Expression of Fyn Kinase Modulates EMT in Oral Cancer Cells

BEN LEWIN*, AMANDA SIU*, CAMERON BAKER, DONGMIN DANG, REBECCA SCHNITT, PEDRAM EISAPOORAN and DANIEL M. RAMOS

Department of Orofacial Sciences, University of California at San Francisco, San Francisco, CA 94143-0422, U.S.A.

Abstract. *Oral squamous cell carcinoma (SCC) is an aggressive tumor with a poor 5-year survival rate. Oral SCC can undergo epithelial to mesenchymal transition (EMT). We previously showed that the epithelial integrin αvβ6 complexes with Fyn kinase in oral SCC to promote EMT. Using immunofluorescence microscopy and Western blotting, we evaluated whether the expression of specific markers of EMT were influenced by modulating serum concentration (ie. growth factors). The SCC cultures were grown under contrasting levels of serum. In low serum (1%), Fyn promoted EMT; whereas suppression of Fyn kinase promoted the epithelial phenotype. However, when the SCC cells were grown in 10% serum, activation of Fyn had the reverse effect. Lastly, cell migration was evaluated under low serum conditions (1% FBS). Activation of Fyn promoted SCC cell migration and its suppression thwarted SCC migration toward FN. These results indicate that the activation of Fyn kinase as well as local growth factor concentration modulate EMT in oral SCC.*

Squamous cell carcinoma (SCC) represents approximately 96% of all oral cancers. Despite advances in surgery, radiotherapy and chemotherapy, prognosis for this disease has not improved in over 60 years: 50% of oral cancer patients do not survive 5 years post diagnosis (1). Factors including epithelial to mesenchymal transition (EMT) may contribute to the poor prognosis associated with oral SCC. β6 Integrin becomes expressed during the mesenchymal transition and overexpression of this integrin confers the motile phenotype on SCC cells (2, 3). It was recently shown that the expression of β6 promotes the mesenchymal pathway; whereas truncation of its cytoplasmic domain promotes the epithelial phenotype (4). A highly characterized marker of the epithelial phenotype is E-cadherin. The localization of E-cadherin on the surfaces of epithelial cells in intercellular contact regions is known as adherens junctions (4). The suppression of E-cadherin expression is regarded as one of the main molecular events responsible for the progression of many cancers.

During the progression from dysplasia to invasive oral cancer, αvβ6 is neoexpressed and enhances migration and invasion both *in vitro* and *in vivo* (5, 6). β6 Integrin has a unique 11 amino acid cytoplasmic extension not seen on other integrin β subunits. It was previously documented that the ligand binding of the β6 integrin results in the activation of Fyn kinase in several different oral cancer cell lines (5). Activation of Fyn is required for full activation of focal adhesion kinase (FAK) and for MAPK phosphorylation (5).

αvβ6 Activates TGFβ1 by binding to its latency activated peptide (LAP/TGFβ1 complex) (7). The coexpression of αvβ6 and TGFβ1 has been seen in a variety of carcinomas and correlates with the overall poor prognosis (8). The role of TGFβ1 in EMT is well established. The process of EMT can be modulated *via* the *C*-terminal amino acids of the β6 subunit (4).

In a previous study it was shown that β6 increases proliferation and the invasive ability of SCC cells (5, 6). It was also illustrated that expression of kinase dead (KD)-Fyn in SCC9β6 cells reduced primary tumor growth and inhibited experimental lung metastases. Therefore, the present study aimed to examine whether changes to Fyn kinase modify EMT.

Materials and Methods

Cell culture. The SCC9 cell line (originally derived from a tongue lesion) was obtained from Dr James Reinwald (Brigham and Woman's Hospital, Harvard School of Medicine) and has been described elsewhere (5). The SCC9β6 cell line was generated in the

*Both authors contributed equally to this work.

