European Journal of Cancer (2014) **50**, 3262–3270

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Original Research

Rapid adherence to collagen IV enriches for tumour initiating cells in oral cancer

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Received 22 May 2014; received in revised form 12 August 2014; accepted 15 September 2014
Available online 10 October 2014

KEYWORDS
Oral cancer
Stem cells
Collagen IV
Rapid adherent cells

Abstract ***Background:*** Although several approaches for identification and isolation of carcinoma cells with tumour initiating properties have been established, enrichment of a population of pure and viable tumour-initiating cells (TICs) is still problematic. This study investigated possibilities to isolate a population of cancer cells with tumour initiating properties based on their adherence properties, rather than expression of defined markers or clonogenicity.

Methods: Several human cell lines derived from oral dysplasia and oral squamous cell carcinoma (OSCC), as well as primary cells derived from patients with OSCC were allowed to adhere to collagen IV-coated dishes sequentially. Rapid adherent cells (RAC), middle adherent cells (MAC) and late adherent cells (LAC) were then harvested and further investigated for their morphology, stem cell-like properties and molecular profile while grown *in vitro* and tongue xenotransplantation in NOD-SCID mice at serial dilutions.

Results: RAC showed significantly higher colony forming efficiency ($p < 0.05$), sphere forming ability, greater migration ability ($p < 0.05$), exhibited longer G2 phase and displayed higher expression of integrin β1 and other stem-cell related molecules as compared to MAC and LAC. RAC induced tongue tumours in NOD-SCID mice with the highest incidence. These tumours were also bigger and metastasised more frequently in loco-regional lymph nodes than MAC and LAC.

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http://dx.doi.org/10.1016/j.ejca.2014.09.010

Conclusions: These findings prove for the first time that OSCC cells with tumour initiating properties can be enriched based on their rapid adhesiveness to collagen IV. This separation procedure provides a potentially useful tool for isolating TICs in OSCC for further studies on understanding their characteristics and drug-resistant behaviour.

1. Introduction

Oral squamous cell carcinoma (OSCC) has poor prognosis due to therapy-resistant loco-regional recurrences and distant metastases [1]. The cancer stem cell theory, although controversial, seems to explain well the behaviour of this disease, and the existence of a sub-population of cells with tumour initiating properties and increased self-renewal has been shown in OSCC [2]. Understanding the biology of tumour initiating cells (TICs) is thus essential for better understanding and more specific targeting of OSCC. For the same reason, robust methods for isolation of TICs are in demand. Numerous strategies for enrichment of TICs have been previously described [2–5]. However, isolating a population of pure and viable putative TICs is still problematic. Fluorescence-activated cell sorting (FACS) is the most common technique to isolate TICs based on specific surface makers, and CD44 has been repeatedly reported to identify human oral cancer stem cells [2,4]. Nevertheless, there is no single marker that can be used for isolation of TICs for any type of cancer, including OSCC, because of tumour heterogeneity and lack of reproducibility [5]. Moreover, the FACS technique requires high-cost, high-speed sorters and high-quality antibodies. Sorting the side population (SP) on the basis of the ability to efflux the fluorescent DNA-binding dye Hoechst 33342, or sorting of cells based on the ALDH1 enzymatic activity has also been used for isolation of TICs, but sometimes only few cells are acquired by these methods, and thus they are hardly of any use for further investigations [5]. In addition, the method of sorting SP cells cannot be applied broadly since not all cell lines contain SP cells [6]. Recent studies that explored more functional, non-invasive alternative methods to FACS analysis, demonstrated that non-adherent sphere formation [3], *in vivo* serial tumour xenotransplantation [7], as well as chemotherapeutic drug resistance [7,8] could be used to enrich a subpopulation with TICs. However, these methods require a long time to yield TICs, and not all cell types and cell lines are able, for example, to grow as spheres. In this study we have assessed the potential of a functional approach for TIC isolation in OSCC, a method previously used for normal epithelium [9]. Although severely disturbed, OSCC has an organisation resembling the normal oral epithelium, including functionally different cell sub-populations: stem cells, transient amplifying cells and terminally differentiated cells [10]. Based on the ability of normal epidermal stem cells to rapidly adhere to basement membrane molecules such as collagen IV due to their differential expression of specific integrins [11], an adhesion assay for their isolation and enrichment was successfully developed in both human [11], murine [12], and rabbit normal epithelium [9]. We have adopted this method of isolating stem cells in normal epithelia for enrichment of TICs in OSCC, and determined whether a sub-population of cancer cells containing putative TICs could be separated over time according to adhesiveness to collagen IV. We report here, for the first time to our knowledge, that sub-populations of cells enriched for TICs, with increased self-renewal and higher tumourigenic potential could be isolated within 10 min using the method of rapid adhesiveness to collagen IV in OSCC.

2. Materials and methods

2.1. Cell culture and generation of primary cells from human samples

OSCC cell lines (Cal, H357 and CaLH3), human dysplastic oral keratinocyte (DOK) cell line and primary oral cancer keratinocytes isolated from patients with OSCC (P1 and P2) were used in this study. Written consent was obtained from all patients, and the project was approved by the Regional Committee for Ethics in Research in West Norway.

2.2. Adhesion to collagen IV

Tissue culture dishes (100 mm, Nunc, Denmark) were coated evenly with 10 μg/mL human collagen IV (Sigma, St. Louis, MO, United States of America (USA)) diluted in 10 mM acetic acid. Single cell suspensions in routine culture medium were allowed to attach to the collagen IV coated dishes in the incubator. Cells that attached within 10 min were collected after trypsinisation and referred to as rapid adherent cells (RAC). Cells that remained unattached within first 10 min were transferred to a new collagen IV-coated dish for additional 30 min in the incubator. Cells that adhered within this period were collected as middle adherent cells (MAC). Remaining unattached cells were then again transferred into a new collagen IV-coated dish for next 4 h. Adherent cells in this time period were collected and referred to as late adherent cells (LAC).

