Paracrine Regulation Of Cancer Stem Cell Populations

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THESIS
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I would like to dedicate this thesis to my brother, parents, faculties and friends who supported me in my academic achievements.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my mentor Dr. Crowe for his support, patience, and mentorship. He has constantly encouraged me and provided a wonderful learning environment. I feel lucky to be able to learn from him and to work under his supervision.

I would like to extend my appreciation to my thesis committee Dr. Adami and Dr. Zhou for their expertise and assistance. They have supported me greatly and were always willing to help.

Thank you for the great experience.

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LIST OF ABBREVIATIONS

Akt	RAC-alpha serine threonine protein kinase
ANOVA	analysis of variance
BCC	basal cell carcinoma
Bmi1	B lymphoma Moloney murine leukemia virus insertion 1
BMP4	bone morphogenetic protein 4
Cbx4	chromobox 4
CD34	cluster of differentiation 34
CD200	cluster of differentiation 200
cRNA	complementary RNA
Cxcl	chemokine CXC ligand
Cxcr	chemokine CXC receptor
DAPI	4’,6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenz(a)anthracene
DNA	deoxyribonucleic acid
DTA	diphtheria toxin A
ERK	extracellular signal regulated kinase
ESC	embryonic stem cell
GFP	green fluorescent protein
IgG	immunoglobulin G
iPS	induced pluripotent stem
K15	keratin 15
Lef	lymphoid enhancer factor

Lgr6	leucine rich repeat containing G protein coupled receptor 6
miR	microRNA
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PI3K	phosphatidylinositol 3'-kinase
PKC	protein kinase C
PLC	phospholipase C
PR	progesterone receptor
PTCH1	Patched 1
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCF	T cell factor
TUNEL	terminal transferase mediated dUTP nick end labeling
Wnt	Wingless integration
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Zfp145	zinc finger protein 14

SUMMARY

Stratified epithelial stem cells are responsible for skin and mucosal renewal and play an important role in homeostasis and wound healing. Lgr6 is a marker for a unique stem cell population giving rise to the different lineages in stratified epithelia. Keratin 15 expression marks a separate population of stem cells in stratified epithelia. CXC family chemokine receptors (Cxcr) are expressed as transmembrane proteins in mammalian epidermal cells. They recognize and specifically bind to chemokines of the CXC family. Recent studies have reported roles for chemokines in inflammation, tumor growth, angiogenesis, invasion, and metastasis in cancer. Selective depletion of the small K15+ stem cell fraction resulted in dramatic reduction of Lgr6+ cells and inhibition of oral tumorigenesis via senescence. Gene expression studies revealed that K15+ cancer stem cells regulate Lgr6+ cancer stem cell expansion via chemokine signaling. Genetic ablation of the chemokine receptor Cxcr2 inhibited cancer stem cell expansion and tumorigenesis via senescence. The effects of chemokines were primarily mediated by PI3K signaling, which is a candidate for targeted therapy in head and neck cancer.

1. INTRODUCTION

1.1 Background

Many tissues are capable of self-regeneration, a property attributed to stem cells. Stratified epithelial stem cells are responsible for skin and mucosal renewal and play an important role in homeostasis and wound healing. Current research is focused on the mechanisms of stem cell mediated tissue regeneration. In addition, stem cells are often a major target of tumor initiation and a main focus in gene therapy. Epidermal stem cells have been identified in interfollicular epidermis and mucosa. Stem cells are located in the basal layer of stratified epithelia, giving rise to the protective layers of stratified epithelial cells. Snippert et al. (2010) identified Lgr6+ cells as a unique stem cell population giving rise to different epidermal lineages.

A separate cell population expresses the stem cell markers keratin 15 and CD34 (Lyle et al., 1998; Lyle et al., 1999). Liu et al. (2003) found that K15 expression marks a stem cell population in stratified epithelium. K15+/CD34+ stem cells have been implicated in multiple cellular processes (Oro et al., 1997; Brown et al., 1998; Gat et al., 1998; Grachtchouk et al., 2000; Waikel et al., 2001; Santos et al., 2002; Bojovic et al., 2015).

CXC family chemokine receptors (Cxcr) are expressed as transmembrane proteins in stratified epithelial cells. They recognize and specifically bind to chemokines of the CXC family. CXC chemokine receptor 2 (Cxcr2) is induced during wound healing, especially during the inflammatory, epithelialization, and

angiogenic phases (Zaja-Milatovich et al., 2008). Engelhard et al. (1998) suggested that CXC chemokines integrate inflammatory and reparative processes during wound healing.

Recent studies have reported roles for chemokines in tumor growth, angiogenesis, invasion, and metastasis in multiple cancers (Han et al., 2012). Chemokine and chemokine receptor mRNA expression was detected in both normal oral keratinocytes and cancer cell lines, with significantly higher expression in oral cancer (Khurram et al., 2014). Warner et al. (2008) demonstrated that chemokines induce tumor cell invasion. Sui et al. (2014) reported that Cxcr2 correlated with lymph node metastasis and poor clinical outcome in esophageal cancer patients. High expression of Cxcr2 in laryngeal squamous cell carcinoma was associated with lymph node metastasis and decreased survival (Han et al., 2012). Moreover, Qian et al. (2014) found that Cxcr2 silencing markedly inhibited migration and invasion of oral squamous cell carcinoma (OSCC) cells in vitro and in vivo. In the same study Cxcr2 silencing showed no effect on proliferation of OSCC cells, but led to morphological changes and decreased lamellipodial structures. Warner et al. (2008) also reported that blockade of Cxcr2 signaling, but not of Cxcr1, inhibited oral squamous cell carcinoma.

1.2 **Significance**

Paracrine signaling between cancer stem cell populations is a novel concept. The ability of a small cancer stem cell population to regulate proliferation and tumorigenesis of a second larger cancer stem cell population via paracrine

signaling suggests that targeting of the smaller population will have dramatic therapeutic effects. Characterization of these signaling pathways will lead to novel targeted therapies for oral cancer patients.

1.3 **Hypothesis**

K15+ stem cells regulate expansion of the Lgr6+ stem cell population via chemokine signaling in tumorigenesis.

1.4 **Specific Aims**

Specific Aim 1. To determine the effects of Cxcr2 null mutation on proliferation, expansion, and programmed cell death of Lgr6+ oral cancer stem cells regulated by K15+ cancer stem cell chemokine signaling.

Specific Aim 2. To determine the effects of Cxcr2 null mutation on Lgr6+ oral cancer stem cell gene expression regulated by K15+ cancer stem cell chemokine signaling.

2. REVIEW OF LITERATURE

2.1 **Stratified epithelial differentiation**

Stem cells have high capacity for self-renewal in permanently renewing tissues such as the hematopoietic system, mucosal lining, and the epidermis (Jones and Watt, 1993). During gastrulation phase of the vertebrate embryo, the primitive ectoderm ultimately gives rise to epidermis and oral mucosa (Coucouvanis and Martin, 1995). Prior to the onset of stratification at embryonic day 9.5, the single layer of epithelium expresses keratin 5, keratin 14, and p63 proteins (Byrne, 1994; Yang et al., 1999). Yang et al. observed the crucial role of p63 in the ectodermal development of newborn mice. The lack of functional p63 resulted in absence of stratified and pseudostratified epithelia resulting in no hind limbs and mammary glands, lack of hair follicles and teeth, and truncated forelimbs. p63-/- mice underwent dehydration and died several hours after birth. The epidermis failed to express many differentiation markers such as keratin 5, keratin 10, keratin 14, loricrin, filaggrin, and involucrin, indicating a fundamental defect in stratified epithelial lineage development.

