available at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/arob

Cancer progression is associated with increased expression of basement membrane proteins in three-dimensional *in vitro* models of human oral cancer

Keerthi K. Kulasekara [a], *Ochiba M. Lukandu* [a], *Evelyn Neppelberg* [a,b], *Olav K. Vintermyr* [a,c], *Anne Christine Johannessen* [a,c], *Daniela Elena Costea* [a,*]

[a] *Section of Pathology, The Gade Institute, University of Bergen, Norway*
[b] *Department of Clinical Odontology, University of Bergen, Norway*
[c] *Department of Pathology, The Gade Institute, Haukeland University Hospital, Norway*

ARTICLE INFO

Article history:
Accepted 18 July 2009

Keywords:
Laminin-332
Collagen IV
Fibronectin
Oral
Cancer
Fibroblasts

ABSTRACT

Background: Although basement membrane was traditionally considered an inert barrier that tumour cells had to cross before invasion into the surrounding stroma, recent studies suggest that basement membrane components are not only degraded during tumour progression, but also newly synthesised at the invasive front.
Objective: This study aimed at evaluating (1) the expression of basement membrane proteins in human oral carcinogenesis and (2) the role that epithelial–mesenchymal interactions play on it, by using an *in vitro* oral cancer progression model.
Material and methods: *In vitro* three-dimensional (3D) organotypic cultures of normal, early neoplastic and neoplastic human oral mucosa were developed by growing primary normal human oral keratinocytes, dysplastic human oral keratinocytes (DOK cell line), and neoplastic human oral keratinocytes (PE/CA-PJ15 cell line) on type I collagen biomatrices, with or without primary fibroblasts isolated from normal human oral mucosa. The cultured tissues were immunohistochemically assessed for the expression of the major basement membrane proteins laminin-332, type IV collagen, and fibronectin.
Results: Expression of laminin-332, type IV collagen, and fibronectin was gradually more pronounced in neoplastic models when compared to normal mucosa models, and, with the exception of laminin-332, it was further enhanced by presence of fibroblasts. Deposition of type IV collagen at the epithelium–biomatrix interface occurred only in presence of fibroblasts, as well as the extracellular matrix deposition of fibronectin.
Conclusions: These findings, obtained in a 3D *in vitro* model that closely mirrors the *in vivo* human oral cancer progression, show an enhanced basement membrane protein expression during human oral cancer progression that is dependent on the epithelial–mesenchymal environment, respectively the existence of fibroblasts.

* *Corresponding author at*: Section of Pathology, The Gade Institute, Haukeland University Hospital, N-5021 Bergen, Norway. Tel.: +47 5597 3230; fax: +47 5597 3158.
E-mail address: daniela.costea@gades.uib.no (D.E. Costea).
0003–9969/$ – see front matter
doi:10.1016/j.archoralbio.2009.07.004

1. Introduction

Basement membrane is the highly specialised structure of extracellular matrix between epithelium and underlying connective tissue that consists of a dense network of glycoproteins (such as laminin, nidogen and fibronectin), type IV collagen, and proteoglycans.[1] Although it was considered for decades a passive structure that separates epithelia from connective tissues only, later findings have shown that it is also able to influence the behaviour of the adjacent epithelium.[2–4] Basement membrane proteins have been found to affect epithelial cellular processes such as proliferation and differentiation in normal epithelia.[3–5] In neoplasia, breaks and defects of basement membrane were thought to be associated with the invasive behaviour of several types of tumours,[6,7] including oral neoplasia.[8–10] However, although historically considered as an inert barrier that tumour cells had to cross before they invaded the surrounding stroma, it has become recently clear that basement membrane is a rather dynamic structure in the neoplastic process.[11,12] It has been shown that basement membrane components are not only degraded during tumour progression, but also newly synthesised and deposited at the tumour invasive front. For example, overexpression of laminin-332 has been detected at the epithelial–stroma interface in invasive areas of colorectal, gastric and squamous cell carcinomas[13–16] and *in vitro* models of oral squamous cell carcinoma,[17] leading to the hypothesis that it could function as an extracellular ligand helping migration and invasion of tumour cells. Alterations in type IV collagen distribution in pre-malignant and malignant lesions of the oral epithelium have also been reported,[9,10] and its expression by neoplastic cells was found to be correlated with invasion in the connective tissue matrix in *in vitro* experimental models.[12,18] Increased expression of fibronectin was found as well to be associated with the invasiveness and the metastatic propensity of oral squamous cell carcinoma,[10,19] and the embryonic EDA and EDB fibronectin splice variants were found highly expressed in the active tumour stroma close to the invading oral squamous cell carcinoma nests.[20]

Taken together, these studies have shown that new deposition of major basement membrane proteins might be of importance for the invasive process, and suggest that the tumour stroma has a major role in this deposition. However, a more specific description of the dynamics of basement membrane and its dependency on epithelial–mesenchymal interactions during oral human tumour progression has not been studied. Thus, the aim of this study was to investigate the expression of laminin-332, type IV collagen, and fibronectin, and the role of epithelial–fibroblast interactions on their expression pattern during progression towards malignancy, using step-wise *in vitro* cell culture models of oral cancer progression.