Un-fractionated cells that were not separated according to their adhesive properties served as controls.

2.3. Colony and sphere formation assays

Five hundred cells per well were seeded in 6-well plates (Nunc) in 3 mL complete culture medium. After 7–10 days, the wells were examined microscopically and stained with 0.5% crystal violet (Sigma). Colonies were manually counted. For sphere formation assay, 500 cells per well were seeded in 24-well plates (Nunc) with 500 μL routine culture medium containing 1% methylcellulose (Sigma). After 7–14 days, wells were examined microscopically and viable spheres were manually counted. Colony forming efficiency (CFE, %) and sphere forming efficiency (SFE, %) were calculated using the following formula: (average of colonies or spheres per well/500) × 100.

2.4. Tongue xenotransplantation in NOD-SCID mice

NOD-SCID 6–8 week old mice were used and maintained in an isolation facility under a pathogen free environment with standard 12/12 h day and night cycle. RAC, MAC and LAC cells isolated from P1 and P2 were suspended in 50 μL Matrigel (BD Biosciences) and transplanted into the tongue of mice ($n = 91$). All mice in each group were sacrificed at the same time. Tumour formation and loco-regional lymph node metastasis were assessed. All animal procedures were approved by the Norwegian Animal Research Authority.

Cell diameter measurement, cell migration assay, immunohistochemical staining, RNA extraction, polymerase chain reaction (PCR) array, quantitative PCR (qPCR), western blot, flow cytometry and cell cycle analysis are described in Supplementary information.

3. Results

3.1. RAC cells were smaller, more homogenous and had increased self-renewal ability

Quantification of the cell diameter showed that RAC cells were significantly smaller than MAC and LAC (Fig. 1A), both in oral dysplastic and OSCC cell lines, as well as in primary OSCC-derived cells. RAC cells displayed a regular round shape and were more homogenous when compared with MAC and LAC that were bigger, heterogeneous and brighter, as seen under bright field microscopy (Fig. 1B). Assessment of the *in vitro* self-renewal ability showed that RAC had significantly higher clonogenicity when compared with MAC, LAC

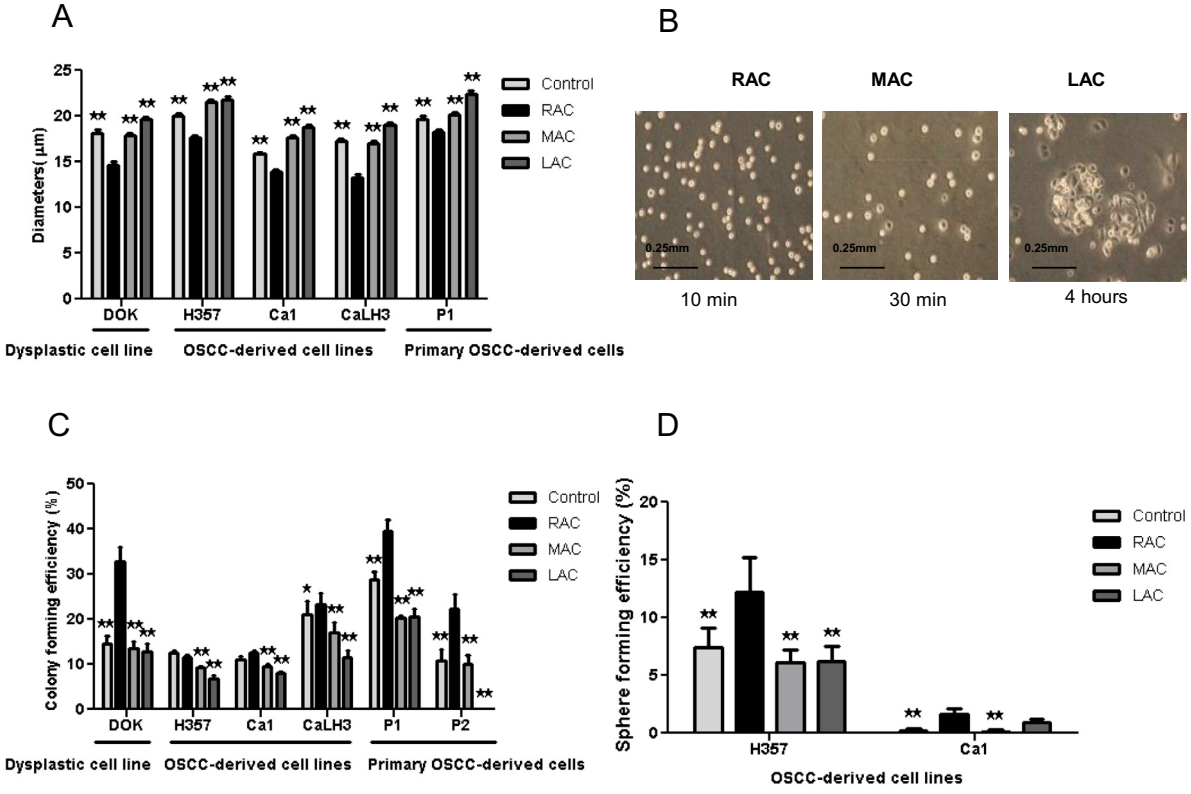

Fig. 1. Cell morphology and *in vitro* self-renewal ability of different sub-populations in dysplastic oral keratinocytes, primary cells isolated from oral squamous cell carcinoma (OSCC) patients and OSCC-cell lines. (A) Quantification of cell diameter. (B) Phase-contrast images of different cell sub-populations isolated based on differential subsequent adhesion to collagen IV-coated dishes (200× magnifications). (C) Quantification of colony formation ability. (D) Quantification of sphere formation efficiency. Values are the mean ± standard error (SEM) ($n \geq 3$). $^{**}P < 0.01$. $^{*}P < 0.05$ when compared with rapid adherent cells (RAC).

