Regulation of Multicellular Spheroids by MAPK and FYN Kinase

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Abstract. *Understanding of the biology of oral squamous cell carcinoma (SCC) has not progressed significantly in the past 60 years, with 5-year survival remaining at approximately 50%. The epidemic of Human Papilloma Virus and its associated SCC warrants a renewed emphasis on fully understanding this disease. We previously used the 3-dimensional multicellular spheroid (MCS) model system to evaluate SCC behavior more accurately. In this study, we determined that SCC growth in MCS approximates epithelial to mesenchymal transition. Organization of an MCS requires the full-length β6 integrin subunit and its maintenance requires mitogen-activated protein kinase (MAPK). Limiting FYN kinase activation results in the down-regulation of E-cadherin, β-catenin and an increase in expression of N-cadherin and SNAIL. These results indicate that the microenvironment and growth patterns in an MCS are complex and require MAPK and FYN kinase.*

Approximately 48,330 Americans will be diagnosed with oral or pharyngeal carcinoma this year. While the prognosis for these patients has improved slightly over the past two decades, the 5-year survival rate remains at 60%, which is likely due to the presence of advanced disease at the time of diagnosis (1). Although head and neck carcinoma makes up only about 4% of all tumors, the morbidity and mortality associated with this disease is disproportionate in relation to its incidence. At the molecular level, oral cancer progression occurs through a multistep process by which an accumulation of genetic mutations and subsequent clonal expansion results in a tumor. We previously showed that the epithelial integrin, αvβ6, is neo-expressed in oral SCC (2). The β6 integrin cytoplasmic tail activates FYN kinase; which lies upstream of the RAS-Rapidly Accelerated Fibrosarcoma-Mitogen-Activated Protein Kinase (RAS-RAF-MAPK) pathway. In the absence of the 11 *C*-terminal amino acids of the β6 integrin, FYN kinase is unable to be phosphorylated resulting in RAS-RAF-MAPK shutdown (2).

The auto-phosphorylation of focal adhesion kinase (FAK), which is localized to focal contacts, is also regulated by the β6 cytoplasmic tail, as is the control of transcription factors NOTCH and Signal Transducer and Activator of Transcription 3 (STAT3) (3). Focal contacts are rich in signaling kinases including SRC family kinases (SRC, FYN, and YES) and FAK (3). Tumor cells acquire signals from the extracellular matrix in part, through cell surface receptors such as the integrin family. However, little is known about the factors affecting phenotypic changes associated with the invasive and metastatic behavior of oral cancer. During invasion, the cells adhere to, partially degrade/remodel, and invade through the basement membrane into the underlying interstitial stroma. Invasion involves interactions between the extracellular matrix and adhesion receptors (integrins).

In this study, we used Multicellular Spheroids (MCS) as a model to investigate the effect of cell–cell contact in a 3-dimensional tumor microenvironment. MCS are three-dimensional structures that are grown on nonadhesive surfaces and have been shown to be more representative of human tumor growth than two-dimensional monolayer cultures (4, 5). In this study, we hope to further understand how growth in a 3-dimensional environment modulates the signaling cascade of oral SCC cells.

Methods and Materials

Cell culture. The SCC9 cell line was derived from a base of tongue lesion and was a generous gift from Dr. James Rheinwald (Brigham and Woman's Hospital, Harvard School of Medicine). The SCC9β6 and SCC9CAFyn cell lines were established in our laboratory by stable transfection of SCC9 cells with cDNAs for the full-length β6 integrin subunit or a constitutively active Fyn, respectively. The

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SCC9β6KDFyn cell line was established by transducing the SCC9β6 cells with the cDNA for a kinase-dead FYN. The constitutively active FYN and kinase dead FYN cDNAs were a generous gift of Dr. H. Kawakatsu (University of California, San Francisco, CA, USA). The cDNA for the full-length β6 was generously provided by Dr. Dean Sheppard (University of California, San Francisco, CA, USA). Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Generation of multicellular spheroids. Tissue culture plates (Santa Cruz Biotechnology, Dallas, TX, USA) were treated with a 0.6% agarose solution and air dried overnight at room temperature under UV light. Cells were harvested from monolayer cultures with 0.25% trypsin-EDTA and rinsed twice with 1X Phosphate-Buffered Saline (PBS) to remove serum. The cell suspension was pipetted onto agarose-coated plates (Sigma-Aldrich, St. Louis, MO), for 48 h at 37˚C in 5% CO_2 in serum-free medium, and allowed to form MCS. In some experiments, U0126 (5 or 10 μM; Sigma-Aldrich), a selective inhibitor of MAPK kinases MEK1 and MEK2, was added for 48h.