2. Materials and methods

2.1. Tissue material

Normal human oral mucosa samples ($n = 17$) were obtained from redundant oral tissue removed during wisdom tooth extraction at Department of Oral Surgery, Faculty of Dentistry, University of Bergen, Norway. Informed consent was sought from the patients prior to obtaining the tissue material. The study was approved by the Ethical Committee of Western Norway and included tissues obtained only from clinically healthy donors.

2.2. Cell culture procedures

Primary normal oral keratinocytes (NOK) and fibroblasts (NOF) were isolated from normal human oral mucosa as described elsewhere.[21] NOK were routinely grown on plastic surfaces (Nunc, Naperville, IL, USA) with no feeding layers, in Keratinocyte-Serum Free Medium (KSFM, GibcoBRL, Grand Island, NY, USA) supplemented with 1 ng/ml human recombinant epidermal growth factor (GibcoBRL), 25 μg/ml bovine pituitary extract (GibcoBRL), 20 μg/ml L-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL), 100 μg/ml streptomycin (GibcoBRL), 0.25 μg/ml amphotericin B (GibcoBRL), 6 μg/ml fluconazole (Pfizer, Amboise, France). NOF were grown in Modified Eagle's Medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal calf serum (Sigma, St. Louis, MO, USA), 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 6 μg/ml fluconazole. The commercially available dysplastic keratinocyte cell line from the European Cell Culture Bank: human (Caucasian) dysplastic oral keratinocyte (DOK) cell line, accession no. 94122104 has been used in this study. This cell line was established from a tongue dysplasia. It was reported not to form tumours in nude mice, and considered to have a transformed but not fully malignant phenotype.[22] Later it was shown that the DOK cell line harbours p53 mutations.[23] DOK cells were routinely grown on plastic surfaces without feeding layers, in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% foetal calf serum, 20 μg/ml L-glutamine, and 5 μg/ml hydrocortisone. The oral cancer derived-PE/CA-PJ 15 cell line, accession no. 961211230, that has been previously successfully used in organotypic cultures of oral neoplasia, was also used.[16] It has been isolated from a well-differentiated buccal squamous cell carcinoma and it was reported to form tumours in nude mice with the histology similar to the native carcinoma.[17] PE/CA-PJ 15 were routinely grown in Iscove's Modified Dulbecco's Medium (Sigma) supplemented with 10% foetal calf serum and 20 μg/ml L-glutamine. DOK cells in passage 29 and PE/CA-PJ 15 cells in passage 9 were used for the experiments.

2.3. Cultivation of three-dimensional organotypic models of human oral mucosa

The multi-step model of oral epithelial tumour progression was obtained by growing normal (NOK, $n = 7$ cell strains isolated from different patients), early neoplastic/dysplastic (DOK) and neoplastic (PE/CA-PJ 15) oral keratinocytes on top of collagen type I biomatrices using a protocol well established in our laboratory.[24] In order to study the role of epithelial–mesenchymal interactions on basement membrane protein expression two types of organotypic cultures were prepared and used in this study: (1) organotypic monocultures in which each different type of keratinocytes was seeded on simple

collagen biomatrices and (2) organotypic co-cultures, in which keratinocytes were seeded on collagen biomatrices populated with NOF ($n = 7$ cell strains isolated from different patients). The organotypic cultures were grown in serum free culture medium: 3 vol. Dulbecco's Modified Eagle's Medium (Sigma)/ 1 vol. Ham's F 12 (Sigma), supplemented with 0.4 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Novo Nordisk, Bagsværd, Denmark), 20 μg/ml transferrin (Sigma), 50 μg/ml L-ascorbic acid (Sigma), 1 mg/ml linoleic acid-albumin (Sigma), 200 μg/ml penicillin, 200 μg/ml streptomycin, 0.5 μg/ml amphotericin B, 6 μg/ml fluconazole, 20 μg/ml L-glutamine. The cultures were lifted at air–liquid interface and harvested after 10 days, formalin fixed and paraffin embedded or fresh-frozen, as previously described.[24]

