Supplementary Data

Induction of Human Epithelial Progenitor Expansion by FOXM1

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Table S1 – Absolute qPCR Primer Sequences

Gene	Primer Sequence	Size(bp)
CEP55-F	TGAAGAGAAAGACGTATTGAAACAA	112
CEP55-R	GCAGTTTGGAGCCACAGTCT	
CORN-F	TCACTGTTGCAGCATGAGTTC	90
CORN-R	TGGCAAGGCTGTTTCACC	
DSG3-F	CCGAATCTCTGGAGTGGGAA	164
DSG3-R	GCCCAAGGACTAGATGTAGA	
EGFP-F	AAAGACCCCAACGAGAAGC	76
EGFP-R	CCATGCCGAGAGTGATCC	
FOXM1A-F	TGGGGAACAGGTGGTGTTTGG	120
FOXM1A-R	GCTAGCAGCACTGATAAACAAAG	
FOXM1B-F	CCAGGTGTTTAAGCAGCAGA	279
FOXM1B-R	TCCTCAGCTAGCAGCACCTTG	
FOXM1C-F	CAATTGCCCGAGCACTTGGAATCA	287
FOXM1C-R	TCCTCAGCTAGCAGCACCTTG	
IVL-F	TGCCTGAGCAAGAATGTGAG	83
IVL-R	TTCCTCATGCTGTTCCCAGT	
K1-F	CGGAACTGAAGAACATGCAG	128
K1-R	CATATAAGCACCATCCACATCC	
K2E-F	GCCTCCTTCATTGACAAGGT	138
K2E-R	GCTGTCGATATACCCCTGGA	
K2P-F	TTGCCTCCTTCATCGACAA	128
K2P-R	GGATTCAAAACAAGGCTCCA	
K3-F	AGTTTGCCTCCTTCATTGACA	108
K3-R	TGCCTGAGATGGAACTTGTG	
K4-F	TCCTGAAGGTCCTCTATGATGC	134
K4-R	GTACTGGGCACGGACCTC	
K5-F	CTCCCCTCAACCTGCAAAT	126
K5-R	TCTGCTGCTCCAGGAACC	
K6A-F	CAAGGCCCAATATGAGGAGA	135
K6A-R	GCAATCTCCTGCTTGGTGTT	
K7-F	CAGGACCCTCAATGAGACG	128
K7-R	ACTGCGCCTTGACCTCAG	
K8-F	GATGAACCGGAACATCAGC	119
K8-R	CATCCTTAATGGCCAGCTCT	

Gene	Primer Sequence	Size(bp)
K9-F	TGGTGACCAAGATGAGACAAA	113
K9-R	AGGCTTCATCGGGAGTCTG	
K10-F	AAACCATCGATGACCTTAAAAATC	134
K10-R	GCGCAGAGCTACCTCATTCT	
K12-F	ACGAGCTGACCCTGACCA	106
K12-R	CGGAAGCTTTGGAGCTCAT	
K13-F	AGTCCCAGCTGAGCATGAA	100
K13-R	CTGCTGATGAGTCCCTGGAT	
K14-F	CGACCTGGAAGTGAAGATCC	124
K14-R	GTCCACTGTGGCTGTGAGAA	
K15-F	TGACCTGGAGGTGAAGATCC	124
K15-R	GATGGTGGTGGCCATGAT	
K16-F	ATCGAGGACCTGAGGAACAA	124
K16-R	GGGCCAGTTCATGCTCATAC	
K17-F	GGTGAAGATCCGTGACTGGT	112
K17-R	CACGGTGGCTGTGAGGAT	
K18-F	TGATGACACCAATATCACACGA	112
K18-R	GGCTTGTAGGCCTTTTACTTCC	
K19-F	GCCACTACTACACGACCATCC	126
K19-R	CAAACTTGGTTCGGAAGTCAT	
K20-F	CCTCAAAAAGGAGCATCAGG	108
K20-R	ATGATGACGCCAAGGTTCA	
K23-F	GCAGACACAGTACAGCACGAA	113
K23-R	CCTCCTCATAGTGGGAGATGA	
NES-F	CGTTGGAACAGAGGTTGGA	103
NES-R	TGTAGGCCCTGTTTCTCCTG	
p75NTR	TCATCCCTGTCTATTGCTCCA	110
p75NTR	TGTTCTGCTTGCAGCTGTTC	
VIM-F	AGGTGGACCAGCTAACCAAC	123
VIM-R	TTTCGGCTTCCTCTCTCTGA	
CD44-F	tgtgggcagaagaaaaagcta	82
CD44-R	cctctccgttgagtccactt	
POLR2A-F	GCAAATTCACCAAGAGAGAC	73
POLR2A-R	CACGTCGACAGGAACATCAG	
YAP1-F	CCCAGATGAACGTCACAGC	83
YAP1-R	GATTCTCTGGTTCATGGCTGA	