Antibodies. Rabbit monoclonal antibodies to E-cadherin (clone 24E10), N-cadherin (clone 4061), phosphorylated-p44/43 MAPK (ERK1/2) (Thr202/Tyr204) (CST 9101), and to β-actin (clone13E5) were purchased from Cell Signaling Technology Inc., (Danvers, MA, USA). Rabbit polyclonal antibody to β-catenin was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (clone7074) and HRP-conjugated anti-mouse antibodies (CST #7076) were purchased from Cell Signaling Technology Inc.

Western blot. Cells were grown as either a monolayer or MCS in serum-free mediumfor 48 h prior to lysing. Cells were rinsed with ice-cold 1X PBS and lysed. Total protein was extracted from cells with RIPA lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.2% sodium-dodecyl-sulfate 1% deoxycholic acid, 1% Triton X-100, and complete protease inhibitor tablet). Lysate was generated by suspending MCS in RIPA lysis buffer, vortexing, and then forcing MCS suspension through a 30-gauge needle. Monolayer lysate was generated by pipetting RIPA lysis buffer over the culture and scraping with a cell scraper. Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane using a semi-dry apparatus (Bio-Rad, Hercules, CA, USA). Membranes were incubated with monoclonal antibodies overnight at 4˚C. After washing, the blots were incubated with HRP-conjugated anti-rabbit antibodies for 40 minutes. The blots were then treated with ECL substrate (Thermo Scientific Pierce; Rockford, IL, USA) and exposed to x-ray film (Santa Cruz Biotechnology).

Results

FYN and MAPK differentially modulate spheroid formation by oral SCC cells. MCS formation approximates tumor cell growth *in vivo*. The SCC9, SCC9β6, SCC9β6KDFyn and SCC9CAFyn cells were plated onto agarose-coated dishes and allowed to form 3-dimensional spheroids for 48 h. Each cell line formed its own characteristic MCS. We previously showed that the SCC9β6KDFyn cell line formed the largest MCS (6). In the current study, the SCC9β6KDFyn MCS were seven times as large as those formed by SCC9β6, SCC9 and SCC9CAFyn cells (Figure 1A).

The MAPK pathway evokes an intracellular signaling cascade in response to extracellular stimuli such as heat and stress. It can also influence cell division, metabolism and cell survival. The cells were plated in the presence of the MEK inhibitor U0126 (10 μM) and allowed to grow for 48 h and then analyzed using Image J (v1.6) (7). When incubated in the presence of U0126, MCS growth was enhanced compared to control cultures: SCC9CAFyn increased 8-fold; SCC9 and SCC9β6 increased 6-fold and the SCC9β6KDFyn MCS increased by 30% Figure 1B). The results indicate that FYN and MAPK pathway exert a negative effect on the size of the MCS.

Differential expression of SNAIL in MCS. The transcriptional repressor SNAIL sits at the vortex of several signaling pathways promoting the epithelial-to-mesenchymal transition (EMT). The role of SNAIL in MCS formation was investigated. Recently, we showed that when grown as MCS, the SCC9β6KDFyn cell line switched from an E-cadherin to an N-cadherin cell–cell adhesion profile (6). Neo-expression of N-cadherin has been associated with invasive phenotype and EMT (8, 9). The SCC9, SCC9β6, SCC9CAFyn and the SCC9β6KDFyn cells were grown as MCS for 48 h either in the presence or absence of the MEK inhibitor, U0126 (Figure 2). Interestingly, only the SCC9β6KDFyn cells expressed detectable levels of SNAIL (Figure 2) and blocking the activation of MAPK suppressed this expression by 90%. When grown as MCS, the SCC cells expressing the full length β6-integrin with a kinase-dead Fyn appear to be EMT-ready as determined by the expression of SNAIL in this study and expression of N-cadherin in our previous study (6).