The stratified epithelium is a differentiating system composed of four layers: basal, spinous, granular, and cornified. The basal layer is composed of cells linked by desmosomes and attached to the basement membrane by integrin receptors. When the cells leave the basal layer, keratin 5 and keratin 14 expression is decreased and keratin 1 and keratin 10 proteins are induced. Cells progress into the spinous layer where they express involucrin. Cells continue upward migration into the granular and cornified layers where they express

filaggrin and loricrin. Cornified cells are shed from the surface of the epidermis (Kursad, 1998).

Identifying molecules involved in stem cell fate has led to better understanding in stratified epithelial development and differentiation. Epidermal stem cells can reside in hair follicles and generate multiple epidermal lineages (Oshima et al., 2001). Regulation of epidermal stem cell fate is by proteins involved in the Wnt signaling pathway (Koster, 2002). Wnt glycoproteins activate the pathway by transducing a cytoplasmic signal, which represses the activity of glycogen synthase kinase 3, thereby stabilizing β-catenin. β-catenin translocates to the nucleus where it binds Lef/TCF transcription factors resulting in activation of target genes. Mouse models indicate that β-catenin/Lef activation is required for epidermal differentiation.

A known target of the Wnt signaling pathway is the c-myc proto-oncogene that can induce growth, proliferation, transformation, and apoptosis (Eisenman, 2001). Potential target genes of c-myc are required for cell cycle progression including genes responsible for lipid metabolism, protein and DNA synthesis, and signal transduction involved in stem cell fate decisions (Koster, 2002).

The epithelium is a self-renewing stratified epithelium that protects from environmental pathogens and prevents water loss. Evidence from analysis of tight junctions and null mice indicate that claudins are required for tight junction barrier function of preventing dehydration (Furuse, 2002). Dehydration defects have been observed in claudin 1 null mice similar to inactivated p63 transcription factor. In addition, claudin 1 null mice failed to develop stratified epithelia, teeth,

hair follicles, and mammary glands and died similar to p63 null mice (Mills et al., 1999). Claudin 1 and p63 might be linked in a common regulatory pathway causing null mice to die after birth due to dehydration from loss of barrier function (Lopardo et al., 2008).

Stratified epithelial development depends on transcription factor p63, which is a member of the p53 family. miR-203 specifically targets human and mouse p63 3'-UTR's. miR-203 controls p63-dependent proliferative of epithelial stem cells during keratinocyte differentiation. miR-203 overexpression reduces keratinocyte clonogenic capacity. miR-203 can regulate p63 expression following genotoxic damage in head and neck squamous cell carcinoma cells (Lena et al., 2008). Other evidence suggest that miR-203 directly represses the expression of p63 and regulates transition of basal cells to terminally differentiating suprabasal cells (Yi et al., 2008).

Another regulatory role of p63 is controlling the expression of chromatin-remodeling factors, which control terminal differentiation (Fessing et al., 2011). Polycomb chromatin-remodeling proteins compact chromatin leading to transcription inhibition of gene promoters (Mardaryev et al., 2015). These proteins form two complexes: Polycomb repressive complex 1 and 2 (Simon and Kingston, 2013). The Cbx4 gene belongs to the PRC1 family that maintains epithelial identity by repressing non-epidermal gene expression (Mardaryev et al., 2015). Cbx4 is a direct p63 target and its ablation in mice results in a marked decrease of epidermal thickness and keratinocyte proliferation.

2.2 **Stratified epithelium and stem cells**

Embryonic stem cells play essential role in organ development and tissue repair by differentiating into multipotent stem cells (Xiao et al., 2014). Multipotent stem cells such as epithelial and mesenchymal cells initiate and regulate the development of highly specialized functional tissues and organs (Aufderheide et al., 1987). Under inductive stimuli mesenchymal stem cells can differentiate into non-mesenchymal cell lineages such as endothelial cells, neuronal cells, and keratinocytes (Sasaki et al., 2008). Epithelial stem cells are located in the basal layer of various epithelial tissues and contribute these tissues by asymmetric divisions to generate suprabasal cells of the skin (Plikus et al., 2012).

Several studies point out that human embryonic stem cells (hESCs) might be the most promising cells for tissue engineering based on their ability to differentiate into all somatic lineages in vitro (Reubinoff et al., 2000). Injury can impair skin structure and function (Chua et al., 2016). Different strategies have been used to differentiate human embryonic stem cells along the keratinocyte lineage using cytokine strategies (Chua at al. 2016; Schuldiner et al., 2000; Green et al., 2003). Keratinocyte progenitors were generated from hESCs by stage-specific application of ascorbic acid, retinoic acid, and BMP4. The resulting cells expressed keratin 14 and p63 and the method was determined to be efficient for producing keratinocyte progenitors that for tissue engineering (Hanquing et al., 2016). Currently, functional epidermal and mucosal substitutes have limitations for clinical use (Guenou et al., 2009; Chua et al., 2016).

Embryonic stem cells can differentiate into all embryonic germ layers. They may also be a source of epithelial cells for transplantation and regeneration

of damaged epithelium. The donor-derived cells to produce transplantable epithelia cell sheets could be allogeneic or autologous (Yoshida et al., 2011). Studies have reported several procedures to differentiate embryonic stem cells and induced pluripotent stem cells (iPS) into epidermal keratinocytes. Takahashi and Yamanaka demonstrated that iPS cells could be generated from adult fibroblasts (Takahashi et al., 2006; Takahashi et al, 2007; Okita et al., 2007; Yamanaka, 2007).

Sakurai et al. (2011) established a culture technique for both induction and differentiation of epithelial progenitors from mouse iPS cells. These progenitors expressed p63 and keratin 14 in the basal layer of the stratified epithelium. Yoshida et al. (2011) successfully obtained keratin 14 and p63 double positive epithelial progenitor cells from mouse iPS cells which produced polarized multilayer epithelial cell sheets in vitro. These cells may be useful for studying the pathophysiology of genetic disorders, drug screening, and personalized medicine.

2.3 **Stratified epithelial stem cells and transformation**

Tissues can repair by re-establishing their continuity or regenerate by replacement of damaged tissue (Cerqueira et al., 2012). Depending on their histological features, most tissues undergo continuous or cyclic processes of regeneration in order to maintain homeostasis (Coletti et al., 2013). Following injury, keratinocytes move between the blood clot and the dermis forming a well vascularized granulation tissue (Friedl et al., 2009). Keratinocytes proliferate and migrate followed by keratinocyte differentiation (Simpson et al., 2011). Scar

tissue is formed as a result of remodeling phase during which collagen type III is replaced by collagen type I, forming large abnormal collagen fibrils arranged in parallel bundles (Martin et al., 1997). Although some studies show the relative contribution of stem cells subpopulations in skin wound healing, the involvement of different stem cell populations is still under investigation (Li et al., 2012). Wound repair defects can lead to severe health issues especially in elderly and immunosuppressed individuals (Reed et al., 1985). Shafer and Werner reviewed an early hypothesis stating that chronic inflammation and repair are a precondition for tumorigenesis and concluded that cancer can be referred as a non-healing wound (Shafer et al., 2008).

