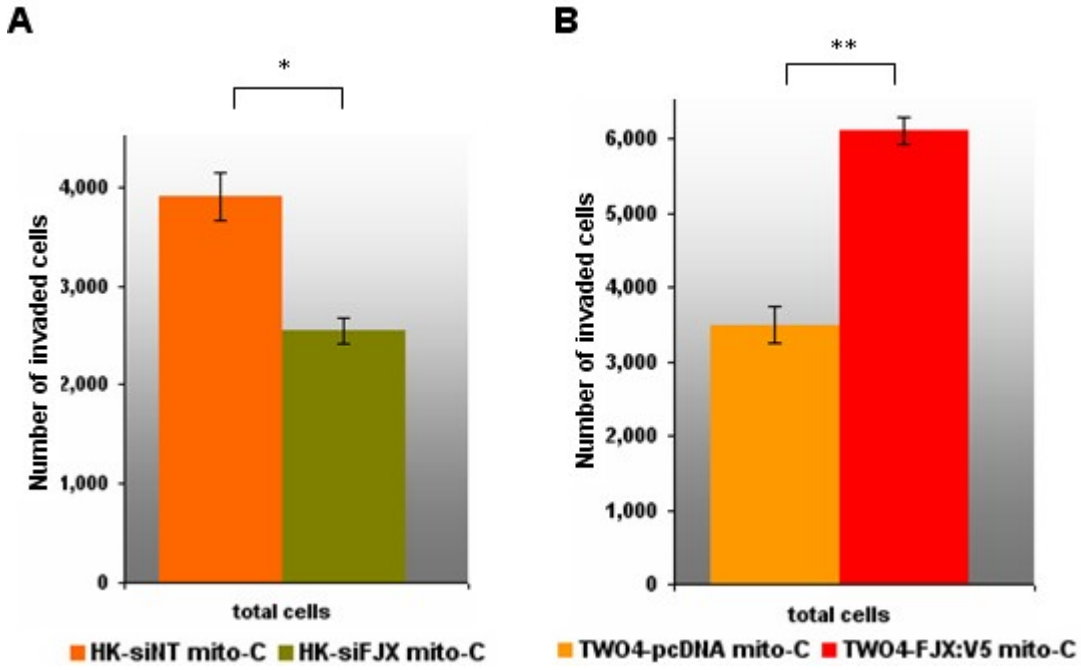

Figure 3.12 | FJX1 enhanced cell invasion. FJX1 knockdown decreased (A) while FJX1 overexpression increased (B) number of invaded cells through Matrigel, as measured by invasion assay. *P<0.05, **P<0.01 (P-value of unpaired two tailed t-test).

3.5.5 FJX1 increased anchorage-independent cell growth

Colony-formation assay was performed to assess the contribution of FJX1 in anchorage-independent cell growth. HK1 cells could not form colonies in soft agar despite several attempts, confirming previous observation (Wong et al., 2010). Hence, the assays were only done in a gain-of-function manner using TW04 and SUNE1 cells. Overexpression of FJX1 in both TWO4 and SUNE1 cells resulted in an increased number of colonies in soft agar (Figure 3.13).