Phosphorylation of MAPK modulates E-cadherin expression in SCC spheroids. E-Cadherin is a critical component of desmosomes. Not only are these cell–cell junctions physical structures used to maintain the architecture of the epithelial sheet but also the individual components of the junctions have multiple signaling properties. During tumor invasion, desmosomes disassemble; E-cadherin is degraded resulting in EMT. As the cell–cell contacts disassemble, the cells become more fibroblast-like in their shape. When grown as an MCS, the SCC9, SCC9β6, SCC9CAFyn cell lines all expressed the same relative level of E-cadherin (Figure 3). In contrast, the SCC9β6KDFyn cell line expressed 10% as much E-cadherin as expressed by the other cell lines (Figure 3). This suggests that the activation of FYN kinase is essential for E-cadherin expression when SCC cells are grown as MCS. We previously reported that the SCC9β6KDFyn cell line, when grown as MCS, expressed N-cadherin, rather than E-cadherin (6). To investigate the role MAPK plays in E-cadherin expression in MCS, the cultures were incubated in the

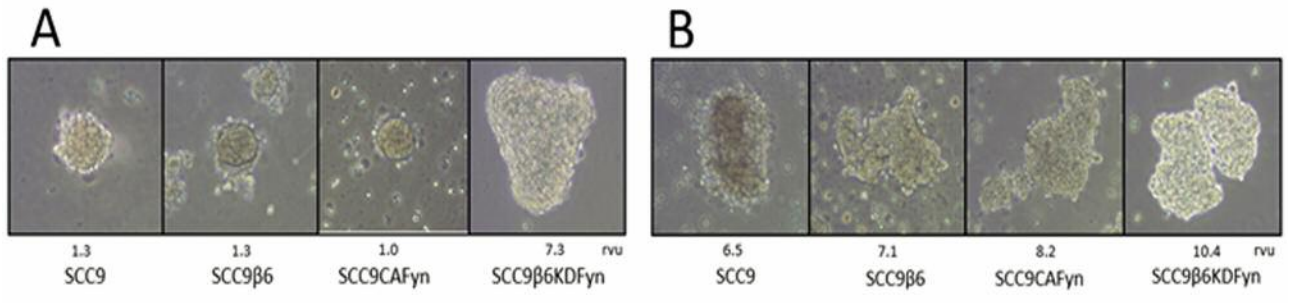

Figure 1. *FYN kinase and Mitogen –activated protein kinase (MAPK) differentially modulate spheroid formation by oral Squamous cell carcinoma (SCC) cells. A: SCC9 (β6-negative), SCC9β6 (express full length β6), SCC9CAFyn (β6-negative with constitutively active FYN and the SCC9β6KDFyn (express full length β6 with a kinase dead FYN) cells were grown on agarose-coated plates for 48 h to promote multicellular spheroid (MCS) formation. The MCS were photographed and assigned relative value units (rvu) using NIH ImageJ. SCC9, SCC9β6 and SCC9CAFynMCS had relatively equal MCS size, while the SCC9β6KDFyn MCS were considerably larger. B: SCC9, SCC9β6, SCC9CAFyn and the SCC9β6KDFyn cells were grown as MCS in the presence of MAPK/extracellular protein kinase kinases inhibitor U0126. The MCS were analyzed by NIH ImageJ and assigned relative rvu. Treatment with U0126 increased MCS size.*

