Signaling Mechanisms Controlling Bony Invasion and Bone Destruction in Oral Squamous Cell Carcinoma

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Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in

Cancer Biology
December, 2016
Nashville, Tennessee

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This dissertation is dedicated to my family, blood relatives and not, who have given me the support to succeed and excel. And to my mother and my husband, who expect nothing less and nothing more, thank you for being my unwavering foundation.

ACKNOWLEDGEMENTS

This research would not have been possible without significant financial support from the NIH Grant CA163499 (J.A.S. & S.A.G.), VA Merit Award, DOD W81XWH-15-0622 (J.A.S), Core funding 1S10RR027027631 (uCT) and Vanderbilt Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, DK59637 and EY08126), VITCR Funding UL1TR000445 (CTSA Award Voucher #VR16993) (S.A.C & J.A.S.), Initiative to Maximize Student Diversity Award 2R25GM062459 (S.A.C.). I would also like to recognize my PI, Julie Sterling for her scientific advisement as well as her personal support and advice. As a successful woman in Science, she has been vital in communicating the unspoken culture in academic research to me. Also, special thanks are given to Linda Sealy, the chair of my committee, director of IMSD, and an invaluable resource for conflict resolution, academic guidance, career development and honest advice. My success at Vanderbilt would not have been possible without her support. I am also grateful for my two other committee members, Scott Guelcher and Stephen Brandt, for their professional input and advice. I am very appreciative that they allowed me the freedom to explore my dissertation project while also supporting my professional goals. I would also like to recognize the numerous people who helped me, both directly and indirectly, to achieve my goals, by being available to answer questions and give advice, donating materials and supplies, and also training me on equipment and techniques. Lastly, I want to give a special thank you to my "sister-wives", Denise Buenrostro and Ushashi Dadwal. These women have been instrumental in my personal growth and success. They have given many hours of their time to listen to me give mock presentations, practice more effective ways of communication, discuss project ideas and challenges, vent about life's cruelties, and more. Their perspectives and experiences have made me a more well-rounded scientist and individual in general.

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ABBREVIATIONS

BBS1, bardet-biedl syndrome 1

Dhh, desert hedgehog

DKK1, dickkopf-related protein 1

ECM, extracellular matrix

FAK, focal adhesion kinase

Fz, frizzled

GANT58, gli antagonist 58

GLI, glioma-associated oncogene homolog

GSK3, glycogen synthase kinase 3

Hh, hedgehog

HNSCC, head and neck squamous cell carcinoma

IFT88, intraflagellar transport protein 88

IGF6, insulin-like growth factor 6

Ihh, indian hedgehog

KIF3A, kinesin family member 3A

LiCl, lithium chloride

Lrp5/6, LDL receptor related protein 5/6

MMP, matrix-metalloproteases

NPDC1, neural proliferation differentiation control gene-1

OPN, osteopontin

OSCC, oral squamous cell carcinoma

PPS, poly-propylene sulfide

Ptch, patched

PTHR, parathyroid hormone receptor

PTHrP, parathyroid hormone-related protein

RANKL, receptor activator of nuclear factor kappa-B ligand

RhoA, rho GTPase A

ROCK1/2, rho-associated coiled-coil-containing protein kinase 1/2

Shh, sonic hedgehog

SIS3, selective inhibitor of smad3

SMO, smoothened

TAZ, tafazzin

TGFβ, transforming growth factor beta

TIBD, tumor-induced bone disease

TRAP, tartrate-resistant acid phosphatase

USP7, ubiquitin-specific protease -7

Wnt, wingless-related integration site

YAP, yes-associated protein

CHAPTER 1

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC)

Head and Neck Squamous Cell Carcinoma. Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer worldwide[1]. These tumors arise in the oral cavity, pharynx, nasal cavity, sinuses and salivary glands[2]. HNSCC has a higher incidence in men -almost two-fold- as compared to women. This is mostly attributed to lifestyle differences, though a genetic predisposition, especially in persons of Asian descent, has been found[3]. Use of alcohol, chewing tobacco and smoking are the strongest predictors of HNSCC, especially when these risks are combined[4]. Other important risk factors include poor oral heath, occupational/radiation exposure and endemic contributions from diet, namely use of betel quid or maté[5, 6]. More recently, HPV status, particularly HPV-16, has been significantly correlated with HNSCC and is now known to be a causative agent[7, 8]. The contribution of HPV status on increasing HNSCC incidence is observed mostly in oropharyngeal tumors, as compared to tumors of the oral cavity, or sinuses[8]. It is important to note that oral cavity tumors comprise the majority of HNSCC, with reports citing up to 50%, many of which arise in the tongue[2, 9]. These tumors are known as Oral Squamous Cell Carcinoma, and will be the focus of the work presented in this text.

OSCC etiology and pathology. OSCC is defined as cancer arising from the lips, first two-thirds of the tongue, buccal mucosa, gingiva, as well as the floor of the mouth and the soft/hard palate[8, 9]. OSCC etiology is not well understood, but it is generally accepted that many OSCC arise as the final stage of a series of oral conditions over the course of many years, starting with leukoplakia and ending with carcinoma[9]. Leukoplakia is a relatively common oral lesion that presents as a

white raised patch that cannot be scraped away but usually self resolves[10]. Dysplasia in leukoplakia is uncommon, but may progress to erythroplakia, which presents as a red raised lesion with clearly defined borders and increased vasculature[11]. Dysplasia in erythroplakia is common and at biopsy have been known to be malignant in persistent/advanced cases[12]. Leuoplakia and erythroplakia are known precursors for OSCC, although some OSCC arise without prior lesions or directly from leukoplakia[13].

Like many other tumors, OSCC presents with varying degrees of differentiation. Patients with well differentiated OSCC have the best prognosis of survival compared to moderately/poorly differentiated OSCC[14]. Gene signatures also vary among OSCC tumors. Mutations affecting p53, Ras, EGFR, and p16 have all been implicated as initiators and/or drivers of OSCC[15]. Some mutations can be grouped based on ancestry, but it is not known whether this is correlative or causative. Additionally, more research is needed to delineate the feasibility of successfully targeting these markers in patients.

Similar to other solid tumors, OSCC is staged according to tumor size, lymph node involvement, and presence of metastases. OSCC is rarely symptomatic in early stages and as such is often not diagnosed until Stage III[16]. Additionally, the vast majority of OSCC patients demonstrate immune suppression at the site of the tumor, further delaying symptoms and diagnoses[17]. While distant metastases best predict overall survival, invasion status remains the most important prognostic indicator for treatment failure and recurrence[18].

Invasion patterns of OSCC. OSCC invasion is characterized into two main types, erosive and infiltrative[19]. Erosive invasion presents pathologically as an invasive front, where tumor cells appear to a uniform front with a clear line of demarcation between the tumor and the host stroma[18, 19]. Molecularly, OSCC tumors with erosive invasion express high levels of cell to cell

adhesion proteins that are known to be associated with a less aggressive tumor cell phenotype, more indicative of epithelial cells[20, 21]. In contrast, infiltrative invasion presents pathologically as "finger-like" projects into surrounding tissue[19]. Histologically, individual as well as small clusters of tumors are observed ahead of the tumor front and can invade independently of the main tumor mass[22, 23]. Unlike single cell metastasis, these clusters can remain bound in groups, and are thought to contribute to secondary tumors, relapse, recurrence and local/distance metastases[23]. Molecularly, OSCC tumors with infiltrative invasion are known to be associated with a more aggressive tumor cell phenotype, indicative of mesenchymal cells. Patients whose tumors invade in an infiltrative manner respond less favorably to treatment and have less positive outcomes[24].

Current OSCC treatments. Unfortunately, OSCC treatment options have not significantly changed for almost 30 years[25]. Surgical resection remains the primary treatment option for OSCC, followed by radiation therapy[26]. Adjuvant treatments, such as platinum-based chemotherapy and hydroxyurea, are also available[27]. Most recently, the first and only targeted treatment for OSCC was FDA approved. This therapy, called Cetuximab, is a humanized anti-EGFR antibody and is used alone to treat metastatic disease, or in combination with other treatments for advanced or recurrent disease[28]. Nearly 75% of HNSCC overexpress EGFR, making it a suitable target for Cetuximab[29]. Unfortunately, many advanced stage tumors treated with Cetuximab quickly develop resistance which leads to patient relapse[30]. This is especially evident if local metastases to the lymph nodes and/or mandible are present.

OSCC recurrence and survival. The current therapies available for HNSCC patients remain limited, and over 40% of OSCC patients suffer from tumor relapse and recurrence after treatment[31]. OSCC patients whose tumors recur have poor prognoses and significantly lower

rates of overall survival[32, 33]. While the five-year survival rates of all OSCC patients is almost 55%, patients who recur have 5-year survival rates of only 30%, while patients without recurrence have upwards of an 80% 5-year survival rate[33].

Bone Biology

Physiological bone remodeling. Post-pubescent skeletons undergo constant, albeit low levels of remodeling. The two main cell types that maintain bone homeostasis are osteoblasts and osteoclasts. Osteoblasts are differentiated bone forming cells derived from mesenchymal stem cells, while osteoclasts are differentiated bone resorbing cells derived from monocytes. These cell populations have directly opposing functions that regulate one another in a tightly controlled feedback loop[34]. Osteoblasts express receptor activator of NFkB ligand (RANKL) on their cell surface which binds to RANK on pre-osteoclasts and facilitates their differentiation. As these cells differentiate, they fuse into large multinucleated cells. Differentiated osteoclasts secrete metalloproteinase enzymes that are tartrate resistant, and are known as tartrate resistant acid phosphatase (TRAP) positive cells[35]. Other hallmarks of a functional osteoclast include a "zone of attachment" or sealing zone, where the plasma membrane of the cell adheres to the bone surface through use of integrin mediated podosomes and a resorption pit, the space directly under the osteoclast. The ruffled border, which increases the surface area of the cell that can directly contact the bone, as well as Cathepsin K secretion are also required for resorption. Cathepsin K is a protease known to catabolize collagen as well as elastin. Combined with the acidic gradient produced in the resorption pit, TRAP, Cathepsin K, and other secreted enzymes, such as matrix metalloproteinases (MMPs) degrade the bone ECM, allowing dissolution of the inorganic component of bone, hydroxyapatite[36].

Importantly, bone resorption releases and activates large amounts of growth factors and cytokines, which are normally sequestered in the ECM. These released signaling molecules have several effects, including contributing to osteoblastogenesis and inducing osteoprotegerin (OPG) expression. OPG is a soluble receptor for RANKL, known as a decoy receptor because it binds free RANKL and therefore blocks RANK/RANKL binding, thus preventing further osteoclastogenesis. This coupling of osteoblast activity to osteoclast activity allows for balanced levels of bone remodeling, where bone is resorbed at about the same rate that it is made[37].

During development, both growth and ossification of the skeletal system is achieved largely via Hedgehog (Hh) signaling (Figure 1). Hh signaling is a highly conserved morphogen-based signaling pathway consisting of the activating receptor, Smoothened (Smo), and three ligands, Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh (Dhh). The receptor Patched (Ptch) is inhibitory, and downregulates Hh signaling. Early in development, cellular patterning of the limb bud is controlled predominantly by Shh. Acting as a classical morphogen, secretion of Shh forms a spatial and temporal gradient which controls cellular differentiation, polarization, and proliferation[38]. In contrast, Ihh is the predominant ligand governing Hh signaling for its role in bone formation.

Proper endochondral ossification is essential for long bone formation. Ihh controls endochondral ossification through a dynamic feedback loop that also controls growth plate development[39]. In this process, Ihh secretion from pre-hypertrophic chondrocytes at the ends of the bones induces expression of parathyroid hormone-related protein (PTHrP) in periarticular chondrocytes[40-42]. PTHrP is secreted and diffuses along the growth plate, which increases chondrocyte proliferation in the growth plate region, and induces them to deposits large amounts of collagenous extra-cellular matrix (ECM), extending the length of the developing bone. Beyond this region, levels of PTHrP drop and chondrocytes no longer proliferate but instead undergo hypertrophy and then

apoptosis[43]. Following, the resulting matrix and empty space is invaded by vasculature which is followed by osteoblast mediated mineralization, completing ossification.

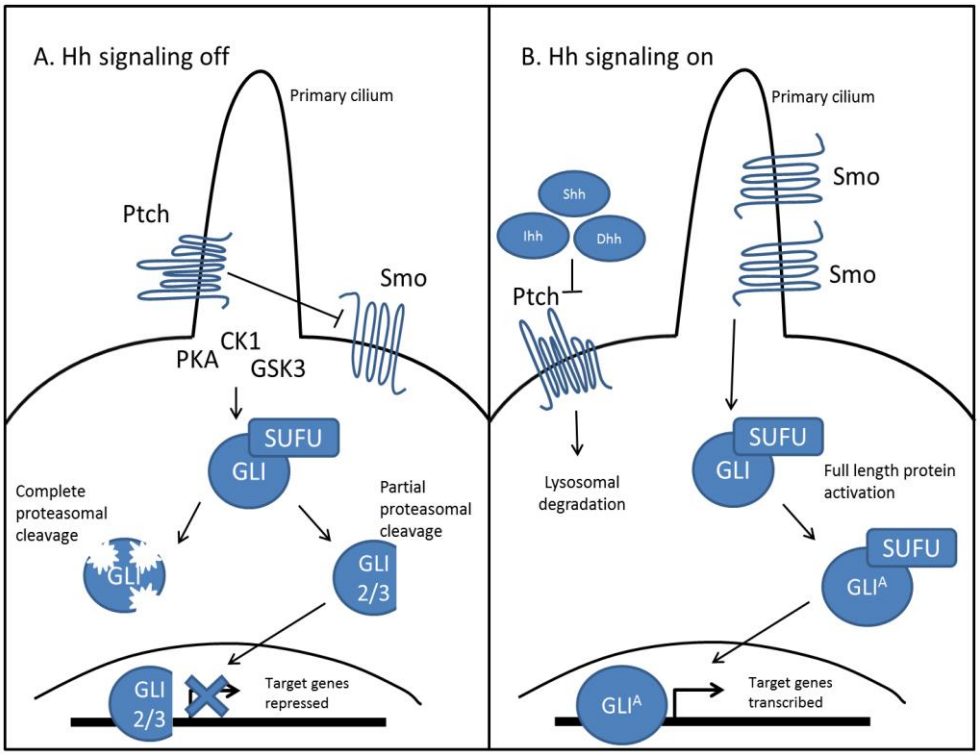


Figure 1. Canonical Hedgehog signaling in mammals is ligand dependent. **(A)** In the absence of Hh ligands, Ptch accumulates in the primary cilium and inhibits the function of Smo. Ptch facilitates the activation of several kinases (CK1, PKA, GSK3), at the base of the primary cilium, which differentially phosphorylate Gli protein. This can lead to complete degradation of Gli protein by the proteasome, as well as partial cleavage of Gli2 and Gli3. Partially cleaved Gli is translocated to the nucleus and functions as a transcriptional repressor for Hh target genes. **(B)** In the presence of Hh ligands, Shh, Ihh, or Dhh binds to Ptch, which induces its lysosomal degradation. This relieves its inhibitory effect on Smo, which accumulates in the primary cilium and prevents degradation of Gli proteins. Activated Gli protein is translocated to the nucleus and functions as a transcriptional activator for Hh target genes.

Non-physiological bone remodeling. Loss of Ihh during bone development has been shown to have detrimental effects on bone elongation and ossification[39]. One group demonstrated that loss of Ihh leads to premature chondrocyte hypertrophy, resulting in significantly shortened limbs lacking ossification. Additionally, levels of osteoblasts were also significantly reduced[44, 45]. Several groups have demonstrated that Hh signaling is critical for osteoblast differentiation, which is important for chondrogenesis and cartilage vascularization and thus endochondral ossification [46-48]. Intramembranous ossification also requires Hh signaling. Ihh null mice present with underdeveloped calvaria that show reduced ossification[39, 49]. Work done to understand this phenotype has demonstrated that Ihh regulates intramembranous ossification via osteogenic differentiation, and to some extent, proliferation[50]. Additionally, in these models, Ihh levels regulate BMP2/4 expression, suggesting that these pathways cooperate to control intramembranous ossification[50]. These findings appear to be central to Hh signaling, as partial loss of Gli3, which mainly functions as a repressor for Hh signaling, leads to increased ossification of calvarial bone. In these mouse models, it was observed that loss of one Gli3 allele led to craniosynostosis of the lambdoid sutures in the skull[51, 52].

Genetic Hh signaling defects also disrupt normal bone remodeling. Evidence for this includes the finding that Ptch1 deficient patients show increased bone mass due to increased osteoblast differentiation[53]. Conversely, in a mouse model utilizing activated Hh signaling in mature osteoblasts, bone density and quality were shown to be significantly reduced. This was found to be caused indirectly; in mature osteoblasts Hh signaling induces PTHrP expression which upregulates RANKL, increasing osteoclastogenesis[54]. This finding is physiologically relevant, as Shh is found to be upregulated in mature osteoblasts at fracture sites. Hh signaling in mature osteoblasts has been shown to be key for several processes, such as osteoblast proliferation and

differentiation, as well as healing by mediating vascularization[55-57]. Normal bone remodeling can also be disrupted extrinsically when Hh signaling is abnormally regulated in tumor cells that are also near the bone microenvironment.

Tumor induced bone disease. Advanced stage tumors, as well as aggressive tumors have an increased propensity to metastasize to distant sites of the body. Along with the lung and the liver, the bone is a common site of metastasis, where tumors in the bone disrupt normal bone remodeling and cause TIBD[58]. In patients, TIBD causes bone pain, hypercalcemia, and an increased risk of fracture, along with reduced skeletal mass and bone lesions[59]. Many tumor types, including breast, prostate, and lung, metastasize to bone via the circulatory system, while other tumors such as head and neck cancer invade directly into the facial bones from surrounding tissues. Primary bone tumors, such as osteosarcoma and multiple myeloma, also cause bone disease. While the molecular mechanisms controlling bone-disease varies by tumor type, all of the above-mentioned tumors dysregulate normal bone remodeling and induce excessive bone resorption in a process coined "The Vicious Cycle"[60]. In the vicious cycle, tumor cells that arrive at the bone microenvironment respond to chemical and physical cues by secreting factors that stimulate bone resorption. Cytokines such TGFβ and BMP present due to normal bone remodeling can stimulate tumor cells[61]. Additionally, our group has shown that the rigidity of the bone matrix can stimulate gene expression via mechanotransduction signaling[62]. As outlined in Figure 2, stimulated tumor cells, in a cell type specific manner, re-activate Hh signaling, which can be achieved by activating mutations in canonical Hh signaling receptor proteins Smo and Ptch, or non-canonically through other signaling pathways that lead to Gli activation such as TGFβ, PI3K/AKT, ERK and others. Increased Gli levels lead to expression and secretion of parathyroid

hormone-related protein (PTHrP), which increases RANKL production in osteoblasts, stimulating osteoclastogenesis.