Correspondence to: Daniel M. Ramos, DDS, Ph.D., Department of Orofacial Sciences, University of California, 513 Parnassus Ave, S612, Box 0422, San Francisco, CA 94143-0422, U.S.A. Tel: +1 4155024905, Fax: +1 8007836653, e-mail: Daniel.ramos@ucsf.edu

Key Words: Integrin, αvβ6, Fyn kinase, EMT, oral SCC, serum.

host laboratory (9, 10). The full length β6 cDNA was a gift of Dr Dean Sheppard (University of California at San Francisco, UCSF). The cDNAs of both the KD-Fyn and the constitutively active Fyn (CA-Fyn) were provided by Dr H Kawakatsu (UCSF) and were stably expressed in SCC9β6 and the SCC9 cell lines, respectively using the Retro-X system (Clontech, Mountain View, CA, USA) (5, 6). An empty vector was expressed in the SCC9 and the SCC9β6 cells to establish SCC9SN and SCC9β6SN cell lines (5). Cells were routinely cultivated in Dulbecco's modified Eagle's medium (DMEM) using 10% fetal bovine serum.

Immunofluorescence microscopy. 2×10^5 cells/ml were seeded on fibronectin (FN)-coated glass coverslips (5 μg/ml) for 24 hours, serum-free and fixed with 3% paraformaldehyde and then permeabilized using 0.1% Triton X-100. The cells were incubated with antibody for 1 hour and rinsed with PBS, then incubated with biotin-conjugated goat anti-mouse IgG (1:50) for 30 min followed by fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:100) (Amersham, Piscataway, NJ, USA) for 30 min at, rinsed and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Reagents. Rabbit polyclonal antibodies to cytokeratin and anti-E-cadherin antibodies (clone She-78-7) were from Zymed Laboratories (South San Francisco, CA, USA). Mouse monoclonal antibodies to N-cadherin antibody (clone GC-4 catalogue C3865), vimentin (clone V9, catalogue V6630) and β-actin (AC-72) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting. Cells were serum-starved for 24 hours, detached from tissue culture plates and reseeded onto FN (10 μg/ml) for 24 hours. The cells were then lysed in Nonidet P-40 lysis buffer (1.5% Nonidet P-40, 150 mM NaCl, 0.2% SDS, 1 mM EDTA, 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 50 mM NaF) and protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Micron Separation Inc; Westborough, MA, USA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA) (11). The membranes were then developed using the ECL chemiluminescence kit (Amersham) and bands detected by X-ray film. The blots were assigned relative value units (rvu) using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Serum experiments. To investigate the possible role of serum as a modifier of EMT, the cells were grown overnight at 37˚C either in DME plus 1% or 10% serum. After 24 hours, the cells were washed with PBS and lysed in 200 mM octylglucoside, 50 mM Tris, 1 mM Mn^{2+}, 5 mM EDTA and protease inhibitors. The cell lysate was spun down at 10.000×*g* for 10 min at 4˚C and protein concentration was determined. The proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting for E-cadherin, N-cadherin, and vimentin.

Migration assay. The lower surface of an 8 μm pore-size Transwell® filter was coated with the 5 μg/ml of FN for 1 hour at 37˚C then 2×10^5 tumor cells were plated onto the upper surface. The lower compartment contained 300 μl of fibroblast-conditioned medium as a source of chemoattractants. The cultures were placed at 37˚C overnight in the presence of 5% CO_2. The filters were fixed, and the upper surface wiped clean and stained with crystal violet. The filters were then air dried and evaluated visually and the number of cells crossing the membrane were counted. Five random fields/filter were evaluated.

Results

Activation of Fyn regulates keratin expression. All four cell lines (SCC9SN, SCC9β6, SCC9CAFyn and SCC9β6KDFyn) were plated on FN (5 μg/ml) and evaluated for expression of the epithelial marker, keratin, using immunofluorescence microscopy. The poorly invasive SCC9SN cells expressed a dense network of keratin fibers which obscured the entire cytoplasm giving the SCC9SN cells a sheet-like appearance (Figure 1A). In contrast, the SCC9CAFyn and the SCC9β6 cells appeared most often as single cells and did not react with keratin antibodies (Figure 1B and 1C, respectively). The SCC9β6kdfyn cell line (Figure 1D) was more cobblestone-like in appearance and reacted highly with anti-keratin antibodies. This demonstrates the interdependence between Fyn kinase and keratin expression in oral SCC cells.