Much effort has focused on identifying stratified epithelial stem cells markers at the tissue level. The hair follicle bulge region is a defined stem cell niche in the skin (Fuchs et al., 2008). Ohyama et al. (2006) characterized the molecular signature of hair follicle bulge cells. This included tenascin C, CD200, keratin 15, and keratin 19. Some markers, including Bmi-1 and Zfp145, were expressed only by human bulge cells and were not detected in rodent cells (Kloepper et al., 2008). Furthermore, human squamous cell carcinomas (SCCs) express stratified epithelial stem cell markers (Jensen at al., 2008).

The epidermis is maintained throughout the adult life and protects from a wide range of environmental assaults such as ultraviolet radiation, chemical carcinogens, and viruses (Owensand et al., 2003). This places the epidermal keratinocytes at high risk of acquiring oncogenic mutations. Epidermal stem cells can self-renew and generate progeny that can undergo terminal differentiation

(Watt et al., 2001). Because of the process of terminal differentiation, most cells that acquire mutations are lost and more than one genetic lesion is required for tumor development (Owensand et al., 2003). For these reasons long term stem cells have the ability to accumulate genetic hits that can result in a tumor. If such a cell undergoes clonal expansion, there is a higher probability that its progeny will contain additional mutations (Brash et al., 1997).

Many studies have discussed overlap between developmental and oncogenic pathways. The Wnt/β-catenin, Notch, p63, c-myc, and Hedgehog pathways have been identified as important regulators of stratified epithelial stem cells, and are also involved in epithelial cancers (Ambler et al., 2009). Human basal cell carcinomas (BCCs) are linked to constitutive Hedgehog pathway activity (Daya-Grosjean et al., 2005; Athar et al., 2006). Half of human BCCs have mutations in the Hedgehog antagonist Patched (PTCH1). PTCH1 mutations were associated with the familial form of nevoid BCCs (Oro et al., 1997). It has been proposed that hair follicle cells give rise to BCC (Hutchin et al., 2005). Inhibiting Wnt/β-catenin signaling in the presence of constitutive Hedgehog signaling suppressed BCC formation. Based on these results the authors suggested a Hedgehog-independent role for Wnt/β -catenin signaling in BCC formation (Hoseong et al., 2008). Other studies have demonstrated that p63 is important for tumor formation. Keyes et al. (2006) showed that p63 heterozygous mice are resistant to spontaneous or chemically-induced tumors. c-myc overexpression can result to spontaneous tumor formation, and the deletion of c-myc in basal cell results tumor resistance (Oskarsson et al., 2006).

Deletion of Notch 1 gene increases sensitivity to tumor development in mice (Lefort et al., 2007). Thelu et al. (2002) demonstrated that Notch signaling is upregulated in differentiating layers of epidermis. Notch signaling is linked to stratified epithelial differentiation and is a known tumor suppressor pathway in oral cancer.

2.4 Chemokines and tumorigenesis

Chemotactic cytokines, which are also referred as chemokines are small proteins (8–10 kDa) that are classified in four groups based on the position of the cysteines adjacent to the amino terminus (Balkwill et al., 2004). Chemokines bind to chemokine receptors. Cell migration is directed by chemokine signaling to a chemokine receptor. The receptors are transmembrane G protein coupled receptors that bind multiple chemokines (Balkwill et al., 2003). CXC family chemokine receptors (Cxcr) recognize and specifically bind to chemokines of the CXC family. CXC chemokine receptor 2 (Cxcr2) is induced during wound healing, especially during the inflammatory, epithelialization, and angiogenic phases (Zaja-Milatovich et al., 2008).

Functional chemokine receptors have been found on leukocytes, endothelial, and epithelial cells. Chemokine receptor expression depends on cell lineage, differentiation, and chemokine concentration in the presence of inflammation in the tumor environment (Muller et al., 2001). Human studies have identified more than 18 chemokine receptors and more than 50 chemokines (Balkwill et al., 2004).

Chemokines and their receptors have an important biological role in malignant cells. Cancers present with complex chemokine network that influence leukocyte immune infiltrate, tumor cell growth, survival, migration, and angiogenesis (Balkwill et al., 2003). Tumors are composed of epithelial cells as well as non-malignant stromal cells such as macrophages, lymphocytes, endothelial cells, fibroblasts, eosinophils, granulocytes, and natural killer cells (Negus et al., 1997, Pollard et al., 2004). Cancer cells can express chemokine receptors and can respond to the chemokine gradient themselves (Murphy et al., 2001). Malignant cells from at least 23 types of human cancers have been found to express Cxcr4, making it the most common receptor expressed by cancer cells (Balkwill et al., 2004). Cxcr4 expression can be increased by microenvironmental signals. Dhawan et al. (2002) described the CXC chemokines Cxcl1 and Cxcl8 as mainly produced by melanoma cells, which shows higher level of Cxcr2 receptors for this chemokines. Vandercappellen et al. (2010) examined the functional role of chemokine ligands in melanoma progression. They found that Cxcl4 inhibition reduced proliferation, chemotaxis, and invasiveness in vitro.

Giuliano et al. (2014) demonstrated that Cxcr1 and Cxcr2 regulate tumor and endothelial cell proliferation. They showed evidence that the pathway stimulates cancer cell proliferation, tumor inflammation, and angiogenesis. Another recent study showed that Cxcr2+ tumor cells have prognostic value for predicting overall survival of hepatocellular carcinoma patients (Li et al., 2015). The study concluded that the combination of Cxcl1 and Cxcr2 expression levels

predict poor prognosis and the Cxcr2-Cxcl1 axis can regulate liver cancer inflammation.

Other studies (Spaks et al., 2016; Spaks et al., 2017) confirmed the prognostic and diagnostic value of CXC chemokines as biomarkers. They analyzed the Cxcl4 levels in fifty patients with early stage of non-small cell lung cancer who underwent pulmonary resection. Their data showed that patients with high Cxcl4 expression experienced poor clinical outcomes. High levels of Cxcl4 were associated with tumor vascularity leading to relapse. These studies pointed out two important possibilities for the Cxcl gradient in the tumorigenic microenvironment. First, CXC chemokine ligands produced in other tissues may bind to Cxcr expressed by tumor cells. Second, Cxcl can be produced by the tumor or tumor infiltrating immune cells. The studies found significant differences in chemokine concentrations for those patients that have good clinical outcomes. These results support the immunoediting theory, which states that patients without recurrence experience tumor elimination with active involvement of immune cells compared to patients going through tumor escape with immunosuppression and tumor spread (Teng et al., 2015; Kim et al., 2007).