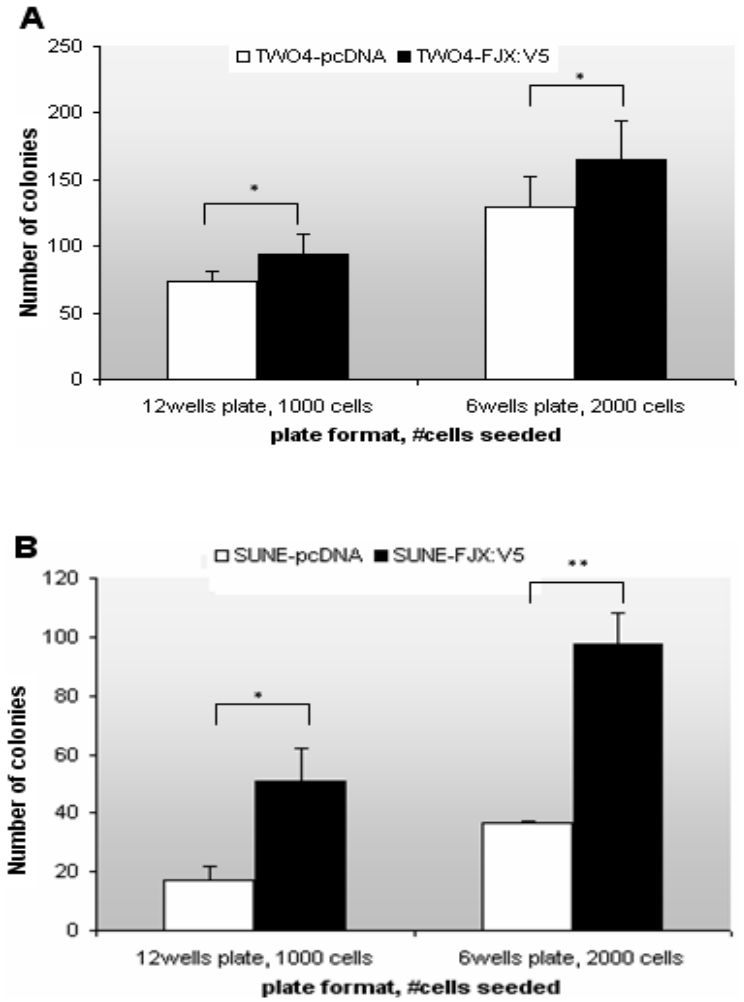

Figure 3.13 | FJX1 facilitated anchorage-independent growth. TWO4 (A) and SUNE1 (B) cells transfected with FJX1 showed increased ability to form colonies in soft agar. Bars represent mean, while error bars represent standard deviation. **P*<0.01, ***P*<0.001 (*P*-value of unpaired two tailed *t*-test).

3.5.6 FJX1 was not involved in epithelial-to-mesenchymal transition and cellular differentiation

The enhancement of cell invasiveness by FJX1 might be explained in terms of change in epithelial or mesenchymal state of the cells, a developmental regulatory program known as Epithelial-to-Mesenchymal Transition (EMT). Therefore, the total levels of several EMT markers, as well as their cellular localization after knockdown or overexpression of FJX1 were determined using Western blotting and immunocytochemistry, respectively. No apparent change in total level (Figure 3.14) and localization (Figure 3.15) of the epithelial (β-catenin, E-cadherin, ZO-1) and mesenchymal (vimentin, snail, twist) markers was observed. For knockdown of FJX1 in HK1 cells, change in vimentin level could not be assessed as parental HK1 cells express undetectable level of vimentin.

In addition, to examine whether FJX1 is involved in cellular differentiation, the protein levels of keratinocyte differentiation markers CK5 and CK10 were determined. No obvious difference in the staining pattern or intensity between FJX1 cells and vector control cells was observed, demonstrating that FJX1 did not alter cellular differentiation *in vitro* (Figures 3.15 and 3.16).