Cell–cell contacts and Fyn activation. A tumor suppressor function has been assigned to E-cadherin (11). The loss of E-cadherin-mediated cell adhesion correlates with the loss of the epithelial morphology and with the acquisition of metastatic potential by the carcinoma cells. The oral cancer cells SCC9SN, SCC9CAFyn, SCC9β6 and SCC9β6KDfyn were next placed on FN (5 μg/ml) for 8 hours and evaluated for expression of the epithelial-specific marker E-cadherin. The epithelial-like, SCC9SN cells were positive for E-cadherin on their entire membrane surface and gave the cells a uniform, honeycomb, sheet-like appearance (Figure 2). These cells had E-cadherin positive cell–cell contacts (Figure 2). The effect of constitutive activation of Fyn kinase was evaluated next for the localization of E-cadherin. The localization of E-cadherin in the SCC9CAfyn cells was significantly diminished. Although the majority of the cells lacked any expression of E-cadherin, a small central cluster contained a small compact accumulation of E-cadherin expression cells. (Figure 2B). When the SCC9β6 cells were grown on FN for 8 hours, they did not form cell–cell contacts and were negative for E-cadherin (Figure 2C). However, the epithelial phenotype was recovered in SCC9β6KDfyn cell line which was positive for E-cadherin. When the SCC9β6 cell line was grown on FN for 8 hours, the cells did not form cell–cell contacts and were negative for E-cadherin staining (Figure 2C). However, recovery of the epithelial phenotype was detected when the SCC9β6KDfyn cells were found to be positive for E-cadherin (Figure 2D). These results indicate that one response of the cells to Fyn kinase activity is differential expression of E-cadherin.

