# Organotypic culture of normal, dysplastic and squamous cell carcinoma derived oral cell lines reveals loss of spatial regulation of CD44 and p75[NTR] in malignancy.

Running Title: **CD44, p75[NTR], CD24 and ALDH in oral cell lines**

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Keywords: Oral Cancer; Oral Dysplasia; Organotypic culture; cancer stem cells; Differentiation

Abstract: (250 words)

Oral squamous cell carcinomas (OSCC) often arise from dysplastic lesions. The role of cancer stem cells in tumour initiation is widely accepted yet the potential existence of pre-cancerous stem cells in dysplastic tissue has received little attention. Cell lines from oral diseases ranging in severity from dysplasia to malignancy provide opportunity to investigate the involvement of stem cells in malignant progression from dysplasia. Stem cells are functionally defined by their ability to generate hierarchical tissue structures in consortium with spatial regulation. Organotypic cultures readily display tissue hierarchy *in-vitro* hence in this study we compared hierarchical expression of stem cell associated markers in dermis based organotypic cultures of oral epithelial cells from normal tissue (OKF6-TERT2), mild dysplasia (DOK), severe dysplasia (POE-9n) and OSCC (PE/CAPJ15). Expression of CD44, p75[NTR], CD24 and ALDH was studied in monolayers by flow cytometry and in organotypic cultures by immunohistochemistry. Spatial regulation of CD44 and p75[NTR] was evident for organotypic cultures of normal (OKF6-TERT2) and dysplasia (DOK and POE-9n) but was lacking for OSCC (PE/CA PJ15) derived cells. Spatial regulation of CD24 was not evident. All monolayer cultures exhibited CD44 ,p75[NTR], CD24 antigens and ALDH activity (ALDEFLUOR[®] assay), with a trend towards loss of population heterogeneity that mirrored disease severity. In monolayer, increased FOXA1 and decreased FOXA2 expression correlated with disease severity, but OCT3/4, Sox2 and NANOG did not. We conclude that dermis based organotypic cultures give opportunity to investigate the mechanisms that underlie loss of spatial regulation of stem cell markers seen with OSCC derived cells.

Introduction

The role of cancer stem cells in solid tumour initiation, growth and metastasis is widely accepted and has been the subject of numerous reviews (1) (2), yet the potential existence of pre-cancerous stem cells in dysplastic tissue has received little attention. Oral squamous cell carcinoma (OSCC) often arise from dysplastic lesions with various grades of severity; malignant progression from dysplasia varying between 6% and 36% (3). Early genetic aberrations in oral SCC involve p53 and pRb pathway abrogation (4) (5) (6) and result in cell immortalisation, but the genetic and epigenetic heterogeneity observed in tumours is a cumulative process (7) which may be evident in pre-cancerous dysplastic tissue.

Cancer stem cells with tumorigenic properties in immunodeficient mice have been successfully isolated from head and neck tumours by using fluorescence-activated cell sorting (FACS) to gate for cell surface antigens (CD44) (8), enzyme activity (Aldehyde dehydrogenase) (9) and small molecule transporter activity (Hoechst 33342 exclusion) (10). When they are cultured *in-vitro*, the tumorigenicity of head and neck tumour derived cells is influenced by media composition (10). Similarly, the proportion of CD44 positive cells in a hypopharynx derived SCC cell line (Gun-1) was also dependent on cell culture media composition (11). There is much work yet to be undertaken before routine isolation and *in-vitro* expansion of cancer stem cells from oral tumours can be realised and still further work before we are able to selectively expand potentially pre-cancerous stem cells from dysplastic oral lesions.

Cell lines from oral lesions bearing different clinical features provide opportunity for comparative evaluation of the tumorigenic role played by cells that display stem-like phenotypes *in-vitro*. Prerequisite to this goal is characterisation of selected cell lines for stem-cell content and establishment of robust organotypic culture techniques that permit establishment of the hierarchical tissue organisation that defines stem-cell functionality (7). In the current study, four commercially available oral cell lines have been selected to provide a range of source tissue dysfunction ranging from normal (OKF6-TERT2), mild to moderate dysplasia (DOK), severe dysplasia (POE-9n) and OSCC (PE/CA PJ15). These cell lines were initially characterised in monolayer culture by flow cytometry, then were grown at the air/liquid interface on a dermis based 3-dimensional culture system to promote organotypic patterns of differentiation that were studied by immunohistochemistry. Four potential cancer stem cell markers were the focus of this initial investigation: CD44, p75[NTR], CD24 and Aldehyde dehydrogenase (ALDH).

CD44 is a cell surface receptor for hyaluronic acid that is present in the majority of head and neck tissues (12). Expression of CD44 in primary tumours of head and neck SCC is highly variable (13), and the CD44 positive cell population from these sites contains both tumorigenic and non-tumorigenic cells (8). p75$^{NTR}$ (or CD271 or p75$^{NGF}$) is a member of the Tumour Necrosis Factor receptor (TNFR) superfamily that is a low-affinity receptor for all neurotrophins (14) (15). Accumulated evidence suggests that p75$^{NTR}$ is a putative cancer stem cell marker for OSCC (16, 17) and that interaction with locally produced neurotrophins may be of relevance to OSCC development (18) (19). CD24 is a cell surface sialoglycoprotein and cancer stem cell marker (20) (21) (22), the expression of which has not been fully evaluated for oral SCC. Aldehyde dehydrogenases (ALDH) are a family of NADP$^{+}$ dependent detoxifying enzymes that facilitate tumour resistance to chemotherapy (23). ALDH1 expression correlates with poor patient outcome (24) and is strongly associated with OSCC cancer stem cells with elevated expression of Oct4, Sox2 and Nanog transcription factor genes (25) (26).

In the current study we have used flow cytometry to define the proportion of CD44, p75$^{NTR}$, CD24 and ALDH positive cells in four cell lines that originate from oral tissues that displayed a range of morphologies from normal, through dysplasia to SCC. A qPCR array was used to quantify relative expression of Oct3/4, Sox2 and Nanog by the four cell lines. The cell lines were subsequently grown at the air/liquid interface (to induce differentiation) in a human dermis based organotypic culture model (termed Human Oral Mucosal Equivalent; HOME) and studied immunohistochemically for their distribution of CD44, p75$^{NTR}$, CD24 and ALDH antigens. Collectively, the results show that Human Oral Mucosal Equivalent culture techniques are required to recapitulate a normal pattern of CD44, p75$^{NTR}$, CD24 and ALDH expression in vitro from cells that would otherwise lack hierarchical expression of these cancer stem cell associated markers in monolayer culture.