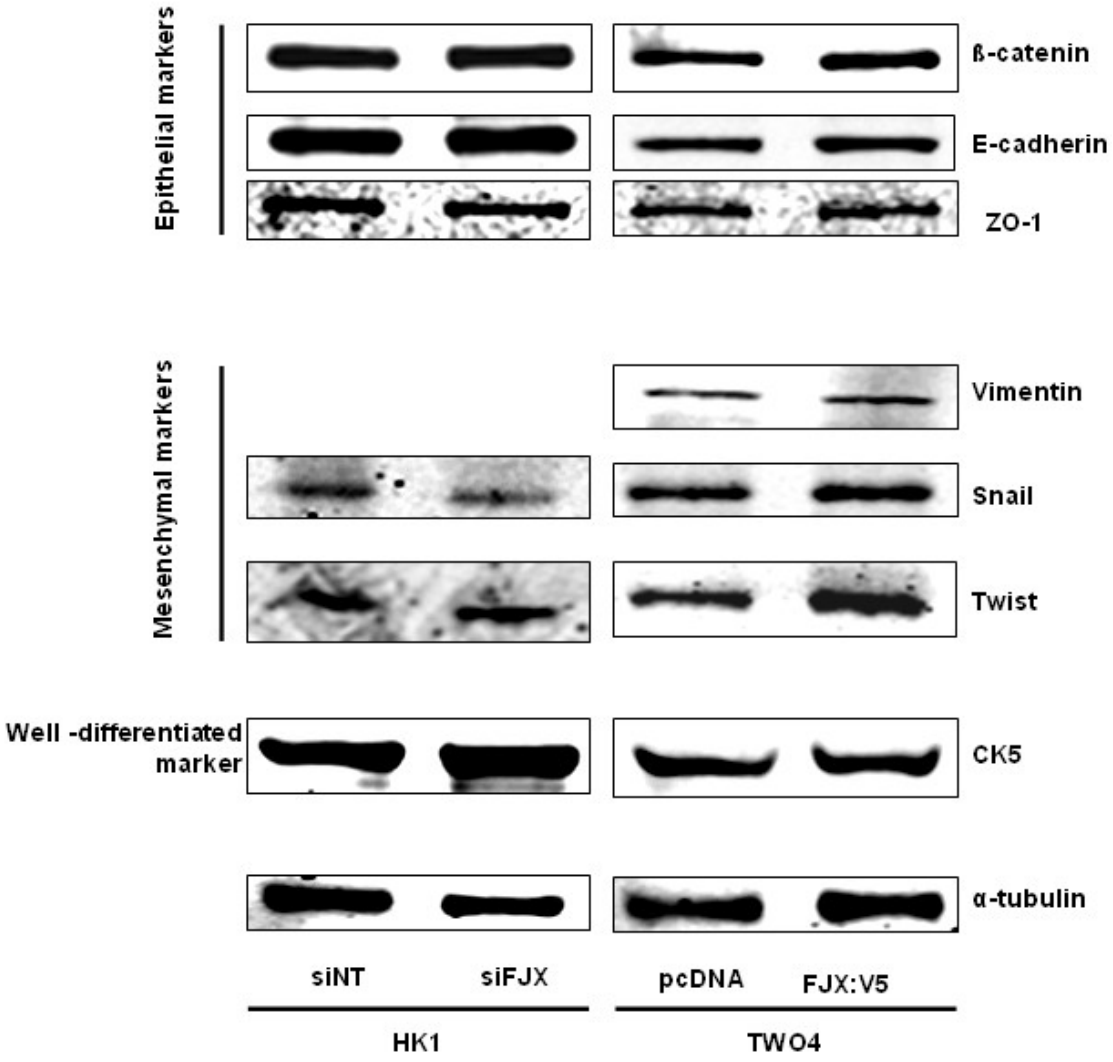

Figure 3.14 | Change in FJX1 level was not accompanied with change in EMT and differentiation marker levels. Neither knockdown nor overexpression of FJX1 caused any significant change in level of epithelial (β-catenin, E-cadherin, ZO-1), mesenchymal (vimentin, snail, twist) or differentiation (CK5) markers. The change in vimentin level in HK1 cells could not be determined as the parental cells express very low vimentin.