2.4. *Immunohistochemistry*

Five μm thick formalin-fixed, paraffin-embedded sections were deparaffinised in xylene for 30 min, thereafter hydrated through a graded series of alcohol of decreasing concentrations. After washing for 10 min in 1% Tween Tris-buffered saline (TBS; 0.005 M Tris–HCl, 0.001 M Tris–Base, 0.15 M $NaCl_2$), the tissue sections were subjected to microwave treatment and/or protease enzyme treatment. Sections were microwave treated at 750 W for 9 min first and then for 15 min at 500 W in 10 mM citrate buffer pH 6 (DAKO, Glostrup, Denmark), followed by enzymatic treatment with proteinase K 20 μg/ml (Merck, Darmstadt, Germany) diluted in TBS for 10 min in a humidified chamber for retrieval of laminin-332, and in antigen retrieval solution pH 6 (DAKO) followed by enzymatic treatment with proteinase K (DAKO, ready to use) for 2 min in a humidified chamber for type IV collagen retrieval. Proteinase K (DAKO, ready to use) for 4 min was used as the only antigen retrieval treatment for fibronectin. After cooling and washing, the sections were incubated with primary antibodies at room temperature in a humidified chamber for 60 min. Anti-type IV collagen (monoclonal mouse, clone CIV22, DAKO) and anti-laminin-332 (monoclonal mouse, clone D4B5, Chemicon International, Temecula, CA, USA) antibodies were diluted (1:50 and 1:400, respectively) in 1% BSA in TBS, while anti-fibronectin antibody (rabbit polyclonal, Sigma) was diluted (1:400) in antibody diluent (DAKO). After washing, endogenous peroxidase activity was quenched with peroxidase block solution (EnVision+ System, DAKO) for 5 min and the secondary antibody conjugated with horseradish peroxidase labelled polymer (EnVision+ System, DAKO) was applied for 30 min. The presence of antigen was visualised with DAB (3,3′-diaminobenzidine, DAKO) for 10 (laminin-332 and fibronectin) and 20 min (type IV collagen). The slides were counterstained with haematoxylin (DAKO), dehydrated through an ascending graded series of alcohol, xylene and then mounted with an alcohol soluble mounting medium (Eukit, DAKO). Native normal human oral mucosa were used as positive controls for tests with all the antibodies. Sections treated with antibody diluent instead of primary antibody were used as negative controls. Specimens stained with various monoclonal antibodies were of the same isotype, serving as a negative control for each other. All the experiments were performed 3 times, each in duplicate.

2.5. *Evaluation of the immunohistochemical staining*

The epithelial compartment, the epithelial–matrix interface, the extracellular matrix compartment and stromal fibroblasts were each evaluated separately. Epithelium was divided into a basal cell layer (BCL) and a superficial cell layer (SCL). The basal cell layer consisted of one to two cell layers that were nearest and perpendicularly arranged on the epithelial–matrix interface. The rest of the epithelial compartment overlying BCL was designated as SCL. The sections were assessed semi-quantitatively as negative (no staining), mild (5–30%), moderate (30–70%) and strong (70–100%), by scoring the percentage of positive cells per microscopic field (for the epithelial compartment and stromal fibroblasts), by evaluating the extent of the stained area per microscopic field (for the extracellular matrix compartment), and the length of the stained epithelial–matrix interface per microscopic field (for the epithelial–matrix interface). In order to avoid false positive staining or other artefacts, the peripheries of tissue sections were routinely excluded from the analysis.

3. Results

3.1. *Enhanced expression of laminin-332 with tumour progression and independent on epithelial–mesenchymal interactions*

In organotypic models of normal human oral mucosa in absence of fibroblasts, laminin-332 was found to be expressed in the cytoplasm of scattered basal cells. When human fibroblasts were included in the matrix, laminin-332 was found to be expressed by almost all cells in the epithelial basal layer, and as a narrow and discontinuous deposition at the epithelial–matrix interface (Fig. 1 and Table 1). When DOK cells were grown on top of matrices in the absence of fibroblasts, laminin-332 immunoreactivity was observed intracellularly in scattered cells throughout the whole epithelium, and as a discontinuous extracellular deposition at the epithelial–matrix interface. Although a slightly more pronounced deposition of laminin-332 was observed immediately close to the invasion front, its pattern of expression did not change with addition of human fibroblasts in the matrix (Fig. 1 and Table 1). When PE/CA-PJ 15 neoplastic cells were grown on top of matrices in absence of fibroblasts, intracellular expression of laminin-332 was detected in almost all epithelial cells, especially in the basal cell layer (Fig. 1). A discontinuous deposition of laminin-332 was detected along the epithelial–matrix interface in these models. The pattern of laminin-332 expression did not change when PE/CA-PJ 15 neoplastic cells were grown on top of matrices with human fibroblasts. The fibroblasts and the matrix compartment did not show any laminin-332 immunoreactivity in any of the above-mentioned models. With the exception of inducing extracellular deposition of laminin-332 in the normal human oral mucosa model, human fibroblasts did not have a significant effect on epithelial cells in relation to the pattern of expression and deposition of laminin-332. In control normal human oral tissues laminin-332 was present as a continuous deposition along the epithelial basement membrane.