Materials & Methods

Cell Lines

Cell lines were purchased from the European Collection of Cell Cultures (ECACC) (Sigma, St Louis, MO) and the Rheinwald Laboratory, Harvard Medical School, and unless stated otherwise, were cultured in accordance with the supplier's instructions. Human neoplastic oral keratinocytes (PE/CA-PJ15 cell line, ECACC accession no. 961211230, received at passage 7, (27)) were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Grand Island, NY; #12440) containing 4 mM L-glutamine, and supplemented with 10% Foetal Calf Serum (FCS) (Invitrogen; # 10099) and 100 U/mL of penicillin, 100 μg/mL of streptomycin and 250 ng/mL of amphotericin B (Antibiotic-Antimycotic) (Invitrogen; # 15240). Human dysplastic oral keratinocytes (DOK, ECACC accession no. 94122104, received at passage 23, (28)) were grown in Dulbecco's Modified Eagle Medium (D-MEM) (Invitrogen; # 11995) supplemented with 10% FCS, 10.3 μM hydrocortisone (Sigma #H2270) and Antibiotic-Antimycotic. Human severe dysplastic oral keratinocytes (POE-9n, received at passage 4, (29)) and hTERT transfection immortalised normal human keratinocytes (OKF6-TERT2, received at passage 16, (29)) were grown in "keratinocyte serum-free medium (Invitrogen K-sfm)" (Invitrogen; #17005-042) containing 25 μg/ml bovine pituitary extract (BPE) (Invitrogen; #13028-014), 0.2 ng/ml human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) (Invitrogen; #10450-013), supplemented with $CaCl_2$ solution to a final concentration of 0.4mM and with Antibiotic-Antimycotic.


ALDEFLUOR® assay optimisation

Aldehyde dehydrogenase (ALDH) activity was assessed using the ALDEFLUOR® assay (StemCell Technologies, Durham, NC, USA) in accordance with the manufacturer's instructions after optimisation. The proprietary ALDEFLUOR® assay is designed and optimised for detection of haematopoietic and progenitor cells in human blood and required further optimisation for use with oral epithelial cell lines. The ALDEFLUOR® assay was optimised using PE/CA PJ15 cells at passage 16. Dose-response profiles were attained for two ALDH inhibitors: 4-diethylaminobenzaldehyde (DEAB, Sigma; #D86256) at 1.5 mM, 15 mM, 30 mM and 90 mM and for tetraethylthiuram disulfide (Sigma; #T1132) at 1.5 mM and 15 mM. ALDH inhibitor concentrations in 100% dimethyl sulphoxide (DMSO, Sigma; #D8418) were directly substituted for the 1.5 mM DEAB in 95% ethanol supplied with the proprietary ALDEFLUOR® kit. Optimal inhibition of ALDH activity with DEAB was obtained using 30mM DEAB; Tetraethylthiuram disulfide gave negligible ALDH inhibition. Optimised ALDEFLUOR®

assay conditions were: 30 minute exposure in the dark to activated ALDEFLUOR® substrate (Bodipy-aminoacetaldehyde) in ALDEFLUOR® kit reaction buffer at 37°C in the absence or presence of 30 mM DEAB (giving a final DEAB concentration of 0.6mM in the ALDEFLUOR® reaction mixture).

Multicolour Flow-cytometric analysis (FACS)

Multicolour flow-cytometry was performed on discrete replicate cultures of OKF6-TERT2 (n=4 replicates), DOK (n=3), POE-9n (n=3) and PE/CA PJ15 (n=3). Sub-confluent OKF6-TERT2 (passage 27 to 29), DOK (passage 36 to 40), POE-9n (passage 19 to 20) and PE/CA PJ15 (passage 22 to 23) cells were detached from 75 cm$^2$ tissue culture flasks (Corning Inc, Lowell, MA; #430641) with 8 mL TrypLE™ Express (Invitrogen; # 12604), collected into phosphate buffered saline (PBS, Amresco, Solon, OH; #E404) containing 10% FCS, pelleted (by centrifugation at 400 g for 5 minutes), resuspended, counted and aliquoted into 1.5 ml microfuge tubes. Following two washes with PBS, a LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen # L34957) was used according to manufacturer's instructions. After fluorescent loading of dead cells, cells were resuspended in 0.1mL 1% BSA (Sigma; # A7906) in PBS then exposed overnight in the dark at 4 °C to appropriate combinations of 4 μL APC-anti-human CD44 (clone G44-26, BD Bioscience, San Jose, CA; #559942), 4 μL PE-anti-human CD271 (otherwise known as anti-p75$^{NGFR}$) (clone ME20.4, BioLegend, San Diego, CA; #345105), PerCP-Cy™5.5-anti-human CD24 (Clone:ML5, BD Bioscience; #561647) or the relative isotype controls: 4 μL APC-mouse IgG2b κ (clone 27-35, BD Bioscience; #555745), 4 μL PE-mouse IgG1 κ (clone MOPC-21, BioLegend; #400111), PerCP-Cy™5.5-mouse IgG2a κ (BD Bioscience; #550927). Subsequently, the ALDEFLUOR® assay was performed using the optimised conditions above with a reaction volume of 0.3mL. Immunofluorescent labelled cells were pelleted, and resuspended in 0.4 mL ALDEFLUOR® reaction buffer to give approximately 2x 10$^6$ cells/mL.

Sample analysis was by a Gallios 10-colour 3-laser flow cytometer (Beckman Coulter, Brea, CA; #775106). One hundred thousand events were recorded for each sample. Data analysis was performed using Kaluza™ software (version 1.2, Beckman Coulter). ALDEFLUOR® assay thresholds were established using the DEAB positive sample and immunotyping thresholds were established using isotype control samples. The R-computing language was used to compile plots of flow cytometry results (R Development Core Team [[http://www.R-project.org]]). One way analysis of variance (ANOVA) with Turkey's honestly significant difference (HSD) post-hoc tests (homoscedasticity confirmed by Levene's test) was conducted using IBM SPSS for Windows, Rel20.0.0.

Human Oral Mucosa Equivalent (HOME) culture

Patient involvement, data management and tissue handling procedures received ethical review and approval from Hospital and University Human Research Ethics Committees (QUT ID:3865H; UQ ID: 2009001069). The study was run in strict accordance to the principles outlined in the Declaration of Helsinki (2008).