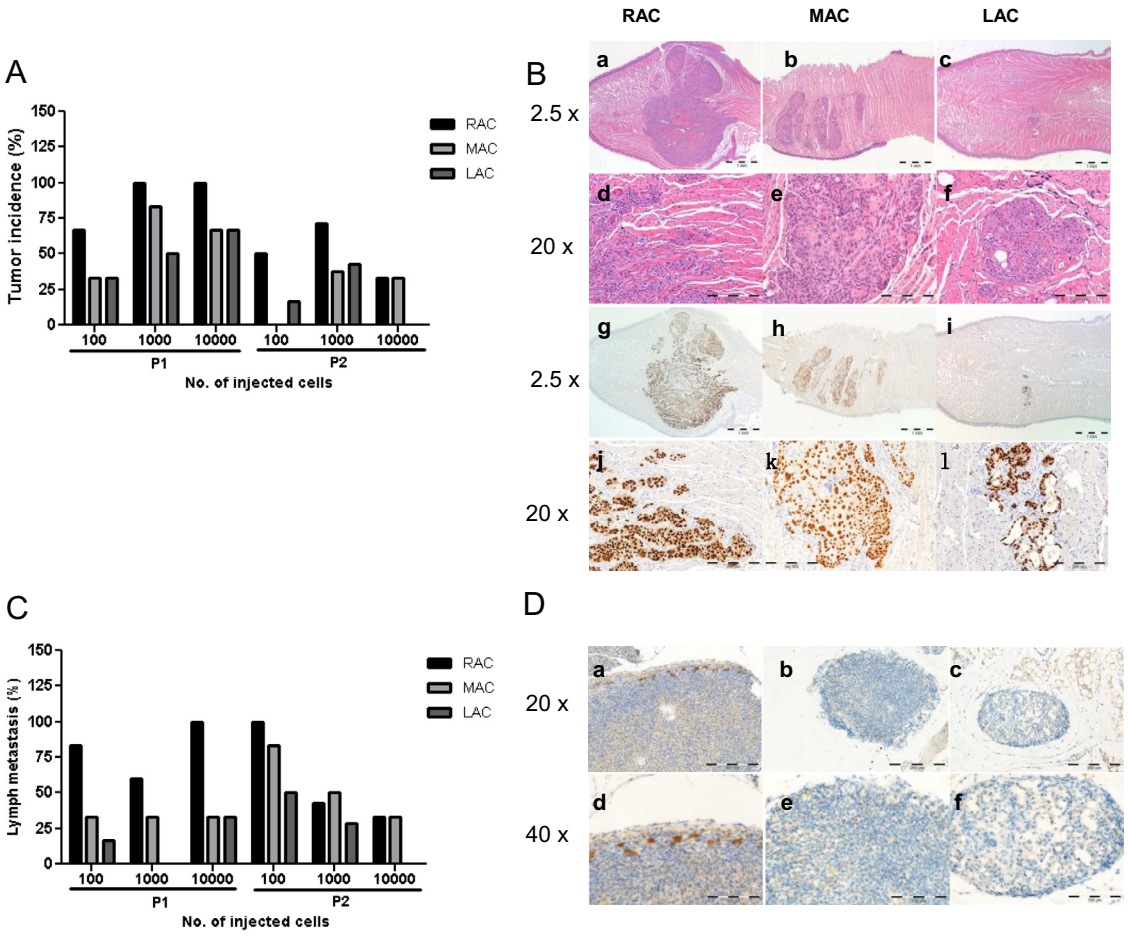

Fig. 2. Tumour initiation and loco-regional metastasis after tongue xenotransplantation in NOD-SCID mice of different sub-populations in primary cells derived from oral squamous cell carcinoma (OSCC) patients. (A) Quantification of tumour incidence. (B) Haematoxylin and eosin (H&E) staining and immunohistochemistry staining of p53 of tongue tumours after orthotopic tongue injections with 102 cells of rapid adherent cells (RAC), middle adherent cells (MAC) and late adherent cells (LAC) isolated from primary OSCC-derived cells (P1). Top two panels: H&E staining for tongue tumours with low magnification (2.5×) and high magnification (20×); Low two panels: immunohistochemistry staining of p53 for tongue tumours with low magnification (2.5×) and high magnification (20×). (C) Quantification of the loco-regional lymph node metastasis. (D) Immunohistochemistry staining of p53 for lymph nodes after orthotopic tongue injections with 102 cells of RAC, MAC and LAC isolated from primary OSCC-derived cells (P1) with low magnification (20×) and high magnification (40×).

and control cells for oral dysplastic cells, most of the OSCC cell lines, and for primary OSCC-derived cells (Fig. 1C and S). Sphere formation assay showed that RAC formed higher number of oro-spheres than MAC, LAC and control cells in OSCC cell lines (Fig. 1D).