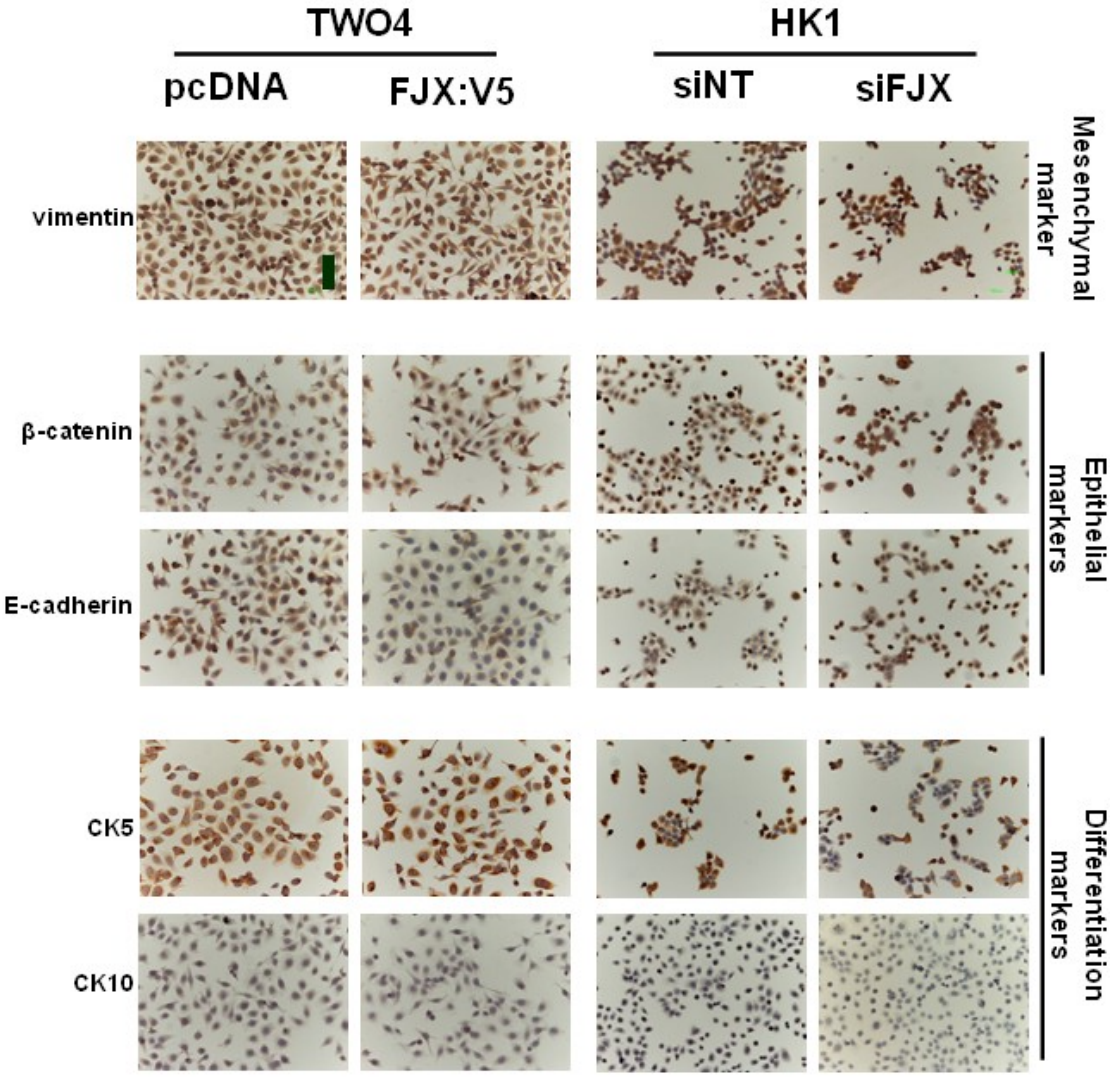

Figure 3.15 | Change in FJX1 level was not accompanied with change in localization and staining intensity of EMT markers. Neither knockdown nor overexpression of FJX1 caused any significant change localization and staining intensity of epithelial (β-catenin, E-cadherin), mesenchymal (vimentin) and differentiation (CK5, CK10) markers. All images were taken at the same maginification. Scale bar in vimentin TWO4-pcDNA (top left) represents 100μm.