Methods & Materials

Retroviral transduction

The retroviral pSIN-MCS (empty vector), pSIN-EGFP and pSIN-EGFP-FOXM1B constructs were described (1-3). PhxA cells were plated in 10 cm dishes and transfected with 10 µg plasmids (pSIN-MCS, pSIN-EGFP or pSIN-EGFP-FOXM1B) using FuGENE 6 reagent (Roche). Transfected PhxA were incubated for 48 hours and then sub-cultured in selection medium with puromycin (3 ug/ml) (Sigma Aldrich) and maintained for 1 week prior to supernatant collection. To infect oral keratinocytes in a 3T3-feeder layer system, keratinocytes were co-cultured with mitomycin-C-treated 3T3-feeder cells and transfected/selected PhxA cells. Initially, mitomycin-C-treated 3T3-feeder cells were plated at a density of $5x10^3/cm^2$. The next day, oral keratinocytes were seeded at a density of ~2000 cells/cm^2 for non-sorted keratinocyte transduction or at 50~1000 cells/cm^2 for flow-sorted keratinocytes used in clonogenic assays. Two hours after keratinocyte plating, mitomycin treated PhxA cells (2 µg/ml for 2 hrs) were plated on top at a density of ~$1x10^5$ cells/cm^2 and polybrene was added at a final concentration of 5 µg/ml (Day 0 of retroviral transduction). Normal growth medium (RM+), without polybrene, was replaced the next day (Day 2). On Day 3, PhxA-3T3 feeder layer was gently removed with PBS and freshly prepared mitomycin C-3T3-treated feeders were added at a density of $1.8x10^4/cm^2$.

Cell cycle synchronisation

To synchronize cells at G1/S phase by double thymidine block, $2x10^5$ cells were plated in 6 cm dishes. Semi-confluent dishes were incubated for 16 hours in culture medium containing 2 mM thymidine (Sigma Aldrich, Dorset UK) and reverted back to normal culture medium for 9 hours. Thereafter, cells were treated again with 2 mM thymidine for further 12-16 hours. Release from the second thymidine block was performed by washing twice with PBS and replacing with complete growth medium. Cell cycle profile was confirmed by FACS.

Cell counting and clonogenic assays

Cells were counted using CAsy® Cell Counter (Innovatis). Each sample was prepared three times in CAsyTon® (Innovatis) buffer and triplicate measurements of 200 µl sample volume were taken each time. Viable cells were measured by excluding cells smaller that 10 µm in size. Population doublings were calculated as follows: PD = $\log_2$ [(N1/N0] where N1 = total yield and N0 = initial number of seeded keratinocytes. Clonogenicity percentages were calculated as follows: Clonogenicity % was determined by the percentage of initial keratinocytes plated that were able to form visible colonies after 12 days in culture. For colony forming assays, keratinocyte cultures were washed once with PBS

and were fixed in 4% (v/v) formaldehyde in PBS for 20 minutes at room temperature. Then, cells were washed 1x with PBS and stained with 1% Rhodamine B (Sigma) in PBS for 30 minutes at room temperature. Individual colony size was digitally measured using a scientific quantification software pack available in the Adobe Photoshop CS4 Extended version. Clonogenic assays were carried out in 6 replicate wells for each sample and keratinocyte colonies were measured twice.