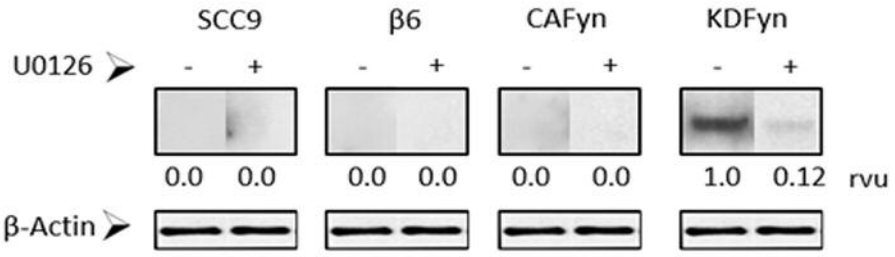

Figure 2. *Differential expression of SNAIL in multicellular spheroids (MCS). SCC9, SCC9β6, SCC9CAFyn and SCC9β6KDFyn cells were grown as MCS in the presence or absence of the MAPK/extracellular protein kinase (MEK) inhibitor U0126 (10 μM). Only SCC9β6KDFyn MCS expressed SNAIL and its expression was significantly suppressed by incubation with the MEK inhibitor U0126.*

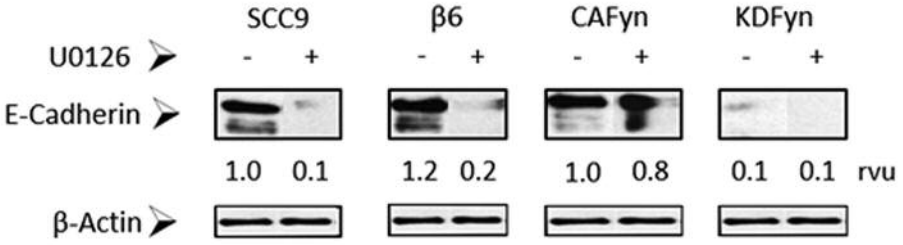

Figure 3. *Phosphorylation of Mitogen-activated Protein Kinase (MAPK) modulates E-cadherin expression in squamous cell carcinoma (SCC) multicellular spheroids (MCS). SCC9, SCC9β6, SCC9CAFyn and SCC9β6KDFyn cells were grown as MCS for 48 h in the presence or absence of the MAPK/extracellular protein kinase (MEK) inhibitor U0126. SCC9, SCC9β6, SCC9CAFyn MCS expressed equivalent levels of E-cadherin. Barely detectable levels of E-cadherin were seen in the SCC9β6KDFyn MCS. When grown in the presence of the MEK inhibitor, E-cadherin was suppressed completely in the SCC9β6KDFYN, by 90% in SCC9 and SCC9β6 MCS. Expression of E-cadherin by SCC9CAFyn MCS was suppressed by 20%.*

presence of U0126 (Figure 3). Expression of E-cadherin expression was reduced by 90% in the SCC9 and SCC9β6 MCS, whereas E-cadherin expression by the SCC9CAFyn MCS was suppressed by 20% (Figure 3), The low-level expression of E-cadherin by SCC9β6KDFyn cells was not affected by the addition of the MEK inhibitor, U0126 (Figure 3). Optimal expression of E-cadherin by the SCC9, SCC9β6, and SCC9CAFyn-derived MCS requires active MAPK.

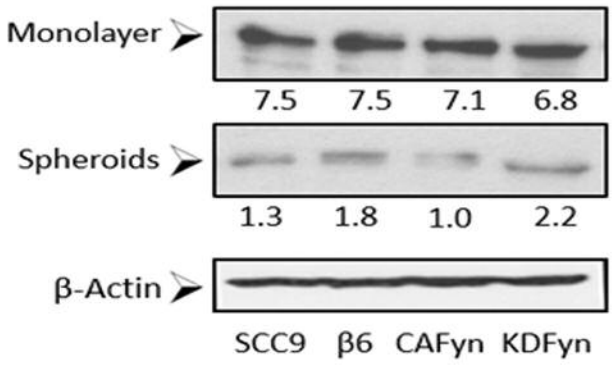

Figure 4. *Expression of β-catenin is suppressed by Multicellular Spheroid (MCS) formation. SCC9, SCC9β6, SCC9CAFyn and the SCC9β6KDFyn were grown as MCS in the presence or absence of the MAPK/extracellular protein kinase (MEK) inhibitor U0126. The MCS were processed for western blotting and assigned relative value units (rvu). A dramatic reduction in β-catenin expression was seen in all the MCS compared with cells when grown as a monolayer.*

Constitutively active FYN provides E-cadherin with relative immunity to MAPK suppression. These results suggest that expression of E-cadherin is mediated by both MAPK and FYN kinase.