Additional signaling factors such as interleukins 6/8/11, and TNF-α, also increase osteoclastogenesis in a similar manner, but have not been shown to be directly dependent on Hh

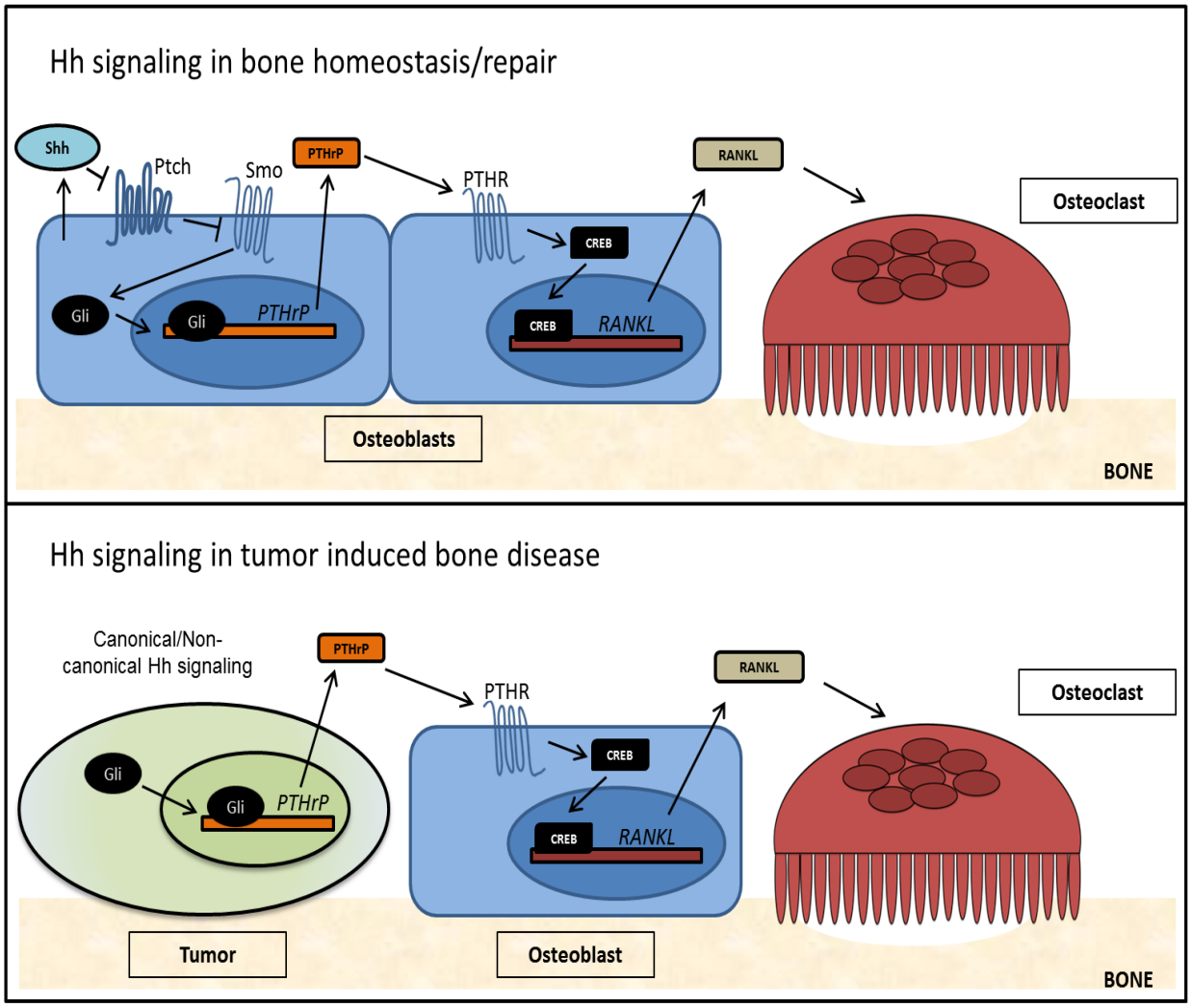


Figure 2. The Role of Hh Signaling in Bone **(A)** Hh signaling is active in normal bone remodeling and repair. Osteoblast derived Hh ligand activates Hh signaling cell autonomously as well as non-cell autonomously. *PTHrP*, a target of Hh signaling, is transcribed and PTHrP protein is secreted from the cell. Secreted PTHrP binds to its receptor PTHR on the surface of osteoblasts and activates the transcription factor CREB. *RANKL*, a target of PTHrP/PTHR signaling is expressed and RANKL protein is presented at the cell surface of osteoblasts, where it induces osteoclastogenesis and supports bone resorption. **(B)** Tumor-derived Hh signaling disrupts normal bone remodeling and induces TIBD. Tumor cells re-activate Hh signaling by canonical and/or non-canonical mechanisms, increasing Gli activity and Hh target genes. *PTHrP is* over expressed in these cells, and large amounts of PTHrP protein is secreted, which activates the PTHR receptor on osteoblasts. Following, large amounts of RANKL are produced by the osteoblasts, which lead to increased osteoclastogenesis and excessive bone resorption.

signaling[63-66]. Ultimately, as an increased number of osteoclasts mature and resorb bone, the released growth factors from the ECM further stimulate tumor cells, which causes a positive feedback loop. Tumor cells in the bone microenvironment uncouple osteoblast/osteoclast signaling, and lead to increased bone resorption as well as impair bone quality.

In breast cancer metastases to bone, this process is mediated by Hh signaling. Using bone metastatic MDA-MB-231 breast cancer cells, we have shown that TGFβ stimulation increases expression of Gli2 and PTHrP, which controls bone destruction[66]. In an experimental model of metastasis, tumor cells injected intracardiacally home to the skeleton where they proliferate and induce bone destruction. We show that when Gli2 expression is repressed using a plasmid over-expressing Gli2 repressor, both PTHrP expression and bone destruction are significantly reduced[67]. In this system, Gli activity does not seem to be regulated canonically, as bone tropic MDA-MB-231 cells do not express the Smo receptor nor do they respond to cyclopamine treatment[66]. A similar observation can be seen in malignant melanoma. Interestingly, while the melanoma cell lines used showed Smo and Ptch gene expression, Gli2 was found to be a transcriptional target of TGFβ signaling. Using 1205Lu melanoma cells that express high levels of Gli2, the authors showed that loss of Gli2 using shRNA led to a decreased incidence of bone metastases as well as smaller lesions[68].

Not surprisingly, primary bone tumors such as chondrosarcoma and osteosarcoma also dysregulate bone remodeling. Chondrosarcoma tumors originate from cartilage and are thought to arise due to activating mutations in Hh signaling[69]. Studies have shown that many primary chondrosarcomas express all canonical Hh signaling proteins, as well as PTHrP[69-73]. Using these tissues in primary organ cultures, one group showed that Hh signaling was constitutively active due to loss of PTCH1/2, activation of Smo or a combination of both. Additionally, using the Hh inhibitor,

triparanol, in a xenograph mouse model, the authors observed decreased proliferation, cellularity, and tumor size[69].

Aberrant Hh signaling inducing TIBD is also observed in head and neck tumors. Many OSCC tumors show activated Hh signaling as measured by immunohistochemical staining of Hh ligands in patient biopsies[74]. Levels of Hh related proteins also correlate with poor prognosis[75]. This is observed in a subset of OSCC patients whose tumors have invaded into the mandible and begins to proliferate in the bone, stimulating bone destruction[19, 76]. One group in Japan demonstrated that loss of Shh in OSCC cells decreased tumor volume as well as bone destruction in an *in vivo* model of bone destruction[77]. Here tumor cells stably expressing shRNA against Shh were inoculated into the tibia of immunocompromised mice. Our group has also observed and demonstrated OSCC dependency on Hh signaling. We utilized an orthotopic model of mandibular invasion and bone destruction, where tumor cells are injected into the masseter muscle and invade directly through underlying tissues into the mandible. Using this model, we injected human OSCC cells stably expressing shRNA against Gli2 and observed that loss of Gli2 decreased both bony invasion and bone destruction in male athymic mice. These studies highlight the importance of Hh signaling on TIBD and suggest that Hh signaling components are viable targets to prevent TIBD.

OSCC Invasion into the Mandible

In patients with OSCC, the mandible is the primary site of TIBD. The mandible is required for mastication and swallowing, but also plays an important structural role in ensuring proper speech, and breathing.

Anatomy of the mandible. The mandible, working with the maxilla, are load bearing bones, where the human jaw can generate biting forces upwards of 1,000 Newtons[78]. Mandibles contain mostly cortical bone, a dense, rigid, and compact bone layer that surrounds, protects, and supports

trabecular bone and other tissues inside bone structures. Like other bones, the mandible is innervated with blood vessels and nerves. The core of the mandible houses relatively small amounts of trabecular bone, along with bone marrow. Teeth are anchored in the mandible in the alveolar process, which remodels significantly over the course of a lifetime[79]. The tooth sockets are the primary mode of invasion by OSCC tumors into the mandible[19]. Tumors can also invade via nerve canals or by direct extension where tumors induce resorption at areas of bone contact[19]. OSCC patients whose tumors have invaded into mandibular space fare significantly worse[80].

Factors influencing mandibular invasion. Despite decades of research, factors influencing which tumors will invade bone are limited. Studies show that mandibular invasion correlates with tumor size as well as the site of the tumor[80]. Gingival tumors are closest in proximity to the mandible, and cause mandibular involvement about 50% of the time, but can be erosive or infiltrative tumors, which predict different outcomes for patients[19]. In contrast, the most common site of OSCC in patients is the tongue, which cause mandibular involvement in highly variable rates depending on the study (5% to 30%). It is important to note that regardless of the primary location or invasion status, patients whose tumors have invaded into the mandible suffer the same consequences, serious chronic pain, excessive bone resorption, and a significantly lower 5-year survival[80].

PTHrP regulation and effects. The bone resorption observed in OSCC patients with mandibular invasion occurs in a well characterized feedback system known as the Vicious Cycle[58]. Here, tumor cells that arrive at the bone microenvironment secrete factors that directly and/or indirectly stimulate bone resorption. There are several proteins capable of doing this, including PTHrP, RankL, IL-6, and TNF. PTHrP is expressed by many OSCC tumors and has been shown to be essential for bony invasion using syngeneic animal models[81]. PTHrP is small yet complex signaling protein originally identified for its role in hypercalcemia[82]. PTHrP is a highly

conserved gene of the PTH/PTHR gene family and has three known promoters which generates three isoforms (139, 141, and 173 amino acids) with differing functions[83]. The amino-terminus of PTHrP (Isoform 139) is secreted, and is a binding partner of PTHR, which signals through several pathways, including cAMP/PKA[84]. When PTHrP is transcribed without an export signal (Isoform 141), it remains in the cytosol and is shuttled into and out of the cell nucleus. This is regulated by both the cell cycle and the status of receptor signaling in the cell. Many studies report that intracellular PTHrP is shuttled to the nucleus in response to cell viability, and functions to prevent apoptosis[85-89]. The last isoform of PTHrP is least understood and is thought to signal completely independently of the other two isoforms. In OSCC patients, PTHrP expression correlates with a positive invasion status, and independently serves as a poor prognostic indicator[90].

Hedgehog signaling in OSCC. PTHrP expression is canonically regulated by Hh signaling, and this is also observed in many OSCC tumors[91]. *In vitro*, many OSCC cell lines have been shown to express Hh ligands, namely Shh, as well as the Hh effector proteins Gli1 and Gli2[75, 92]. Some studies highlight the role of aberrant Hh signaling as a driver of OSCC tumorigenesis. One study demonstrated that OSCC induced by the oral carcinogen 4NQO, had significantly higher levels of Shh, and further showed that significantly elevated levels of Shh could also be found in dysplastic lesions[93]. Many studies of OSCC investigate the effects of Hh signaling on promoting tumor progression, such as by increasing angiogenesis or by suppressing the immune compartment[18, 94-96]. Using immunohistochemical staining, Shh expression has been found to be linked to invasion, as levels of Shh correlate significantly with MMP-9 expression[97, 98]. Generally, the implications of Hh signaling in OSCC has been limited to correlation studies using patient samples and data. Though these are informative in predicting prognoses and identifying relevant targets,

molecular mechanisms explaining Hh signaling and its effects in OSCC is still largely unstudied. In our studies, we investigate the effect of Hh signaling in OSCC on mediating expression of PTHrP, which is required for tumor invasion into bone. The Hh effector protein Gli2, is expressed in nine of the ten OSCC lines tested and plays a major role in tumor invasion into the bone.

TGFβ signaling in OSCC. It is important to note that while Hh signaling is highly conserved, there are non-canonical mechanisms that can activate Hh signaling downstream of its receptor and induce expression of Hh related target genes[99]. In addition to increasing Hh components, these signaling systems may or may not retain their individual signal transduction profile, which makes understanding signaling mechanisms quite difficult. TGFβ signaling is one such signaling pathway that aberrantly increases Hh targets downstream of the Hh receptor, Smo[66, 67, 100]. Canonical TGFβ signals through dimerization and activation of TGFβ receptors, which increases phosphorylation of Smad2 and Smad3, which are translocated to the nucleus and increases expression of TGFβ target genes. In OSCC, and other tumor types, TGFβ activation increases Gli2 transcription by directly binding to the promoter region of Gli2 and inducing expression[99]. TGFβ signaling in OSCC has also been shown to induce expression of c-myc (a known driver oncogene in OSCC), and miR-455-5p, both of which have been implicated in supporting invasion in OSCC[101]. Even so, there are several studies with negative and/or conflicting reports involving TGFβ. In a cohort of 89 clinical OSCC samples, TGFβ was not found to correlate with known invasion biomarkers[102]. Non-canonical TGFβ signaling, which signals through p38/MAPK, can also increase Hh activation and contribute to OSCC tumorigenesis. MAPK activation phosphorylates MEF2C, which can transcribe Gli2[99]. Additionally, p38/MAPK activation was found to correlate with increased cell proliferation and decreased cell apoptosis when OSCC cells are treated with a GABRP agonist[103]. Collectively, it is clear that the role of TGFβ on

modulating OSCC is specific to the context of its activation. In our system though, the direct effect of TGFβ on increasing Hh activation is clear in several different OSCC cell lines tested.

Wnt signaling in OSCC. Wnt signaling is another highly conserved developmental signaling pathway with major roles in many organ systems including bone homeostasis, stem cell maintenance and cancer initiation[104, 105]. Wnt signaling is activated when Wnt ligands bind to and activate the Wnt receptor complex of Frizzled and LRP5/6 causing release of the Wnt effector protein, beta-catenin, from an inhibitory complex. Stabilized beta-catenin is then translocated to the nucleus and initiates transcription of target genes. There have been several studies highlighting Wnt target genes and/or beta-catenin as a mediator for oncogenic signaling. A recent study from 2016 demonstrated that during oral carcinogenesis, negative regulators of Wnt are found to be significantly silenced by gene methylation, highlighting it as an important epigenetic modification in OSCC[106]. Other data stress the importance of beta-catenin and downstream targets for OSCC invasion and migration, independent of upstream regulation[107, 108]. We have observed similar findings, where Wnt activation at the levels of beta-catenin transcribes genes important for OSCC bony invasion, such as Gli2.

Mechanical signaling in OSCC. Mechanical signaling is another pathway OSCC cells exploit during tumorigenesis and tumor progression. Mechanotransduction allows cells to sense and respond to changes in the ECM by converting mechanical forces into biochemical signals[109]. This primarily occurs through integrin signaling and subsequent downstream activation of FAK, Rho and ROCK and eventually leads to cytoskeletal rearrangements, activation of growth factor receptor signaling, and/or changes in protein trafficking and transports[110]. The oldest published work on OSCC ECM components have been found to be important in OSCC, especially in the context of OSCC invasion and metastasis[23]. For example, one group has shown that fibronectin

in the ECM contributes to cancer cell migration, while another group demonstrated that the ECM protein, thrombospondin, and increased MMP 3/9/11/13 expression via integrin signaling[22, 97, 111]. These studies are very recent and demonstrate the novel signaling mechanisms controlling OSCC. Other studies demonstrate that ECM components and the resulting mechanical signaling, are essential for many cell processes, such as directional migration, filopodium formation, and single cell metastases[22, 112, 113]. Unfortunately, there have been very few studies investigating the mechanisms of mechanical signaling in OSCC. Most of the research done have been correlative studies, either *in vitro* or through the use of patient samples. Our group has investigated the role of mechanical signaling on increasing Gli2 activation in OSCC grown on ECM of differing rigidities. Similar to other groups, we observed changes in FAK, Rho, and ROCK in response to changes in ECM. Collectively, Hh, TGFβ, Wnt, and mechanical signaling all play an important role in regulating OSCC.

Gli family regulation. The effector signaling molecule of Hh signaling is Gli, which is activated in canonical Hh signaling, but is also regulated by a number of non-canonical signaling mechanisms. There are three known members of the Gli family; Gli1, Gli2, and Gli3. Each Gli protein has specific functions, but is also known to overlap, oppose, and regulate one another in a context specific manner. The Gli family members are zinc finger proteins that can bind directly to DNA. The consensus promoter sequence is GACCACCCA-like. Gli1 is the best studied member of the Gli family and functions as a transcriptional activator of downstream target. Gli1 mRNA is constantly made and translated, but Gli1 protein is quickly degraded via ubiquitination-mediated proteasome degradation. When Hh signaling is activated, Gli1 protein is stabilized and very quickly accumulates in the cell, where it is shuttled to the primary cilium for activation in a process that is still very much not understood[114]. Activated Gli1 is then shuttled to the nucleus where it

transcribes genes important for cell cycle regulation, such as Cyclin D2, as well as Patched, which serves as a negative regulator of Hh signaling.

Gli member downstream targets. Other genes regulated by Gli1 include osteopontin, neural proliferation differentiation control gene-1 (NPDC1), ubiquitin-specific protease -7 (USP7), and insulin like growth factor binding protein-6 (rIGF-6)[115]. Gli2 binds to the identical consensus region as Gli1, yet can be extensively regulated independently of Gli1. Gli2 mRNA expression can potentially be regulated by a large number of transcription factors, including members of the JUN/FOS signaling family, MYC, CREB, and E2F, among others[116]. Gli2 has several alternate start sites, which leads to five distinct isoforms, some of which require proteasome processing for activation[117]. Four of the isoforms function as transcriptional activators, but the remaining isoform encodes truncated Gli2 protein that functions as a transcriptional repressor. Activated Gli2 transcribes Gli1 and Ptch, but can also regulate expression of HHIP1, BCL2, BMI1, SNAIL, SLUG, and TWIST[118]. Many of these targets serve a potential, but unstudied, role in OSCC tumorigenesis. It is important to note that while Gli1 and Gli2 have the potential to regulate many of the same targets, context specific signaling cascade status, as well epistatic regulation, leads to a complex yet interconnected target profile that varies between cell types[119]. Gli3 is processed as a truncated protein and functions as a transcriptional repressor of Gli genes. Gli3 has not been identified in OSCC with oncogenic potential, but may serve in the capacity of a tumor suppressor[120]. More recently, important roles for Gli3 signaling has been identified in early development, especially in the context of tissue polarization[121].