3.2. RAC had increased in vivo tumourigenic and metastatic ability in primary OSCC-derived cells

A total of 91 mice were injected with different cell subpopulations isolated from two patients with OSCC, at serial dilutions (100, 1000 and 10,000 cells). RAC showed increased tumour initiating ability when compared to MAC and LAC (Fig. 2A and Table S1), while MAC and LAC had lower tumour initiation ability, and this was more obvious at higher dilutions. Tumour growth curves showed that RAC induced the biggest tongue tumours with the fastest tumour growth (Fig. 3). Of note, only tumours developed by RAC exhibited small islands of invading cells at the tumour front (Fig. 2B – a, d, g and j), while MAC (Fig. 2B – b, e, h and k) and LAC (Fig. 2B – c, f, i, and l), when they developed tumours, had a less aggressive pattern of invasion, more of the 'pushing' type tumour front, with no single cells or small islands invading the surrounding musculature. Examination of the loco-regional lymph nodes (Fig. 2D) demonstrated the presence of p53-positive human cells at a higher rate in the lymph nodes of mice injected with RAC (Fig. 2D – a and d), than in the mice injected with MAC (Fig. 2D – b and e) or LAC (Fig. 2D – c and f).

3.3. RAC showed higher expression of stem cell-related markers

Flow cytometry analysis of integrin β1 expression, a known stem cell marker in normal epidermis and a collagen IV receptor when heterodimerised with integrin α2

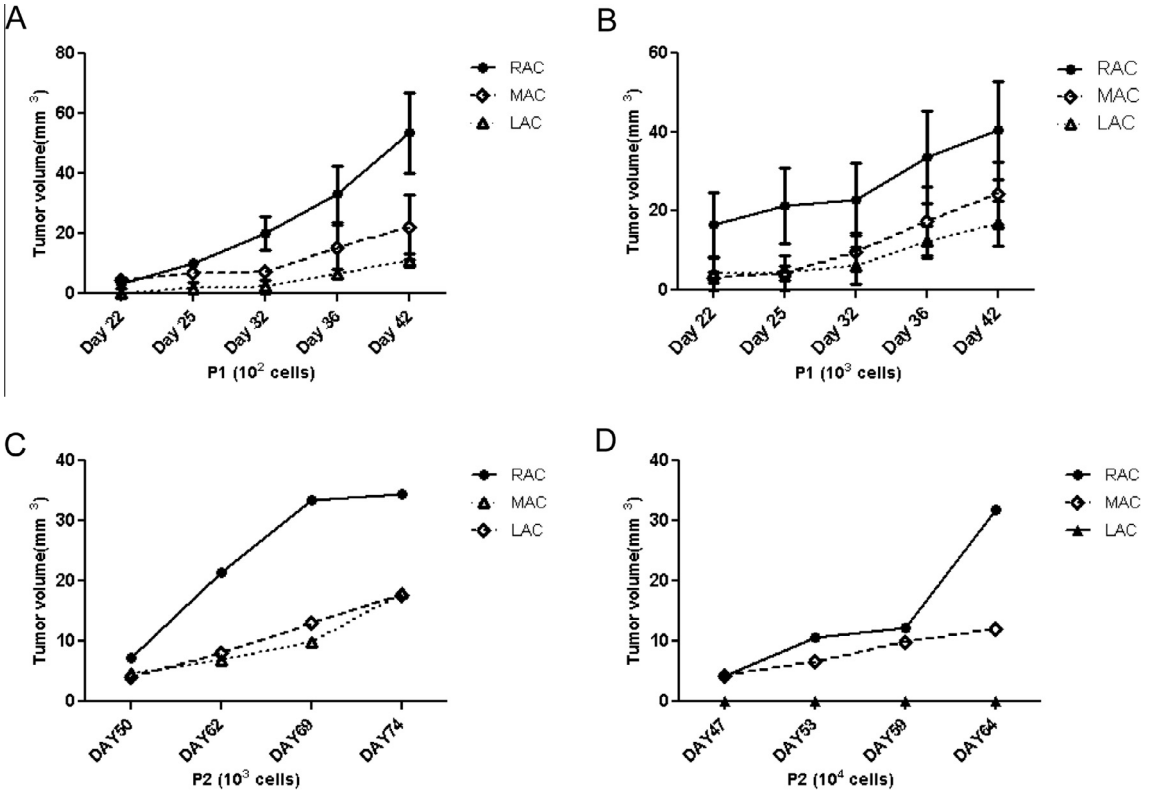

Fig. 3. Tumour growth rates when different numbers of cells from two primary oral squamous cell carcinoma (OSCC)-derived cells injected into the tongue of NOD-SCID mice. (A) Tumour growth rates when 102 cells from P1 were injected. (B) Tumour growth rates when 103 cells from P1 were injected. (C) Tumour growth rates when 103 cells from P2 were injected. (D) Tumour growth rates when 104 cells from P2 were injected. Values in (A) and (B) are the mean ± standard error (SEM).