Expression of β-catenin is suppressed by formation of spheroids. β-Catenin is the central signaling molecule of the canonical WNT pathway, where it activates target genes in a complex with LEF/TCF transcription factors in the nucleus. The regulation of β-catenin activity is thought to occur mainly on the level of protein degradation. In our study, all four cell lines expressed approximately equivalent levels of β-catenin when grown as a monolayer (Figure 4). However, when the cells were cultured as MCS, there was a significant decrease in β-catenin expression by all four cell lines (Figure 4. The expression of β-catenin was reduced by 87% in SCC9, 76% in SCC9β6, 86% in SCC9CAFyn and 68% in SCC9β6KDFyn cells. This suggests that growth as MCS alters available β-catenin. Formation of spheroids reduces the level of available β-catenin which positively correlates with the E-cadherin profile. When grown as a monolayer, all the cells expressed a high level of β-catenin. However, when grown as MCS, expression of β-catenin significantly decreased in all four cell lines.

Phosphorylation of MAPK is enhanced by the activation of Fyn kinase. The SCC cells were grown as a monolayer for 48 h, lysed and evaluated by western blotting. When grown as a monolayer, the SCC9β6, SCC9β6KDFyn and SCC9CAFyn cell lines expressed similar levels of MAPK, whereas the SCC9 cells, which lack β6, expressed roughly

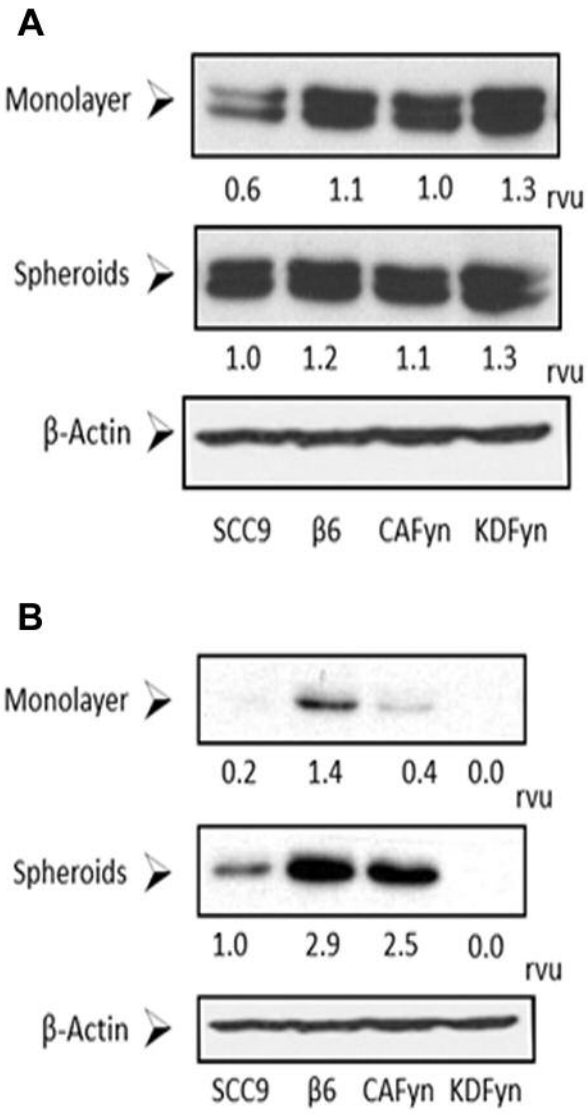

Figure 5. *Phosphorylation of Mitogen-Activated Protein Kinase (MAPK) is enhanced by the activation of FYN kinase. When grown as monolayers, the SCC9β6 cell line expressed the highest level of phosphorylated pMAPK, followed by SCC9CAFyn and SCC9 cells. No expression of pMAPK was seen in the SCC9β6KDFyn cells. When grown as MCS, the expression of pMAPK increased in all cell lines with the exception of the SCC9β6KDFYN cells which remained negative.*

half as much total MAPK (Figure 5A). The same was true when cells were grown as MCS (Figure 5A). No difference in expression of total MAPK was detected between growth as a monolayer or MCS (Figure 5A).