3. MATERIALS AND METHODS

Mouse breeding and procedures. Mouse strains and experimental procedures were approved by the institutional animal care committee. C57Bl/6-*Cxcr2*^{tm1Rmra}/J, B6.Cg-Tg(tetO-DTA)1Gfi/J, Tg(KRT14-Cre)1Amc/J, and B6;SJL-Tg(Krt1-15-cre/PGR)22Cot/J;*Gt(ROSA)*$^{26Sortm1(rtTA,EGFP)Nagy}$/J mutant mouse strains were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were crossed to create K15CrePR;GFP;DTA, K14Cre;Cxcr2+/+, and K14Cre;Cxcr2f/f offspring (n=20 for each group). K15 lineage tracing mice were injected with 200 μg of the progesterone receptor antagonist RU486 to express GFP and the reverse tet activator in K15+ cells. Mice received 25 μg dimethylbenzanthracene in ethanol applied topically to oral mucosa twice weekly. Control mice received ethanol vehicle only. K15CrePR;GFP;DTA mice received 2 mg/ml doxycycline in drinking water. The latency, number, and volume of tumors were recorded for each animal. In separate experiments, 10^3 K15CrePR;GFP SCC cells were injected subcutaneously in Matrigel into NU/J mice which were treated with 60 mg/kg buparlisib (PI3K inhibitor), 1 mg/kg trametinib (MEK inhibitor), 240 mg/kg MK2206 (Akt inhibitor), or vehicle (n=5/group). Tumors from 9 month old mice were fixed in 4% formaldehyde in PBS, flash frozen and stored at -80^0 C, or trypsin dissociated and cryopreserved in liquid nitrogen.

Immunohistochemistry. Fixed tumor tissue was dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections were deparaffinized and stained with hematoxylin and eosin. For immunohistochemical analysis, sections

were blocked using 10% normal serum and incubated with anti-PCNA or $p16^{INK4A}$ antibodies overnight at room temperature. After washing, sections were incubated with biotinylated secondary antibody at room temperature for 10 minutes. After additional washing, sections were incubated with streptavidin-conjugated horseradish peroxidase for 10 minutes at room temperature. Antigen–antibody complexes were detected by incubation with peroxide substrate solution containing aminoethylcarbazole chromogen. Data were analyzed by ANOVA.

Cell death analysis. Tumor tissue sections were incubated with terminal deoxynucleotidyl transferase and dUTP-fluorescein for 1 h at 37 °C according to manufacturer's recommendations (Roche Applied Sciences, Indianapolis, IN, USA). After washing, apoptotic cells were visualized by fluorescence microscopy. The percent fluorescent cells was determined using quantitative image analysis software. Data were analyzed by ANOVA.

Fluorescence activated cell sorting. GFP+ and GFP- SCC cells were dissociated by trypsinization, washed in PBS, and sorted by flow cytometry (Beckman Coulter MoFlo). Data were analyzed by ANOVA.

RNA extraction and gene expression profiling. Total RNA was extracted from sorted K15CrePR;GFP+ cells using a commercially available kit (RNEasy, Qiagen, Germantown, MD). The integrity of rRNA bands was determined by northern gel electrophoresis. Total RNA (1 μg) with spike in controls was reverse transcribed followed by second strand synthesis. The double stranded cDNA was used as the template for in vitro transcription. T7 RNA polymerase and

biotinylated nucleotide analogue/ribonucleotide mix was used to synthesize cRNA which was purified, fragmented, and hybridized to Affymetrix mouse genome GeneChip. Raw gene expression scores analyzed for quality control and normalized to the relative hybridization signal from each experiment.

Western blot. Protein was extracted from tumors in 1X Laemmli buffer. 75 μg total cellular protein was separated by SDS-PAGE. Proteins were electroblotted to PVDF membranes. Blots were incubated with antibodies to Cxcr2, pAkt1, total Akt1, pERK1, total ERK1, pPI3K, total PI3K, pPKC, total PKC, pPLCγ, total PLCγ, or β-actin for 16 hours at 4° C. After washing, blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Bands were visualized by the enhanced chemiluminescence method, normalized to β-actin expression, and quantitated by image analysis software.

Senescence associated β-galactosidase activity. Frozen tumor sections were fixed for 5 minutes in phosphate buffered 2% formaldehyde and 0.2% glutaraldehyde at room temperature. Sections were washed for 5 minutes in phosphate buffered saline and incubated in 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$ in 40 mM citric acid/sodium phosphate buffer (pH 6) at 37^0 C for 16 hours. Sections were washed in PBS and counterstained with nuclear fast red solution.

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

11/17/2016

David Crowe
Center for Molecular Biology & Oral Diseases
M/C 860

Dear Dr. Crowe:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 11/17/2016.**

Title of Application:	Role of Stem Cells In Epithelial Carcinogenesis
ACC NO:	15-226
Original Protocol Approval:	12/1/2015 **(3 year approval with annual continuation required).**
Current Approval Period:	11/17/2016 **to** 11/17/2017

Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol.

[redacted]

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO/*ss*
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088

4. RESULTS

We selectively ablated K15+ cancer stem cells in oral SCC by crossing the K15CrePR;GFP mouse with tetO-DTA animals. Following RU486 injection, K15+ cells in this mouse express the reverse tetracycline activator which drives diphtheria toxin A expression in this fraction when doxycycline is added to drinking water. SCC with depletion of K15+ cancer stem cells failed to grow (93 mm^3 vs. 460 mm^3; $P<0.002$; Fig. 1A) compared to control tumors. The number of metastatic lymph nodes was reduced in cancer stem cell depleted tumors (21% vs. 59%; $P<0.006$; Fig. 1B,D,F). Cancer stem cell depletion induced marked terminal differentiation and regression of SCC (31%; $P<0.001$; Fig. 1C,E) compared to control tumors as determined by histopathology. Senescence associated β-galactosidase activity was increased in cancer stem cell depleted SCC (66% vs. 0.9%; $P<0.00002$; Fig. 1G,H) compared to control tumors. Expression of the senescence marker $p16^{INK4A}$ was increased in cells from cancer stem cell depleted SCC (49% vs. 2.3%; $P<0.00001$; Fig. 1I,J) compared to control tumors. Doxycycline induced ablation reduced the K15+ cancer stem cell population to 0.01% ($P<10^{-6}$; Fig. 1K,L). The Lgr6+ cancer stem cell population was reduced to 0.04% ($P<10^{-7}$; Fig. 1M,N). These results indicate that K15+ cancer stem cells are responsible for the dramatic expansion of Lgr6+ cancer stem cells observed in oral SCC.