[11], revealed a significantly higher expression in RAC than MAC and LAC in all OSCC cell lines and primary cells (Fig. 4A and B). Immunohistochemical staining for P75NTR, a surface marker more recently related with stemness in normal oral epithelium showed higher expression in RAC than MAC and LAC for all cell lines (Fig. 4C). Western blot for a self-renewal related molecule, BMI1, revealed a higher level in RAC compared to MAC or LAC (Fig. 4D). To characterise more precisely the expression of stem cell-related molecules in the three cell sub-populations, 84 genes related to stem cell properties were analysed using a PCR array in primary OSCC-derived cells (P1). Unsupervised hierarchical clustering revealed distinct separation of RAC from LAC (Fig. 5A). In addition, MAC also clustered separately from LAC (Fig. 5B), but not from RAC (Fig. 5C). Pair-wise significance analysis of microarrays (SAM) analysis showed that genes related to the stem cell properties were differently expressed in RAC and MAC compared to LAC (Table S2). Compared to LAC, RAC were found to have significantly (FDR = 0) up-regulated expression of *ABCG2*, *CCND2*, *PPARG*, *FGF2*, *DVL*, *RPL13A*, *GJA1 and GDF* (Table S2). Differential gene expression pattern between RAC, MAC and LAC was further independently verified by quantitative real-time PCR (qRT-PCR) using TaqMan assays. In line with the PCR array results, RAC expressed significantly higher mRNA level of *FGF2*, *PPARG*, *CCDN2*, *TERT* and *RPL13A* (Fig. 5D) than LAC. The expression of *BMP1* was found to be significantly lower in RAC than LAC (Fig. 5D). Furthermore, RAC expressed significantly higher levels of *NGFR* and *POU5 F1* than LAC (Fig. 5E), however, no significant differences of *CD44*, *BMI-1*, and *PDPN* mRNA expression were found in RAC, MAC and LAC (Fig. 5E).

3.4. RAC displayed migratory behaviour and higher viability than FACS sorted CD44 high cells

Flow cytometry analysis after staining the three cell sub-populations for DNA content showed an increase in the proportion of cells in G2-phase of the cell cycle in RAC compared to MAC and LAC for all cell lines (Fig. 6A). Transwell migration assay showed that RAC had significantly higher rate of migration than MAC and LAC, consistent for all cells examined in the study (Fig. 6B and C).

When compared with TICs isolated based on the established method of FACS sorting for CD44 [13], the viability of RAC was significantly higher than that of CD44high cells (Table 1).

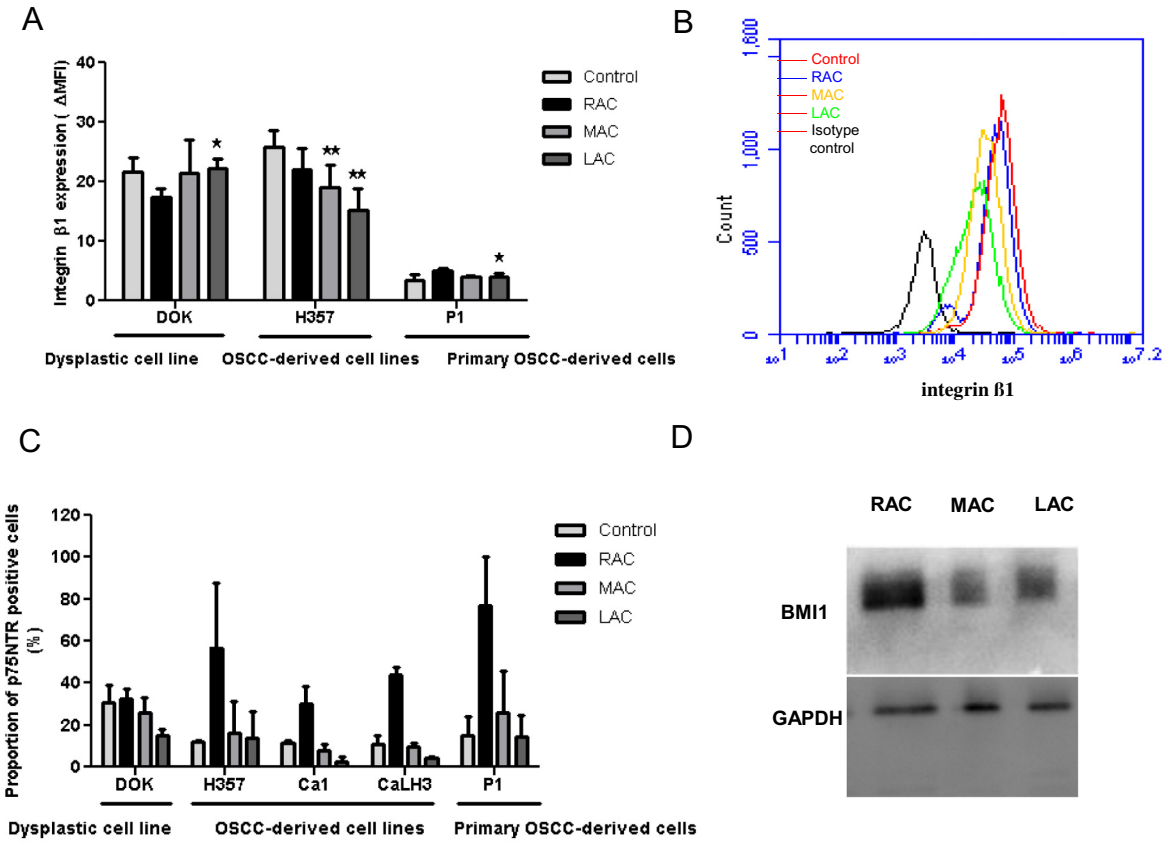

Fig. 4. Expression of stem cell-related markers in different sub-populations in dysplastic oral keratinocytes, primary cells isolated from oral squamous cell carcinoma (OSCC) patients, and OSCC cell lines. (A) Quantification of integrin β1 expression. (B) Flow cytometry analysis for β1-integrin expression in H357. (C) Quantification of proportion of P75NTR positive cells in rapid adherent cells (RAC), middle adherent cells (MAC) and late adherent cells (LAC) from oral dysplastic and OSCC cell lines, as well as primary OSCC-derived cells. (D) Western blot for BMI1 in RAC, MAC and LAC from OSCC-derived cell line H357. Values are the mean ± standard error (SEM) ($n \geqslant 3$). $^{**}P < 0.01$. $^{*}P < 0.05$ when compared with RAC. MFI, median fluorescence intensity.