Full thickness normal human skin was obtained with informed patient consent from an elective surgical abdominoplasty procedure and grafted with a modified Watson dermatome under aseptic conditions. Split thickness skin was processed into de-epidermised dermis (DED) as previously described (30) but without sterilisation or osmotic decellularisation. Briefly, split thickness skin was washed with PBS containing Antibiotic-Antimycotic (Invitrogen; # 15240) and cut into 2 cm squares and incubated for 18 hours at 37 °C in sterile 1 M sodium Chloride solution (Ajax Finechem, #Univar 465) to achieve a gentle dermal / epidermal separation by pealing with curved forceps. The DED was incubated at 4°C for 48 hours in excess volumes of three descending concentrations of antibiotics in D-MEM (Invitrogen; # 11995): 320ng/mL gentamycin plus 4x Antibiotic-Antimycotic (400 U/mL of penicillin, 400 µg/mL of streptomycin and 1 mg/mL of amphotericin B); followed by 2x Antibiotic-Antimycotic only (200 U/mL of penicillin, 200 µg/mL of streptomycin and 500 ng/mL of amphotericin B); followed by 50 U/mL penicillin G and 50 U/mL streptomycin sulphate only (Invitrogen; # 15070-063).

Before HOME culture, the DED was pre-incubated for 2 hours in Green's media consisting: a 3:1 ratio of DMEM (Invitrogen; #10313021) and Ham's F12 medium (Invitrogen; #11765-054) supplemented with 10% v/v FCS (Invitrogen; # 10099), 2 mM L-glutamine(Invitrogen; #25030149), 180 µM adenine (Sigma; #A9795), 100 µM non-essential amino acids (glycine, L-alanine, L-aspargine, L-aspartate, L-glutamate, L-proline, L-serine) (Invitrogen; #11140-050), 5 µg/mL insulin (Sigma; #I9278), 5 µg/mL transferrin (Sigma; #T8158), 0.2 µM triiodo-L-thyronine (T$_3$)(Sigma; #T6397), 10 ng/mL human recombinant EGF (Invitrogen; #PHG0311), 0.4 µg/mL hydrocortisone (Sigma; #H0888), $1.2 \times 10^{-10}$ M cholera toxin (Sigma; #C8052), 50 U/mL penicillin G and 50 U/mL streptomycin sulphate (Invitrogen; # 15070-063). The DED was orientated papillary surface uppermost in 6-well plates (Corning; #3516) and 1 cm$^2$ internal diameter stainless steel rings were applied with pressure. Sub confluent cultures of OKF6-TERT2, POE-9n, DOK and PE/CA PJ15 were detached from 175 cm$^2$ tissue culture flasks (Corning; #431080) with 20 mL 0.05% Trypsin / 0.02% EDTA (Invitrogen; # 25300), collected into DMEM (Invitrogen; #10313021) supplemented with 10% v/v FCS (Invitrogen; # 10099), then pelleted

by centrifugation at 130 g for 5 minutes, resuspended and counted. HOME cultures were seeded with 2.5 x 10$^6$ of OKF6-TERT2 (passage 28), DOK (passage 37), POE-9n (passage 13) or PE/CA PJ15 (passage 17) cells suspended in 0.5 mL of Green's media. Finally, 2 mL of Green's media was applied to the wells surrounding the HOME cultures which were incubated at 37 °C for 24 hours. On day 2 the stainless steel rings were removed and the Green's media was replenished. HOME cultures were maintained in submerged culture for a further 5 days and then lifted to the air/liquid interface by transferral onto stainless steel grids and application of approximately 7 mL of Green's media. HOME cultures were maintained at the air/liquid interface for 14 days with regular media changes.

Immunodetection of ALDH1a1, CD44, p75$^{NTR}$, CD24, cytokeratin 13 and cytokeratin 19

Immunohistochemical staining for ALDH1a1, CD44 p75$^{NTR}$ and CD24 followed the conventional single stain technique (31). Paraffin-embedded 5 μm sections were dewaxed by three 5 minutes immersions in xylene then rehydrated in descending ethanol concentrations: 100%, 95%, 70% and 0% for 5 minutes each. Heat-induced epitope retrieval (HIER) utilised 10 mM sodium citrate, 0.05% Tween 20, pH 6.0 in a Decloaking Chamber™ (Biocare Medical, Concorde, CA, USA) set to provide 125 °C for 30 seconds then return to 90 °C. After ambient cooling, slides were washed with tris buffered saline (TBS) (Amresco Inc., Solon, OH; #K859) and demarked (Dako Australia Pty. Ltd, Vic AU; #S200230). Triplicate TBS buffer washes separated each subsequent step. Endogenous peroxidase activity was blocked in Peroxidazed 1 (Biocare Medical; # PX968) for 10 minutes, sections were blocked by 15 minute incubation in Background Sniper (Biocare Medical; # BS966) and then incubated at 4 °C overnight with primary antibody. The primary antibodies were: Aldh1a1 rabbit monoclonal (EP1933Y) 1:200 dilution (Biocare Medical; #CME351), p75$^{NTR}$ mouse monoclonal (O.N.469) 1:200 dilution (Santa Cruz Biotechnology, CA, USA; #sc-71695), CD24 mouse monoclonal (SN3b) 1:50 dilution (Biocare Medical; #CME323) and CD44 mouse monoclonal (156-3C11) 1:750 dilution (Biocare Medical; #CM318). Aldh1a1 antibody was detected by a 45 minute incubation with MACH 2™ Universal HRP-Polymer (Biocare Medical; # RHRP520) while p75$^{NTR}$ , CD24 and CD44 antibodies were detected by sequential 15 minute incubations with MACH 1™ Mouse Probe (Biocare Medical; # UP537) and MACH 1™ Universal HRP-Polymer (Biocare Medical; # M1U539). Sections were stained with Betazoid DAB chromogen (Biocare Medical; #BDB900) for 2 minutes then counterstained for 20 seconds with haematoxylin, dehydrated in alcohol, mounted and scanned using a ScanScope CS™ (Aperio, CA, USA).

The immunofluorescent labelling protocol for cytokeratins 13 and 19 followed a similar workflow with the following exceptions. After HIER, the sections were blocked with 10% donkey

serum (Jackson ImmunoResearch Laboratories Inc, PA, USA; #017-000-121) and incubated at 4 °C overnight with primary antibody diluted in 10% donkey serum. The primary antibodies were: cytokeratin 13 mouse monoclonal (1C7) 1:50 dilution (Santa Cruz Biotechnology; # sc-58721) and cytokeratin 19 mouse monoclonal (BA17) 1:200 dilution (Santa Cruz Biotechnology; # sc-53258). Primary antibody immunolabelling was achieved with AlexaFluor®555 donkey anti-mouse 1:400 dilution (Invitrogen; #A31570) together with 6 μg/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (Sigma; #D9542) to label nuclei; 90 minute incubation at room temperature in the dark. After the wash steps, sections were mounted overnight in ProLong Gold® antifade reagent (Invitrogen; #P3634). Sections were imaged using a LSM 710 confocal microscope running ZEN2010 software (Carl Zeiss MicroImaging GmbH, Jena, Germany).