Oncogenic Gli in OSCC. In OSCC, there are very few published reports investigating Hh/Gli signaling. Hh/Gli signaling is a known oncogene in several tumor types, especially as a driver mutation in basal cell carcinoma[122]. In many other tumors, such as breast, prostate, lung, brain,

pancreatic and colon cancer, Hh/Gli signaling serves as an important oncogenic signaling pathway and can promote tumorigenesis[123-127]. Specifically, in OSCC, two independent studies have found that Hh signaling factors serves as a prognostic indicator as Ptch and Gli correlate with lymphatic metastasis, tumor reoccurrence and poor prognoses[74, 98]. Molecularly, Hh/Gli signaling in OSCC is known to be involved in invasion and EMT, though specific signaling mechanisms are not reported. In terms of OSCC invasion into bone, one group demonstrated that Hh/Gli signaling from tumor cells can directly influence osteoblast signaling *in vitro*, suggesting that early on, tumor in bone establishment may depend on osteoblastic specific signaling mechanisms[128].

PTHrP gene structure. Hh/Gli signaling is also essential in OSCC in the context of bone resorption, as Gli2 is known to be involved in controlling PTHrP expression. Transcriptional regulation of PTHrP has been studied extensively since its discovery, but has not been investigated recently. The PTHrP gene contains three distinct promoters and nine exons, leading to 15 possible mRNA transcripts. It is not known whether Gli protein directly binds to the PTHrP promoter, but there are several Gli binding sites upstream of the transcriptional start site of the second promoter.

PTHrP gene regulation. A seminal review published in 2010 discussed the known regulators of PTHrP[129]. Ets was the first transcriptional activator of PTHrP to be discovered, which regulates heavily in the P3 promoter and functions cooperatively with SP-1. Additional work done by Dittmer's group also identified Smads as an important binding partner of Ets in the P3 promoter. Lastly, ras/MapK signaling is also known to directly regulate PTHrP expression, though this regulation is most often observed in squamous cell carcinomas which suggests tissue specific regulation of PTHrP. Binding partners important for ras/MapK mediated PTHrP expression are not known. In fact, mutations in Sp-1, and Smad binding sites do not prevent ras/MapK induction

of PTHrP expression. These findings strongly suggest that other signaling pathways must be active to facilitate an increase in PTHrP, which highlights Gli as a possible regulator. In OSCC, we have several lines of evidence that support direct regulation of Gli2 on PTHrP transcription. OSCC require Gli2 to increase PTHrP promoter activity and mRNA expression and we have shown that this involves both Smad2/3 and p38/MapK. However, promoter mapping and promoter mutation experiments would need to be done to definitively prove this Gli2 binding to the PTHrP promoter.

CHAPTER II

HEDGEHOG AND TGFβ SIGNALING CONVERGE ON GLI2 TO CONTROL BONY INVASION AND BONE DESTRUCTION IN OSCC

Introduction

Oral Squamous Cell Carcinoma (OSCC) is a subset of head and neck squamous cell carcinomas (HNSCC), and accounts for the majority of cases[2, 9]. Known risk factors include smoking, alcohol, and chewing tobacco use[4]. Tumor resection remains the primary treatment for OSCC, although neoadjuvant and adjuvant therapies are often used[130]. These include radiation therapy, chemotherapy, neck dissection to remove lymph node metastases, and most recently, the EGFR inhibitory antibody, Cetuximab[28, 131]. These invasive tumors infiltrate cervical lymph nodes and the mandible, which is significantly correlated with increased local recurrence and decreased overall survival[32]. Tumors that invade into the mandible disrupt normal bone remodeling, and cause large amounts of bone destruction, chronic pain, and impairs eating and speaking abilities. These patients require surgical resection of the tumor-laden bone, which includes mandible reconstruction, followed by radiation therapy and other adjuvant treatments, including chemotherapy[132]. Unfortunately, OSCC patients have a 30-40% local recurrence rate, which leads to significantly reduced survival rates[33].

A syngeneic model of oral cancer demonstrated that PTHrP is required for OSCC invasion into the mandible[81]. PTHrP, a known regulator of tumor-induced bone disease, is expressed at low levels in healthy adult tissues, and thus it is a potential target for drug treatment[60, 133]. Unfortunately, the molecular mechanisms that control PTHrP expression in OSCC are not known. In long bone formation, PTHrP is regulated by canonical Hedgehog (Hh) signaling, where receptor proteins, Smoothened (Smo) and Patched (Ptch) control Gli protein and downstream target genes.

However, in the context of cancer, Gli can also be regulated by non-canonical Hh signaling[99], through direct Gli activation stemming from AKT, MEK, or S6K1 activity[134, 135]. Additionally, Gli can be regulated transcriptionally through other signaling pathways, where TGFβ and Wnt signaling can increase expression and induce activation of Gli2 independent of its upstream regulators[100, 136]. In OSCC, PTHrP expression can be stimulated by activation of Gli2 using TGFβ and our clinical data shows that Gli2 expression correlates with bony invasion. Here, we demonstrate that PTHrP expression is regulated by Gli2 and both Gli2 activity and PTHrP expression are controlled concomitantly through Hh and TGFβ signaling. Using an orthotopic *in vivo* model, we validate that bony invasion and bone destruction are regulated by PTHrP through modulation of Gli activity.

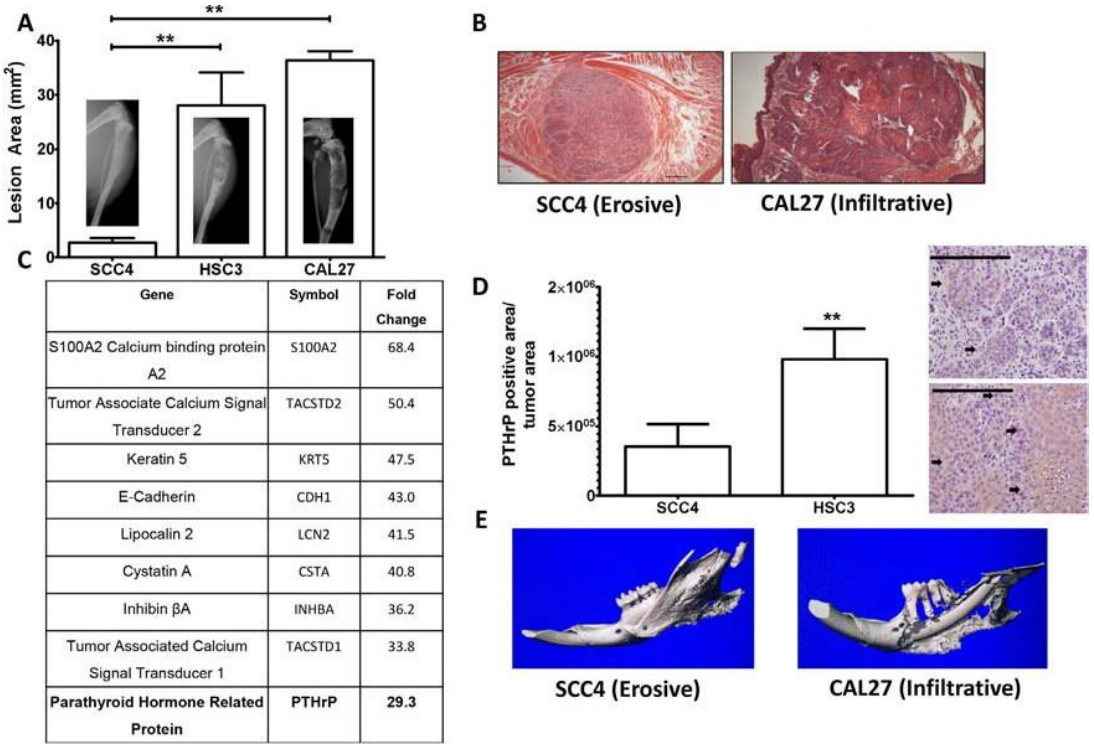
Results

PTHrP mRNA levels predict bony invasion and bone destruction in vivo. OSCC commonly invades with an erosive or infiltrative pattern. To discern between soft tissue invasion potential and bony invasion potential we utilized a well-established model of bone destruction, where tumor cells are inoculated directly into the tibia and allowed to establish for four weeks. Mice are then sacrificed and tumor burden and trabecular bone loss quantified. Using this model, we injected three human OSCC cell lines (SCC4, CAL27, and HSC3) and found that SCC4 cells showed minimal bone destruction. In contrast, CAL27 and HSC3 cells showed significantly more bone destruction (Figure 3A) while tumor burden remained similar to SCC4 cells (Figure 4A & 4B). To identify differences between OSCC cells capable of inducing destruction of mandibular bone compared to those that are incapable, we utilized a genome-wide microarray comparing SCC4 and CAL27 cells (Figure 3B and Table 1-2) to identify genes associated with bone destruction. SCC4 and CAL27 cells were derived from Caucasian males, who have the highest incidence of OSCC

in the United States[2]. Additionally, the site of origin for both cell lines were the tongue, correlating well with the finding that a considerable number of patients with OSCC of the tongue develop mandibular invasion[19]. Our micro-array analyses identified genes over-expressed in the CAL27 cells compared to the SCC4 cells, with several genes being involved in calcium signaling. Of notable interest was Parathyroid Hormone-related Protein (PTHrP) which showed 30-fold higher expression in bony invasive CAL27 cells (Figure 3C). PTHrP and Gli2 expression levels in CAL27 and SCC4 cells were verified using qRT-PCR along with eight additional OSCC cell lines. Nine of the ten cell lines tested expressed Gli2 and PTHrP (Figure 5A & 5B). To verify these differences *in vivo*, we used immunohistochemical staining to measure PTHrP and found that PTHrP secretion significantly correlated with the bony invasive HSC3 cells, while SCC4-injected tibiae showed very little PTHrP secretion (Figure 3D and Figure 6). In addition to bone destruction, PTHrP levels can also predict bony invasion[90]. To verify this correlation *in vivo*, we used an orthotopic model of bony invasion, where tumor cells are injected into the masseter muscle adjacent to the mandible and directly invade into the surrounding tissues. SCC4 cells have minimal destructive effect on mandibular bone, while CAL27 cells induce significant amounts of bone loss (Figure 3E). These findings correlate OSCC PTHrP levels with bony invasion and bone destruction in two separate models.

OSCC express PTHrP in a Gli2-dependent manner. We evaluated the contribution of Gli2 to regulation of PTHrP expression in OSCC using Gli Antagonist 58 (GANT58), a small molecule inhibitor specific to Gli[137]. Gli2 inhibition significantly decreased PTHrP expression at both basal and stimulated levels in several OSCC lines (Figure 7A–7C). Additionally, Gli2 is sufficient to increase PTHrP expression, as Gli2-SA662 overexpression induced by plasmids significantly increased PTHrP expression (Figure 7D & 7E) [138]. We observed that wild-type over-expression

Gli plasmids does not lead to increased PTHrP promoter activity (Figure 8A), but because Gli2 expression is undetectable in many adult tissue compartments (except for basal/ stem cell compartments), we surmise that cells with Gli2 activity have reactivated normally silenced signaling mechanisms, highlighting its potential importance in OSCC tumorigenesis[75]. To determine if Gli2 is required for PTHrP expression, we used shRNA to stably knock-down Gli2 expression. Loss of Gli2 prevented stimulated PTHrP expression (Figure 7F) but did not affect cell viability (Appendix A). Together, our findings suggest that Gli2 modulates PTHrP and is required for its expression.



Gene	Symbol	Fold Change
S100A2 Calcium binding protein A2	S100A2	68.4
Tumor Associate Calcium Signal Transducer 2	TACSTD2	50.4
Keratin 5	KRT5	47.5
E-Cadherin	CDH1	43.0
Lipocalin 2	LCN2	41.5
Cystatin A	CSTA	40.8
Inhibin βA	INHBA	36.2
Tumor Associated Calcium Signal Transducer 1	TACSTD1	33.8
Parathyroid Hormone Related Protein	**PTHrP**	**29.3**

Figure 3. PTHrP mRNA levels predict bony invasion and bone destruction in two mouse models.
(A) Tumor model of bone destruction. X-ray data of SCC4 cells injected into the tibiae of athymic mice show significantly less bone destruction than HSC3 and CAL27 cells. (**B)** H&E stained histological sections of SCC4 and CAL27 tumors taken from tip-of-the-tongue injections in athymic mice show morphological differences. SCC4 cells bear an erosive phenotype, while CAL27 cells bear an infiltrative phenotype. (**C)** PTHrP is upregulated almost 30-fold in bony invasive OSCC. The top ten upregulated genes as determined from a genome wide microarray study comparing mRNA levels of CAL27 cells (bony invasive OSCC) vs SCC4 (non-bony OSCC). PTHrP is highlighted because it is known to be essential for OSCC bony invasion. (**D)** PTHrP levels correlate with bony invasion status. SCC4 cells injected into the tibiae of athymic mice show low PTHrP staining by IHC, while HSC3 cells show significantly larger amounts of PTHrP expression, as denoted by the black arrows. (Images at 40X, scale bar is 200μm) (**E)** Orthotopic model of bony invasion. Representative μCT scans of mandibles dissected from mice bearing tumors from masseter muscle injections. SCC4 cells show minimal bone destruction and small amounts of new bone formation, while CAL27 cells show extensive bone destruction.

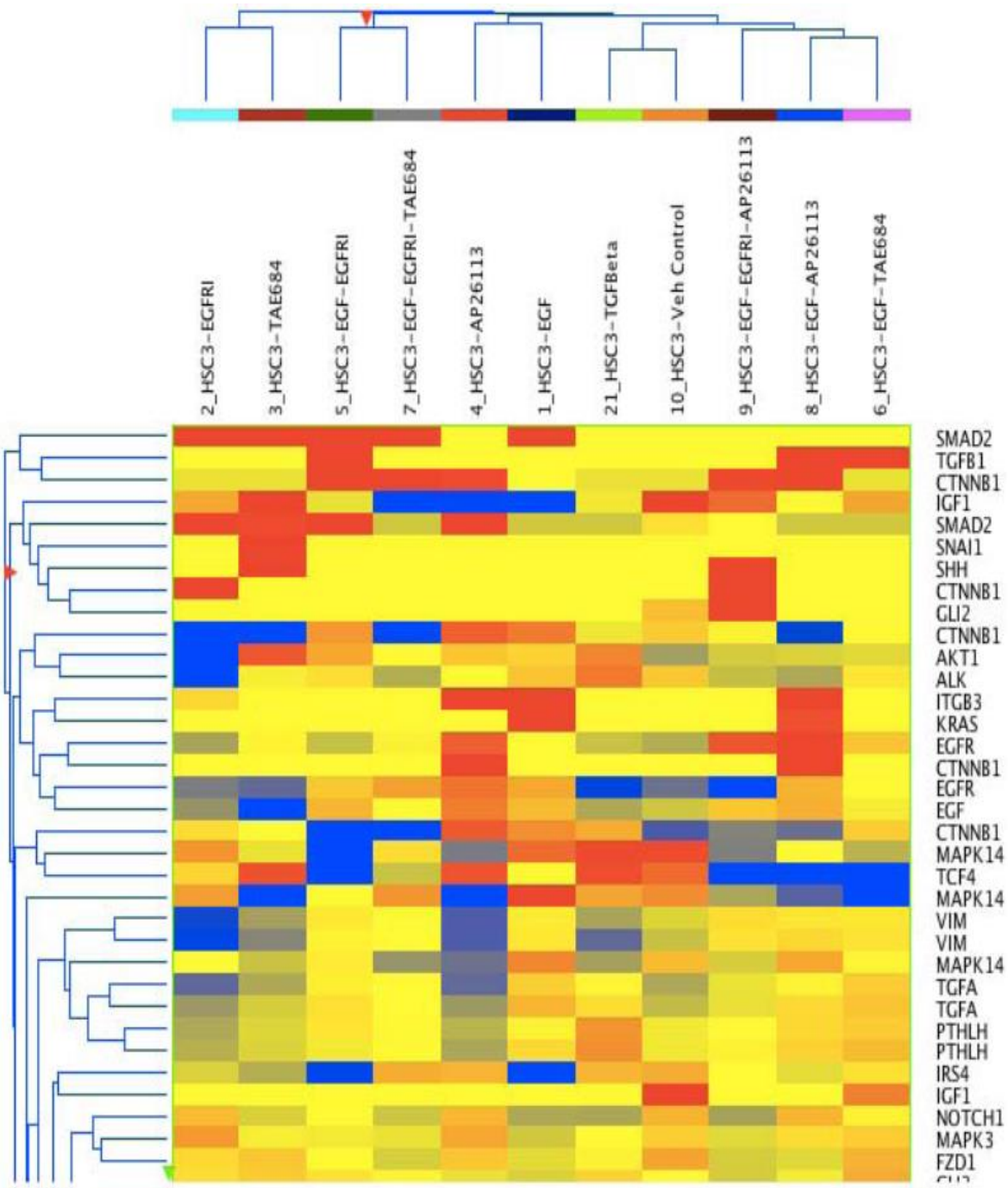


Table 1. HSC3 microarray data

mRNA expression of the indicated genes is shown via heat map, where gray represents no change in gene expression compared to control. Light blue and dark blue indicate downregulation in the two and four-fold range respectively, while orange and red indicate upregulation in the two and four-fold range, respectively.