4. Discussion

Many studies have showed that extracellular matrix (ECM), as an essential component of both normal and cancer stem cell niches, plays a crucial role in maintaining stem cell properties [14]. Collagen IV is an important ECM component and its expression pattern in the basement membrane of OSCC was associated with cervical lymph node metastasis as an important predictor of the metastatic aggressiveness of OSCC [15]. The present study describes, for the first time to our knowledge, the use of a simple, non-invasive and functional method to yield viable TICs in OSCC, based on rapid adherence to collagen IV. We show here that OSCC cells that adhered to collagen IV coated plates within 10 min displayed a number of stem cell properties, including small cell size, high *in vitro* self-renewal ability, high *in vivo* tumour initiating ability, high expression of stem cell markers as well as a high proportion of cells in the G2 cell cycle phase and high migratory capability. Previous studies suggested that cell size was related to cell phenotype, cell differentiation and proliferative potential in human keratinocytes [16]. The finding that RAC were smaller in size and showed higher expression of stem cell-related markers is in line with the previous study on human corneal epithelium which showed that small cells were expressing stem cell-associated markers and the small cell size represented one of the important stem cell properties while larger cells were more differentiated cells [17]. From the stem-cell markers assessed in this study, integrin β1 is of particular importance. It is well known that integrin β1 functions as the major receptor for basement membrane components such as collagen IV when heterodimerised with integrin α2, and laminin 5 when heterodimerised with integrin α3 [18]. Early studies showed that FACS for cells highly expressing integrin β1 can be used to isolate normal human epidermal stem cells from transit amplifying cells, since the cells with the highest level of integrin β1 expression showed the greatest colony formation ability [11]. In corroboration with these studies performed on normal epithelium, the present study shows that in a malignant epithelium the cells displaying the highest level of integrin β1 (RAC) also had the highest colony formation.