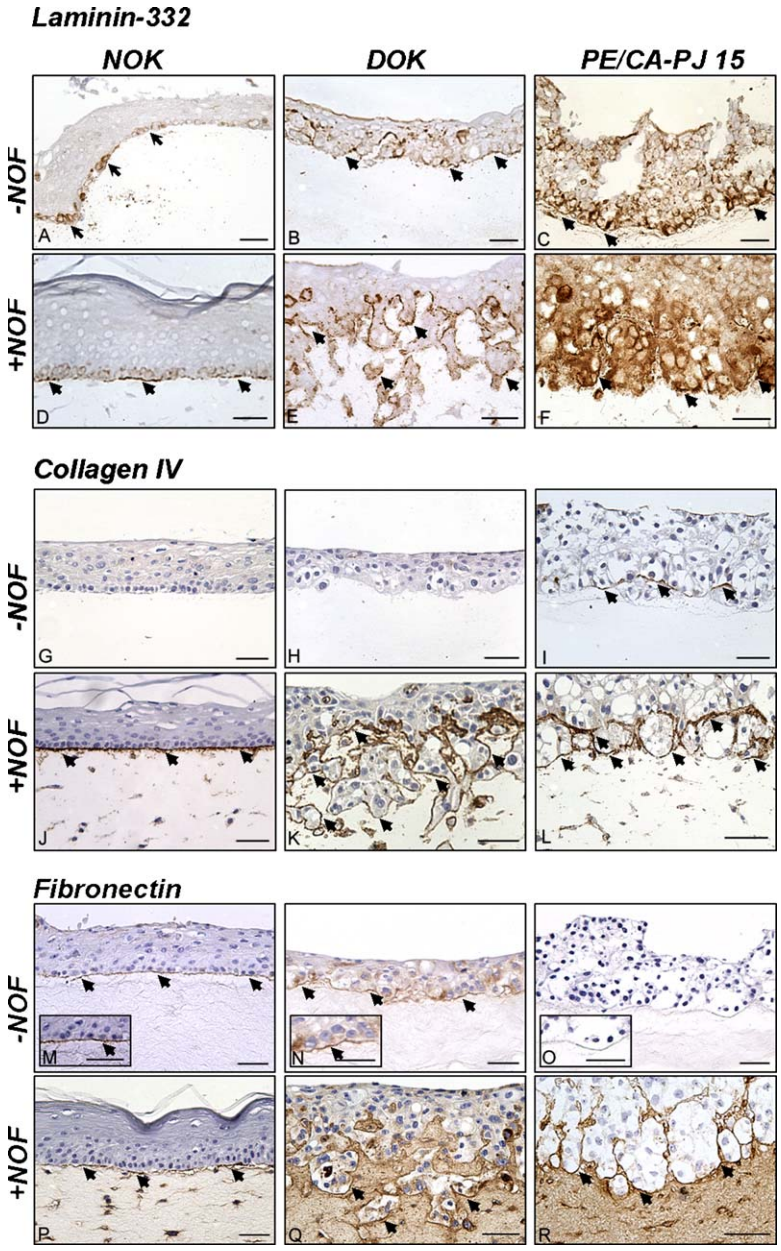
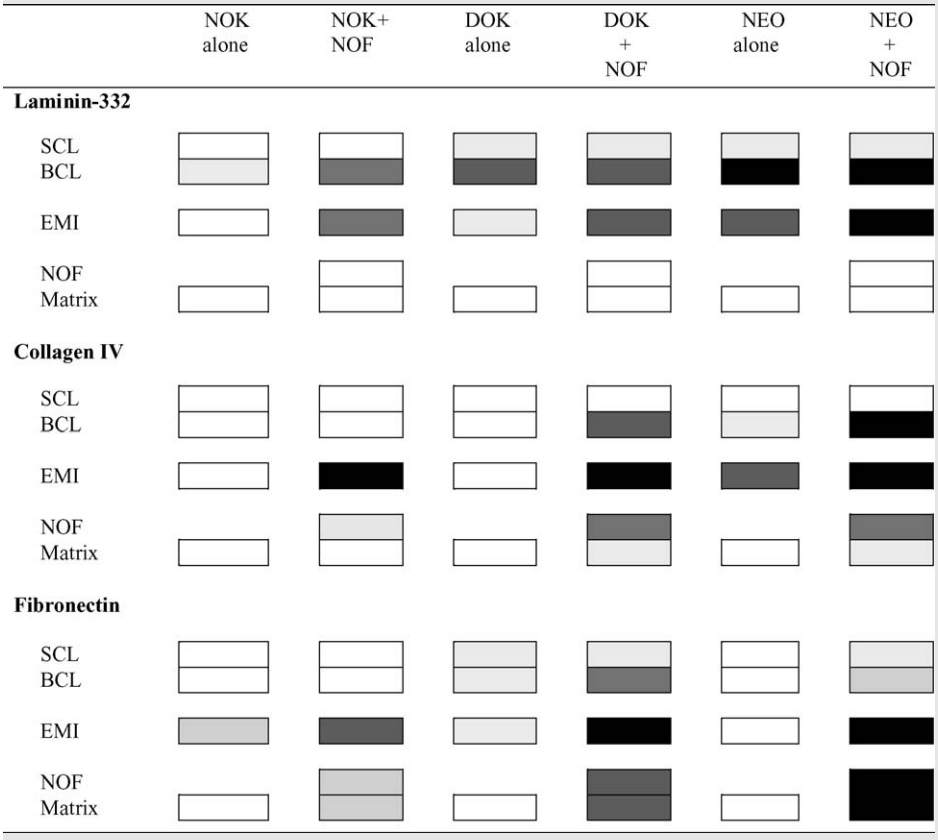