<u>Results</u>

*OKF6-TERT2, DOK, POE-9n and PE/CA PJ15 cell populations exhibit different proportions of ALDH, CD44, p75$^{NTR}$ and CD24 positive sub-populations in monolayer culture.*

Oral cell lines from normal tissue (OKF6-TERT2), mild dysplasia (DOK), severe dysplasia (POE-9n) and OSCC (PE/CA PJ15) were cultured as submerged monolayers and studied by flow cytometry for their relative proportion of ALDH, CD44, p75$^{NTR}$ and CD24 positive sub-populations. At least three discrete cultures of each cell line were analysed on separate occasions. Figure 1 demonstrates use of the ALDEFLUOR $^{®}$ assay to define ALDH positive populations in each cell line by using 0.6 mM DEAB to abolish ALDH activity (Figure 1, a, b, c, d) in order to establish the threshold fluorescence above which ALDH positive cells are defined (Figure 1, e, f, g, h). The percent of viable cells that were ALDH positive for each line were 61±13%, 84±3%, 73±4%, and 93±1% for OKF6-TERT2, DOK, POE-9n and PE/CA PJ15, respectively (Table 1). Importantly, use of the ALDEFLUOR $^{®}$ assay revealed increased aldehyde dehydrogenase activity that corresponded with the overall trend of increased disease severity of the source tissue for the cell lines from normal, through dysplasia to OSCC.

Table 1 shows the mean percent gating of viable OKF6-TERT2 , DOK, POE-9n and PE/CA PJ15 cells by CD44, p75$^{NTR}$, CD24 immunofluorescent labelling and ALDH enzyme activity (ALDEFLUOR$^{®}$ assay). Comparison of ALDEFLUOR$^{®}$ assay results across the four cell lines by one-way ANOVA revealed significant differences in mean percent of viable cells that were positive for ALDH activity (ANOVA; $F_{(2065,2842)} = 7.965$, $p = 0.07$). Post-hoc comparisons using the Turkey HDS test indicated that the mean percent ALDH positive populations for OKF6-TERT2 cells (61±13%) were significantly smaller in magnitude than for DOK (84±3%) or PE/CA PJ15 cells (93±1%). Similar analysis revealed significant differences in mean percent of populations labelled for CD24 (ANOVA; $F_{(13571,14813)} = 32.767$, $p < 0.01$) or p75$^{NTR}$ (ANOVA; $F_{(3361,5498)} = 4.720$, $p = 0.03$) but not CD44 (ANOVA; $F_{(43,75)} = 1.741$, $p = 0.228$). Post-hoc comparisons indicated that the mean percent CD24 positive populations of OKF6-TERT2 cells (18±15%) was significantly smaller in magnitude than for DOK (87±5%), POE-9n (97±1%) or PE/CA PJ15 cells (70±8%). Similar comparisons indicated that mean percent p75$^{NTR}$ positive populations of PE/CA PJ15 cells (54±17%) were smaller in magnitude than for POE-9n cells (98±1%).

Co-expression of CD44, p75$^{NTR}$ and CD24 by OKF6-TERT2, DOK, POE-9n and PE/CA PJ15 cells did not define sub-populations within the ALDH positive population for any of the cell lines irrespective of the gating strategy implemented (data not shown). Figure 2 compares the trends in percent co-

expression of CD44, p75[NTR], CD24and ALDH by OKF6-TERT2, DOK, POE-9n and PE/CA PJ15 cells in submerged monolayer culture. Greater heterogeneity of co-expression data was observed for normal tissue derived cells (OKF6-TERT2) than for diseased tissue derived cells (DOK, POE-9n and PE/CA PJ15).

*Stem Cell Transcription Factor PCR-array analysis of OKF6-TERT2, DOK, POE-9n and PE/CA PJ15 cell monolayer cultures.*

RT[2] Profiler™ PCR array for Stem Cell Transcription Factors (Qiagen #PAHS-501A) was used primarily to compare expression of NANOG, SOX2 and OCT3/4 (POU5F1) by DOK, POE-9n and PE/CA PJ15 cells relative to OKF6-TERT2 cells. Expression of NANOG, SOX2 and OCT3/4 were not elevated in any of the disease tissue derived cells relative to the control cells (OKF6-TERT2). Expression of OCT3/4 was equivalent for all of the cell lines, whereas SOX2 and NANOG were reduced in both DOK and PE/CA PJ15 cells. No correlation between source tissue disease severity and negative fold change of NANOG, SOX2 or OCT3/4 was observed.

From a total of 87 gene products analysed by the RT[2] Profiler™ PCR array for Stem Cell Transcription Factors, the numbers of up-regulated genes (with fold change greater than 2) compared to OKF6-TERT2 (control) for DOK, POE-9n and PE/CA PJ15 cell lines were 19, 16 and 23, respectively, and the numbers of down-regulated genes (with fold change less than 0.5) for DOK, POE-9n and PE/CA PJ15 cell lines were 27, 33 and 26 respectively (data available upon request). Interestingly, up-regulation of FOXA1 and down-regulation of FOXA2 both correlated with increased disease severity of source tissue. Positive correlation between source tissue disease severity and gene product up-regulation was also observed for LMXB1 (LIM homeobox transcription factor 1 beta). In all, four gene products were up-regulated in DOK, POE-9n and PE/CA PJ15 cells relative to control (OKF6-TERT2): FOXA1 (forkhead box A1), HOXA10 (homeobox A10), HOXA9 (homeobox A9) and PCNA (proliferating cell nuclear antigen).