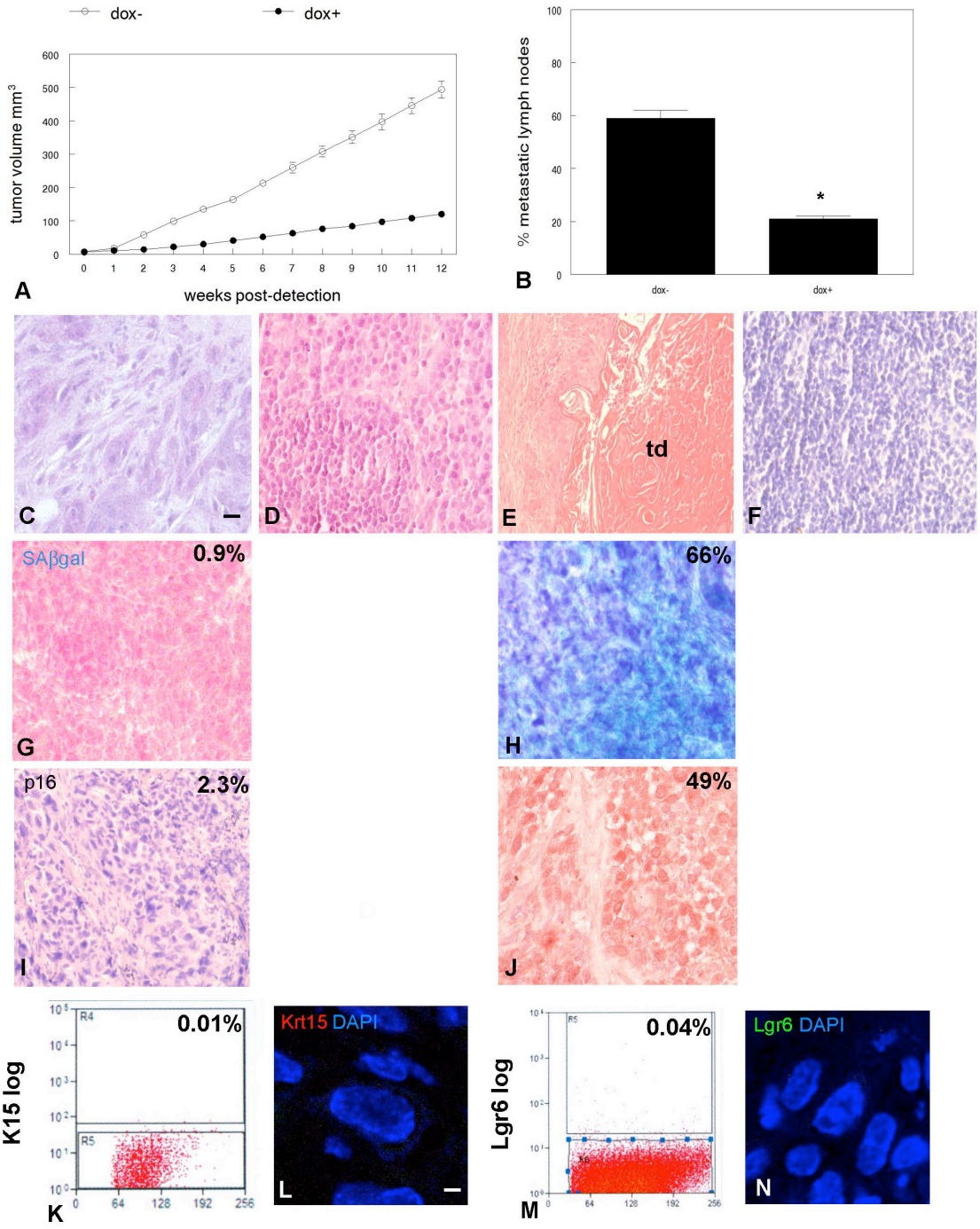
dox-
dox+
tumor volume mm3
weeks post-detection
A
% metastatic lymph nodes
dox-
dox+
B
C
D
E
td
F
SAβgal
0.9%
G
66%
H
p16
2.3%
I
49%
J
K15 log
R4
0.01%
R5
K
Krt15 DAPI
L
Lgr6 log
R5
0.04%
M
Lgr6 DAPI
N

Fig. 1. Selective depletion of K15+ cancer stem cells blocks Lgr6+ cancer stem cell expansion and inhibits SCC tumorigenesis. (A) Tumor volume in K15+ (dox-) and K15- (dox+) SCC is shown by weeks following primary cancer detection. (B) Percent metastatic lymph nodes in K15+ (dox-) and K15- (dox+) SCC is shown 12 weeks after primary cancer detection. Histopathology of primary (C) and metastatic (D) K15+ SCC is shown by hematoxylin and eosin staining. Scale bar = 10 μm. Histopathology of primary (E) and metastatic (F) K15- SCC is shown by hematoxylin and eosin staining. Senescence associated β-galactosidase activity in K15+ (G) and K15- (H) SCC. Nuclei were counterstained with nuclear fast red. Cellular senescence in K15+ (I) and K15- (J) SCC was determined by $p16^{INK4A}$ immunohistochemistry. Nuclei were counterstained with hematoxylin. (K) Quantitation of K15+ cells from K15- SCC was determined by flow cytometry. K15 log and forward scatter linear scales are shown. (L) Absence of K15+ cancer stem cells in K15- SCC is shown by immunofluorescence microscopy. Nuclei were counterstained with DAPI. (M) Quantitation of Lgr6+ cell fraction from K15- SCC was determined by flow cytometry. (N) Absence of Lgr6+ cancer stem cells in K15- SCC is shown by immunofluorescence microscopy. Scale bar = 2 μm.

Gene expression profiling studies of K15+ and Lgr6+ cancer stem cells revealed increased expression of chemokines Cxcl1 (16 fold), Cxcl3 (5 fold), and Cxcl5 (3 fold) in K15+ cancer stem cells compared to Lgr6+ cells ($P<0.001$; Fig. 2A). These 3 chemokines bind to the Cxcr2 receptor which was expressed at 9 fold higher levels in Lgr6+ cancer stem cells ($P<0.004$). These results suggest that paracrine chemokine signaling from K15+ cancer stem cells drives expansion of the Lgr6+ population. To test this hypothesis, we genetically ablated Cxcr2 expression in mucosal epithelium using K14Cre;Cxcr2f/f mice to selectively target stratified epithelium. Mice with Cxcr2 ablation in mucosal epithelium largely failed to develop SCC using our DMBA carcinogenesis protocol (23 mm^3 vs. 443 mm^3 by 10 weeks after tumor detection; $P<10^{-7}$; Fig. 2B) compared to control tumors. These SCC lacking Cxcr2 expression also largely failed to develop lymph node metastases (65% vs. 6%; $P<10^{-6}$; Fig. 2C,E,G) compared to control tumors. SCC lacking Cxcr2 expression also exhibited terminal differentiation by histopathologic analysis (59% vs. 5%; $P<0.0001$; Fig. 2D,F) compared to control tumors. Senescence associated β-galactosidase activity was increased in SCC without Cxcr2 expression (53% vs. 1.7%; $P<0.00009$; Fig. 2H,I) compared to control tumors. These results indicate that control of Lgr6+ cell expansion by K15+ cancer stem cells in SCC is mediated by chemokine signaling.