Fig. 1 – Immunoreactivity for laminin-332, type IV collagen and fibronectin in *in vitro* step-wise organotypic models of oral cancer progression, in presence and absence of fibroblasts. Normal, early neoplastic and established neoplastic human oral mucosa models were generated by growing organotypically NOK (A, D, G, J, M, P), DOK (B, E, H, K, N, Q) and PE/CA-PJ 15 (C, F, I, L, O, R) cells on top of simple collagen I matrices (A, B, C, G, H, I, M, N, O) or in the presence of normal human oral fibroblasts (NOF) (D, E, F, J, K, L, P, Q, R). Empty arrowheads indicate intracellular accumulation of basement membrane proteins while solid arrowheads indicate their extracellular deposition. Scale bar is 50 μm at all magnifications.

3.2. *Enhanced expression of type IV collagen with tumour progression and dependent on epithelial–mesenchymal interactions*

Type IV collagen immunoreactivity could not be detected in the normal mucosa models constructed in the absence of fibroblasts (Fig. 1 and Table 1). With inclusion of fibroblasts in the connective tissue matrices, a distinct linear type IV collagen deposition could be detected along the epithelial–matrix interface, similar to the staining pattern and localisation found in native oral mucosa (Fig. 1 and Table 1). Similarly, type IV collagen could not be detected in DOK models grown on matrices in the absence of fibroblasts, but inclusion of human fibroblasts in these cultures resulted in a strong deposition of type IV collagen as a continuous band at the epithelial–matrix interface, and also in the basal layer of early neoplastic epithelium, as previously shown by our group (Fig. 1, Table 1 and Ref.[12]). In the neoplastic models in absence of fibroblasts an intraepithelial collagen IV deposition was observed in the lower half of the neoplastic epithelium,

Table 1 – Expression of the basement membrane proteins: laminin-332, type IV collagen and fibronectin in *in vitro* stepwise organotypic models of oral cancer progression in presence and absence of fibroblasts. Normal, early neoplastic and established neoplastic human oral mucosa models were generated by growing organotypically NOK, DOK and PE/CA-PJ 15 cells on top of simple collagen I matrices or in the presence of normal human oral fibroblasts (NOF). The expression of collagen IV was assessed by immunohistochemistry. SCL: superficial epithelial cell layer; BCL: basal epithelial cell layer; EMI: epithelial–matrix interface, blank square: no staining; light grey square: mild staining, dark grey square: moderate staining, and black square: strong staining.



especially in close proximity to the epithelial matrix interface (Fig. 1 and Table 1). When normal human fibroblasts were incorporated in the matrix, a stronger type IV collagen immunoreactivity was detected within and around the epithelial cells of the basal layer of the neoplastic epithelium. In both early neoplastic and neoplastic models immunoreactivity for collagen IV could be detected in the stromal fibroblast and in the connective tissue matrix, especially in close association with the invasive nests of neoplastic cells. In control normal human oral tissues type IV collagen was present as a continuous deposition along the epithelial basement membrane and in the basement membrane of blood vessels.

3.3. *Enhanced expression of fibronectin in stromal fibroblasts and extracellular deposition in the connective tissue matrix with tumour progression and dependent on epithelial–mesenchymal interactions*

Immunostaining for fibronectin showed a linear deposition at the epithelial–matrix interface when NOK were grown on top of simple collagen matrices without fibroblasts (Fig. 1). When fibroblasts were incorporated in the matrix of NOK models, in addition to the linear distribution at epithelial–matrix interface, fibronectin was detected within approximately 50% of the fibroblasts and as a mild diffuse staining of the connective tissue matrix (Fig. 1 and Table 1). When DOK cells were grown on matrices in absence of fibroblasts, a very thin extracellular deposition of fibronectin was observed at the epithelial–matrix interface, but also within the epithelial layers (Fig. 1 and Table 1). Inclusion of human fibroblasts in the matrix resulted in increased fibronectin expression at the epithelial–matrix interface of these cultures, and pronounced diffuse fibronectin immunoreactivity in the connective tissue matrix and within the fibroblasts (Fig. 1 and Table 1). Interestingly, fibronectin immunoreactivity could not be detected when PE/CA-PJ 15 neoplastic cells were grown on top of matrices in absence of fibroblasts, but when the collagen matrices were supplemented with NOF, there was a strong discontinuous extracellular deposition of fibronectin within the epithelial compartment, at the epithelial–matrix interface, within the matrix fibroblasts and diffusely in the matrix (Fig. 1 and Table 1). Compared to NOK models with matrices supplemented with fibroblasts, the neoplastic models showed a gradually enhanced immunoreactivity in the matrix (Table 1). In control normal human oral tissues fibronectin was present along the epithelial basement membrane, in the basement membrane of blood vessels and within fibroblasts.