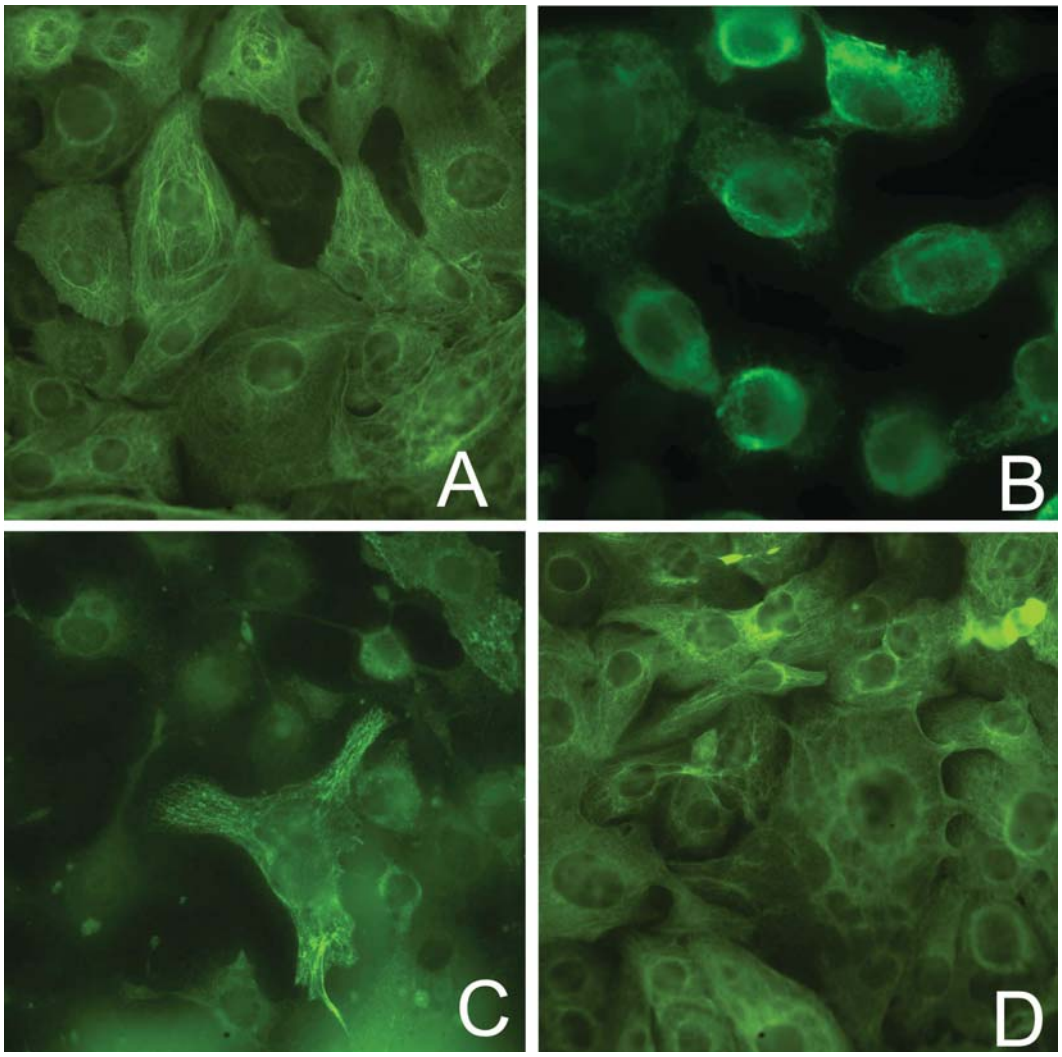

Figure 1. *Expression of keratin intermediate filaments is modulated by Fyn kinase. The SCC9SN (A), SCC9CAFyn (B), SCC9β6 (C) and SCC9β6KDFyn (D) cells were placed on FN substrates for 6 hours, fixed, stained and processed for immunofluorescence microscopy and stained using monoclonal antibodies to cytokeratin. Note the flattened out cells with a robust distribution of cytokeratin filaments when the SCC9SN (A) and SCC9β6KDFyn (D) cells were examined. Note the cohesive appearance of the cells in A and D, and the lack of intermediate filaments in the SCC9CAFyn (B) and SCC9β6 (C) cell lines.*

Serum alters expression of EMT markers. Serum provides a wealth of growth factors and is used as a nutrient source for routine cell culture. To examine whether serum influences the expression of proteins associated with EMT, the cells were grown either in high or low serum conditions (10% or 1%, respectively).

1% Serum: Cells were grown in the presence of 1% serum for 24 hours and then evaluated by Western blot. SCC9SN and the SCC9KDFyn cell lines had high level E-cadherin whereas little expression was seen in the SCC9β6 and SCC9CAFyn cell lines (Figure 3). N-Cadherin was highly expressed by the SCC9β6 and SCC9CA cell lines (Figure 3), and was barely detectable in the lysate from the SCC9SN or SCC9β6kdfyn cells (Figure 3). All 4 cell lines expressed vimentin, although the SCC9β6 and the SCC9CAfyn cell lines expressed more than either the SCC9SN or SCC9β6KDFyn cell lines (Figure 3).

10% serum: The cells were grown in the presence of 10% serum for 24 hours and then evaluated by Western blot. The SCC9β6 and the SCC9CAFyn (Figure 3B) cells had significant expression of E-cadherin; in direct contrast to when the cell were grown in low serum. E-Cadherin could not be detected in the SCC9SN or the SCC9β6KDFyn cells (Figure 3B). The addition of high serum also reversed the expression of N-cadherin. N-Cadherin was highly expressed in the SCC9SN and the SCC9β6KdFyn cell lines, while low