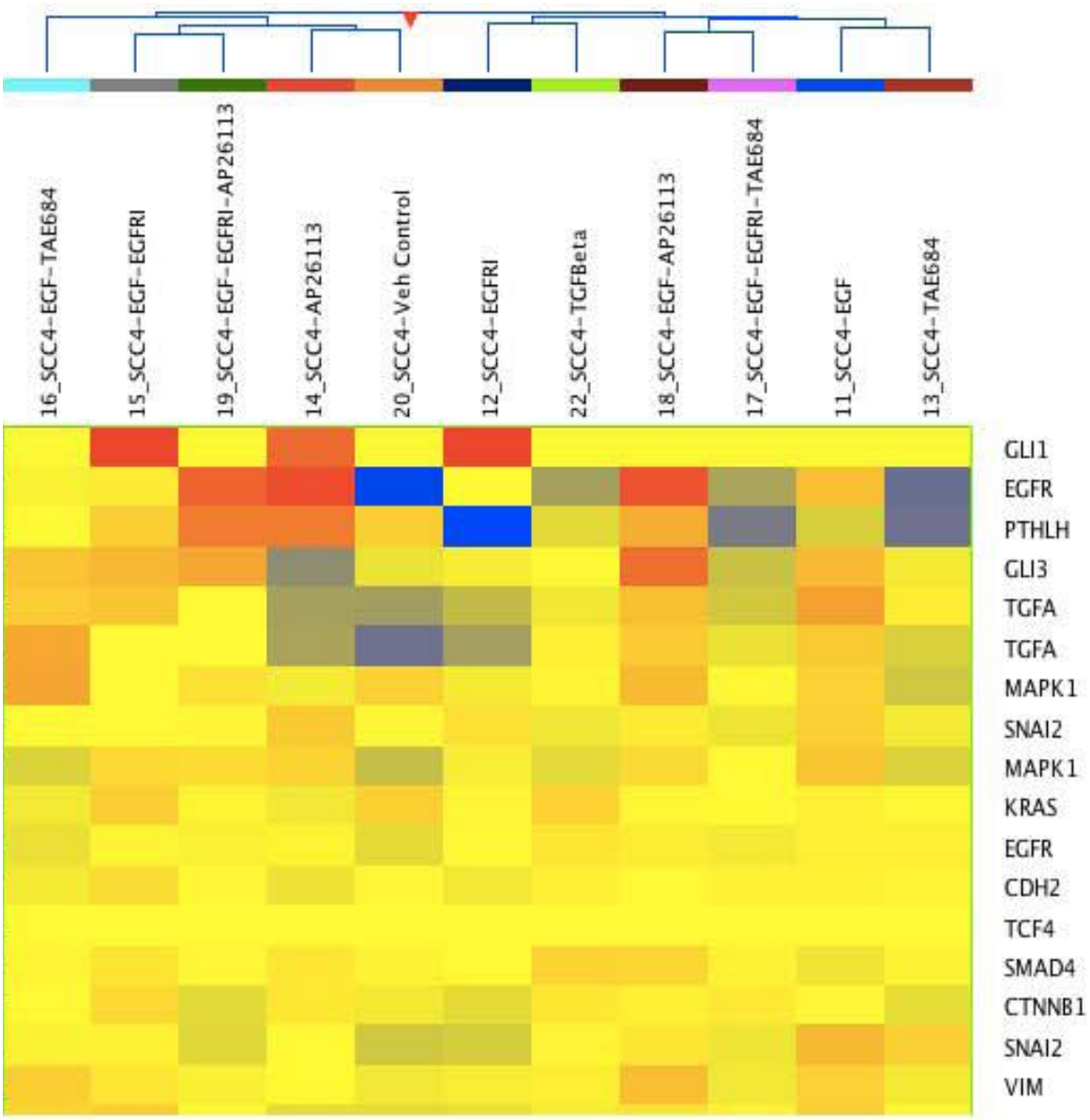


Table 2. SCC4 microarray data

mRNA expression of the indicated genes is shown via heat map, where gray represents no change in gene expression compared to control. Light blue and dark blue indicate downregulation in the two and four-fold range respectively, while orange and red indicate upregulation in the two and four-fold range, respectively.

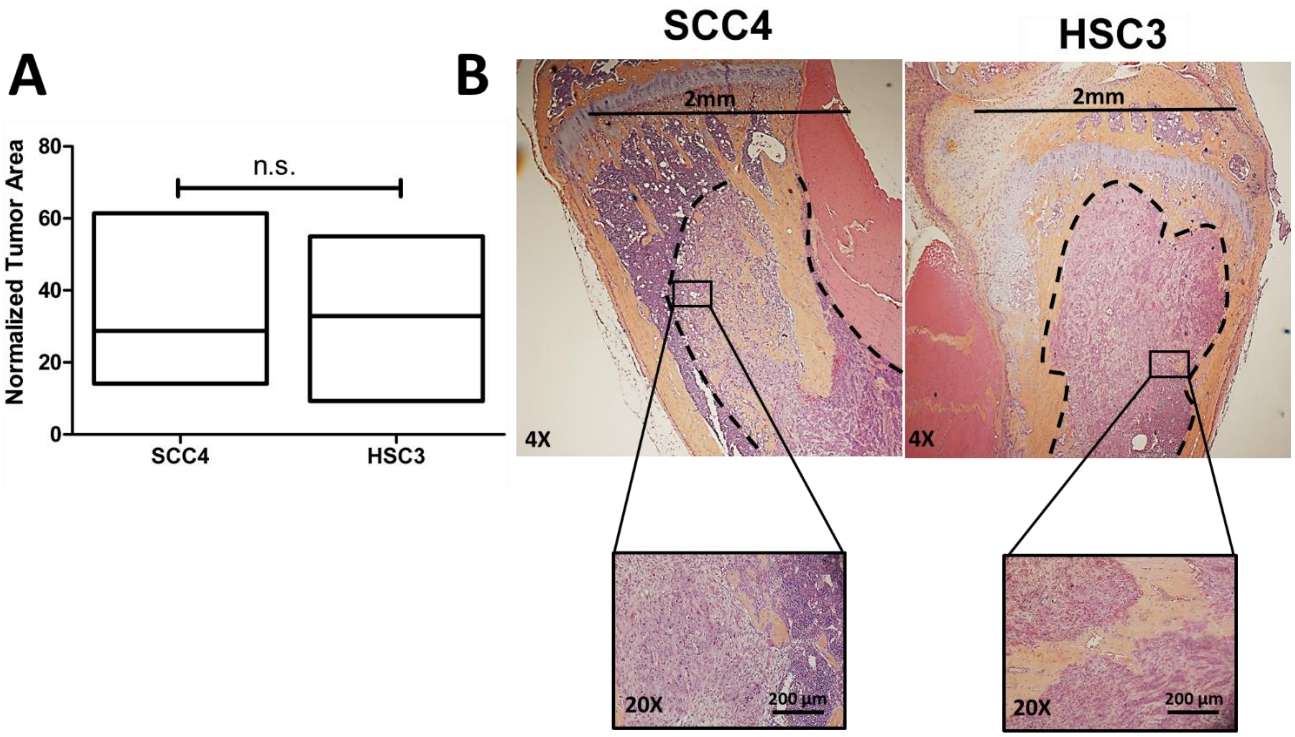


Figure 4. SCC4 and HSC3 tumor burden is not significantly different *in vivo*
(A) SCC4 and HSC3 tumors have comparable tumor area. SCC4 and HSC3 tumors cells injected into the tibia have tumor areas that are not statistically significant by histological analyses. **(B)** Representative images of SCC4 and HSC3 tumor bearing tibias show similar tumor burden. 4X and 20X representative images of H&E stained tibia sections show similar tumor burden.

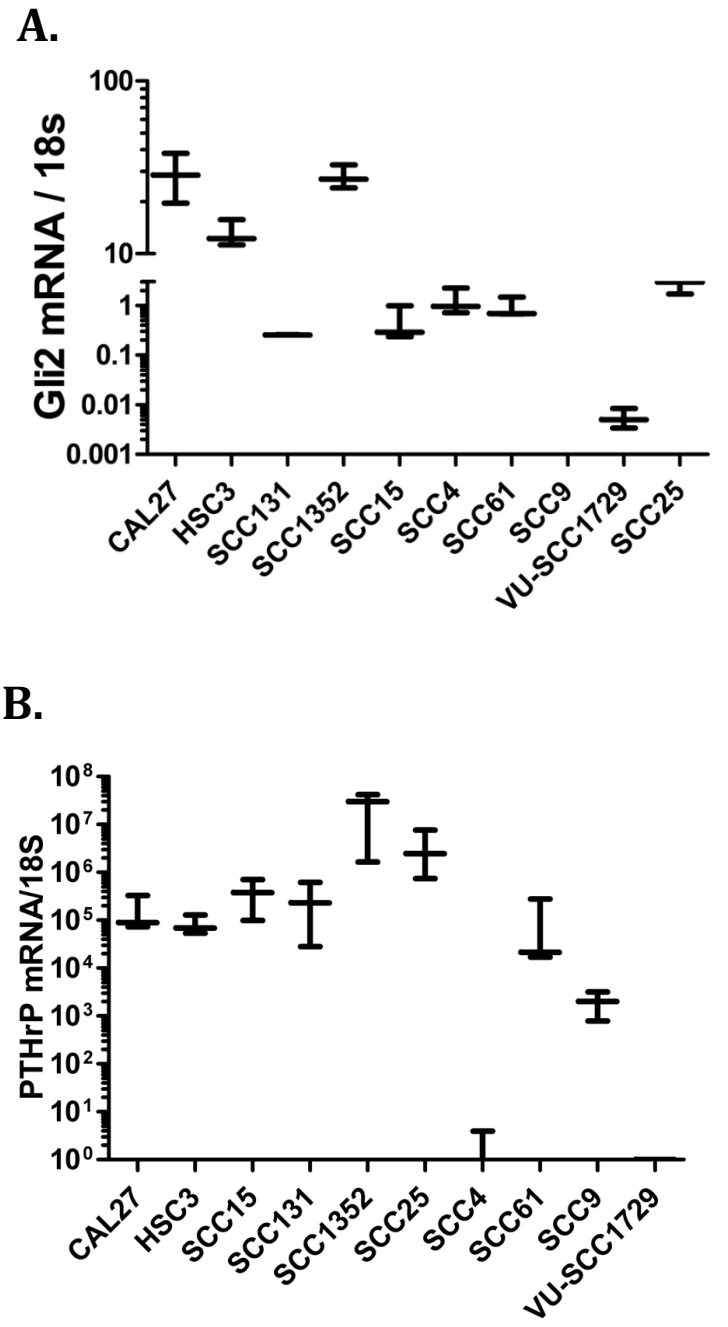


Figure 5. Gli2 and PTHrP is expressed by nine of ten OSCC cell lines tested. (A) OSCC express varying levels of Gli2. By qRT-PCR, all OSCC cell lines tested, except SCC9, expressed
Gli2 mRNA at differing levels when kept in serum free conditions for 24 hours. **(B)** OSCC express varying levels of PTHrP. By qRT-PCR, all OSCC cell lines tested, except VU-SCC1729, expressed PTHrP mRNA at differing levels when kept in serum free conditions for 24 hours.

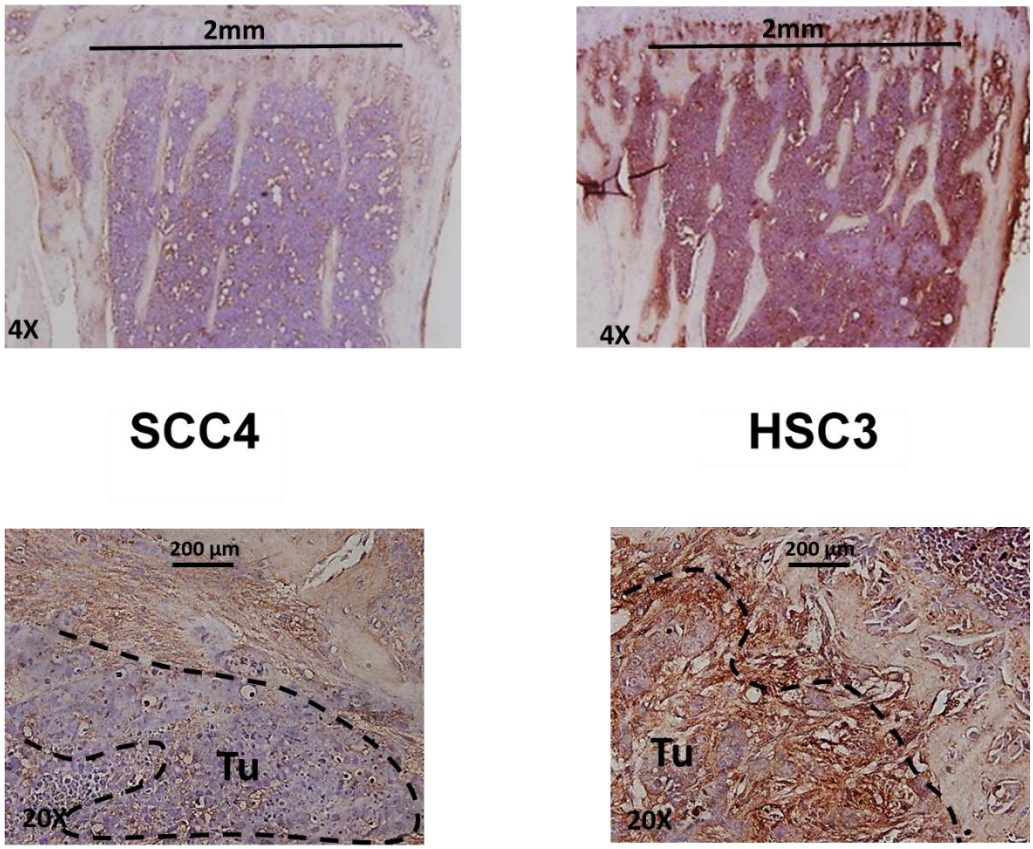


Figure 6. Bony invasive status correlates with PTHrP levels *in vivo*. The bony invasive OSC cell line HSC3 express significantly more PTHrP protein than the non-bony invasive SCC4 OSCC cell line. Representative images (4X and 20X) of SCC4 or HSC3 cells injected into the tibia demonstrate different levels of PTHrP protein as shown by IHC staining. Black dotted lines indicate tumor front.

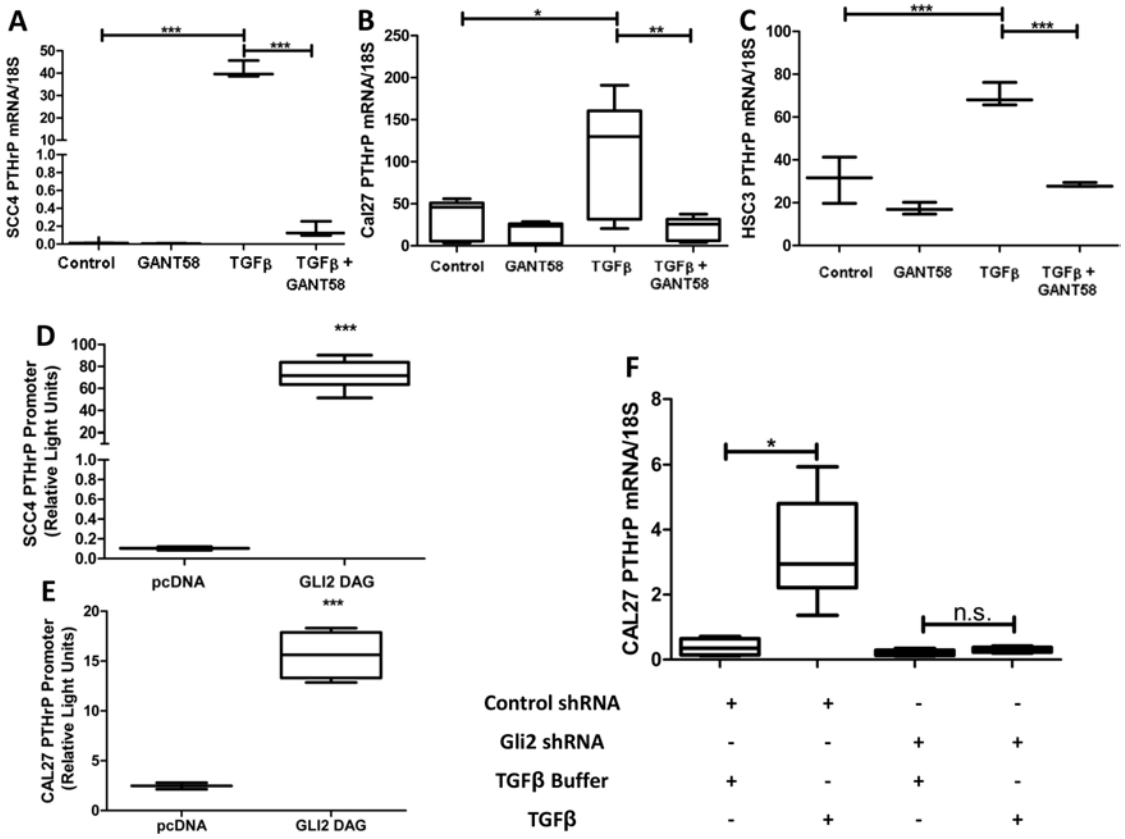
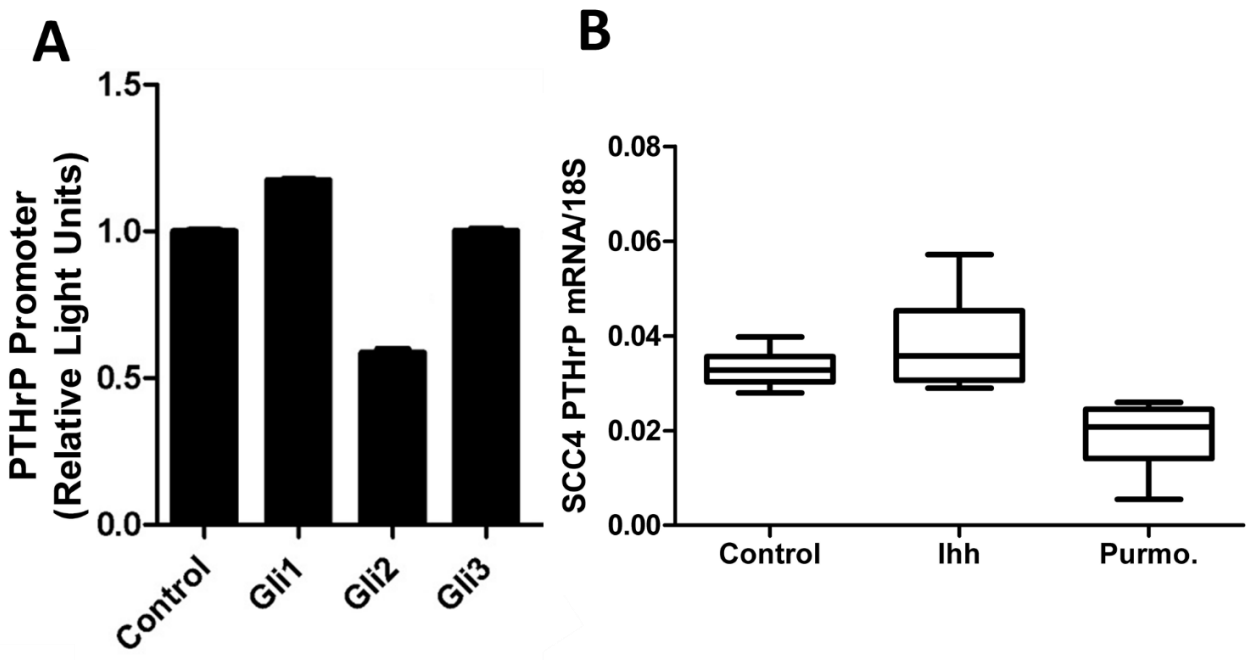


Figure 7. OSCC express PTHrP in a Gli2 dependent manner. (A-C) Gli2 inhibition decreases basal PTHrP expression and suppresses TGFβ induced PTHrP expression. qRT-PCR was used to determine PTHrP mRNA levels of three OSCC cell lines (CAL27, SCC4, and HSC3) that were treated with 10uM of the Gli inhibitor, GANT58 or the solvent control DMSO, with or without the addition of 10ng/ml TGFβ, which induces PTHrP expression. In all groups GANT58 treatment significantly decreased PTHrP expression. (**D** & **E)** Gli2 over-expression significantly increases PTHrP expression. SCC4 and CAL27 cells were co-transfected to overexpress Gli2 SA662 Flag (Gli2 protein resistant to ubiquitination based proteosomal degradation), as well as a PTHrP firefly luciferase reporter plasmid and a constitutively active Renilla luciferase reporter plasmid. 48 hours later cells were harvested and firefly activity quantified. In both lines, Gli2 over-expression increased PTHrP. (**F)** shRNA mediated Gli2 silencing prevents TGFβ induced PTHrP expression. qRT-PCR was used to determine PTHrP mRNA levels in CAL27 cells transfected with non-coding hairpins or a pool of four independent hairpins against Gli2. Both groups showed low basal expression of PTHrP, but when treated with TGFβ, control cells significantly increased PTHrP mRNA expression while Gli2 deficient cells were unable to increase PTHrP mRNA.