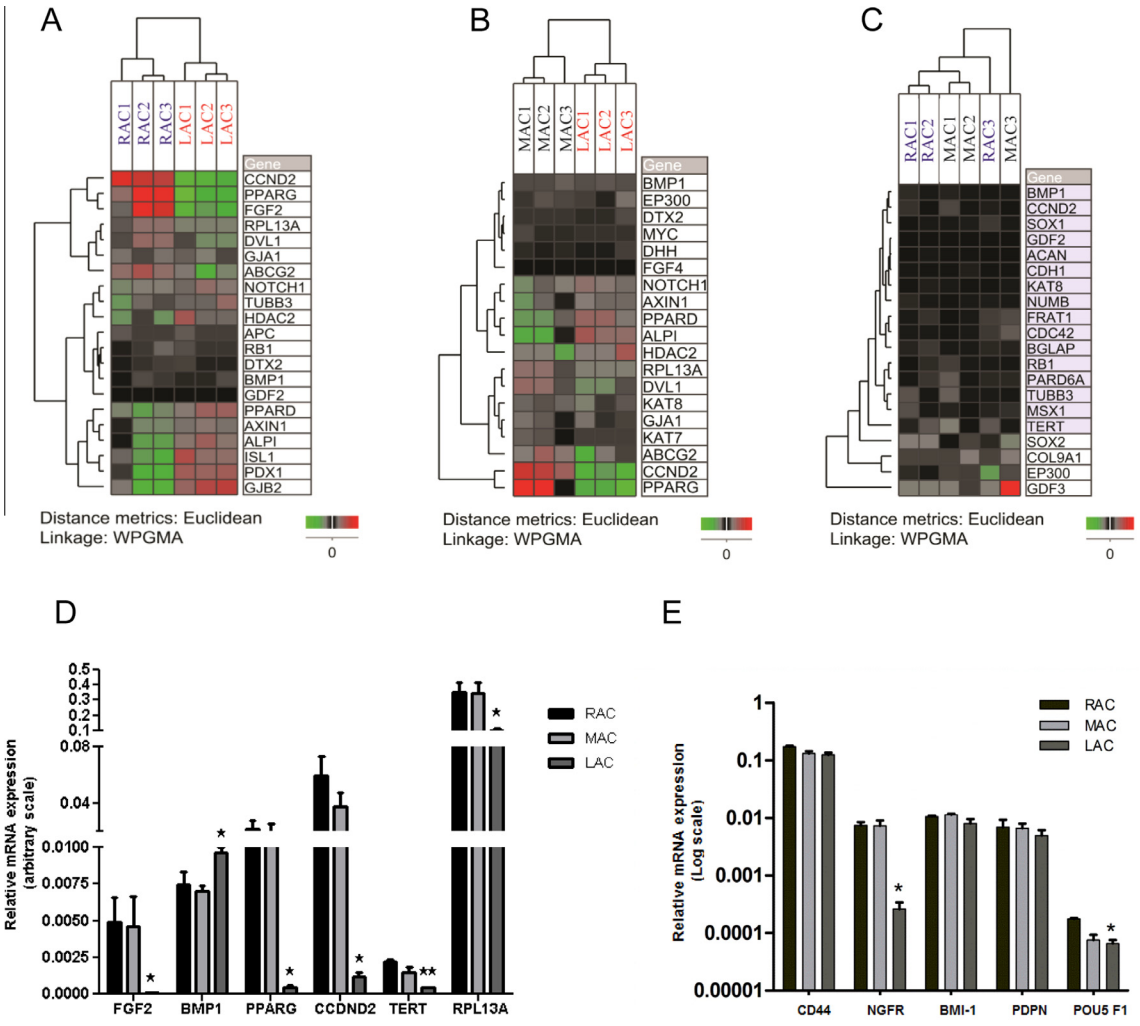

Fig. 5. Expression of stem cell-related markers in different sub-populations in primary cells isolated from primary oral squamous cell carcinoma (OSCC) cells P1. (A) Unsupervised hierarchical clustering of polymerase chain reaction (PCR) array for stem cell-related genes between rapid adherent cells (RAC) and late adherent cells (LAC), middle adherent cells (MAC) and LAC (B) as well as RAC and MAC (C). (D) Quantitative polymerase chain reaction (QPCR) analysis of mRNA level of FGF2, PPARG, CCDN2 TERT and RPL13A in the RAC, MAC and LAC. (E) Quantification of mRNA expression of stem cell-related markers *CD44*, *NGFR*, *BMI-1*, *PDPN* and *POU5 F1* in RAC, MAC and LAC. Values are the mean ± standard error (SEM) ($n \geqslant 3$). $^{**}P < 0.01$. $^{*}P < 0.05$ when compared with RAC.

In addition to increased clonogenicity and sphere formation ability, we show that the brightest integrin β1 cells (RAC) have also the highest tumour initiating abilities. In the present study we used orthotopic tongue xenotransplantation in NOD-SCID mice. This model was chosen because the human xenotransplantation cells are allowed, in the immunocompromised animal models, to develop and form tumours that mirror the phenotypic heterogeneity of the original human tumour, thus being still considered the 'gold standard model' for testing tumour initiating properties [19]. Despite the fact that the most common model for tumourigenesis testing is the subcutaneous xenotransplantation model, we have chosen the orthotopic tongue model, since in this way we could evaluate the loco-regional lymph node metastatic ability, this type of metastasis being the most common in OSCC.

Additional stem cell traits were also shown in this study for the RAC cells, including the higher proportion of cells in G2 phase of the cell cycle, consistent for all cell lines tested. Human malignant epithelial cells with stem-like properties were shown previously to spend a longer time in G2 phase in order to escape apoptosis by activating G2/M checkpoint proteins and thus provide more time for repair of DNA damage [13]. We did not investigate the ability of RAC cells to resist apoptosis compared to MAC and LAC, but based on the cell cycle analysis it is expected that they will show increased resistance. However, this needs to be further tested.

More recently, EMT features were attributed to stem-like cells in carcinomas, including OSCC [20]. We have assessed here the *in vivo* migratory/metastasising potential of the three subpopulations of RAC, MAC and

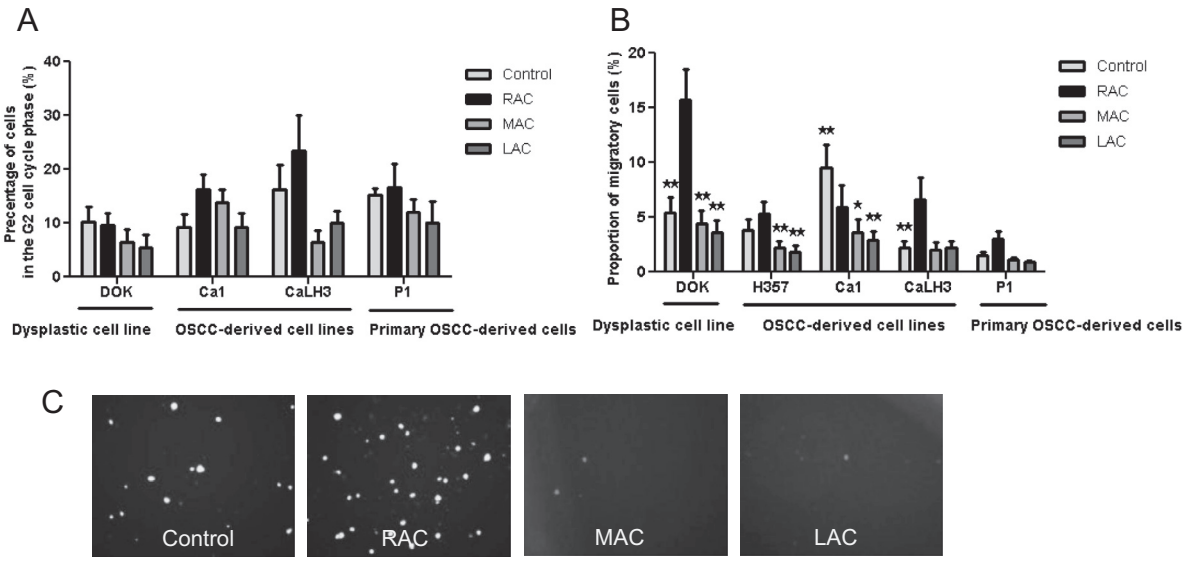

Fig. 6. Cell cycle analysis and cell migration of different sub-populations in dysplastic oral keratinocytes, primary cells isolated from oral squamous cell carcinoma (OSCC) patients and OSCC cell lines. (A) Quantification of the G2 cell cycle phase by flow cytometry analysis. (B) Quantification of the migratory capability. (C) Images of migrated cells amongst different sub-populations (magnification 4×). Values are the mean ± standard error (SEM) ($n \geqslant 3$). $^{**}P < 0.01$. $^{*}P < 0.05$ when compared with rapid adherent cells (RAC).