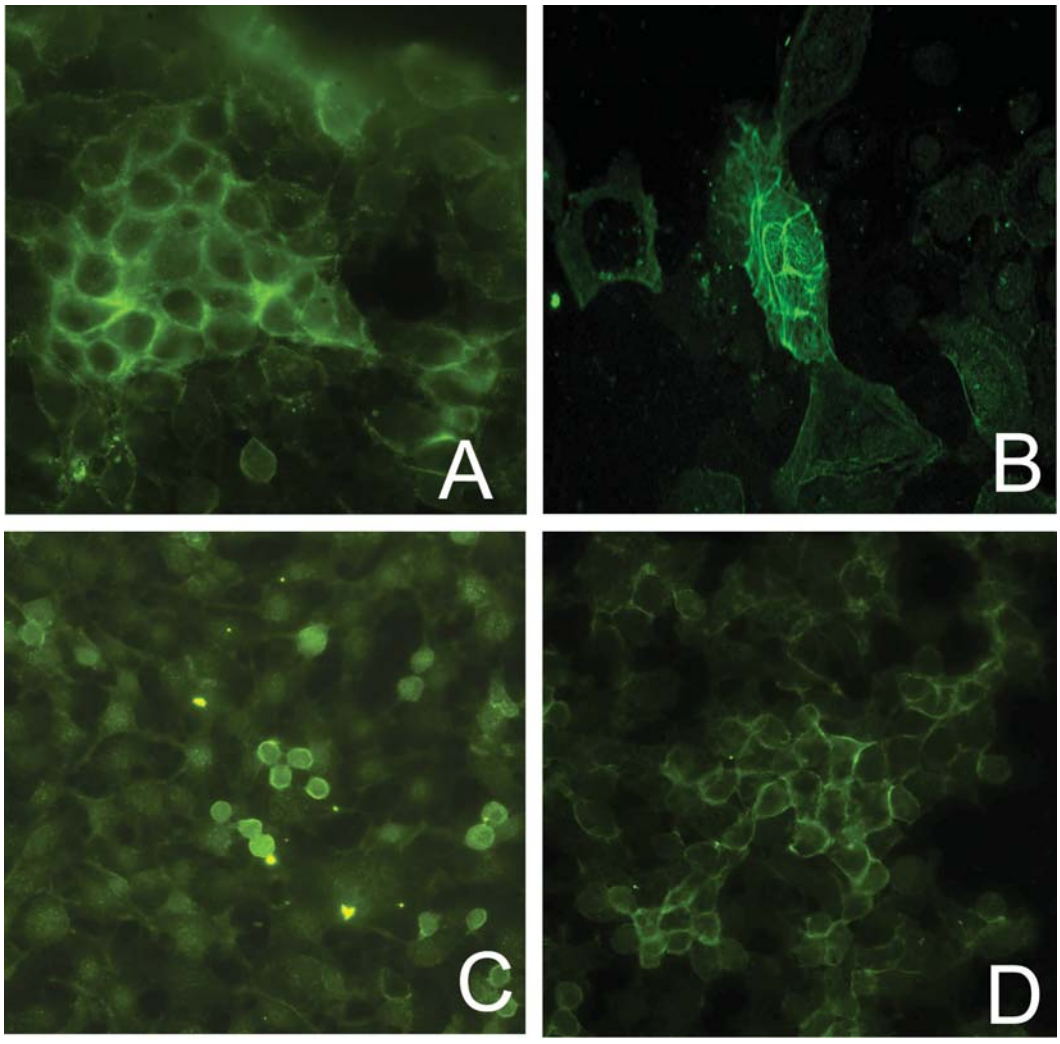

Figure 2. *Expression and localization of E-cadherin is linked to Fyn kinase activation. The SCC9SN (A), SCC9CAfyn (B), SCC9β6 (C) and SCC9β6KDfyn (D) cells were placed on FN for 6 hours and then fixed, stained and processed for immunofluorescence microscopy using antibodies to E-cadherin. Note the robust staining in the SCC9SN (A) and SCC9β6KDfyn (D) cell lines. The SCC9CAFyn cells (B) had low level reactivity with anti-E-cadherin antibodies. Note that in B, the cells were clustered in the center which reacts with anti-E-cadherin antibodies. The SCC9β6 cells (C) were completely void of E-cadherin.*

levels of N-cadherin were detected in the SCC9β6 and the SCC9CAFyn cell lines (Figure 3B). High serum also reversed the expression of vimentin shown by its absence in the SCC9β6 and SCC9CAFyn cell lines. The effect of Fyn kinase was reversed by the presence of 10% FBS.