Canonical Hh activation does not increase PTHrP expression levels
Figure 8. (A) Gli protein overexpression does not increase PTHrP promoter activity. SCC4 cells transfected to overexpress Gli protein do not increase PTHrP promoter activity after 24 hours as shown by DLR assay. **(B)** Exogenous Hh ligand does not increase PTHrP mRNA expression. SCC4 cells treated with Ihh ligand or purmorphamine do not increase PTHrP mRNA expression after 24 hours of treatment.

Hedgehog receptor signaling is required for Gli2 activity and PTHrP expression. As the canonical regulator of Gli2, we examined the role of receptor based Hh signaling on regulating Gli2 activity and PTHrP expression using purmorphamine, a Smo-specific agonist, or cyclopamine, a Smo-specific antagonist. We demonstrate that purmorphamine significantly increased Gli2 promoter activity (Figure 9A & 9B) as well as Gli2 protein activity using luciferase-based reporter assays (Figure 9C). As expected, cyclopamine significantly decreased stimulated Gli2 expression (Figure 9D) and prevented an increase of stimulated levels of PTHrP, highlighting the requirement of canonical Hh signaling for PTHrP expression (Figure 9E). Unexpectedly, purmorphamine did not significantly increase PTHrP expression (Figure 9F & 9G) and activation of Smo using Indian hedgehog protein (Ihh) showed similar results (Figure 8B). To determine if Hh signaling was being silenced by proteasome mediated Gli degradation, we used bortezomib, a

proteasome inhibitor, and measured PTHrP expression. While bortezomib treatment doubled PTHrP levels in cells treated with PURMO, this increase was quite negligible as compared to TGFβ. To investigate this further, we stimulated cells with either TGFβ or PURMO, and then treated them with Actinomyosin D (to prevent transcription) for different time periods. This was done to investigate whether these treatments affected PTHrP mRNA stability. We observed that canonical Hh signaling potently, but very transiently, increases PTHrP expression (Appendix B). Collectively, these observations suggest that Hh signaling is necessary but not sufficient for PTHrP expression, and thus other pathways are required.

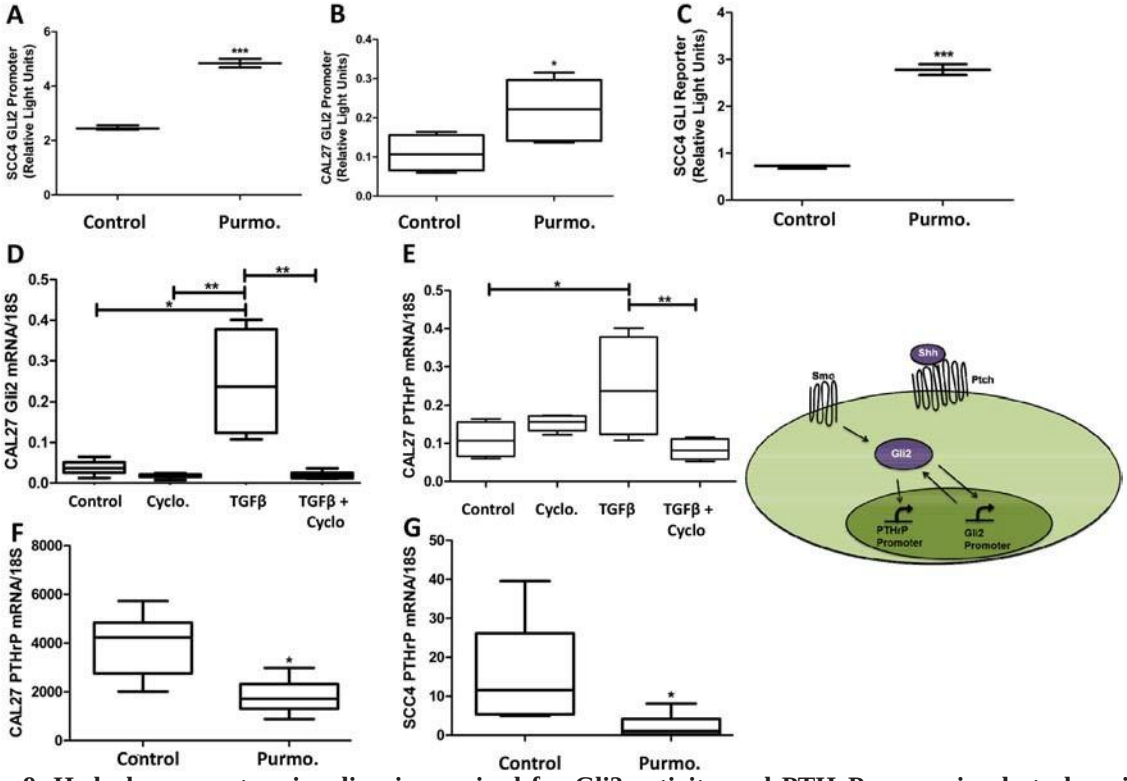


Figure 9. Hedgehog receptor signaling is required for Gli2 activity and PTHrP expression but alone is not sufficient for PTHrP expression. (A & B) Hh stimulation increases Gli2 promoter activity. OSCC cells were transfected using luciferase based reporter plasmids to assay endogenous Gli2 promoter activity and then stimulated with 10uM purmorphamine, resulting in a significant increase in Gli2 promoter activity. (**C)** Hh stimulation increases Gli protein activity. OSCC cells were transfected using luciferase based reporter plasmids to assay Gli2 protein activity, and then stimulated with purmorphamine, resulting in a significant increase in Gli2 protein activity. (**D** & **E)** Canonical Hh signaling is required for both Gli2 and PTHrP expression. qRT-PCR was used to determine PTHrP mRNA levels of CAL27 cells that were treated with 12nM of cyclopamine, or the solvent control DMSO, with or without the addition of TGFβ. Cyclopamine decreased basal PTHrP expression as well as significantly inhibited TGFβ-induced PTHrP expression. Inset demonstrates the dual role of Gli2 protein to increasing Gli2 and PTHrP expression. (**F** & **G)** Hh stimulation is not sufficient to increase PTHrP expression. qRT-PCR was used to determine PTHrP mRNA levels of CAL27 cells that were treated with purmorphamine, which did not induce PTHrP expression, but instead decreased basal levels of PTHrP, suggesting a possible feedback loop.

TGFβ signaling modulates Gli2 and PTHrP expression. Studies on breast cancer metastasis to bone highlight the importance of PTHrP for regulation of bone destruction by a non-canonical Gli2-dependent mechanism through TGFβ signaling[66, 67]. Several lines of evidence support TGFβ signaling induction of Gli independent of Hh signaling[100, 139, 140]. We examined the role of TGFβ on regulating Gli2 and PTHrP and found that TGFβ stimulation significantly increased levels of PTHrP expression (Figure 10A & 10B). Moreover, increased PTHrP expression is facilitated through canonical TGFβ signaling, since the use of Smad protein over-expression plasmids led to a marked increase in PTHrP expression (Figure 10C). Inhibition of canonical TGFβ signaling using the Smad3 inhibitor, SIS3, decreased PTHrP expression (but not significantly), and inhibition of non-canonical TGFβ signaling using the p38/MAPK inhibitor, SB202190, significantly decreased Gli activity and PTHrP expression (Figure 10D & 10E). Importantly, the TGFβ mediated increased of PTHrP expression correlates with an increase in both Gli2 promoter and protein activity levels (Figure 10F & 10G).

Intracellular Wnt signaling increases PTHrP expression through crosstalk with Hh signaling. Wnt signaling plays an essential role in bone biology, and research has demonstrated its significance in supporting metastases to bone[64, 141]. We explored the role of Wnt signaling in OSCC to increasing PTHrP expression using Lithium Chloride (LiCl) to inhibit activity of glycogen synthase kinase-3β (GSK-3), a negative regulator of Wnt signaling. Wnt activation was found to have no effect on PTHrP expression (Figure 11A & 11B). Additionally, qRT-PCR for the Wnt target gene, DKK1, showed no significant changes in expression between control and LiCl-stimulated cells (Figure 11C), suggesting that canonical Wnt signaling is inactive. Since Wnt signaling may play a role in PTHrP expression downstream of GSK-3, we evaluated the role of β-

catenin, the downstream effector protein, on PTHrP expression. We found that β-catenin over-expressing plasmids increased PTHrP expression levels.

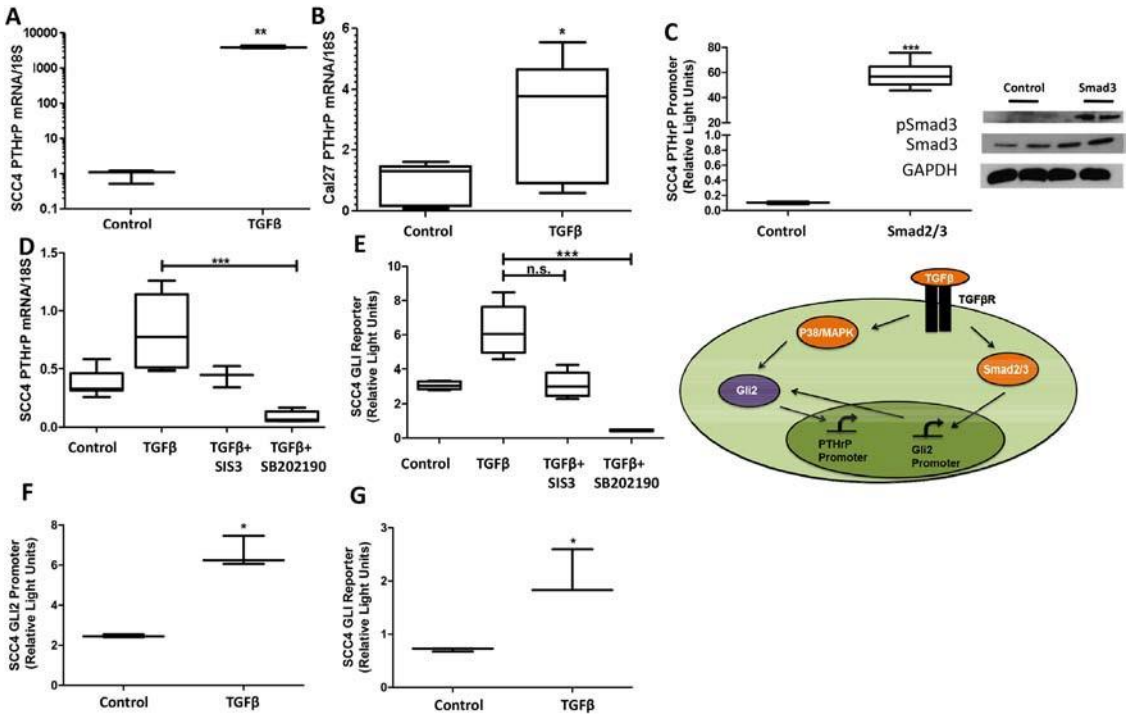


Figure 10. TGFβ signaling modulates Gli2 and PTHrP expression. (A & B) TGFβ signaling increased PTHrP expression. qRT- PCR was used to determine PTHrP mRNA levels of SCC4 and CAL27 cells that were treated with TGFβ or the buffer control. TGFβ treatment significantly increased PTHrP in all cell lines tested. (**C)** Smad2/3 over-expression increased PTHrP expression. SCC4 cells were co-transfected to overexpress equal amounts of Smad2 and Smad3 (see insert for protein confirmation by Western blot), as well as a PTHrP firefly luciferase reporter plasmid and a constitutively active Renilla luciferase reporter plasmid. 48 hours after transfections, cells were harvested and firefly activity quantified. Smad over-expression significantly increased PTHrP promoter expression. (**D** & **E)** Canonical and non-canonical TGFβ inhibition decreased Gli activity and PTHrP expression. qRT-PCR was used to determine PTHrP mRNA levels of SCC4 cells treated with TGFβ, and SIS3, a Smad3 inhibitor, or SB202190, a p38/MAPK inhibitor. While Smad3 inhibition trended to significantly decrease Gli activity and PTHrP expression, only p38/MAPK inhibition significantly decreased both. (**F** & **G)** TGFβ signaling increased Gli2 promotor and protein activity. SCC4 cells were co-transfected with an endogenous Gli2 promoter construct, or a Gli2 protein reporter construct, as well as a constitutively active Renilla luciferase reporter plasmid. 24 hours after transfections, cells were treated with TGFβ or the buffer control and harvested 24 hours later before firefly activity was quantified. Both promoter and protein activity of Gli2 was significantly increased with TGFβ signaling. Inset demonstrates the dual role TGFβ signaling has on increasing Gli2 at the level of mRNA as well as protein.

PTHrP levels decrease when two β-catenin inhibitory proteins, dominant negative TCF4 and ICAT, are over-expressed (Figure 11D). There are several β-catenin/TCF4 binding sites on the promoter region of Gli2[142], so we tested the role of β-catenin to increasing Gli2 promoter

activity using luciferase-based reporter assays and found that β-catenin significantly increased Gli2 promoter activity (Figure 11E). We tested the requirement of intracellular Wnt signaling for PTHrP expression using the small molecule Wnt inhibitor VUWS113, which led to a significant reduction in stimulated levels of PTHrP (Figure 11F). Surprisingly, direct inhibition of Wnt signaling at the receptor level using Sclerostin significantly increased PTHrP expression (Figure 12), which highlights abnormal Wnt signaling and indicates possible downstream crosstalk with

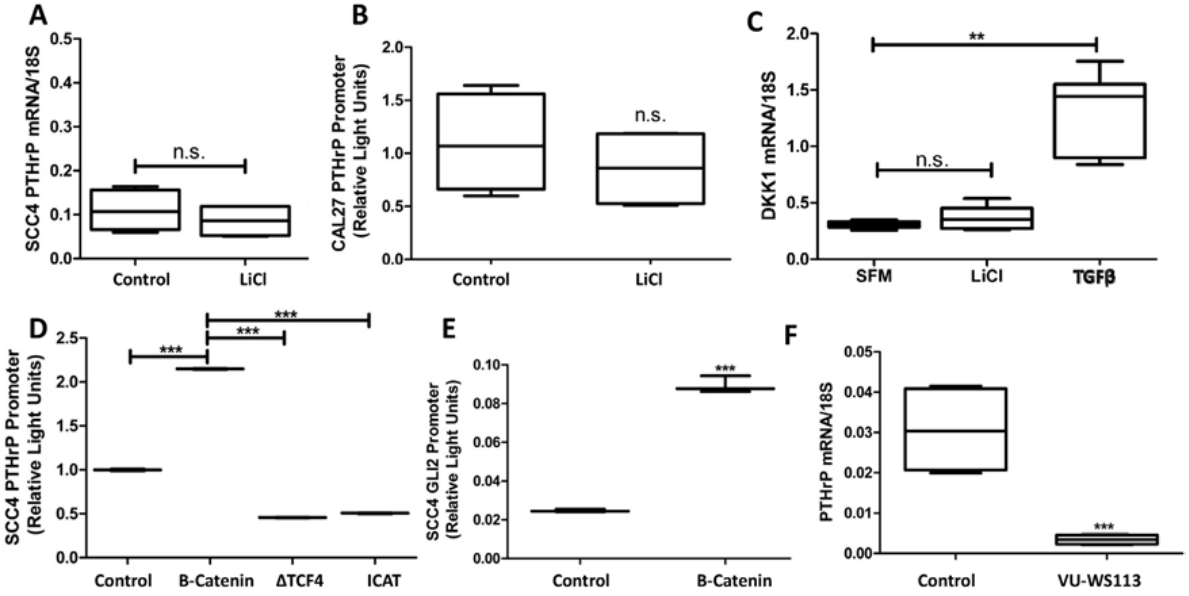


Figure 11. Intracellular Wnt signaling increases PTHrP expression through crosstalk with Hh signaling. (A & B) Wnt signaling activation is insufficient to increase PTHrP expression. SCC4 cells were treated with LiCL and PTHrP mRNA measured by qRT-PCR. Li Cl treatment does not significantly increase PTHrP expression. Similar results were seen for PTHrP promoter activity by dual-luciferase assays in CAL27 cells treated with LiCl for 24hours. (**C.)** Canonical Wnt signaling is not active in OSCC. CAL27 cells were treated with Li-Cl, TFGβ, or control for 24 hours before being harvested for qRT-PCR. Active Wnt signaling, indicated by higher levels of the β-catenin target gene DKK1, is observed with TFGβ but not Li-Cl stimulation. (**D)** Intracellular Wnt signaling modulates PTHrP expression. SCC4 cells were transfected to express a PTHrP firefly luciferase reporter and a constitutively active Renilla luciferase reporter plasmid in combination with a β-catenin protein expressing plasmid, a dominant negative TCF4 protein expressing plasmid, or an ICAT protein expressing plasmid. Intracellular Wnt activation using β-catenin led to a significant increase in PTHrP promoter activity; while inhibitors of β-catenin decreased PTHrP promoter activity. (**E)** β-catenin over-expression increases Gli2 promoter activity. SCC4 cells were transfected to express an endogenous Gli2 promoter firefly luciferase reporter and a constitutively active Renilla luciferase reporter plasmid in combination with a β-catenin protein expressing plasmid. 48 hours after transfection, cells were harvested and firefly activity quantified, which demonstrated a significant increase in Gli2 promoter activity, quite similar to that of PTHrP promoter activity. (**F)** Intracellular Wnt inhibition decreases PTHrP expression. qRT-PCR was used to determine PTHrP mRNA levels of CAL27 cells that were treated with or without 10uM of the CK1 inhibitor, VU-WS113. Treatment with VU-WS113 significantly decreased PTHrP expression.