*Immunohistology of cultures of OKF6-TERT2, DOK, POE-9n and PE/CA PJ15 cells.*

Figure 3 (a,b,c,d) shows the gross morphology of HOME cultures of OKF6-TERT2, DOK, POE-9n and PE/CA PJ15 cells after 14 days culture at the air/liquid interface to induce cellular differentiation. The degree of epithelialisation of the dermis was: PE/CA PJ15 > DOK > POE-9n > OKF6-TERT2. Culture at the air/liquid interface promoted a normal pattern of differentiation for each cell type as demonstrated by supra-basal expression of cytokeratin 13 in each HOME culture (figure 3 e,f,g,h). Cytokeratin 13 immunolabelling was most ordered with OKF6 cells which gave even staining of the outer layers, whereas cytokeratin 13 immunolabelling was most disordered with PE/CA PJ15 cells for which individual stained cells were distributed throughout the supra-basal epidermal layers. POE9n cells gave intense cytokeratin 13 immunolabelling as a broad band just below the outermost layer of cells, whilst for DOK cells cytokeratin 13 immunolabelling was discontinuous and restricted to the outermost layers. Cytokeratin 19 immunolabelling has been shown previously to occur in the basal layers of non-keratinising oral epithelia (32). Cytokeratin 19 immunolabelling was completely absent from DOK and PE/CA PJ15 HOME cultures, but intense immunolocalisation for cytokeratin 19 was seen throughout the epidermis of OKF6-TERT2 and POE9n HOME cultures (figure 3 i,j,k,l). Importantly, expression of CD44 (figure 3 m, n, o, p) and p75$^{NTR}$ (figure 3 q,r,s,t) was closely associated with the basal layers of cells but not solely at the basal layer adjacent to the dermal basement membrane. Immunolabelling for both CD44 and p75 was most widely distributed throughout the epithelium of PE/CA PJ15 HOME cultures. All HOME cultures exhibited positive CD24 immunolabelling (figure 3 u,v,w,x) throughout the epithelium, with DOK and POE-9n HOME cultures giving more intense staining than OKF6-TERT2 and PE/CA PJ15 HOME cultures. Very low immunolocalisation of ALDH1a1 (figure 3 y,z,$\alpha$,$\beta$) was observed in the basal layers of each HOME culture, where individual cells tended to be stained.

Discussion

The current study examined application of 3D organotypic culture (HOME) of a carefully selected range of cell lines to investigate cancer stem cell involvement in the progression of oral dysplasia to squamous cell carcinoma. The key finding of this work was that HOME culture gave appreciably more information than monolayer cultures on the hierarchical regulation of cells *in-vitro*, information that is crucial in properly interpreting empiric data on the relative expression of candidate stem cell markers by various cell lines *in-vitro*.

HOME culture of oral epithelial cells at the air/liquid interface uses techniques that have been optimised for other epithelial cells by several research groups over many years (30) (33) (34). Key features of HOME culture using intact acellular dermis include: retention of an intact basement membrane, promotion of hierarchical epithelial tissue formation, and good compatibility with established immunohistochemical techniques. HOME culture of oral cell lines from normal tissue (OKF6-TERT2), mild dysplasia (DOK), severe dysplasia (POE-9n) and OSCC (PE/CA PJ15) for 7 days gave ordered multilayer epithelial structures that showed spatial regulation of differentiation associated cytokeratin 13.

In keeping with the high levels of CD44 and p75$^{NTR}$ expression seen for each cell line in monolayers, all HOME cultures gave intense immunolabelling for CD44 and lesser immunolabelling for p75$^{NTR}$. With exception of PE/CA PJ15 cells, immunolocalisation of CD44 and p75$^{NTR}$ was spatially restricted to layers close to the basement membrane, confirming that in HOME cultures CD44 and p75$^{NTR}$ antigens do localise to the presumed stem cell niche and indicating that special regulation of CD44 and p75$^{NTR}$ expression is operative in HOME cultures. Basal layer bound epithelial cells were optimally immunolocated by p75$^{NTR}$, indicating this antigen as a good marker of cells derived from this niche for future investigations. The comparative absence of spatial regulation of CD44 and p75$^{NTR}$ in HOME cultures of PE/CA PJ15 cells is of key importance since this is the defining feature that separates the dysplasia derived cells from the OSCC derived cells. Hypothetically, stochastically acquired loss of spatial regulation of stem cell functionality could underlie progression of oral dysplastic lesions to oral SCC. In which case, greater understanding of these spatial regulatory mechanisms could lead to the development of more robust prognostic indicators for oral epithelial dysplasia.

Standard submerged monolayer culture conditions for oral cell lines from normal tissue (OKF6-TERT2), mild dysplasia (DOK), severe dysplasia (POE-9n) and OSCC (PE/CA PJ15), gave an overall

trend of increasing ALDH activity for cell lines derived from normal tissue, through dysplasia to OSCC. Expression of three other stem cell associated antigenic markers, CD44, p75[NTR] and CD24 did not share this correlation with disease severity. A notable observation was that that the overall heterogeneity in co-expression of the stem cell associated antigenic markers investigated was more prominent for normal tissue derived OKF6-TERT2 cells than either the dysplastic lesion (DOK and POE-9n) or OSCC (PE/CA PJ15) derived cell lines (figure 2). Expression of stem cell associated transcription factors OCT4, Sox2 and NANOG in head and neck derived ALDH-1 positive cells have been reported previously (25) (26), however, our analysis by qPCR array did not reveal correlation between OCT3/4, Sox2 or NANOG and source tissue disease severity.

The qPCR array analysis of stem cell associated transcription factor expression by OKF6-TERT2, DOK, POE-9n, and PE/CA PJ15 cells grown under standard conditions (data available upon request) will benefit future research with these cell lines. Of interest to the current investigation is the correlation between source tissue disease severity and up-regulation of FOXA1 and down-regulation of FOXA2 expression by the cell lines. High expression of FOXA1 and absence of FOXA2 has been documented for normal oral tissue (35) and evidence for the role of forkhead box proteins in cancer is accumulating (35) (36), with FOXA1 being an established marker of luminal A type breast cancer (37), and FOXA2 functioning as a suppressor of lung tumour metastasis (38).

Two apparent anomalies are presented in the data. The first relates to immunolabelling of HOME cultures for cytokeratin 19, the expression of which typically occurs in the basal layers of non-keratinising oral epithelia (32). Cytokeratin 19 immunolocalisation was intense and ubiquitous for HOME cultures of OKF6-TERT2 and POE-9n cells yet completely absent from HOME cultures of DOK and PE/CAPJ15 cells. This difference appears to be intrinsic to the cell lines, reflecting either differences in tissue source and clonal selection, or a progressive adaptation to routine culture in serum free media (OKF6-TERT2 and POE-9n) versus serum rich media (DOK and PE/CA PJ15). All HOME cultures were maintained for several population doubling times in serum rich Green's media which supports growth of each cell line, indicating that the absolute presence or absence of cytokeratin 19 immunolabelling was not attributable to a transient change in culture media. Further investigation of this may be relevant to future work but is beyond the scope of this text.