Densitometric quantification of Western blots. Densitometric analysis using the public domain ImageJ software (developed at the US National Institutes of Health and available on the Internet at http://rsbweb.nih.gov/ij/index.html) was performed to quantify the Western blots. The most notable changes due to increasing serum concentration to 10% was the 10-fold decrease in E-cadherin by the SCC9SN cell line countered by the 10-fold increase seen in the SCC9β6 cell line (Figure 3C). Elevated serum also resulted in an 80% decrease in E-cadherin expression by the SCC9β6KD cells with an equivalent increase in expression in the SCC9CAFyn cells (Figure 3C). The expression of N-cadherin was elevated 5-fold in both the SCC9SN and SCC9β6KDFyn cells lines and was decreased a similar amount in the SCC9β6 and SCC9CAFyn (Figure 3D). Lastly, vimentin expression was abolished in the SCC9β6 and SCC9CAFyn by growth in 10% serum (Figure 3E). Essentially every marker of EMT was reversed by modulating serum concentration. These results indicate that EMT is regulated by serum factors.

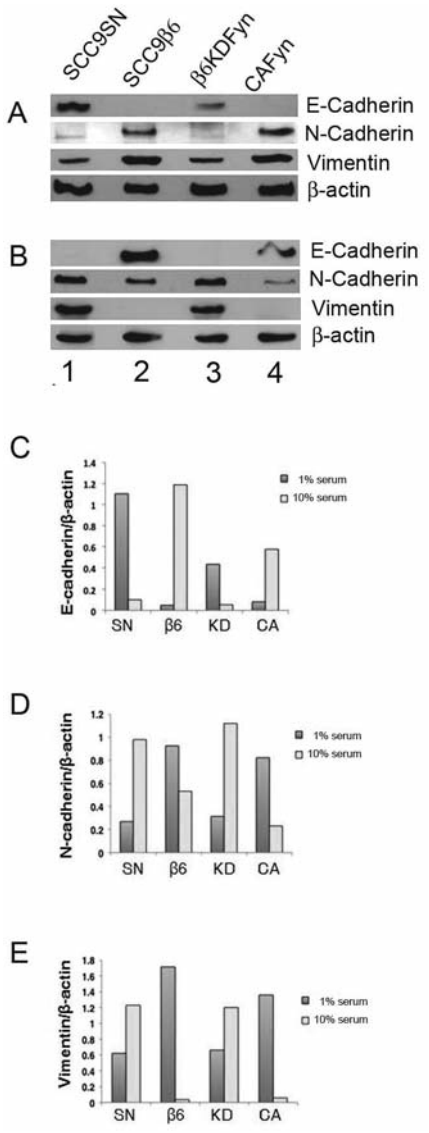

Figure 3. *Modulation of EMT by serum factors. The SCC9SN (lane 1); SCC9β6 (lane 2); SCC9β6KDFyn (lane 3) and SCC9CAFyn (lane 4) cell lines were grown at 37°C for 24 hours in either 1% FBS (A) or 10% FBS (B). The cells were then lysed and proteins separated by SDS-PAGE. The separated proteins were analyzed by Western blot for expression of E-cadherin, N-cadherin, and vimentin. B-Actin was used as a loading control. The bands were scanned using NIH Image and assigned relative value units (rvu).*

Activation of Fyn kinase mediates oral SCC motility. Transwell filer assays were performed to examine SCC migration on FN-coated substrates. SCC9β6 and SCC9β6KDFyn cells were evaluated for their ability to migrate onto FN-coated filters (Figure 4). SCC9SN cells were used as a negative control. Oral SCC cells (2×10^5) were then added to the upper surface of the membrane and placed