Gli. Indeed, indirect modulation of Wnt signaling using LiCl and VU-WS113 have been shown to affect Hh signaling as reported[143]. Together, our data support that Wnt signaling in OSCC is irregular; suggesting that only Wnt activation at the level of β-catenin consistently modulates PTHrP expression, where Gli2 expression is also increased.

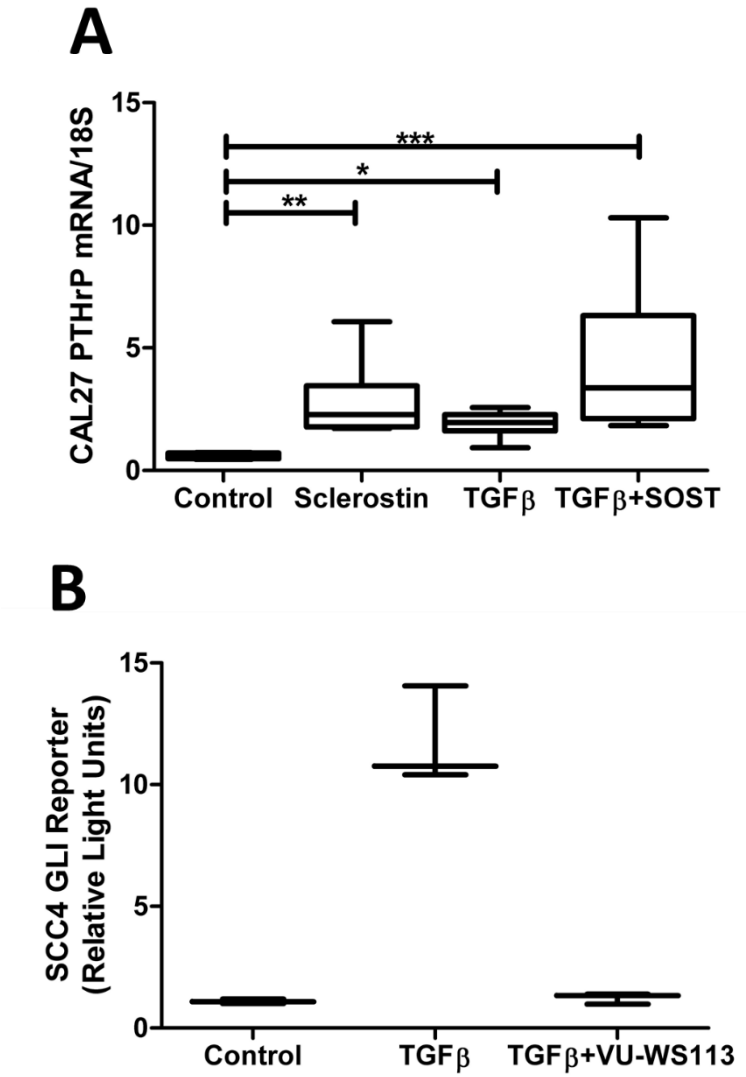
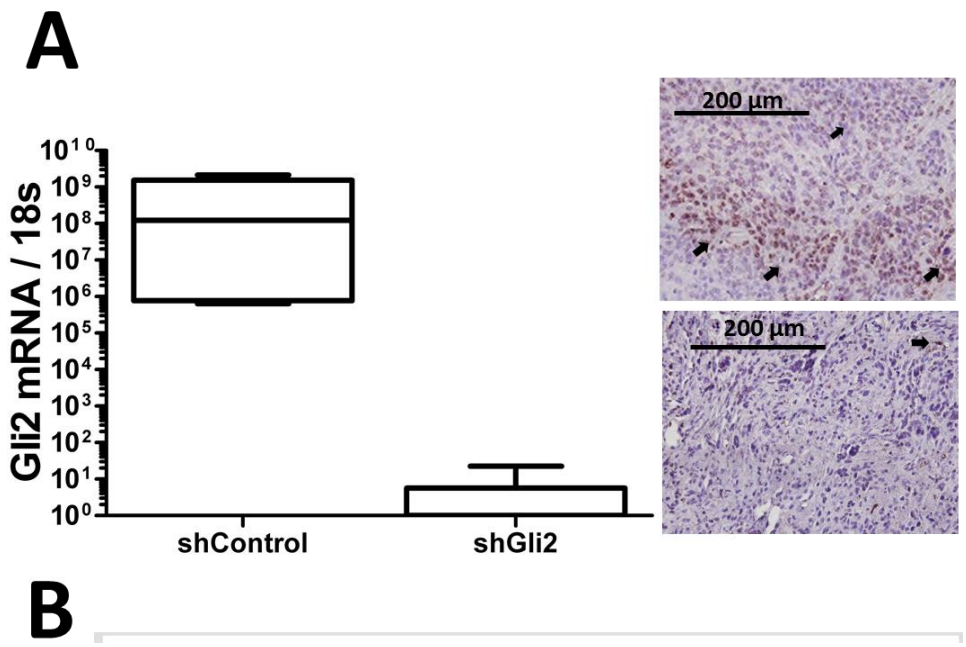


Figure 12. Wnt signaling is dysregulated in OSCC and cross-talks with Hh/Gli signaling. (A) Sclerostin treatment increase PTHrP expression. SCC4 cells treated with Sclerostin to inhibit Wnt signaling at the receptor level show increased levels of PTHrP mRNA after 24 hours of treatment, similar to TGFβ stimulation. **(B)** CK1 inhibition decreases Gli activity. SCC4 cells treated with the small molecule Wnt inhibitor (VU-WS113) show decreased levels of PTHrP mRNA after 24 hours of treatment.

Gli2 expression is required to increase PTHrP expression, bony invasion and bone destruction. To validate that Gli2 is required for bony invasion, we utilized shRNA to stably knock down expression of Gli2 in bony invasive CAL27 cells (Figure 13A). Non-silencing control cells and shGli2 cells were injected into the masseter muscle of male athymic mice and allowed to progress for eight weeks. While both groups developed tumors, control tumors had significantly increased levels of bony invasion as well as bone destruction (Figure 14A). Surprisingly, loss of Gli2 led to smaller tumors, some of which were unable to invade into surrounding tissues. CAL27 shGli2 tumors had significantly lower PTHrP protein levels as determined by IHC (Figure 14B images) and histological data confirmed loss of Gli2 protein in the tumors (Figure 13A images). Additionally, Tartrate Resistant Acid Phosphatase (TRAP staining) confirmed a significantly smaller number of osteoclasts at the tumor-bone interface in mandibular sections (Figure 14C).

Gli2 levels correlate with bony invasion in clinical OSCC samples. OSCC samples from patients were characterized as bony invasive if patients underwent a mandibulectomy or non-bony invasive if patients only underwent soft tissue surgery. Eight samples of both bony invasion and non-bony invasion were used to measure Gli2 mRNA expression levels. By qRT-PCR, patients with bony invasion had significantly higher expression of Gli2 (Figure 14D). Additionally, immuno-histochemical staining of a larger cohort of paraffin embedded OSCC samples showed similar results for Gli2 protein, where Gli2 levels were significantly up-regulated in bony invasive samples (Figure 14E). These findings are clinically relevant, as Gli2 and TGFβ1 are significantly co-expressed in a cohort of 279 head and neck carcinomas catalogued in cBioPortal (Figure 13B)[144].



Co-occurrence of Gene A and Gene B in 279 HNSCC samples (Modified from cBioPortal)		
Gene A	Gene B	p-value
Gli2	TGFβ	0.043

Figure 13. Gli2 knock-down tumors remain Gli2 negative *in vivo* and Gli2 and TGFβ significantly co-occur in HNSCC patients. (A) shRNA against Gli2 significantly decrease Gli2 expression in OSCC. CAL27 cells transfected to stably express shRNA against Gli2 show significantly reduced levels of Gli2 mRNA by qRT-PCR. Representative images of CAL27 tumors show active Gli2 protein in control transfected cells and very little Gli2 protein in shGli2 transfected cells. (Positive staining is denoted by black arrows) **(B.)** Gli2 and TGFβ significantly co-occur in HNSCC patients. Using the cBioPortal resource, a significant correlation was found between Gli2 and TGFβ1 expression in a cohort of 279 patients with head and neck cancer.

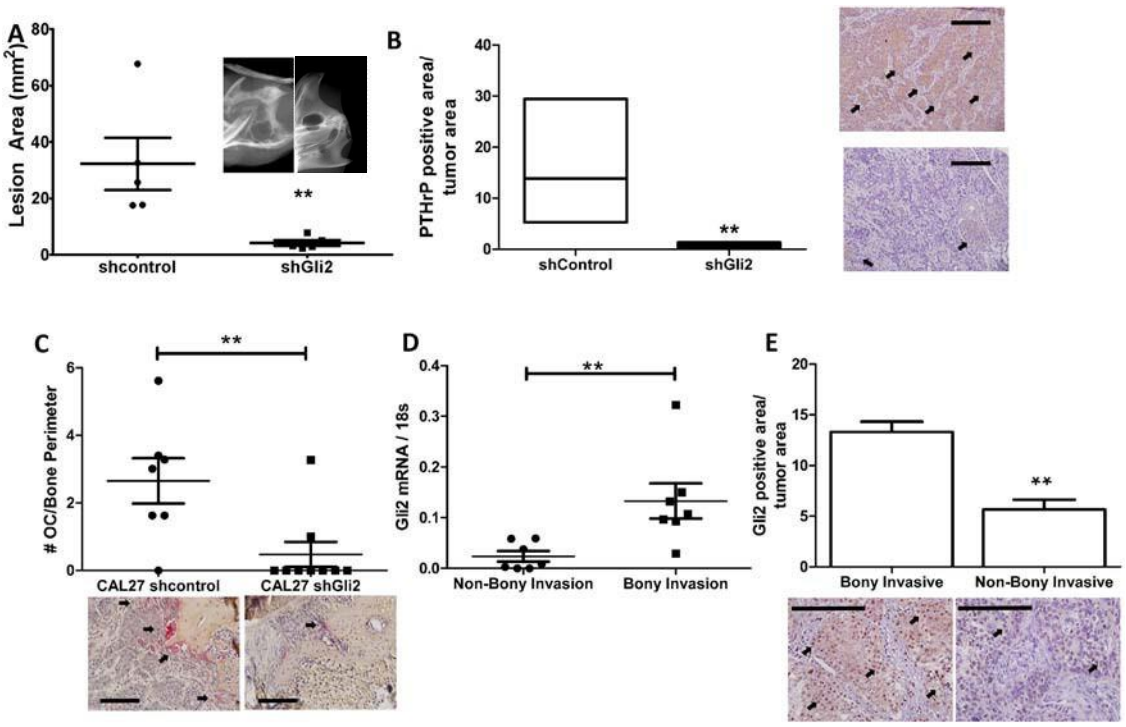


Figure 14. Gli2 is required to increase PTHrP expression, bony invasion and bone destruction. (A) Loss of Gli2 using shRNA decreases bone destruction *in vivo*. CAL27 cells stably expressing shRNA against a non-silencing control sequence or Gli2 were injected into the masseter muscle of male athymic mice and allowed to progress for eight weeks. Mice were then sacrificed and mandibles dissected for high resolution x-rays. Significantly less bone destruction, as measured by lesion area, was observed in the shGli2 group. (**B)** Loss of Gli2 decreases PTHrP levels *in vivo*. CAL27 tumors expressing shRNA against Gli2 show significantly lower PTHrP protein levels by IHC. (Inset is 40X with 200μm scale bars. Arrows denote positive staining) Loss of Gli2 expression is verified using IHC. (**C)** Loss of Gli2 decreases osteoclasts numbers *in vivo*. Tartrate Resistant Acid Phosphate (TRAP) staining was used to identify osteoclasts at the tumor bone interface of mandible sections. We found a significantly larger number of TRAP-positive multinucleated cells in the control group as compared to the shGli2 group. (**D)** Gli2 mRNA is associated with bony invasion in clinical samples. qRT-PCR was done on human OSCC clinical samples, where patients that underwent a mandibulectomy were classified as bony invasive (BI), while samples from patients who did not undergo a mandibulectomy were classified as non-bony invasive (NBI). Samples from the BI group had significantly higher expression of Gli2. (**E)** Gli2 protein correlates with bony invasion in human clinical samples. A larger cohort of clinical samples based on the selection criteria described above was used for IHC against Gli2. We show that Gli2 protein also significantly correlated with bony invasion.

Discussion

We have demonstrated that in OSCC, Gli2 is the central regulator of PTHrP expression (Figure 15). Importantly, we have identified several signaling pathways that control Gli2 at the level of mRNA expression and protein activity. Gli2 directly correlates with PTHrP expression and is both necessary and sufficient for PTHrP expression. Along with Hh signaling, we have identified TGFβ

signaling as a major contributor to Gli activation and we show that both pathways are important for increasing Gli levels and thus PTHrP expression. We have also identified Wnt signaling as an activating pathway for Gli, although this seems to be driven in a non-canonical manner. Targeting Gli2 in OSCC using shRNA significantly decreased PTHrP expression and prevented bony invasion and bone destruction *in vivo*. Finally, our clinical data showing that Gli2 levels correlate with bony invasion strongly supports our findings that Gli2 controls PTHrP expression in OSCC.

Canonical Hh signaling is a highly conserved signaling pathway essential for normal development in higher organisms. In adults, Hh signaling is silenced in almost all tissues, but is often reactivated in tumorigenesis. Basal cell carcinoma and medulloblastoma are Hh-driven tumors, where loss of Ptch leads to constitutive activation of Hh signaling[145]. Additionally, there are reports of canonical and non-canonical Hh signaling in other tumor types, such as breast, lung, prostate, and colon, where Hh signaling is not the driving mutation but is involved in tumor progression and contributes to treatment failure and tumor recurrence[99, 126]. There have also been several published findings on Hh signaling in OSCC, where it is known that Hh signaling contributes to growth, migration and invasion[98, 146]. In our system, OSCC signals through both canonical and non-canonical Hh signaling to increase Gli2 activation and expression of downstream target genes. Alternatively, both TFGβ and Wnt signaling pathways activate transcription of Gli2, and we show that TFGβ increases Gli2 protein activity as well. Our findings highlight the complexity of Gli regulation and PTHrP expression in OSCC and demonstrate the importance of inhibiting Hh signaling at the level of Gli, as compared to upstream receptor-based inhibition.

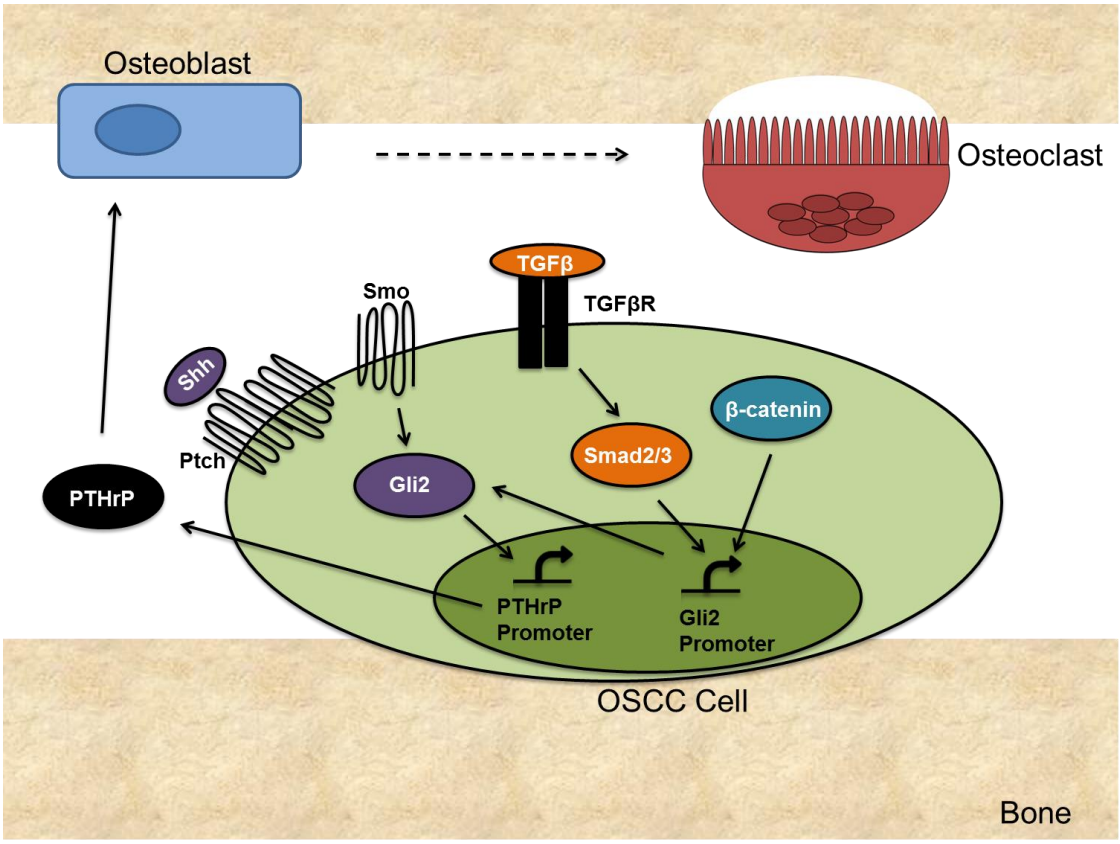


Figure 15. Proposed Mechanism of Gli2 Mediated Bone Destruction in OSCC.
OSCC signals through several signaling pathways to increase Gli2 and PTHrP. Canonical Hh signaling activates Gli2 protein accumulation and increases Gli2 expression, both of which can increase PTHrP expression. TGFβ signaling increases Smad2/3 activation, which increases Gli2 expression. Increased p38/MAPK activity can also increase Gli2 levels by stabilizing Gli2 protein. B-catenin activation increases Gli2 expression and directly and may also indirectly affect Gli2 protein levels through cross-talk. Gli2 increases PTHrP expression in OSC, which results in an increase in osteoblast-mediated osteoclast activation, resulting in large amounts of bone resorption.

The contribution of Wnt signaling to Gli2 and PTHrP activation is also complex. While Wnt signaling in OSCC does not appear to be normal, β-catenin activation can modulate PTHrP expression, and this activation occurs via Gli2. The inability of cells to respond to Wnt stimulation led us to believe that Wnt signaling was not active in these cells, thus, we found it surprising that Wnt inhibition could decrease PTHrP expression. We investigated if this inhibition was independent of Gli2 using qRT-PCR and found that VU-WS113 treatment significantly decreased Gli2 protein activity (Figure 12B), supporting the concept of crosstalk between the two pathways.