Table 1
Comparison of viability for isolating tumour-initiating cells (TICs) by fluorescence-activated cell sorting (FACS) sorting based on CD44 and collagen IV adherent assay. Values are the mean ± standard error (SEM) ($n \geqslant 3$).

Method of cancer stem cell (CSC) enrichment	% of viable cells (average ± SEM)
FACS sorting	
CD44high	76.2 ± 2.57
CD44low	67.17 ± 10.40
Adhesiveness to collagen IV	
Rapid adherent cells (RAC)	89.02 ± 2.84*
Middle adherent cells (MAC)	90.69 ± 2.43
Late adherent cells (LAC)	85.0 ± 6.25

* $P < 0.05$ when compared with RAC.

LAC by quantifying their presence in the cervical lymph nodes 74 days after tongue xenotransplantation. To visualise the human malignant cells present in the lymph nodes we have used IHC staining with a human specific antibody for mutated p53 (the primary cells injected in mice were chosen after sequencing of *TP53* and only cells derived from *TP53* mutated tumours were used for this purpose), that specifically identifies these cells and not mouse cells. Our results showed that RAC cells have the highest *in vivo* migratory/metastasising potential since the rate of cervical lymph nodes with scattered p53-positive human cells was higher in mice injected with RAC than in mice injected with MAC or LAC, fulfilling thus one more criteria recently linked to stemness in carcinomas.

Although isolated from tumours of the same histological type and clinical staging, *in vivo* tumour formation and especially the metastasising ability varied between the two primary cell lines used in the study. For example, no tumours were formed when 100 cells from MAC P2 cells were injected in six mice (Fig. 2A, Table S1). Of interest, we have detected p53-positive human migratory cells in the lymph nodes of five of these mice (Fig. 2C and D, Table S1). This could be a methodological bias, since for comparison reasons we have sacrificed all mice at the same time point, when the RAC tumours were too large and impaired the well-being of the mice. It might be that at this time point the primary tumours formed by the 100 MAC cells injected were too small to be detected, although migratory p53-positive human cells could be histologically detected in the lymph nodes. This might be taken as an indication that migration of malignant cells to the lymph nodes happens quiet early in the process of tumourigenesis, or at least in our experimental model of tumourigenesis.

Taken together, the findings presented here show that the cells rapidly adherent to collagen IV are enriched for cells with stem cell-like properties, and suggest that the method based on the adhesiveness of collagen IV provides a low cost, non-invasive, functional alternative to FACS for isolation and enrichment of TICs in OSCCs. The sub-population being rapidly adherent to collagen IV could be further used to explore stem cell and tumour initiating properties, improving our knowledge of OSCC biology.

Conflict of interest statement

None declared.

Acknowledgements

We thank Gunnvor Øijordsbakken and Edith Fick for excellent technical assistance. We also thank Molecular Imaging Centre (MIC) for outstanding facilities. This study was supported by The Norwegian State Educational Load Fund (Quota Programme), Meltzer's fund (project no. 804513, DS), Bergen Medical Research Foundation (DEC, 2010/11 and JW, 2010) and The Norwegian Cancer Association (2245585, 3294890).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.09.010.

References

[1] da Silva SD et al. Recurrent oral cancer: current and emerging therapeutic approaches. Front Pharmacol 2012;3:149.
[2] Prince ME, Ailles LE. Cancer stem cells in head and neck squamous cell cancer. J Clin Oncol 2008;26(17):2871–5.
[3] Ponti D et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005;65(13):5506–11.
[4] Harper LJ et al. Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. J Oral Pathol Med 2007;36(10):594–603.
[5] Chen SF et al. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. PLoS One 2012;7(2):e31864.
[6] Wu C, Alman BA. Side population cells in human cancers. Cancer Lett 2008;268(1):1–9.
[7] Yu F et al. Let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007;131(6):1109–23.
[8] Tsai LL et al. Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. J Oral Pathol Med 2011;40(8):621–8.
[9] Igarashi T et al. Isolation of oral epithelial progenitors using collagen IV. Oral Dis 2008;14(5):413–8.
[10] Costea DE et al. Cancer stem cells – new and potentially important targets for the therapy of oral squamous cell carcinoma. Oral Dis 2006;12(5):443–54.
[11] Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993;73(4):713–24.
[12] Bickenbach JR, Chism E. Selection and extended growth of murine epidermal stem cells in culture. Exp Cell Res 1998;244(1):184–95.
[13] Harper LJ et al. Normal and malignant epithelial cells with stem-like properties have an extended G2 cell cycle phase that is associated with apoptotic resistance. BMC Cancer 2010;10:166.
[14] Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 2012;196(4):395–406.
[15] Murakami M et al. Immunohistological investigation of type IV collagen in the basement membrane surrounding the cancer nest (cancer nest membrane) of head and neck squamous cell carcinoma – its relation to frequency of cervical lymph node metastasis. Nihon Jibiinkoka Gakkai Kaiho 1992;95(11):1773–84.
[16] Barrandon Y, Green H. Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc Natl Acad Sci USA 1985;82(16):5390–4.
[17] De Paiva CS, Pflugfelder SC, Li DQ. Cell size correlates with phenotype and proliferative capacity in human corneal epithelial cells. Stem Cells 2006;24(2):368–75.
[18] Alonso L, Fuchs E. Stem cells of the skin epithelium. Proc Natl Acad Sci USA 2003;100(suppl. 1):11830–5.
[19] Cheng L et al. Mouse models for cancer stem cell research. Toxicol Pathol 2010;38(1):62–71.
[20] Biddle A, Mackenzie IC. Cancer stem cells and EMT in carcinoma. Cancer Metastasis Rev 2012;31(1–2):285–93.