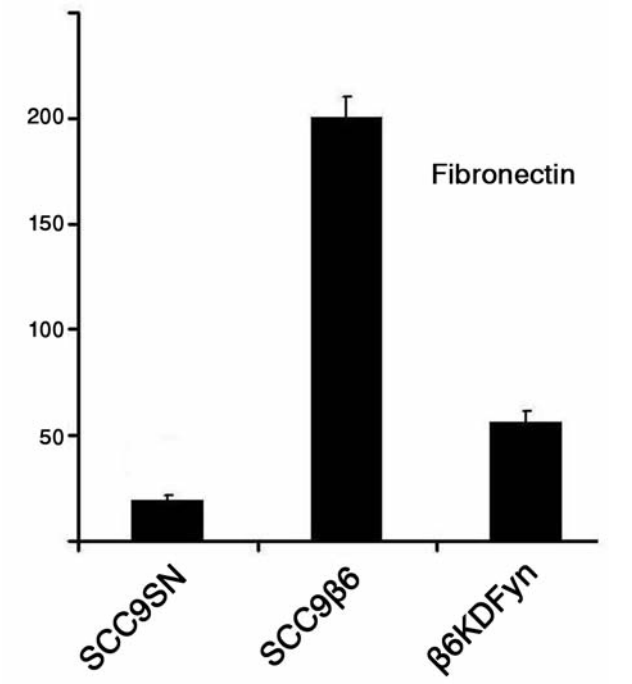

Figure 4. *Fyn activation modulates oral cancer migration. SCC9SN, SCC9β6 and SCC9β6KDFyn cells (2×10^5) were seeded onto the upper surface of an 8-μm pore size Transwell filter in which the under surface was previously coated with 5 μg/ml of FN. The lower compartment contained 300 μl of fibroblast conditioned medium to serve as a chemoattractant. The cells were allowed to migrate for 8 hours. The upper surface was wiped clean and the filters were fixed and stained using crystal violet. The number of cells was counted manually using 5 random fields per filter. The experiment was performed in triplicate and 5 random fields were counted visually per filter.*

at 37˚C with 5% CO_2 for 8 hours. Suppression of Fyn kinase reduced oral SCC migration by 75% (Figure 4). Expression of a kinase dead Fyn significantly impeded oral SCC migration on FN-coated filters (Figure 4). These results were the average of 3 independent experiments, and in each experiment 5 random fields were counted per membrane.

Discussion

The results of this study indicate that the coupling of β6-activated Fyn signaling pathway is required for progression through EMT. Overexpression of the β6 integrin has been reported to be up-regulated in numerous types of carcinomas and is associated with poor prognosis (9). αvβ6 Integrin has been identified in colon, lung and cervical carcinomas and its expression correlates with poor patient survival (8, 9). β6 Integrin confers a poorly differentiated, invasive phenotype on well-differentiated oral SCC cells (5, 6). Fyn activation complexes with and activates FAK and signals through the Raf-ERK/MAPK pathway which promotes EMT (5).

This study investigated whether Fyn kinase can regulate EMT. Lehembre *et al*. (10) showed a direct link between the downregulation of E-cadherin with the subsequent increase in N-cadherin (10). The authors found a direct link between N-cadherin and activation of Fyn kinase (p59) and the onset of EMT (10). Therefore, the present study investigated whether manipulating Fyn kinase modulated EMT.

First, the cells were analyzed for expression of the standard epithelial marker keratin. Both the cell lines with little Fyn kinase activity expressed major levels of E-cadherin and keratin, while simultaneously expressing extremely low levels of vimentin and N-cadherin. In low serum conditions, the SCC9SN and the SCC9β6KDFyn cell lines have the typical profile of epithelial cells. For example they form cohesive 'cobblestone' sheet-like units compared to the more spindle-shaped SCC9β6 and SCC9CAFyn, suggesting that Fyn kinase drives EMT. The SCC9β6 and the SCC9CAFyn cell lines did not express significant levels of E-cadherin or keratin. In addition, the cells were also poorly cohesive and appeared mostly as single cells. These results suggest that the activation of Fyn induces EMT by promoting the mesenchymal-cell type.