Indeed, the mechanism of Wnt inhibition using VU-WS113 is through inhibition of CK1, an inhibitory kinase of β-catenin that has been shown to be a potent inhibitor of Hh signaling[143]. These findings are in line with reports of crosstalk between canonical Hh and Wnt signaling, which has been documented in normal development, wound repair and tumorigenesis[136, 147, 148]. Additionally, inhibition of Wnt signaling at the receptor level using Sclerostin increased PTHrP expression, suggesting that while β-catenin activation increases Gli2 and PTHrP expression, upstream activation of Wnt signaling has an opposite effect. These results are consistent with other literature showing that Hh and Wnt signaling can function as negative regulators of one another[149]. Thus, our data strongly suggests that Wnt regulation of PTHrP occurs at least in part, via Gli2. In several OSCC lines tested, TGFβ stimulation led to an increase in PTHrP expression, with a similar increase in Gli2 transcription and protein activity. This was found to be mediated in a Smad-dependent manner. As there are several published reports demonstrating that the Smad binding sites on the Gli2 promoter are functional, we surmise that Gli2 is a direct target of TGFβ signaling. The contribution of non-canonical TGFβ signaling on increasing Gli2 and PTHrP expression is also in line with other published findings, where several downstream targets of p38/MAPK including MEF2C and NF-kβ can induce expression of Hh ligands as well as Gli[99]. Our group has published on TGFβ mediated activation of Gli2 in other tumor types that do not express the conical Hh signaling receptors, Smo and Ptch. We have found however, that in OSCC, canonical Hh signaling is required for TGFβ stimulation to increase Gli activity and PTHrP expression, suggesting a cooperative or synergistic signaling system. Taken together, our data demonstrates a complex yet interconnected signaling network controlling PTHrP expression. Importantly, these mechanisms all converge on Gli2, highlighting its importance on regulating PTHrP expression. Previous findings have highlighted the importance of PTHrP for bony invasion

in murine OSCC, but until now, the mechanism controlling PTHrP expression remained unknown[81]. Other studies have shown the prognostic value of PTHrP expression for predicting bony invasion using human samples, and we have built upon these findings to both identify and test regulators of PTHrP. It is important to note that Gli2 regulation of PTHrP is observed in other tumors. In tumors that metastasize to bone, PTHrP plays an important role in promoting tumor progression and bone destruction[67]. In a process coined "The Vicious Cycle", metastatic tumor cells arrive at the bone microenvironment and secrete factors that directly or indirectly lead to excessive osteoclast-mediated bone resorption. In bone-metastatic MDA-MB-231 breast cancer cells, PTHrP expression has been shown to be regulated by non-canonical Hh signaling[66]. Similar to OSCC, these cells increase Gli2 levels in response to TGFβ, which leads to an increase in PTHrP. Dissimilar to OSCC however; PTHrP in breast cancer cells can also be modulated by classical Wnt signaling. Additionally, bone metastatic MDA-MB-231 cells do not express canonical Hh receptor signaling proteins, nor do they respond to Hh receptor based inhibition or activation, highlighting the complexity of PTHrP and Gli2 regulation in different systems[66]. OSCC invasion into the mandible is a serious occurrence that significantly impacts patient overall survival and quality of life. These patients have high rates of recurrence at or near the site of the original tumor, emphasizing the need for more effective anti-tumor therapies. Currently, the only FDA approved targeted treatment for OSCC is Cetuximab, a monoclonal antibody against EGFR. While Cetuximab significantly increases overall survival in patients with locally advanced disease, patients with recurrent and/or metastatic disease only survive three months longer when Cetuximab is added to their treatment regimen. Thus, it is clear that other targets are needed. PTHrP expression significantly correlates with bone involvement in OSCC patient samples and previous studies have identified PTHrP as an essential component of bony invasion and bone

destruction in OSCC. However, no published work on PTHrP regulation in OSCC exists. Through this study, we have identified key signaling mechanisms that control PTHrP-mediated bony invasion. The contributions of Hh, TGFβ, and Wnt signaling on Gli2 strongly suggest that bony invasion is regulated in a multifaceted system. The variability of receptor signaling control among OSCC cell lines highlights the importance of targeting the common downstream mediator, Gli2. Our work highlights mechanisms that can contribute to the observed clinical failures of Smo inhibitors, and we have demonstrated the value of targeted downstream Gli inhibitors to prevent treatment resistance and disease recurrence[150]. We have provided sufficient evidence to support that Gli2 controls bony invasion and bone destruction in OSCC via regulation of PTHrP. Our findings highlight the feasibility of using Gli2 inhibitors to prevent mandibular invasion and bone destruction in OSCC patients and should be evaluated further as a possible adjuvant treatment.

Materials and Methods

Cell lines

The OSCC cell lines, SCC4, CAL27, and HSC3 were provided by Cara Gonzales at UTHSCSA, and were purchased from American Type Culture Collection (ATCC). The remaining OSCC cell lines, SCC9, SCC15, SCC25, SCC61, SCC131, SCC1352, and VU1729 were donated by Dr. Stephen Brant at Vanderbilt University. Cell lines were tested for mycoplasma and if positive, were treated with BM Cyclin (Roche). Cell lines were cultured in 50% Dulbecco's modification of Eagle's medium and 50% Nutrient Mixture F12 (DMEM/F12) (ThermoFisher Scientific), supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories) and 1% penicillin/ streptomycin (Mediatech). Cells were maintained at 37°C with 5% CO2.

Genome wide microarray

Total RNA was extracted from OSCC cells stimulated with TGFβ or the buffer control using RNA Stat 60 reagent (AMSBIO) according to the manufacturer's protocol. The RNA was sent to the UTHSCSA Microarray

Core facility and used as a template for double-stranded cDNA synthesis, followed by biotin-labeled cRNA synthesis. The cRNA was fragmented and hybridized to the U133A GeneChips overnight at 45°C in a rotating incubator. Hybridized cRNA was fluorescently labeled with anti-biotin antibodies and streptavidin phycoerythrin dye conjugate on a programmable microfluidics workstation. The probe arrays were scanned twice and the stored images were analyzed using the GeneChip MAS 5.0. Signal intensities were normalized and scaled using MAS 5.0 for comparison analysis of experimental and baseline arrays. Significantly up-regulated and downregulated genes were identified by MAS 5.0.

Drug treatments

All drug treatments were carried out in serum free DMEM/F12 for 24 hours. TGFβ and Sclerostin (R&D Systems) were used at 10ng/ml and 1-2ug/ml respectively. TGFβ buffer (5%BSA in 4mmol HCl) was used as a control. GANT58 and Lithium Chloride (SigmaAldrich) were used at 10μM and 20mM respectively. Purmorphamine and SIS3 (EMD Millipore) were used at 10μM. Cyclopamine (LC Labs) was used at 12nM. SB202190 (Tocris) was used at 10μM. VU-WS113, a less cytotoxic derivative of Pyrvinium, was a gift from Dr. Ethan Lee at Vanderbilt University.

Transfections Cells were transfected per manufacturer's instructions using Lipofectamine 2000 (ThermoFisher Scientific). Briefly, cells were incubated overnight in OptiMem (ThermoFisher Scientific) before being transfected at a ratio of 1.25 ug DNA to 1ul Lipofectamine 2000. Fresh media (complete with FBS and antibiotics) was added to cells the following morning. Transiently transfected cells were harvested 48 hours after transfections, while stably transfected cells were

maintained in antibiotic media. 400ug/ml G418 or 125 ng/ml puromycin were used for two weeks to select for transfected cells. Thereafter, cells were cultured in antibiotic maintenance media, which was 200ug/ml G418 or 62.5 ng/ml puromycin.

Plasmids for stable transfections

Gli2-SA662 Flag was a gift from Vladimir Spiegelman from the University of Wisconsin Medical School. The Gli2 protein produced from these plasmids harbor a single point mutation, changing the serine at amino acid position 662 to alanine. This enables resistance to proteasomal degradation by ubiquitination [21]. Three pooled shRNA constructs against Gli2 and the non-silencing control (Origene) were used to knock-down Gli2 expression. DNA amounts between groups were held constant.

Plasmids for transient transfections

The pRL Renilla Luciferase Control Reporter plasmid (Promega) and the PTHrP promoter plasmid were used as described [48]. The Gli2 promoter and protein reporter plasmids, as well the β-catenin plasmids were also a gift from Vladimir Spiegelman. The Smad2/3 protein plasmids were a gift from the laboratory of Dr. Harold Moses at Vanderbilt University.

Quantitative real-time PCR

Cells were harvested by direct lysis and total RNA extracted using the RNeasy Mini Kit (Qiagen). The qScript cDNA synthesis kit (Quanta, VWR) was used to synthesize cDNA from 1ug RNA. Validated Taq-Man primers from (ThermoFischer Scientific) were used to measure gene expression in triplicate using the 7500 Real-Time PCR System from Applied BioSciences (ThermoFisher Scientific). Absolute gene expression was quantified using a standard curve and 18s was used as an internal control.

Western blots

Briefly, cell lysate was harvested using RIPA Buffer (Sigma) supplemented with protease and phosphatase inhibitors (Roche). 20ug of protein were loaded per well and gels were run using Nu Page supplies (Novex by Life Technologies) before being transferred to PVDF membranes. Membranes were blocked in TBS with .1% Tween-20 and 5% BSA or 5% non-fat dry milk for 1 hour. Primary antibody incubations of GAPDH, Smad3 and phospo Smad3 (Cell Signaling) were done overnight at four degrees under gentle agitation, and the secondary antibody, anti-rabbit IgG (Cell Signaling) was incubated for one hour at room temperature under gentle agitation. Membranes were exposed on x-ray film using Western Lightening PlusECL (Perkin Elmer).

Animal studies

All animal studies were carried out in compliance with the Vanderbilt University Institutional Animal Care and Use Committee and the National Institutes of Health guidelines. Intra-tibial injections 5x105 cells were injected into the left tibia of 4-6-week-old athymic male mice from Harlan Laboratories. The right tibia was used as a PBS injection control. Weekly x-rays using the XR-60 digital radiography system from Faxitron were done to monitor tumor progression. At the end of the experiment, mice were sacrificed and hind limbs dissected for ex vivo analyses.

Masseter muscle injections 1x106 cells were injected into the left masseter muscle (parallel to the mandible) of 4-6-week-old athymic male mice from Harlan Laboratories. The right muscle was used as an injection control. Mice were weighed weekly to assess tumor burden. Drug treatments began once tumors were palpable (~10 days). At the end of the experiment, mice were sacrificed and mandibles were dissected for ex vivo analyses.

Clinical OSCC samples

Dr. Kim Ely reviewed patient charts to identify OSCC patients that underwent a mandibulectomy as compared to those that underwent soft tissue removal, which was used to acquire 30 total OSCC

samples on histological slides from the Translational Pathology Shared Resource Core at Vanderbilt. 16 available matching macrodissections were acquired from the Vanderbilt-Baker Head and Neck Bio-repository, and processed for qRT-PCR as described.

Immunohistochemical staining

Mandible specimens were dissected and fixed in 10% neutral-buffered formalin (Fisher Scientific) for 48 hours at four degrees. Mandibles were then decalcified in 10% EDTA for 10 days at room temperature under agitation and embedded in paraffin. Mandible sections (5-7μm thickness) were stained with hematoxylin & eosin, orange G, and phloxine to measure tumor burden. Antibody staining against Gli2 (Novus Biologicals at 1:250) was used to measure Gli2 protein expression. Antibody staining against PTHrP (Jack Martin at 1:400) was used to measure PTHrP protein expression. Unlabeled goat IgG and rabbit IgG from Santa Cruz (1:400) were used as control primary antibodies. HRP linked rabbit anti-goat or goat anti-rabbit and ImmPACT NOVA RED from Vector Laboratories was used to detect staining. TRAP staining was used to measure osteoclast numbers. All slides were examined under an Olympus microscope at 20X and 40X and images (taken using Olympus DP71 camera and software) were quantified using Metamorph software (Molecular Devices, Inc.) for thresholding and region of interest (ROI) analysis.

Immunohistochemical analyses

Histological images from HRP-labeled antibody staining were uploaded into Metamorph. For each image, the bottom incisor was used as a landmark. The area of tumor was traced using region of interest analyses, then, positive staining was quantified for each slide using a representative threshold (based on the positive control). For PTHrP, positive staining is marked by tumor specific brown staining. For Gli2, positive staining is marked by tumor specific nuclear brown staining. Nonspecific staining from the IgG control was used measure background staining. This value was

subtracted from each slide to normalize values. The resulting value represents positive staining, which is then divided by the total area of the image yielding percent positive staining. Similarly, tumor burden in tibiae were assessed using H&E staining, where tumor area is quantified and divided by the total area (total marrow space of tibia from the growth plate to near mid-shaft).

Statistical analyses and replicates

All *in vitro* experiments were done in triplicate with a minimum n =3 samples. For intra-tibial injections, n=8 mice per group. For masseter muscle injections, n=10 mice per group. All statistical analyses were done using InStat v3.03 software from GraphPad Software. All values are presented as mean ± SEM where * denotes $p<.05$, ** denotes $p<.01$ and *** denotes $p<.001$.

CHAPTER III

GLI INHIBITION, USING LOCALLY DELIVERED ENCAPSULATED GANT58, PREVENTS MECHANOTRANSDUCTION MEDIATED ORAL CANCER INVASION INTO BONE

Introduction

Oral Squamous Cell Carcinoma (OSCC) accounts for most Head and Neck Squamous Cell Carcinomas (HNSCC) and has a five-year mortality rate of 64% [2, 9]. Invasion status remains the most important prognostic factor for recurrence and overall survival. This is especially evident in patients with mandibular invasion, which predicts poor prognoses[32]. Patients with bony invasion require surgical resection, along with additional therapies such as radiation, chemotherapy, and immunotherapy[28, 130, 131]. Even so, many patients suffer from tumor recurrence, which significantly decreases mortality. Advancements in OSCC treatments are limited, in part, by a fundamental lack of knowledge concerning how and why OSCC invades into mandibular bone. OSCC cells that invade the bone can express factors such as Parathyroid Hormone-related Protein (PTHrP) and Receptor Activator of Nuclear Factor-B Ligand (RANKL), which indirectly and/or directly stimulate excessive osteoclast-mediated bone destruction[60, 81, 90, 151]. While the mechanisms behind this expression in OSCC had remained unclear, our group recently demonstrated that PTHrP expression in bony invasive OSCC cells is driven by the transcription factor Gli2. The expression of Gli2 requires both Hedgehog (Hh) and TGFβ signaling. In this study, we investigated how the tumor-bone microenvironment affects this Hh-TGFβ/Gli2 signaling cascade, with the goal of identifying anti-tumor targets. In both our work and that of others, levels of PTHrP significantly correlate with bony invasive status and are markedly increased at the tumor bone interface[90, 152].

Since we have previously demonstrated in other tumor types that PTHrP and Gli2 expression are regulated by the bone microenvironment, we reasoned that the bone microenvironment may regulate Gli2 expression in invasive OSCC cells,[99, 100, 136, 153] and may contribute to the ability of OSCCs to invade the bone. One aspect of the bone that has been associated with the regulation of Gli2/PTHrP is the rigidity of the bone extracellular matrix (ECM) which is 1,000 times more rigid than soft tissue[154]. Numerous reports highlight the contributions of a rigid ECM to increasing tumor progression and our group has shown that a rigid ECM regulates the bone-destroying phenotype of bone metastatic breast cancer[62]. Mechanotransduction allows cells to sense and respond to changes in the ECM by converting mechanical forces into biochemical signals[155]. A major regulator of both Gli2 and mechanical regulation is the primary cilia. Canonical Hh signaling requires primary cilium to recruit and activate Gli proteins, suggesting that the increased activation of Gli2 is linked to the increase in ciliogenesis. In this study, we investigate how the rigid microenvironment regulates Gli2. Here we show that OSCC cells require a rigid ECM to increase Gli2 activation and PTHrP expression. This mechanism occurs through mechanically transduced signaling, specifically at the levels of ROCK1/2 activation and importantly, downstream to myosin. Surprisingly, cells grown on compliant matrices, with rigidities similar to collagen, do not respond to Hh or TGFβ stimulation. These results are specific to the Hh-TGFβ/Gli2 signaling axis, as protein levels of other pathways such as EGFR signaling remain unchanged across the two matrices. Importantly, inhibiting mechanotransduction at the level of myosin prevents increases in both primary cilium and Gli2 activation. Additionally, we find that bone rigid facilitates the expression of genes important for generating primary cilium, such as BBS1 and KIF3A. Lastly, direct Gli2 inhibition using encapsulated GANT58 *in vivo* mimics the effects of compliant matrices, where tumor-bearing

mice treated with GANT58 show significantly decreased bone destruction and PTHrP expression. These studies demonstrate the important role of ECM rigidities on modulating Gli2 activation, and highlight the feasibility of using GANT58 in a localized delivery system to prevent OSCC invasion into the bone.

Results

Bone rigidity increases basal and stimulated expression of the osteolytic gene, PTHrP and its transcriptional activator, Gli2. While OSCC invasion into the mandible is associated with tumor recurrence and lower overall survival[33], very little is known about how OSCC cells respond to ECM rigidities similar to bone. To investigate this, we seeded OSCC cells onto 2D films with matrix rigidities matching collagen in the basement membrane (compliant) or cortical bone (rigid)[156]. Under serum free conditions, cells grown on rigid films significantly upregulated expression of Gli2 (Figure 16A) and PTHrP (Figure 16B) by qRT-PCR. Using Western Blots for nuclear Gli2, we also confirm that levels of active Gli2 protein is increased on rigid films as compared to compliant films (Figure 16C). To determine if these effects would be additive to stimulated levels of Gli2 and PTHrP, we used TGFβ to increase Gli2 activation and measured PTHrP expression by qRT-PCR. Surprisingly, OSCC cells grown on compliant films do not significantly increase PTHrP mRNA levels when stimulated with TGFβ (Figure 16D). This finding contrasts with cells grown on rigid films, which dramatically increase PTHrP mRNA expression. Additionally, Western Blots for EGFR, a commonly overexpressed oncogenic protein in OSCC[157], remains unchanged across both rigidities (Figure 16E), suggesting that the changes in gene expression due to rigidity are specific to the Hh-TGFβ/Gli2 signaling axis.