Antibodies

The antibodies used were rabbit polyclonal anti-FOXM1 (C-20, Santa Cruz), rabbit monoclonal anti-GAPDH (Abcam), mouse anti-p63 (4A4; Santa Cruz), mouse monoclonal anti-Ki-67 (DakoCytomation, Cambridgeshire, UK), mouse monoclonal anti-EGFR (clone H11; Dako Cytomation), mouse monoclonal anti-cytokeratin 19 (CRUK), mouse monoclonal anti-Involucrin (Sy5; Abcam), mouse monoclonal anti-cytokeratin 13 (Abcam), mouse monoclonal anti-cytokeratin 16 (CRUK), mouse monoclonal antil-p75NTR (Millipore), rabbit polyclonal anti TGAse-1 (Covalab-UK), mouse monoclonal anti-Filaggrin (Monosan), mouse monoclonal anti-integrin β1 (Santa Cruz), and rabbit anti-β-tubulin (Santa Cruz). Secondary antibodies used were polyclonal rabbit anti-mouse immunoglobulin/HRP (DakoCytomation), and polyclonal goat anti-rabbit immunoglobulin/HRP (DakoCytomation). Fluorescence conjugated antibodies were all supplied by Molecular Probes, Invitrogen, Paisley, UK: Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, and Alexa Fluor 568 goat anti-mouse. The cell surface marker specific antibodies used for flow cytometry are described below.

Organotypic Culture on Collagen Matrix

Organotypic keratinocyte cultures on collagen matrix were constructed by seeding, for each culture, $0.5x10^6$ primary normal keratinocytes on top of 700 µl collagen gel prepared the day before on ice by mixing 7 vol. of 3.40 mg/ml rat tail collagen type I (Collaborative Biomedical), 2 vol. reconstitution buffer (261 mM NaHCO3, 150 mM NaOH, 200 mM N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid) pH 8.15, 1 vol. DMEM 10x (Sigma) and 1 vol. FCS (Invitrogen) containing $0.5x10^6$/ml primary normal oral fibroblasts. The cultures were grown in serum-free RM+ medium (DMEM:Ham's F-12/3:1) supplemented with 1 mM hydrocortisone, 0.8 mM insulin, 0.25 mM transferrin, 0.25 mM L-ascorbic acid, 30 mM linoleic acid, 15 mM bovine serum albumin, 2 mM L-glutamine (all from Sigma). The cultures were harvested on day 10 of co-culture. One half of each culture was snap frozen in isopentane prechilled in liquid nitrogen and the other fixed in 4% buffered formalin pH 7.15 and embedded in paraffin.

Figure Legends

Figure S1: A, N/TERT or B, HeLa cells were released from double thymidine cell cycle block (G1/S synchronization) and mRNA was harvested at regular time intervals and levels of FOXM1A, B and C isoforms were quantified using absolute qPCR as described previously (see Methods & Materials). Only FOXM1B isoform showed a significant upregulation during cell cycle re-entry in both cell types. C, FACS-sorted primary oral keratinocytes with p75NTR low or high were harvested after 3 days for qPCR to determine the expression levels of FOXM1C mRNA. RS stands for random sorted populations. All values are fold expression relative to $p75NTR^{lo}$. Each bar represents the mean ± SEM of two independent experiments performed in triplicates. No significant difference in FOXM1C expression was found in any of the sorted populations. D, Genomic DNA from transduced human keratinocytes was harvested either 3 or 10 days after retroviral transduction to determine the viral transgene copy number by absolute qPCR. 10 ng of genomic DNA (gDNA) were used for qPCR analysis for the detection of EGFP transgene copy number. No significant difference in EGFP transgene copy number was found between day 3 and 10 cultures. E, Immunofluorescence staining of endogenous FOXM1 protein (green) in primary normal human oral keratinocytes (NHOK) and an oral SCC cell line (UK1). Nuclei were counterstained with DAPI (blue). White arrow heads indicate paired foci of FOXM1 protein within the nuclei of cycling NHOK cells and numerous foci within both cycling and non-cycling UK1 cells. F, Clonogenic assays for CD44-sorted CA1 and 5PT oral SCC cell lines at day 7 and 12 after FACS sorting. Cells were stained with Crystal Violet for colony visualisation and densitometry quantification of colony growth. G, Graphical representation of the colony-size in G. **P<0.05, **P<0.01, ***P<0.001.*