4. Discussion

In this study we report (1) an enhanced expression of laminin-332, type IV collagen and fibronectin that accompanies human oral cancer progression *in vitro*. This might seem contradictory with the classical view and earlier *in vivo* findings that in oral dysplasia and oral squamous cell carcinoma multifocal breaks of BM that correlate to malignancy grade in their extent are present. However, more recent studies show that in OSCCs the BM breaks co-exist with new synthesis and deposition of BM proteins within the cytoplasm of budding carcinoma cells and in the adjacent stroma of the invasion front. In addition, we have also shown (2) an important role for epithelial–mesenchymal interactions in the expression and deposition of collagen IV and fibronectin, and (3) a minor role for epithelial–mesenchymal interactions for laminin-332 expression. To the best of our knowledge, such a correlation between expression of major basement membrane proteins and tumour progression in an *in vitro* step-wise model of human oral mucosa has not been previously reported, although a role for epithelial–mesenchymal interactions in deposition and ultrastructural organisation of basement membrane proteins like type IV, type VII collagens and laminin, has been shown in three-dimensional cultures of normal skin[25–27] and oral mucosa.[18,24]

In our tumour progression model the degree of oral keratinocyte transformation was associated with a stronger expression and deposition of laminin-332, in line with previous findings on archival human oral tissues that showed high expression of laminin-332 in basal epithelial cells of oral dysplasia and OSCC[17,28] and with previous *in vitro* observations that PE/CA-PJ 15 cells expressed laminin-332 mainly within the cells at the invasive front.[17] The last study had also shown that the presence of tumour-derived fibroblasts was crucial in organising a fibrillary deposition of laminin-332 in the stroma adjacent to the invading cells. In our study, as well as in another very recent study,[29] normal human fibroblasts had a less modulatory effect on laminin-332 expression by transformed keratinocytes, suggesting that the origin of fibroblast (tumour versus normal stroma) may be of importance for accurate *in vitro* restoration of the *in vivo* epithelial–mesenchymal interactions. This hypothesis needs however to be further tested on matched pairs of tumour-derived fibroblasts and normal fibroblasts from the same patient, and we are now in the process of running of a such a comparative study.

Immunohistochemical localisation of type IV collagen at the basement membrane area of normal, dysplastic and oral cancer tissues has been reported previously in human archival tissues,[6,8] as well as in animal models of oral squamous cell carcinoma progression.[30] However, these studies documented a decreased expression or a focal loss of type IV collagen at the basement membrane area, in relation to tumour progression. In contrast to those studies we could detect a continuous and pronounced type IV collagen deposition at the invasive front of both early neoplastic and established neoplastic oral mucosa models. In addition, our results show, as we also previously suggested[12,18,24] a strong dependency on epithelial–mesenchymal interactions for type IV collagen synthesis and extracellular deposition, in both normal and neoplastic models of oral mucosa. This supports the previous suggestion that collagen IV in normal epithelial basement membrane is mainly derived from fibroblasts from the connective stromal tissue.[31] Nevertheless, our finding that PE/CA-PJ 15 neoplastic cells were able to synthesise and deposit collagen IV in the absence of fibroblasts indicates that an epithelial origin for type IV collagen cannot be excluded, although it might be a feature acquired through malignant progression.[12]

A major observation of this study is that normal keratinocytes were able to synthesise and deposit fibronectin extracellularly at the epithelial–matrix interface area even in the absence of fibroblasts in the matrix. This indicates that normal oral keratinocytes are able to synthesise fibronectin, although in this particular study it was detected in specific culture conditions. This point of view is supported by previous findings that found fibronectin to be expressed in new born rat skin keratinocytes in monolayer culture.[32,33] Therefore, although the present findings are based on an *in vitro* culture model, they raise the question on fibroblasts as the major source of fibronectin, and indicate that the role of keratinocytes in synthesising fibronectin cannot be ruled out. However, the presence of fibroblasts, especially in the neoplastic models resulted in a significant increase in the fibronectin immunoreactivity of the connective tissue matrix of these co-cultures (Fig. 1). Previous studies reporting strong stromal fibronectin immunoreactivity adjacent to the tumour invasive front suggested that in highly invasive oral squamous cell carcinoma deposition of fibronectin could be an invasion associated phenomenon.[10] In our *in vitro* oral cancer progression model not only cultures with fully transformed malignant phenotype but also those with partially transformed phenotype of keratinocytes (DOK cell line), overexpressed fibronectin in the extracellular matrix. This upregulation of fibronectin expression in the extracellular matrix may be an indication of an active desmoplastic tumour stroma.[19] Our observation that matrix fibronectin overexpression is associated with the degree of keratinocyte transformation in the epithelium points to the fact that while fibroblasts play a role in stimulating keratinocytes to grow invasively into the matrix, the transformed keratinocytes modulate the underlying fibroblasts to deposit more fibronectin in the stroma and thereby create a proinvasive stromal microenvironment. However, future studies should investigate the effects of fibroblast activation by transformed keratinocytes on basement membrane protein expression in general, and specifically on synthesis and extracellular deposition of fibronectin and its alternative spliced variants that would throw more light into the understanding of specific mechanisms as to how epithelial–mesenchymal interactions influence the progression of oral squamous cell carcinoma. Nevertheless, the present findings, obtained in a 3D *in vitro* model that closely mirrors the *in vivo* human oral cancer progression, show an enhanced basement membrane protein expression during human oral cancer progression that is dependent on the epithelial–mesenchymal environment, respectively the existence of fibroblasts.