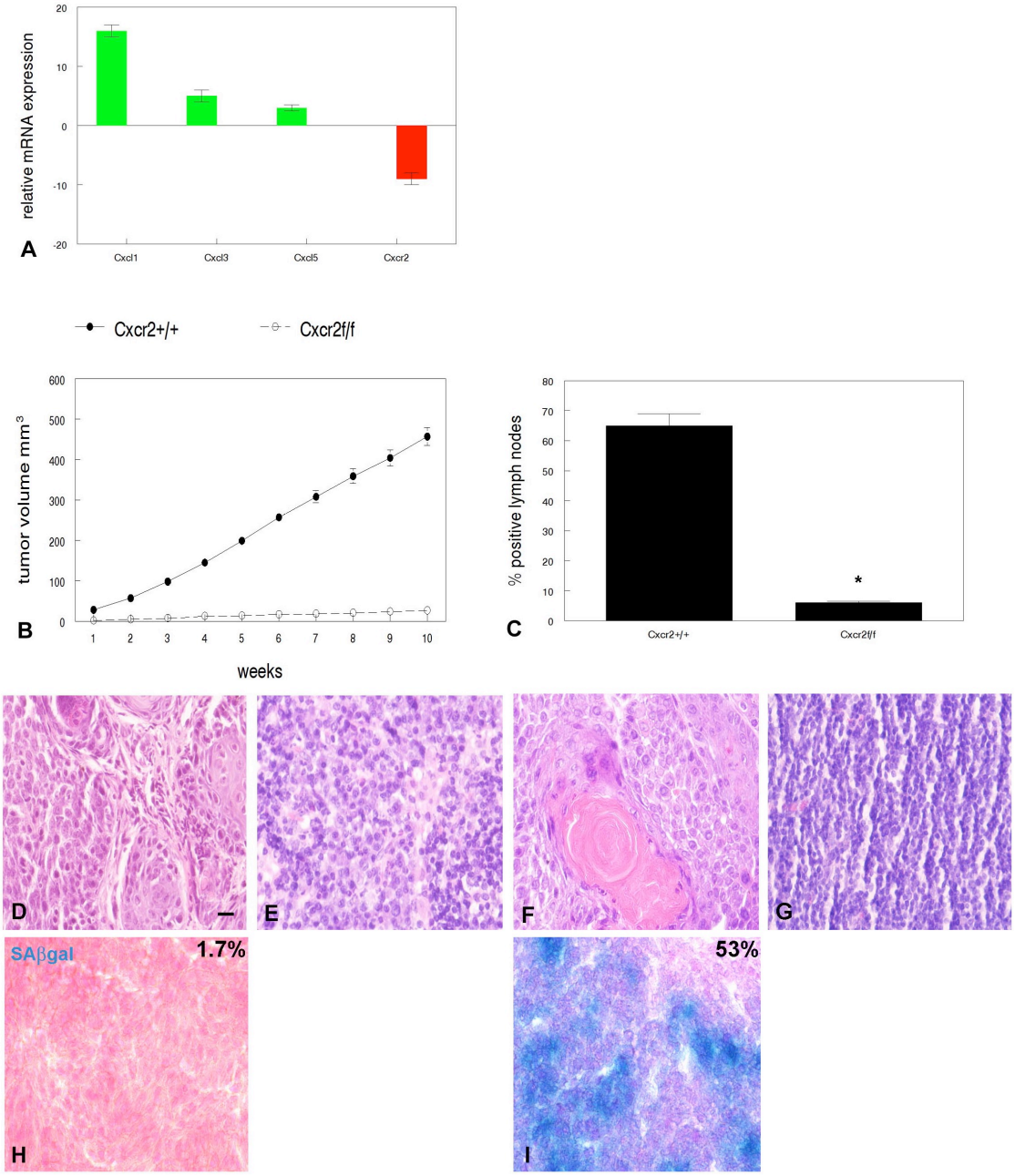
relative mRNA expression
20
10
0
-10
-20
A
Cxcl1
Cxcl3
Cxcl5
Cxcr2
Cxcr2+/+
Cxcr2f/f
tumor volume mm3
600
500
400
300
200
100
0
B
1
2
3
4
5
6
7
8
9
10
weeks
% positive lymph nodes
80
70
60
50
40
30
20
10
0
*
C
Cxcr2+/+
Cxcr2f/f
D
E
F
G
SAβgal
1.7%
H
53%
I

Fig. 2. Inhibition of chemokine signaling from K15+ cancer stem cells blocks primary and metastatic SCC tumorigenesis. (A) Expression of chemokine ligands and Cxcr2 receptor in K15+ vs. Lgr6+ cancer stem cells was determined by microarray analysis and qRT-PCR. (B) Tumor volume in K14Cre;Cxcr2+/+ and K14Cre;Cxcr2f/f SCC is shown by weeks following primary cancer detection. (C) Percent metastatic lymph nodes in K14Cre;Cxcr2+/+ and K14Cre;Cxcr2f/f SCC is shown 10 weeks following primary cancer detection. Error bars indicate SEM. Histopathology of primary (D) and metastatic (E) in K14Cre;Cxcr2+/+ SCC is shown by hematoxylin and eosin staining. Scale bar = 10 μm. Histopathology of primary (F) and metastatic (G) K14Cre;Cxcr2f/f SCC is shown by hematoxylin and eosin staining. Senescence associated β-galactosidase activity in K14Cre;Cxcr2+/+ (H) and K14Cre;Cxcr2f/f (I) primary SCC. Nuclei were counterstained with nuclear fast red.

We examined signaling pathways downstream of Cxcr2 in control and Cxcr2 null SCC by western blot. Cxcr2 protein expression was not detected in Cxcr2 null SCC (Fig. 3). pAkt1 and total Akt1 protein expression was reduced by 2 fold in Cxcr2 null SCC. pERK1 expression was reduced by 2-3 fold, and total ERK1 was reduced by 2-8 fold in Cxcr2 null SCC. pPI3K expression was reduced 2 fold, and total PI3K levels were reduced by 2-4 in Cxcr2 null SCC. pPKC expression was reduced to undetectable levels in Cxcr2 null SCC, as was total PKC levels in some Cxcr2 null samples. pPLCγ expression was undetectable in most samples, and total PLCγ expression was reduced by up to 32 fold in Cxcr2 null SCC. These results indicate that loss of Cxcr2 expression inhibits multiple downstream signaling pathways in SCC.

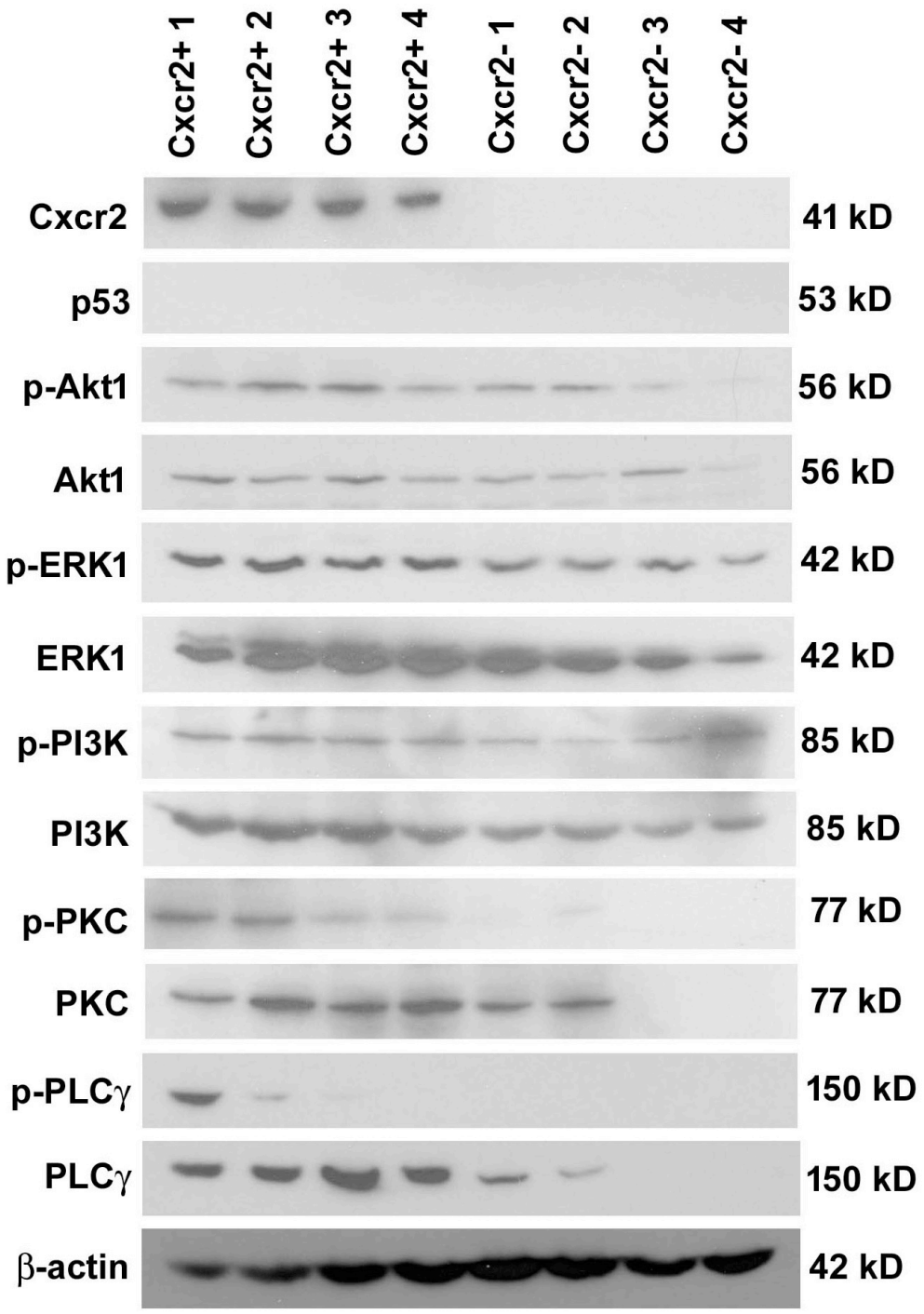
Cxcr2+ 1
Cxcr2+ 2
Cxcr2+ 3
Cxcr2+ 4
Cxcr2- 1
Cxcr2- 2
Cxcr2- 3
Cxcr2- 4
Cxcr2
41 kD
p53
53 kD
p-Akt1
56 kD
Akt1
56 kD
p-ERK1
42 kD
ERK1
42 kD
p-PI3K
85 kD
PI3K
85 kD
p-PKC
77 kD
PKC
77 kD
p-PLCγ
150 kD
PLCγ
150 kD
β-actin
42 kD