Figure S2: Primary human oral keratinocyte stem cell isolation. A, FACS staining profile of primary human oral keratinocytes stained with anti-integrin β1 (CD29) and anti-p75NTR (CD271) antibodies. Gated populations marked as P1, P2, P3 and P4 were used for combined cell sorting analysis. The percentages for P1, P2, P3 and P4 are representative of experiments performed on primary human oral keratinocytes derived from three different donors and were carried out in triplicates. B, Fluorescence microscopy on oral keratinocyte populations immediately after flow sorting to demonstrate successful discrimination between the indicated populations. C, Oral keratinocytes sorted for $p75NTR^{lo\ or\ hi}$ were allowed to grow for 12 days. Keratinocyte colonies were stained with Rhodamine B for quantification of the clonogenic potential. D, Relative colony size and E, cell number were measured as mentioned in materials and methods section after 12 days of culture. RS stands for random sorted control cells. Values are representative of two independent experiments carried out in triplicates. Bars represent the average fold difference from $p75NTR^{lo}$ control samples ±SEM.**P≤0.05,*

***P≤0.005, ***P≤0.001.* F, Clonogenicity % and population doublings (PD) measurements of all sorted oral keratinocytes after 12 days in culture. Values are representative of three independent experiments carried out in duplicate wells, ±SEM for each average value.

Figure S3: A, Western blotting showing an inverse expression relationship between FOXM1 and involucrin protein in early (population doubling 10, PD10, 3 days) and late (PD17, 12 days) passage p75NTR^{lo} primary oral keratinocytes. B, Absolute qPCR analysis of KRT4 in p75NTR$^{lo\ or\ hi}$ cells. Each bar represents the mean ± SEM of two independent experiments performed in duplicates. **P<0.05*

Figure S4: 3D-Organotypic cultures derived from either EGFP or EGFP-FOXM1B transduced primary oral keratinocytes stained with either A and B, epidermal growth factor receptor (EGFR) or C and D, cytokeratin 19 (KRT19). E, Normal human oral mucosa tissue stained with FOXM1 (red) and Ki67 (green). All tissue sections were counterstained with DAPI (blue) for nuclear DNA visualization. F, Digital densitometry measurement of the epithelial thickness (pixel area) in EGFP- and FOXM1B-derived organotypics (n=4; *P<0.05). G, Ki-67 positive cell count (between 100-200 DAPI-positive cells were counted per organotypic) within the suprabasal layers in EGFP- and FOXM1B-derived organotypics (n=4; *P<0.05).

References

1. Teh MT, Wong ST, Neill GW, Ghali LR, Philpott MP, Quinn AG. FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res 2002;62(16):4773-80.
2. Gemenetzidis E, Bose A, Riaz AM, et al. FOXM1 upregulation is an early event in human squamous cell carcinoma and it is enhanced by nicotine during malignant transformation. PLoS ONE 2009;4(3):e4849.
3. Teh MT, Gemenetzidis E, Chaplin T, Young BD, Philpott MP. Upregulation of FOXM1 induces genomic instability in human epidermal keratinocytes. Mol Cancer 2010;9(1):45.

Figure-S1 (Teh et al.)