The second apparent anomaly presented relates to the high levels of ALDH activity reported for monolayer cultures, yet the low antigenic immunolabelling of ALDH1a1 in HOME cultures. Whilst it is possible that ALDH1a1 is down-regulated in all of the cells under HOME culture conditions, we believe this difference is more likely to be due to the difference in detection methods used. The ALDEFLUOR ® assay gives a sensitive and robust assessment of total ALDH enzyme function in live

cells by flow cytometry, but the technique is not readily applicable for HOME cultures. HOME cultures are particularly suited to immunohistological methods but these techniques provide a much less direct assessment of ALDH1a1 enzyme presence than the ALDEFLUOR ® assay of total enzyme activity. The authors avoided cytometric analysis of HOME cultured cells because of numerous practical incompatibilities between these two techniques, such as the need for harsh digestion regimens to release and disaggregate cells from HOME cultures and the consequent low viable cell yield and high potential for selecting sub-populations of cells. Up-scaling of HOME cultures to counter problems of low viable cell yield post-digest is not realistic because such up-scaling of human dermis based culture systems presents numerous difficulties which include poor availability of skin, variation in grafting depth, avoidance of scars or striae, and most importantly, dermal contraction in culture (33).

The HOME culture system combined with careful selection of cell lines that are derived from oral lesions bearing different clinical features provides an ideal investigative tool to study the loss of tissue hierarchy that coincides with the clinical progression of oral epithelial dysplasia to oral squamous cell carcinoma. Long-term HOME cultures maintained for several weeks could provide information on development of the invasive phenotype of OSCC derived cells. The current investigation showed that ALDH enzyme activity delineated source tissue disease severity in four oral cell lines when grown in monolayer, and that loss of hierarchical coordination of CD44 and p75$^{NTR}$ discriminated carcinoma derived cells from non-cancerous cells when grown as HOME cultures.

Ongoing work aims to confirm loss of CD44 and p75$^{NTR}$ hierarchical coordination in HOME culture of other oral SCC cell lines, to investigate the invasive phenotype in these cells, and to explore the mechanism that drives spatial regulation of cells bearing stem cell markers in HOME cultures.

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Figure Legends

Figure 1.

Dot plots of nucleated cells from (in descending rows) OKF6-hTERT, DOK, POE-9n and PE/CA-PJ15 cell cultures incubated with Aldefluor$^{®}$ reagent alone (**e,f,g,h**) or in the presence of 30mM DEAB inhibitor (**a,b,c,d**). Density banded dot plots of Aldefluor$^{®}$ bright cells co-labelled with anti-CD44-APC and/or anti-p75$^{NTR}$-PE are presented in the right hand column (**i,j,k,l**).

Figure 2.

Box and whisker plots showing co-expression data for CD44, p75$^{NTR}$, CD24 and ALDH positive (+) and negative (-) cell populations within (a) OKF6-TERT2 (n=4 replicates), (b) DOK (n=3 replicates), (c) POE-9n (n=3 replicates), and (d) PE/CA PJ15 (n=3 replicates) submerged monolayer cell cultures. The box and whisker plots show median (line) and interquartile range (box), with whiskers extending to 1.5 times the interquartile range. Plots are identified by a co-expression tree and median values ≥1% are given below each plot.