Fig. 3. Loss of Cxcr2 signaling inhibits multiple intracellular signaling pathways in SCC. Expression of activated signaling proteins shown at left were determined in cellular lysates from K14Cre;Cxcr2+/+ and K14Cre;Cxcr2f/f SCC. β-actin expression was used to determine equal protein loading of each lane. Representative blots are shown.

To determine which of multiple signaling pathways was most important in regulating chemokine signaling in SCC, we treated oral SCC transplanted to NU/J mice separately with 3 small molecule inhibitors currently in cancer clinical trials. After 4 weeks of treatment, the PI3K inhibitor buparlisib produced a 64% reduction in tumor volume ($P<0.007$; Fig. 4A) compared to vehicle treated control tumors. The MEK inhibitor trametinib resulted in a 39% reduction in tumor volume, and the Akt inhibitor MK2206 created a 23% reduction. Histopathologic sections of SCC treated with buparlisib or vehicle are shown by hematoxylin and eosin staining in Fig. 4B,C. The proliferating cell fraction was reduced in buparlisib treated tumors (23% vs. 37%; $P<0.04$; Fig. 4D,E) as determined by PCNA immunohistochemistry. Buparlisib treated SCC exhibited increased apoptotic cells (43% vs. 0.4%; $P<0.00002$; Fig. 4F,G) as determined by TUNEL analysis. Buparlisib treated SCC exhibited increased senescent cells (69% vs. 1.1%; $P<0.004$; Fig. 4H,I) as determined by senescence associated β-galactosidase activity. The K15+ cancer stem cell fraction was reduced in buparlisib treated SCC (0.3% vs. 2%; $P<0.03$; Fig. 4J,K). The Lgr6+ cancer stem cell fraction also was reduced in buparlisib treated SCC (0.2% vs. 17.1%; $P<0.0005$; Fig. 4L,M). These results indicate that PI3K is an important mediator of cancer stem cell chemokine signaling in SCC.

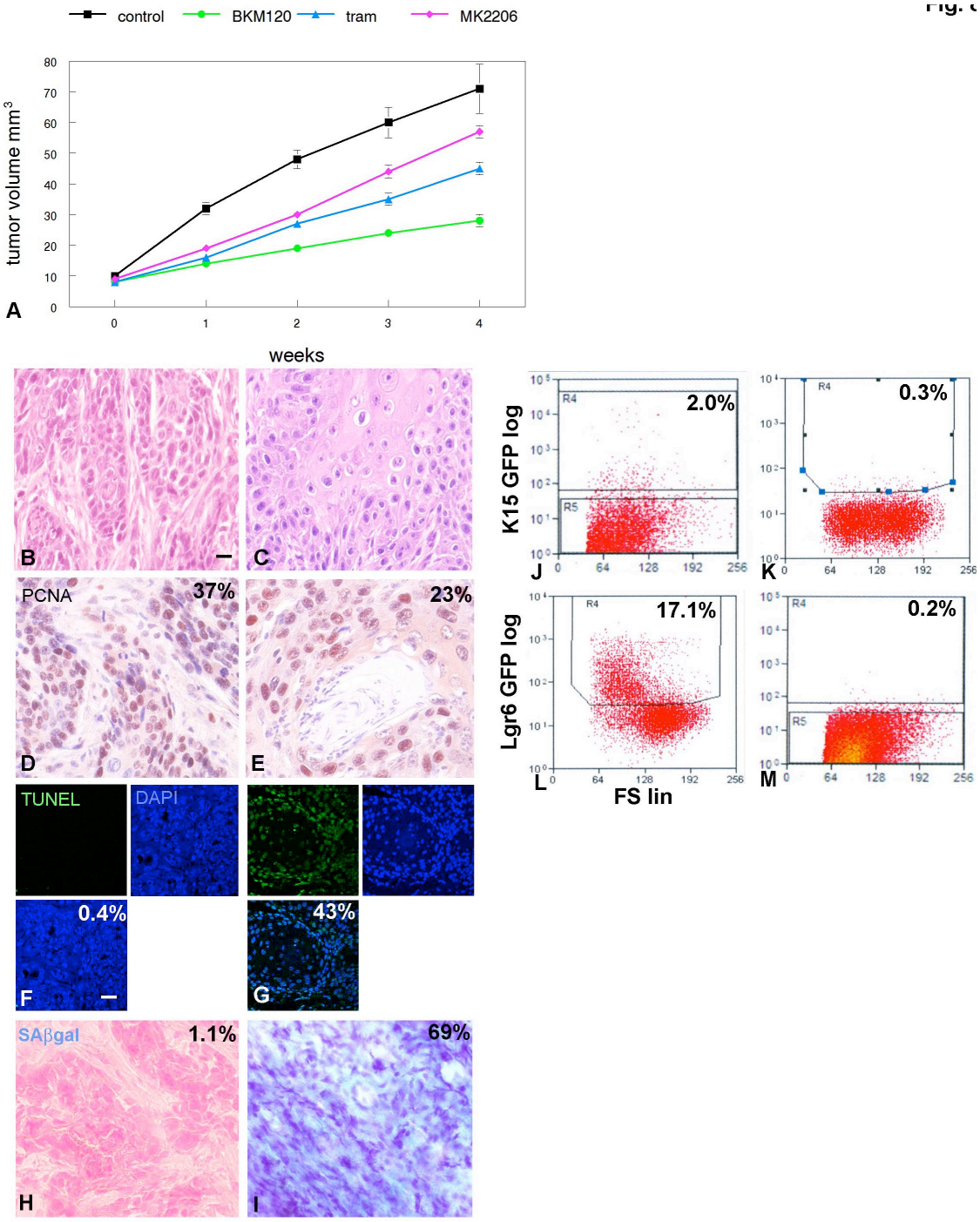
control
BKM120
tram
MK2206
tumor volume mm3
weeks
A
B
C
PCNA
37%
23%
D
E
TUNEL
DAPI
0.4%
43%
F
G
SAβgal
1.1%
69%
H
I
K15 GFP log
Lgr6 GFP log
FS lin
R4
R5
2.0%
0.3%
17.1%
0.2%
J
K
L
M