While the activation of Fyn induced EMT, manipulation of cell phenotype by cell culture in high serum conditions produced an interesting new profile. Interestingly, when these molecules were evaluated in oral cancer cells grown under high serum conditions a different profile emerged. The highly invasive SCC9β6 and SCC9CAFyn cells acquired a more epithelial phenotype as determined by increased E-cadherin and keratin levels with coordinate decreases in vimentin and N-caderin. The poorly invasive SCC9SN and SCC9β6Kdfyn cells acquired a mesenchymal pattern of protein expression. These results indicate that a good portion of the EMT phenotype is regulated by Fyn kinase activation and that the progression through EMT is also regulated partly through serum factors.

Integrin β6 is important for cell motility, an important aspect of cancer invasion. β6 Integrin is important both for cell motility and for signaling, especially through its unique cytoplasmic tail (4). The present study determined that Fyn kinase activation through β6 ligand binding modulates oral SCC migration on FN substrates. Migration of highly invasive, β6 positive oral SCC cells was completely suppressed by inhibiting activation of Fyn kinase. When the same experiment was performed using a high serum content, the SCC9β6 cells were consistently less motile when compared to migration under low serum conditions (data not shown).

Understanding the role of integrin signaling in the process of differentiation and tumor cell invasion is critical to gain a full understanding of the invasive process. The results of this study indicate that the process of EMT or dedifferentiation may be in part a result of Fyn kinase function. The current study evaluated the process of differentiation with respect to expression of the β6 integrin and its role in activating Fyn kinase.

Acknowledgements

This manuscript is dedicated to Ann Sandberg. This work was supported by an Individual Investigator Award from UCSF Academic Senate to DMR and by additional funds supplied by the Department of Orofacial Sciences. Additional support was funded by the Summer Student Research Fellowship, UCSF to RS.

References

1 Silverman SJ: Oral Cancer. Ontario: B C Decker, 1998.
2 Ramos DM, Chen BL, Boylen KB, Stern M, Kramer RH, Sheppard D, Nishimura SL, Greenspan DG, Zardi L and Pytela R: Stromal fibroblasts influence oral squamous cell carcinoma cell's interactions with tenascin-C. Int J of Cancer *72*: 369-372, 1997.
3 Ramos DM, Chen B L, Regezi J, Zardi L and Pytela R: Tenascin-C matrix assembly in oral squamous cell carcinoma. Int J of Cancer *75*: 680-687, 1998.
4 Ramos DM, Dang D and Sadler S: The role of the integrin alpha v beta 6 in regulating epithelial to mesenchymal transition in oral cancer. Anticancer Res *29*: 3-8, 2009.
5 Li X, Yang Y, Hu Y, Dang D, Regezi J, Schmidt BL, Atakilit A, Chen B, Ellis D, and Ramos DM: Alpha v beta 6-Fyn signaling promotes oral cancer progression. J Biol Chem *278*: 41646-41653, 2003.
6 Ramos DM, Wu T, Schmidt B, But M, Regezi J, Jordon R, Atakilit A and Li X: Expression of the integrin αvβ6 enhances invasive behavior in oral squamous cell carcinoma. Matrix Biology *21*: 297-307, 2002.
7 Sheppard D: Integrin-mediated activation of latent transforming growth factor beta. Cancer Metastasis Rev *3*: 395-402, 2005.
8 Hazelbag S, Kenter GG, Gorter A, Dreef EJ, Koopman LA, Violette SM, Weinreb PH and Fleuren GJ: Overexpression of the alpha v beta 6 integrin in cervical squamous cell carcinoma is a prognostic factor for decreased survival. J Pathol *3*: 316-324, 2007.
9 Bates RC: Colorectal cancer progression: integrin alpha v beta 6 and the epithelial–mesenchymal transition (EMT). Cell Cycle *4*: 1350-1352, 2005.
10 Lehembre F, Yilmaz M, Wicki A, Schomber T, Strittmatter K, Ziegler D, Kren A, Went P, Derksen PW, Berns A, Jonkers J and Christofori G: NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. EMBO J *27*: 2603-2615, 2008.
11 Pećina-Slaus N: Tumor suppressor gene E-cadherin and its role in normal and malignant cells. Cancer Cell Int. Oct *14;3(1)*: 1-7; 2003.

Received April 27, 2010
Revised May 16, 2010
Accepted May 24, 2010