When grown as a monolayer, the SCC9β6 cell line expressed the greatest level of pMAPK, followed by SCC9CAFyn and SCC9 cells (Figure 5B). No detectable

pMAPK was detected in the SCC9β6KDFyn cells (Figure 5B). However, when grown as MCS, expression of pMAPK was increased: the SCC9β6 and SCC9CAFyn expressed the greatest level followed by SCC9 (Figure 5B). No pMAPK was detected in the SCC9β6KDFyn cells (Figure 5B). These results indicate that when grown as MCS, MAPK is subjected to increased phosphorylation and this appears to be under the "control" of FYN kinase. In the absence of FYN kinase activity, both the expression and activation of MAPK is absent.

Discussion

Despite advancements in strategies for the treatment of cancer, patient prognosis and mortality rates have improved minimally, with recurrence rates and metastasis remaining as the primary cause of cancer mortality worldwide. EMT is the process by which epithelial derived tumors de-differentiate to a more mesenchymal phenotype. The cells lose epithelial-specific structures that are responsible for their structural adherence. This is also accompanied by a change to an integrin adhesion-receptor profile of these cells. Integrins are cell-surface transmembrane molecules that connect the extracellular environment with the actin/keratin cytoskeleton. No specific pattern of integrin expression has ever been shown to cause tumor cell conversion or invasion directly. However, the expression of the epithelial-specific αvβ6 integrin has been shown to correlate with invasiveness in oral SCC (10) and is associated with a poor prognosis in colorectal cancer (11). Understanding the mechanisms that contribute to the process of cancer development is of fundamental importance when designing new therapeutic strategies.

Using the MCS model of oral cancer, we found that the full-length β6 integrin subunit is required for MCS formation, as in the absence of the 11 cytoplasmic terminal amino acids of β6 integrin, MCS cannot be organized (6). Interestingly, when MAPK activity was suppressed, the size of the MCS increased, indicating that MAPK activation exerts a negative feedback loop on MCS growth. SNAIL, a transcription factor known to modulate and promote EMT, was identified only in SCC9β6KDFyn MCS and this expression was suppressed using the MEK inhibitor U0126. This further indicates that EMT, which is promoted by SNAIL, is actually suppressed by MAPK, as is the epithelial marker, E-cadherin. In the presence of the MEK inhibitor U0126, the expression of E-cadherin was dramatically suppressed (90%) in MCS. Modification of FYN kinase (constitutively active or kinase deficient) rendered the MCS resistant to treatment with U0126. This indicates that MAPK and FYN kinase have antagonistic functions when SCC cells are grown as MCS.

Dysregulation of the WNT/β-catenin signaling pathway is a recurring theme in cancer biology. The delicate balance between β-cateinin and E-cadherin has been re-recognized in our studies. Cell–cell rearrangements such as EMT are due to the dynamic properties of this cadherin complex (12). Dysregulation/delocalization of signaling is important to a number of malignancies, including oral cancer. β-Catenin mutations appear to be a crucial step in the progression of a subset of these types of cancer, suggesting an important role in the control of cellular proliferation or cell death (13). β-Catenin is a subunit of the cadherin protein complex and acts as an intracellular signal transducer in the WNT signaling pathway (14). It is homologous to plakoglobin, a structural component of the desmosome (15). As a component of the cadherin complex, β-catenin can regulate cell growth and adhesion between cells. It may also be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete. The E-cadherin–β-catenin–α-catenin complex is weakly associated with actin filaments (16). Adherent junctions are dynamic, rather than a static link to the actin cytoskeleton.

These results indicate the presence of a complex association between FYN kinase, MAPK and growth in MCS. The cells are put under unique stresses not normally appreciated when cells are grown in a 2-dimensional monolayer. The MCS model results in unique protein associations not seen in monolayers but which are more representative of the *in vivo* experience. E-Cadherin/β-catenin also appear to be intimately involved with the FYN/MAPK signaling and the EMT. Further work is needed to fully understand the depth of the association of spheroid formation with the suppression of epithelial markers and increase in mesenchymal markers in the quest for EMT.

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