Fig. 4. PI3K signaling is the primary mediator of the effects of cancer stem cell chemokines on SCC. (A) Tumor volume of oral SCC treated with the PI3K inhibitor buparlisib, MEK inhibitor trametinib, Akt inhibitor MK2206 or vehicle for 4 weeks. Error bars indicate SEM. Histopathology of SCC from vehicle (B) or buparlisib (C) treated mice was determined by hematoxylin and eosin staining. Scale bar = 10 μm. Proliferating cells from vehicle (D) or buparlisib (E) treated SCC was determined by PCNA immunohistochemistry. Nuclei were counterstained with hematoxylin. Apoptotic cells in SCC from vehicle (F) or buparlisib (G) treated mice was determined by TUNEL analysis. Nuclei were counterstained with DAPI. Senescence associated β-galactosidase activity in SCC from vehicle (H) or buparlisib (I) treated mice. Nuclei were counterstained with nuclear fast red. Quantitation of K15/GFP+ cell fraction from vehicle (J) or buparlisib (K) treated SCC was determined by flow cytometry. GFP log and forward scatter linear scales are shown. Quantitation of Lgr6/GFP+ cell fraction from vehicle (L) or buparlisib (M) treated SCC was determined by flow cytometry.

5. DISCUSSION

K15+ stem cells secreted Cxcl chemokines which were primarily responsible for Lgr6+ cell proliferation and tumor progression. Non-stem glioma cells undergo senescence and secrete pro-angiogenic factors which increase tumor initiation of glioma stem cells (Ouchi et al., 2016). While senescence can lead to a tumor promoting inflammatory microenvironment, the senescence associated secretome is complex and may trigger tumor cell proliferation in the absence of inflammatory signals.

Chemokines mediate numerous functions in tumorigenesis, including proliferation, survival, angiogenesis, epithelial-mesenchymal transition, and metastasis (Albert et al., 2013). Cxcl1 expression was induced in stratified epithelia treated with phorbol ester (Shen et al., 2012), and Cxcl1 itself induced epithelial proliferation (Steude et al., 2002). Cxcl1 and Cxcl3 were expressed in human head and neck squamous cell carcinoma lines and patient tissue (Shintani et al., 2004; Ye et al., 2008; Wolff et al., 2011). Cxcr2 regulated head and neck cancer cell proliferation and migration in cell lines (Khurram et al., 2014). Cxcr2 expression was associated with cervical lymph node metastasis in human head and neck cancer (Qian et al., 2014). Ras transformation of mouse stratified epithelial cells induced Cxcr2 ligands, and these cells failed to form tumors when Cxcr2 expression was inhibited (Catalsson et al., 2009). While paracrine signaling from stromal cells to epithelial tumors has been extensively studied (Feller et al., 2012), our work provides a unique example of high level Cxcl secretion by one stem cell population (K15+) which regulates expansion of a

second stem cell fraction (Lgr6+) expressing high levels of the receptor for these ligands (Cxcr2). Depletion of the K15+ stem cell population or deletion of Cxcr2 receptor expression in Lgr6+ cells dramatically inhibits expansion of the latter population, and blocks tumor growth and metastasis. These studies indicate an important role for Cxcl chemokines and Cxcr2 in human head and neck cancer.

A striking result of our study was marked inhibition of metastasis in Cxcr2 null SCC. Given low levels of apoptosis in these tumors, the metastatic phenotype is difficult to attribute to increased programmed cell death, and our previous studies indicate that apoptosis in primary SCC does not correlate with metastatic phenotype (Bojovic et al., 2013). In contrast, increased senescence and terminal differentiation of SCC provides a likely mechanism for inhibiting metastasis. Reduction in the proliferating cell fraction likely inhibits genomic instability and therefore creation of potentially metastatic clones. This mechanism has therapeutic implications particularly for late evolving metastatic clones.

5.2 Limitations of the study

We examined cancer stem cell chemokine signaling in the present study; it is likely that the cancer stem cell secretome is highly complex and other soluble factors affect tumor cell proliferation, invasion, and survival. We examined targeted signaling pathways downstream of Cxcr2 in Lgr6+ cancer stem cells; there may be additional target genes which influence the observed phenotype of these cells. Our results do not rule out potential autocrine signaling between

K15+ cancer stem cells, or negative feedback loops which may exist between the Lgr6+ and K15+ cancer stem cell populations.

5.3 Future research

Future directions for this project include more complete characterization of the cancer stem cell secretome and elucidation of specific cancer stem cell signaling pathways which could be targets for molecular therapies and personalized medicine.

6. CONCLUSIONS

Selective depletion of the small K15+ stem cell fraction resulted in dramatic reduction of Lgr6+ cells and inhibition of oral tumorigenesis via senescence. Gene expression studies revealed that K15+ cancer stem cells regulate Lgr6+ cancer stem cell expansion via chemokine signaling. Genetic ablation of the chemokine receptor Cxcr2 inhibited cancer stem cell expansion and tumorigenesis via senescence. The effects of chemokines were primarily mediated by PI3K signaling, which is a candidate for targeted therapy in head and neck cancer.

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EDUCATION

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D.M.D 2011 - 2015
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B.S in Biology with Minor Chemistry 2007 – 2010
University of Illinois at Chicago
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LICENSURE AND BOARD CERTIFICATION

Licensed Dentist State of Illinois 2017
Temporary dental training license, Illinois Department of Professional Regulation 2015
North East Regional Board of Dental Examiners and American Board of Dental Examiners 2015
ACLS (Advanced Cardiac Life Support) 2016
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PROFESSIONAL ACTIVITIES

Predoctoral Periodontics Clinical Faculty as a Resident 2015-present
University of Illinois at Chicago

- Teaching and instructing third and fourth year dental students on periodontal evaluations, diagnosis and treatment planning.

Undergraduate Periodontics Specialty Honors Program 2014-2015

- Gain clinical experience in implant placement.
- Gain additional experience in diagnosis and treatment planning.
- Participate in postgraduate seminars and lectures.

Periodontics Teacher's Assistant 2014-2015

- Teach and assist dental students on periodontics fundamentals.

HONORS AND AWARDS

Leo and Wanda Sabien Scholarship 2015
UIC COD Award in the Exceptional Patient Care 2015

PROFESSIONAL ASSOCOATIONS

American Dental Association
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