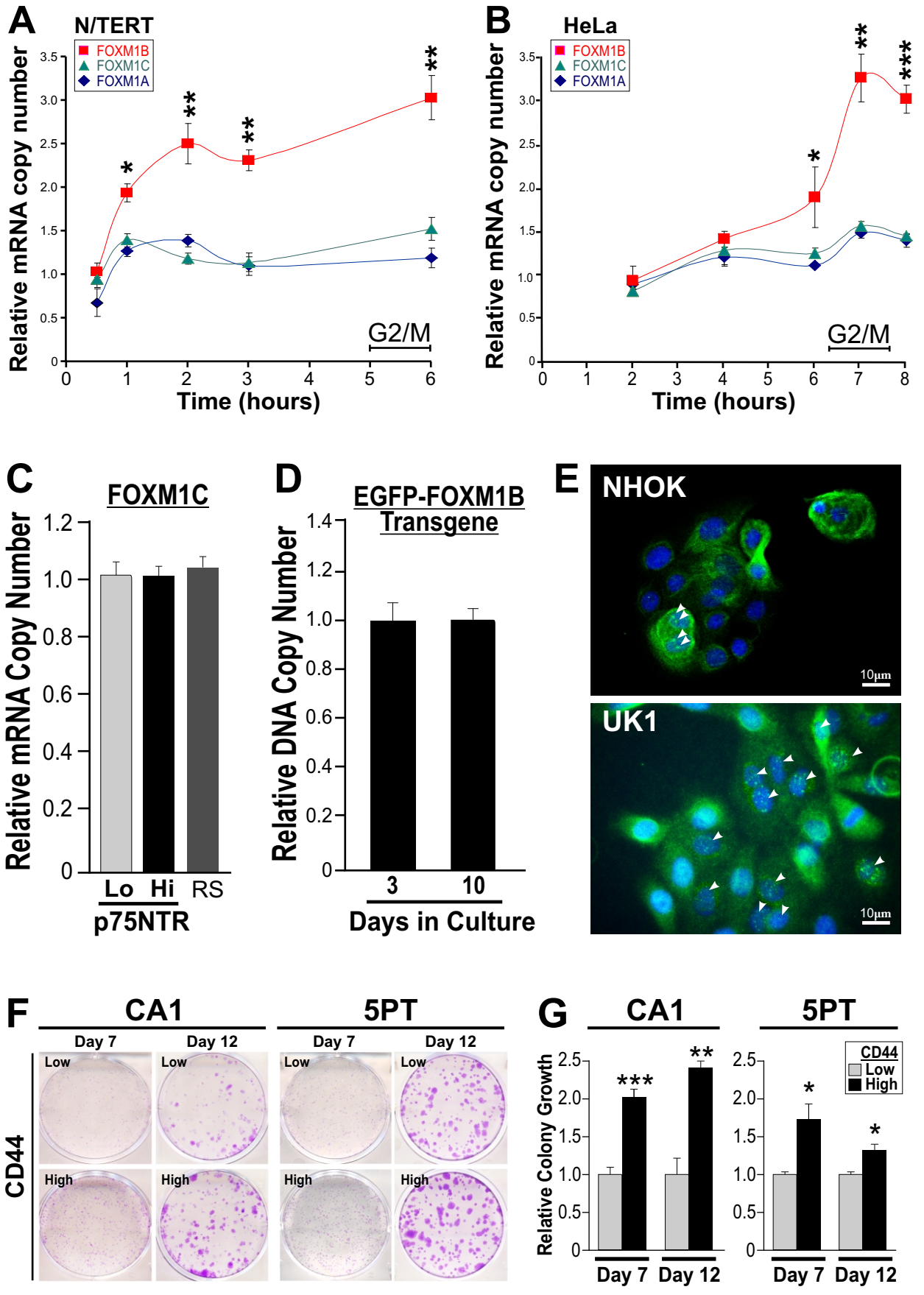

Figure-S2 (Teh et al.)