Figure 3

Expression pattern of CD44, p75$^{NTR}$, CD24 and Aldh1a1 in human organotypic mucosal epithelial (HOME) cultures after 14 days at the air/liquid interface.
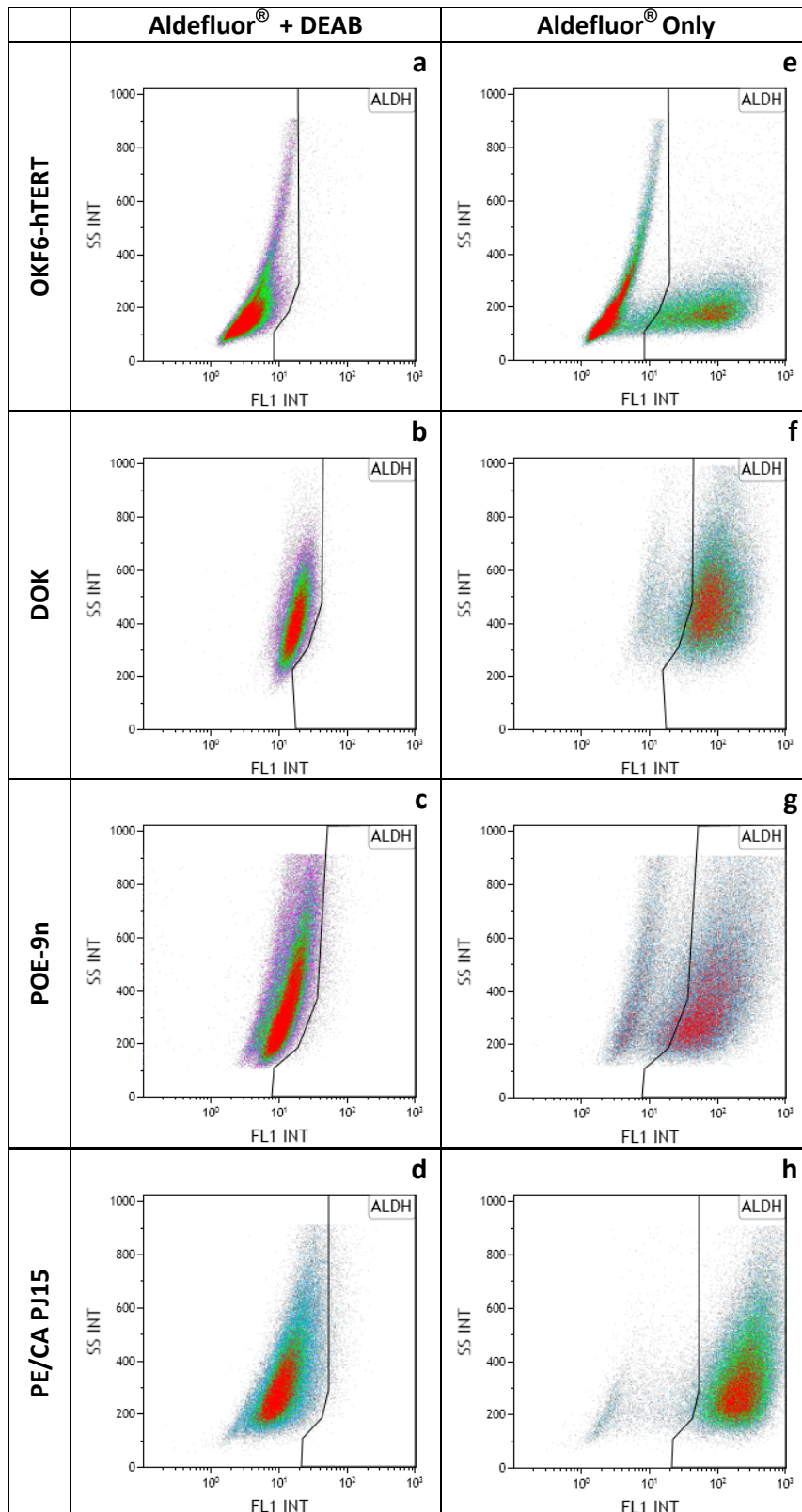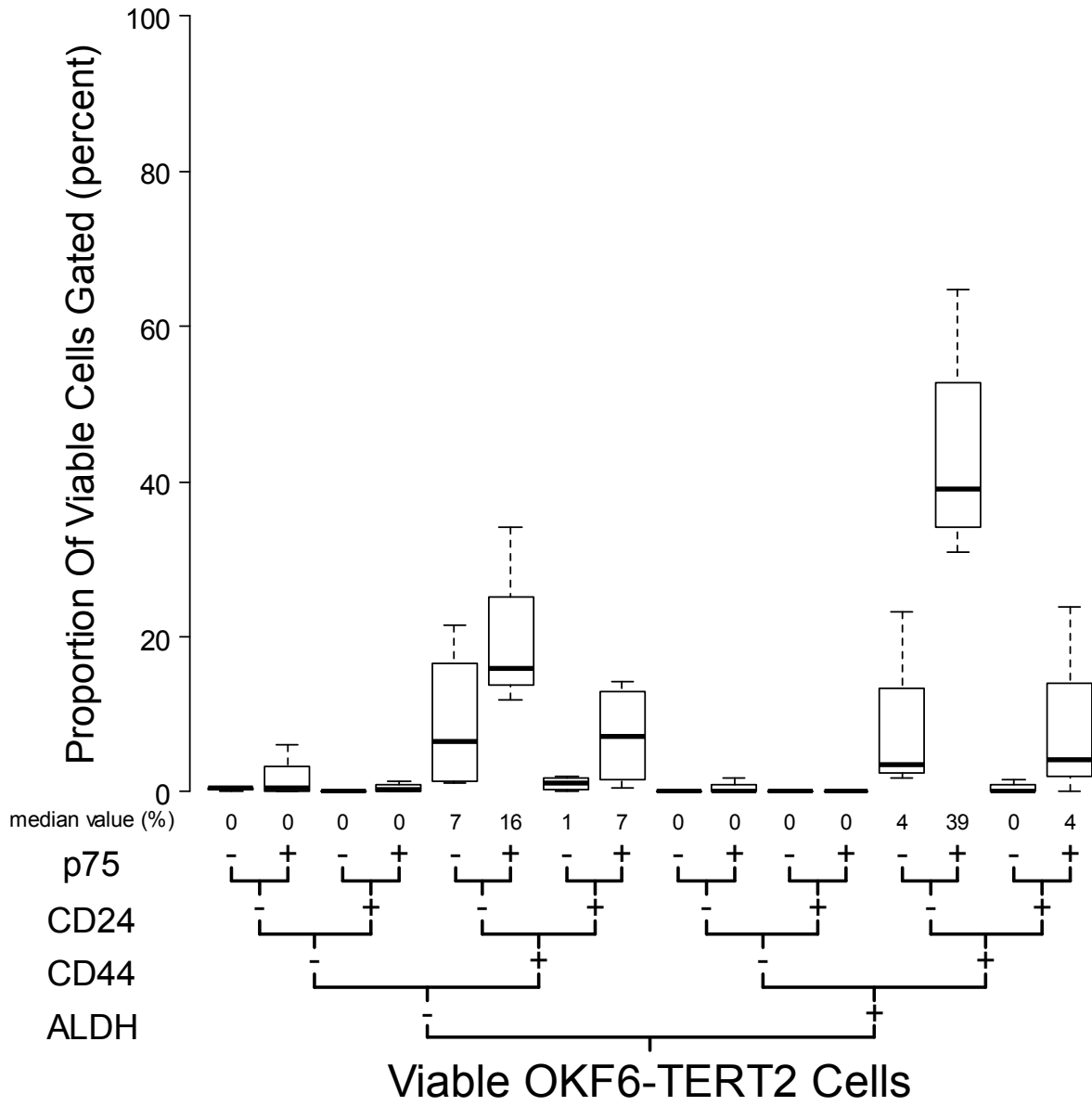
**Figure 1**. Representative density banded dot plots of Live cells from (in descending rows) OKF6-hTERT, DOK, POE-9n and PE/CA-PJ15 cell cultures incubated with Aldefluor® reagent alone (**e,f,g,h**) or in the presence of 30mM DEAB inhibitor (**a,b,c,d**).

**a**

Proportion Of Viable Cells Gated (percent)

| median value (%) | 0 | 0 | 0 | 0 | 7 | 16 | 1 | 7 | 0 | 0 | 0 | 0 | 4 | 39 | 0 | 4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p75 | − | + | − | + | − | + | − | + | − | + | − | + | − | + | − | + |
| CD24 | | | + | | | | + | | | | + | | | | + | |
| CD44 | | | | | + | | | | | | | | + | | | |
| ALDH | | | | | | | + | | | | | | | | | |

Viable OKF6-TERT2 Cells

**b**



Proportion Of Viable Cells Gated (percent)

| median value (%) | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 15 | 0 | 0 | 0 | 0 | 0 | 7 | 5 | 69 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p75 | − | + | − | + | − | + | − | + | − | + | − | + | − | + | − | + |
| CD24 | | | − | + | | | − | + | | | − | + | | | − | + |
| CD44 | | | | | − | | | + | | | | | − | | | + |
| ALDH | | | | | | | | − | | | | | | | | + |

Viable DOK Cells

# c



| median value (%) | 0 | 0 | 0 | 3 | 0 | 1 | 0 | 21 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 69 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| p75 | − | + | − | + | − | + | − | + | − | + | − | + | − | + | − | + |
| CD24 | | | + | | | | + | | | | + | | | | + | |
| CD44 | | | | | − | | | + | | | | | − | | | + |
| ALDH | | | | | | | | − | | | | | | | | + |