Acknowledgements

We thank the staff at Institute of Clinical Dentistry, University of Bergen for their assistance in getting tissue samples. The

technical support from Gunnvor Øijordsbakken (immunohistochemistry) is highly appreciated. This study was supported by The Norwegian Research Council (ACJ and DEC grant no 178601), NORAD Programme (KK) and Quota Programme (OML).

Funding: Norwegian Research Council (A.C. Johannessen and D.E. Costea grant no. 178601), NORAD Programme of the Norwegian Government (K. Kulasekara), and Quota Programme of the Norwegian Government (O.M. Lukandu).

Competing interests: None declared.

Ethical approval: Ethical approval was given by the Research Ethical Committee of Western Norway (ref. no. REKVest 177.04).

REFERENCES

1. Merker HJ. Morphology of the basement membrane. *Microsc Res Tech* 1994;**28**(2):95–124.
2. Smola H, Stark HJ, Thiekotter G, Mirancea N, Krieg T, Fusenig NE. Dynamics of basement membrane formation by keratinocyte–fibroblast interactions in organotypic skin culture. *Exp Cell Res* 1998;**239**(2):399–410.
3. Kim SW, Park KC, Kim HJ, Cho KH, Chung JH, Kim KH, et al. Effects of collagen IV and laminin on the reconstruction of human oral mucosa. *J Biomed Mater Res* 2001;**58**(1):108–12.
4. Segal N, Andriani F, Pfeiffer L, Kamath P, Lin N, Satyamurthy K, et al. The basement membrane microenvironment directs the normalization and survival of bioengineered human skin equivalents. *Matrix Biol* 2008;**27**(3):163–70.
5. Andriani F, Margulis A, Lin N, Griffey S, Garlick JA. Analysis of microenvironmental factors contributing to basement membrane assembly and normalized epidermal phenotype. *J Invest Dermatol* 2003;**120**(6):923–31.
6. Giannelli G, Milillo L, Marinosci F, Lo Muzio L, Serpico R, Antonaci S. Altered expression of integrins and basement membrane proteins in malignant and pre-malignant lesions of oral mucosa. *J Biol Regul Homeost Agents* 2001;**15**(4):375–80.
7. Tomakidi P, Mirancea N, Fusenig NE, Herold-Mende C, Bosch FX, Breitkreutz D. Defects of basement membrane and hemidesmosome structure correlate with malignant phenotype and stromal interactions in HaCaT-Ras xenografts. *Differentiation* 1999;**64**(5):263–75.
8. Kannan S, Balaram P, Chandran GJ, Pillai MR, Mathew B, Nalinakumari KR, et al. Alterations in expression of basement membrane proteins during tumour progression in oral mucosa. *Histopathology* 1994;**24**(6):531–7.
9. Tosios KI, Kapranos N, Papanicolaou SI. Loss of basement membrane components laminin and type IV collagen parallels the progression of oral epithelial neoplasia. *Histopathology* 1998;**33**(3):261–8.
10. Harada T, Shinohara M, Nakamura S, Oka M. An immunohistochemical study of the extracellular matrix in oral squamous cell carcinoma and its association with invasive and metastatic potential. *Virchows Arch* 1994;**424**(3):257–66.
11. Lohi J. Laminin-5 in the progression of carcinomas. *Int J Cancer* 2001;**94**(6):763–7.
12. Costea DE, Kulasekara K, Neppelberg E, Johannessen AC, Vintermyr OK. Species-specific fibroblasts required for triggering invasiveness of partially transformed oral keratinocytes. *Am J Pathol* 2006;**168**(6):1889–97.
13. Pyke C, Salo S, Ralfkiaer E, Romer J, Dano K, Tryggvason K. Laminin-5 is a marker of invading cancer cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas. *Cancer Res* 1995;**55**(18):4132–9.
14. Koshikawa N, Moriyama K, Takamura H, Mizushima H, Nagashima Y, Yanoma S, et al. Overexpression of laminin gamma2 chain monomer in invading gastric carcinoma cells. *Cancer Res* 1999;**59**(21):5596–601.
15. Patel V, Aldridge K, Ensley JF, Odell E, Boyd A, Jones J, et al. Laminin-gamma2 overexpression in head-and-neck squamous cell carcinoma. *Int J Cancer* 2002;**99**(4):583–8.