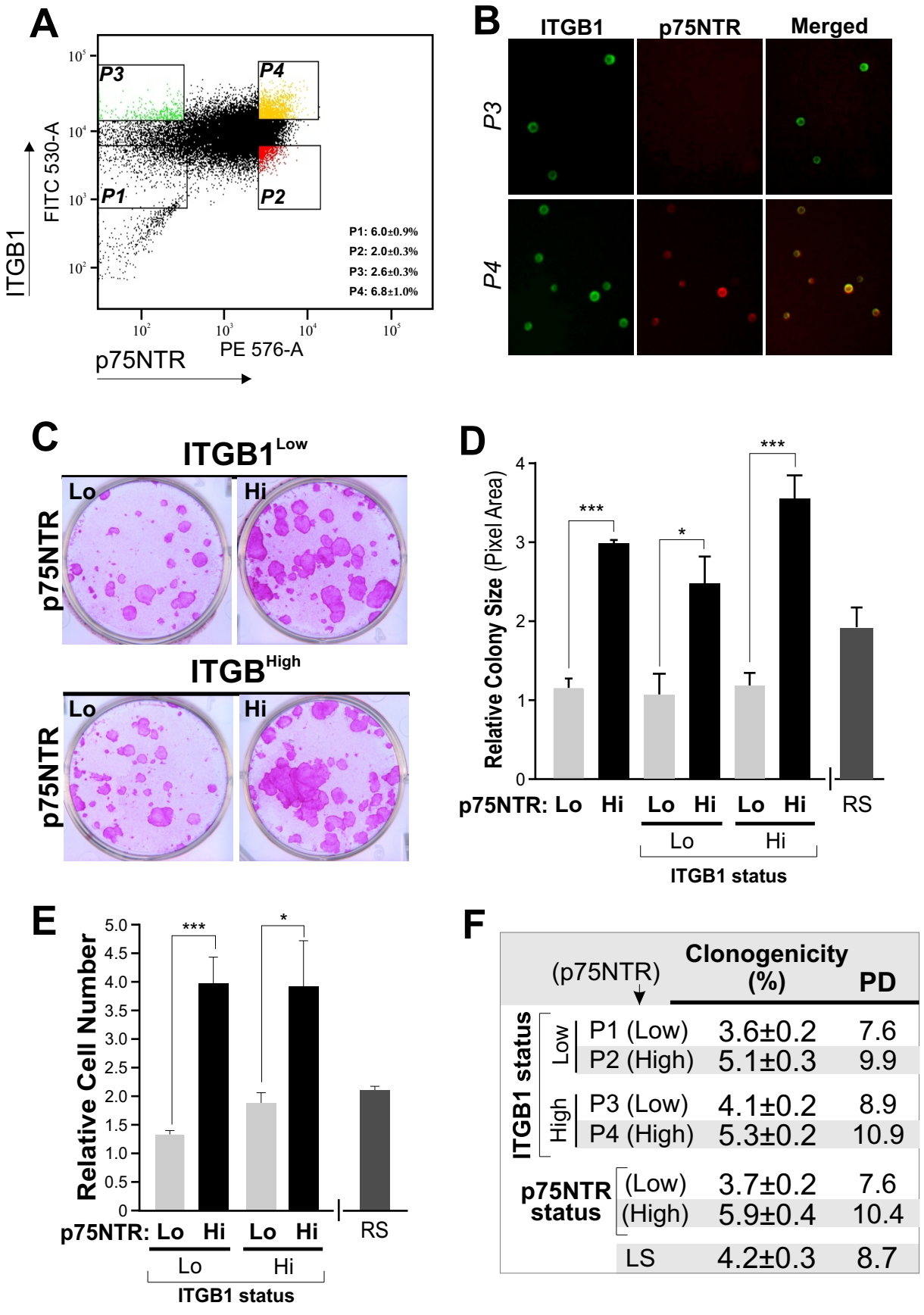

Figure-S3 (Teh et al.)

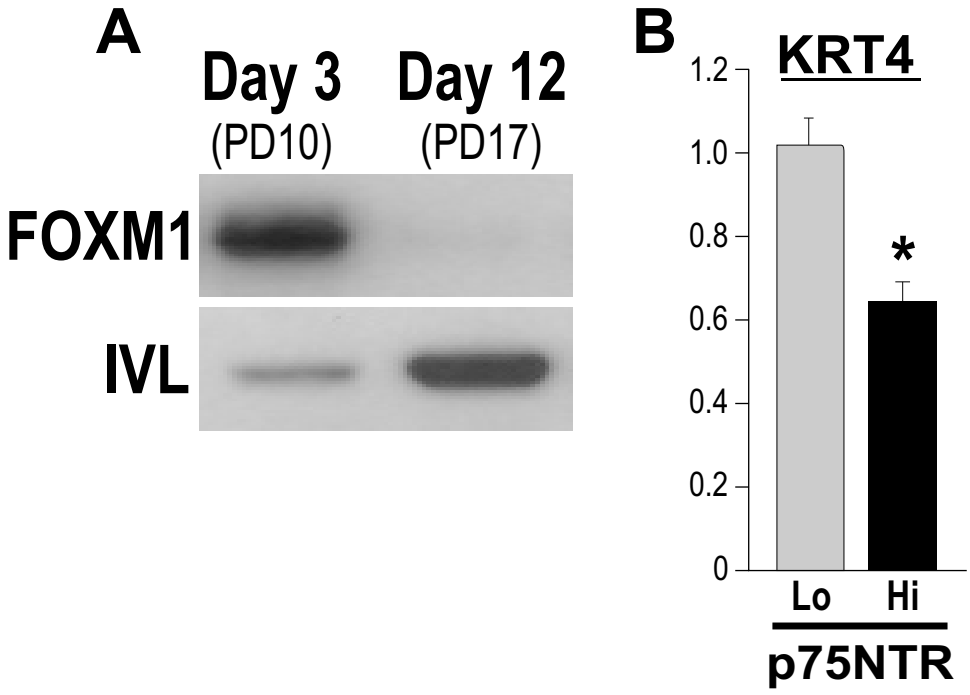

Figure-S4 (Teh et al.)