Viable POE-9n Cells

**d**

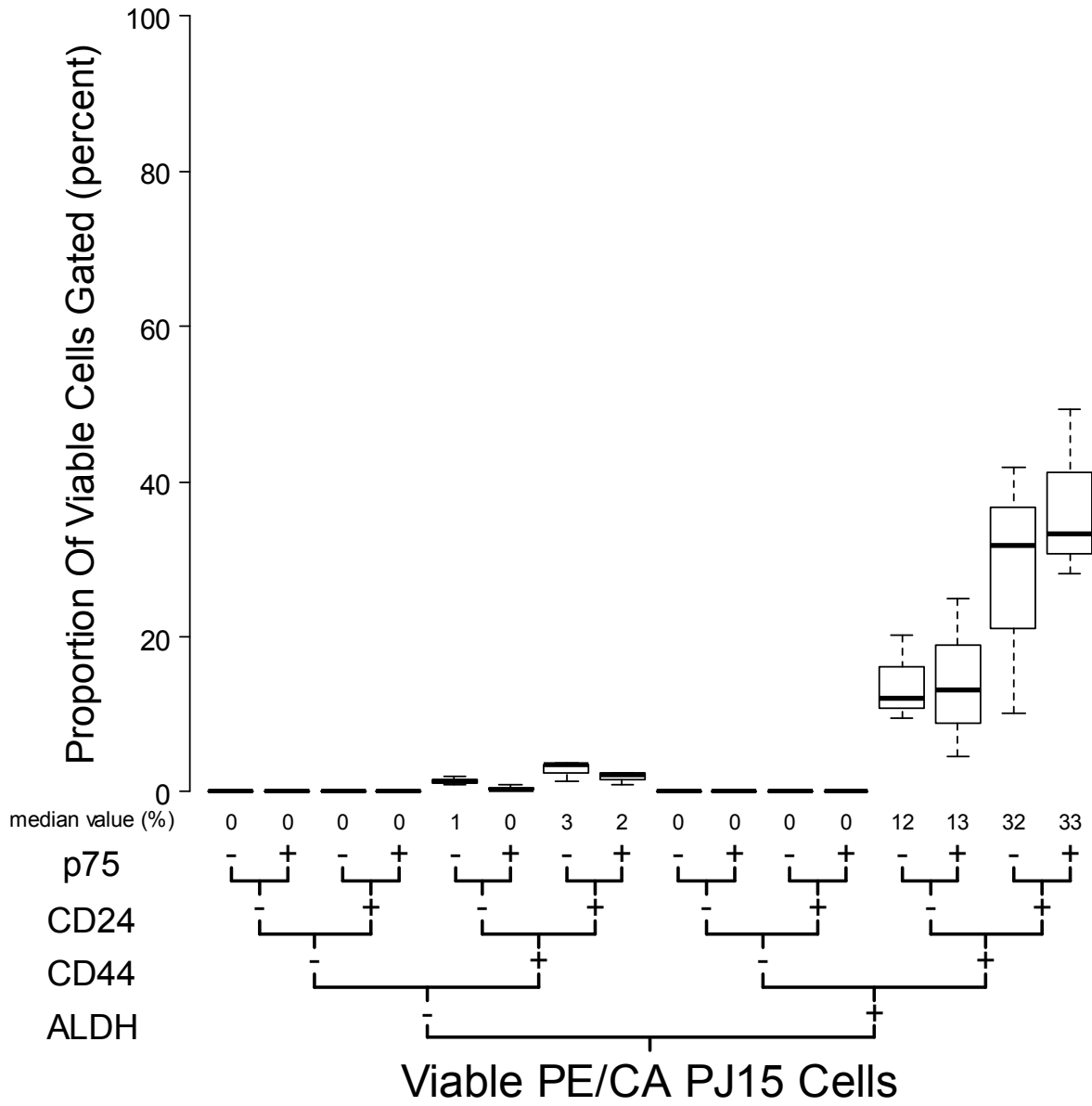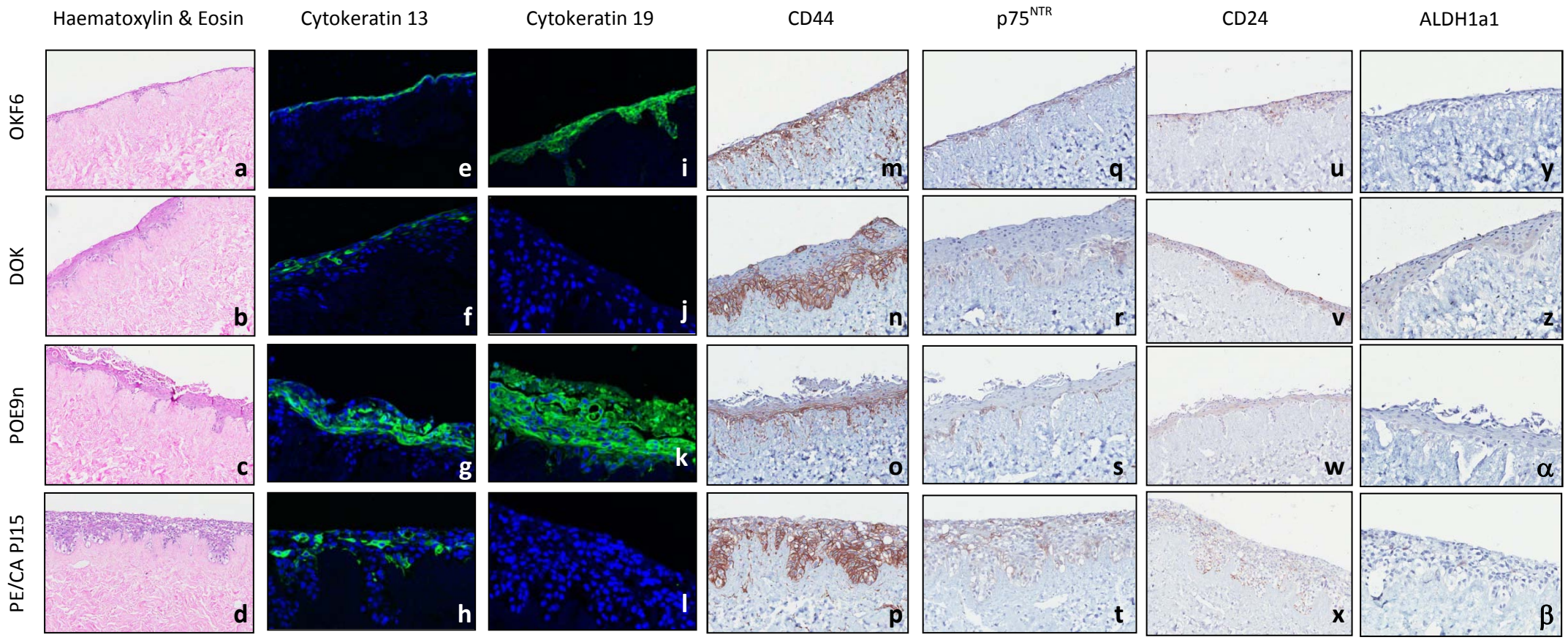**Figure 3** Expression pattern of CD44, p75$^{NTR}$, CD24 and Aldh1a1 in human organotypic mucosal epithelial (HOME) cultures after 14 days at the air/liquid interface.