16. Berndt A, Hyckel P, Konneker A, Katenkamp D, Kosmehl H. Oral squamous cell carcinoma invasion is associated with a laminin-5 matrix re-organization but independent of basement membrane and hemidesmosome formation. clues from an in vitro invasion model. *Invasion Metastasis* 1997;**17**(5):251–8.
17. Berndt A, Borsi L, Hyckel P, Kosmehl H. Fibrillary co-deposition of laminin-5 and large unspliced tenascin-C in the invasive front of oral squamous cell carcinoma in vivo and in vitro. *J Cancer Res Clin Oncol* 2001;**127**(5):286–92.
18. Costea DE, Johannessen AC, Vintermyr OK. Fibroblast control on epithelial differentiation is gradually lost during in vitro tumor progression. *Differentiation* 2005;**73**(4):134–41.
19. Kosmehl H, Berndt A, Strassburger S, Borsi L, Rousselle P, Mandel U, et al. Distribution of laminin and fibronectin isoforms in oral mucosa and oral squamous cell carcinoma. *Br J Cancer* 1999;**81**(6):1071–9.
20. Liu H, Chen B, Zardi L, Ramos DM. Soluble fibronectin promotes migration of oral squamous-cell carcinoma cells. *Int J Cancer* 1998;**78**(2):261–7.
21. Costea DE, Dimba AO, Loro LL, Vintermyr OK, Johannessen AC. The phenotype of in vitro reconstituted normal human oral epithelium is essentially determined by culture medium. *J Oral Pathol Med* 2005;**34**(4):247–52.
22. Chang SE, Foster S, Betts D, Marnock WE. DOK, a cell line established from human dysplastic oral mucosa, shows a partially transformed non-malignant phenotype. *Int J Cancer* 1992;**52**(6):896–902.
23. Burns JE, Clark LJ, Yeudall WA, Mitchell R, Mackenzie K, Chang SE, et al. The p53 status of cultured human premalignant oral keratinocytes. *Br J Cancer* 1994;**70**(4):591–5.
24. Costea DE, Loro LL, Dimba EA, Vintermyr OK, Johannessen AC. Crucial effects of fibroblasts and keratinocyte growth factor on morphogenesis of reconstituted human oral epithelium. *J Invest Dermatol* 2003;**121**(6):1479–86.
25. Lee DY, Cho KH. The effects of epidermal keratinocytes and dermal fibroblasts on the formation of cutaneous basement membrane in three-dimensional culture systems. *Arch Dermatol Res* 2005;**296**(7):296–302.
26. El Ghalbzouri A, Jonkman MF, Dijkman R, Ponec M. Basement membrane reconstruction in human skin equivalents is regulated by fibroblasts and/or exogenously activated keratinocytes. *J Invest Dermatol* 2005;**124**(1):79–86.
27. El Ghalbzouri A, Ponec M. Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components. *Wound Repair Regen* 2004;**12**(3):359–67.
28. Kainulainen T, Autio-Harmainen H, Oikarinen A, Salo S, Tryggvason K, Salo T. Altered distribution and synthesis of laminin-5 (kalinin) in oral lichen planus, epithelial dysplasias and squamous cell carcinomas. *Br J Dermatol* 1997;**136**(3):331–6.
29. Nielsen JD, Moeslund M, Wandall HH, Dabelsteen S. Influences of tumor stroma on the malignant phenotype. *J Oral Pathol Med* 2008;**37**(7):412–6.
30. Kobayashi Y, Nakajima T, Saku T. Loss of basement membranes in the invading front of O-1N, hamster squamous cell carcinoma with high potential of lymph node metastasis: an immunohistochemical study for laminin and type IV collagen. *Pathol Int* 1995;**45**(5):327–34.

31. Fleischmajer R, Perlish JS, MacDonald 2nd ED, Schechter A, Murdoch AD, Iozzo RV, et al. There is binding of collagen IV to beta 1 integrin during early skin basement membrane assembly. *Ann N Y Acad Sci* 1998;**857**:212–27.
32. Gibson WT, Couchman JR, Badley RA, Saunders HJ, Smith CG. Fibronectin in cultured rat keratinocytes: distribution, synthesis, and relationship to cytoskeletal proteins. *Eur J Cell Biol* 1983;**30**(2):205–13.
33. Couchman JR, Gibson WT, Thom D, Weaver AC, Rees DA, Parish WE. Fibronectin distribution in epithelial and associated tissues of the rat. *Arch Dermatol Res* 1979;**266**(3):295–310.