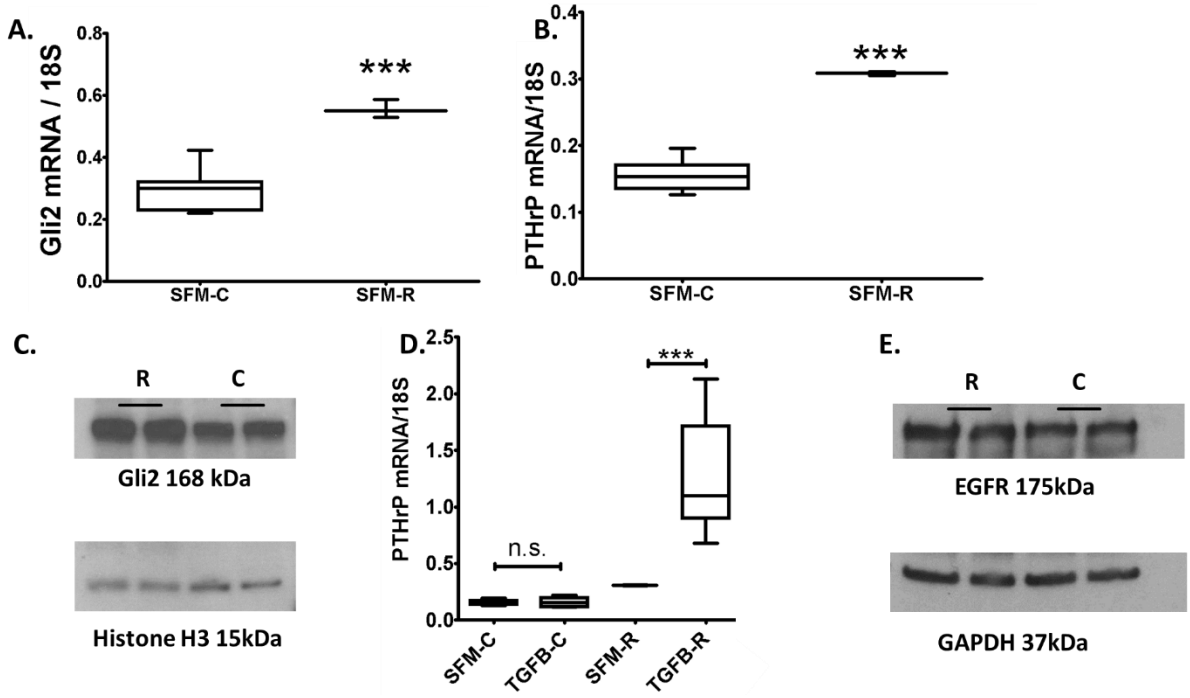


Figure 16. Rigid matrices increase basal and stimulated expression of the osteolytic gene, PTHrP and its transcriptional activator, Gli2. OSCC cells grown on rigid (R) films in serum free media (SFM) significantly increase Gli2 expression **(A)** and PTHrP expression **(B)** by qRT-PCR, as compared to cells grown on compliant films (C**)**. **(C)** OSCC cells grown on compliant films are unable to respond to TGFβ stimulation, while cells grown on rigid films significantly increase PTHrP expression by qRT-PCR. Cells grown on tissue culture poly-styrene (TCPS) increase PTHrP expression when stimulated, as expected. **(D)** Western blots for nuclear Gli2 protein demonstrate an increase in active Gli2 protein when cells are grown on rigid films as compared to compliant films under serum free conditions for 24 hours. **(E)** Changes in extracellular matrix rigidity does not modulate levels of EGFR. As seen by Western Blot, OSCC cells grown on compliant or rigid films do not change levels of EGFR protein, suggesting that the effects of rigidity on increasing Gli2 are specific.

Extracellular matrix rigidity regulates OSCC response to Hh and TGFβ signaling modulation.

To further investigate OSCC cellular responses to changes in rigidity, we treated cells with the Gli antagonist GANT58[137], and found that inhibiting Gli2 in cells grown on compliant films does not significantly alter PTHrP mRNA expression (Figure 17A). However, PTHrP mRNA expression is significantly reduced when cells grown on a rigid, bone-like film. We next examined the mechanism of Gli2 inhibition by GANT58 to better understand how changes in rigidity might

be affecting levels of Gli2 and PTHrP. OSCC cells stimulated with TGFβ and then treated with GANT58 significantly decrease levels of active Gli protein as compared to stimulated cells, as shown by luciferase activity using a Gli-binding site luciferase construct (Figure 17B graph). Additionally, by immunofluorescence, we observe a dramatic decrease in nuclear levels of Gli2 (Figure 17B photos), suggesting that GANT58 prevents nuclear translocation of Gli2 as proposed, while also implying that rigidity increases Gli2 activity and nuclear localization. A similar increase in Gli2 nuclear translocation was also observed when OSCC cells plated on a rigid film were treated with TGFβ, but not when cells seeded on a compliant film were treated with TGFβ. TGFβ signaling is known to increase activation of Gli2 protein and expression of Gli2 mRNA through both canonical and non-canonical signaling[158]. Thus, we used the small molecule inhibitors to selective inhibit Smad 3 (SIS3) or MAPK (SB-202190) Targeting TGFβ signaling using either inhibitor decreased nuclear Gli2 proteins by Western blot (Figure 17C). Similarly, inhibition of canonical Hh signaling using cyclopamine in cells grown on rigid films decreases PTHrP mRNA expression (Figure 17D), indicating that rigidity can alter upstream signaling pathways that regulate Gli2 expression.

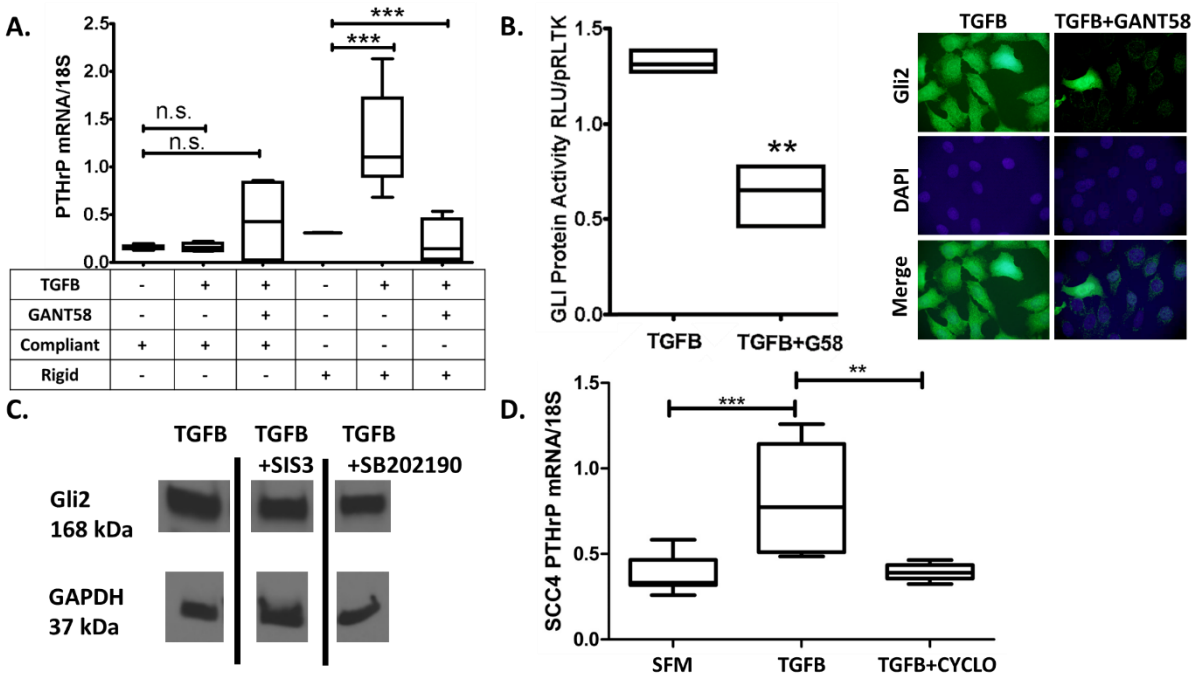


Figure 17. Extracellular matrix rigidity regulates OSCC response to Hh and TGFβ signaling modulation **(A)** TGFβ stimulation is not sufficient to increase PTHrP in the absence of a rigid matrix. OSCC cells grown on compliant films are unable to increase PTHrP expression in response to TGFβ stimulation. On rigid films however, TFGB stimulation significantly increases PTHrP expression. **(B)** Furthermore, Gli2 inhibition using GANT58 significantly reduces PTHrP expression, suggesting that the cellular effects of rigidity are upstream of Gli2. **(C)** TGFβ inhibition decreases Gli2 protein levels on rigid films. OSCC cells grown on compliant films show decreased nuclear Gli2 when treated with TFGB inhibitors. **(D)** Hh inhibition, using cyclopamine, decreases PTHrP expression in OSCC. OSCC cells grown on rigid films remain sensitive to canonical Hh inhibition, where Smoothened inhibition using cyclopamine prevents an increase in stimulated PTHrP levels

Inhibition of upstream mechanotransducers, αv integrins or Rho GTPases, does not prevent stimulated PTHrP expression on rigid films. To investigate the role of mechanotransduction on Gli2 and PTHrP modulation, we used cilengitide, a RGD pentapeptide inhibitor of αv integrins, to prevent integrin binding to the ECM. On compliant films, cells stimulated with TGFβ and treated with cilengitide do not significantly decrease Gli2 or PTHrP expression. Surprisingly, cells grown on rigid films and double treated with TGFβ and cilengitide do not significantly decrease Gli2 and PTHrP mRNA expression. In fact, we observe a significant increase in Gli2 and PTHrP expression as compared to the unstimulated control, which is similar to TGFβ only stimulated group (Figure

18A & 18B). Similar results are observed when OSCC cells are stimulated with TGFβ and treated with Rhosin, a selective reversible and potent inhibitor of RhoA GTPases (Figure 18C & 18D). We then used immunofluorescence staining to visualize levels of Gli2 protein in cells treated with Cilengitide or Rhosin (Figure 18E). Similar to the qRT-PCR data, neither integrin nor RhoA inhibition prevented stimulated PTHrP expression on rigid films. These results, though surprising, suggest that rigidity alters Gli2 and PTHrP expression independently of integrins and RhoA.

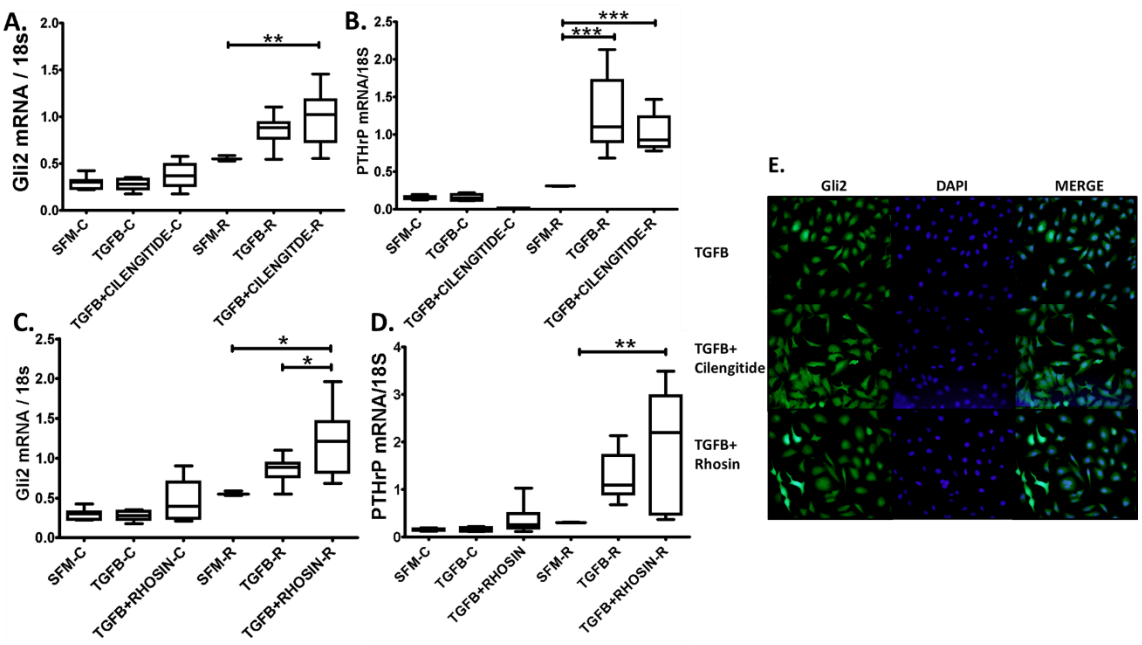


Figure 18. Inhibition of upstream mechanotransducers, alpha 5 integrins and Rho GTPases, does not prevent stimulated PTHrP expression on rigid films.
(A) OSCC cells grown on compliant films do not significantly change Gli2 expression in response to TGFβ stimulation with or without integrin inhibition. Conversely, on rigid films, integrin inhibition increases Gli2 expression. **(B)** OSCC cells grown on compliant films do not change levels of PTHrP in response to TGFβ stimulation nor integrin inhibition. On rigid films, integrin inhibition has no significant effect on PTHrP expression. **(C)** OSCC cells grown on compliant films do not change Gli2 expression in response to RhoA inhibition. On rigid films, RhoA inhibition significantly increases Gli2 mRNA levels. **(D)** OSCC cells grown on compliant films do not change PTHrP expression in response to RhoA inhibition. On rigid films, RhoA inhibition increases PTHrP mRNA levels. **(E)** Inhibition of integrins or RhoA does not prevent nuclear localization of Gli2 in OSCC cells treated with TGFβ and in some cells, leads to increased levels of active Gli2.

ROCK1/2 and non-muscle myosin II are required for PTHrP expression on rigid films. To determine if mechanical regulators downstream of integrin/RhoA activation could modulate Gli2 and PTHrP levels, we investigated the contributions of ROCK1/2, a Rho associated kinase and downstream target of RhoA using the small molecule inhibitor Y-27632 to inhibit ROCK1/2 activity. ROCK1/2 inhibition did not significantly change Gli2 or PTHrP mRNA levels as measured by qRT-PCR on compliant or rigid films (Figure 19A), but on rigid films, ROCK1/2 inhibition significantly decreased PTHrP, not Gli2, mRNA expression (Figure 19B). Western blots for nuclear Gli2 in cells treated with Y-27632 showed a significant decrease in active Gli2 when ROCK1/2 is inhibited, suggesting that ROCK1/2 only regulates Gli2 protein (Figure 19C).

One of the effector proteins of mechanotransduction is non-muscle myosin II. This protein is downstream of ROCK1/2 and is responsible for actin and actomyosin rearrangement due to changes in ECM rigidities. To determine if non-muscle myosin II could modulate rigidity-mediated expression of Gli2 and PTHrP, cells were treated with the myosin II inhibitor, blebbistatin. Blebbistatin significantly decreased both Gli2 and PTHrP mRNA expression (Figure 19D and 19E) and Gli activity using a Gli binding luciferase reporter construct (Figure 19F), similar to what was observed when cells were treated with GAN58. These data suggest that bone-like rigidity can stimulate a signaling cascade that facilitates Gli2 activation that is not activated on compliant matrices (soft tissue).

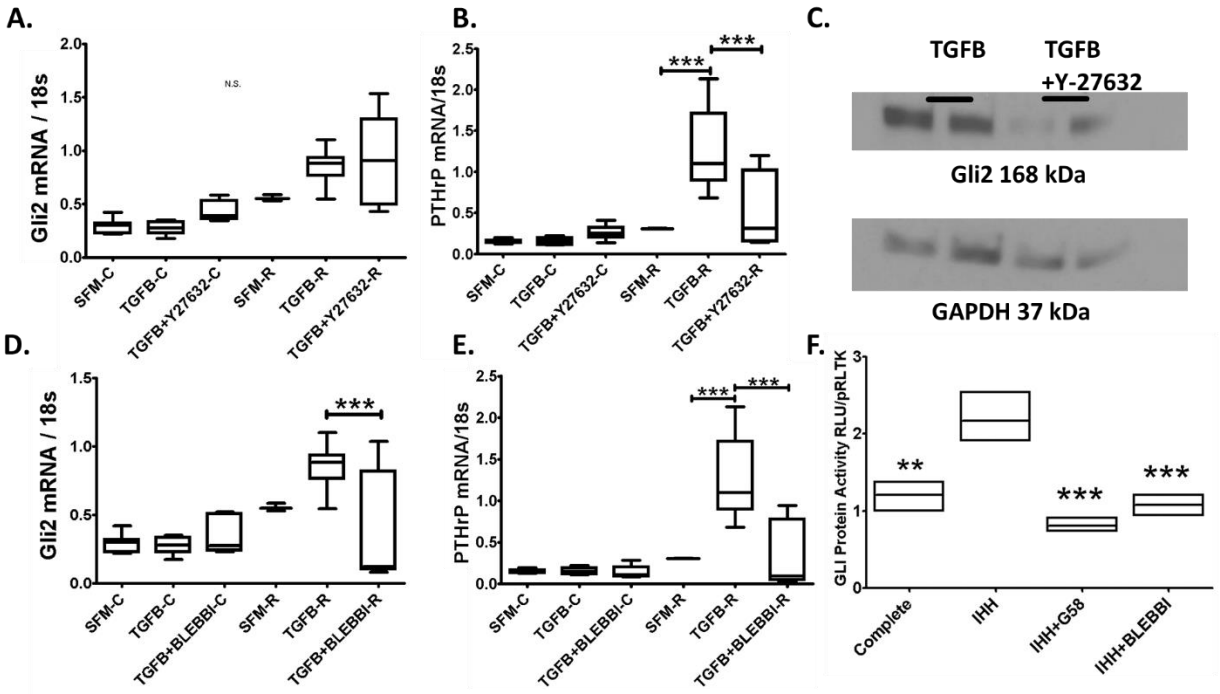


Figure 19. Downstream mechanotransducers, ROCK and Myosin II, are required for PTHrP expression on rigid films

(A) OSCC cells grown on compliant or rigid films do not significantly change Gli2 expression in response to TGFβ stimulation or ROCK inhibition. **(B)** OSCC cells grown on compliant films do not change levels of PTHrP in response to TGFβ stimulation nor ROCK inhibition. On rigid films however, ROCK inhibition significantly decreases PTHrP expression. **(C)** While ROCK inhibition does not change Gli2 mRNA levels by qRT-PCR, by Western Blot Y-27632 decreases levels of nuclear Gli2 in OSCC cells stimulated with TGFβ. **(D)** OSCC cells grown on compliant films do not change Gli2 expression in response to myosin II inhibition. On rigid films however, myosin II inhibition significantly decreases Gli2 mRNA levels. **(E)** OSCC cells grown on compliant films do not change PTHrP expression in response to myosin II inhibition. On rigid films however, myosin II inhibition significantly decreases PTHrP mRNA levels. **(F)** Myosin II inhibition significantly decreases Gli2 protein activity in OSCC cells stimulated with Ihh, similar to the Gli2 antagonist, GANT58.

Bone rigidity increases expression of ciliogenesis related genes. To investigate the link between myosin-mediated cytoskeletal rearrangement in response to ECM rigidity and Gli2 activation, we next measured levels of primary cilium. The primary cilium is known to both initiate and respond to changes in the ECM rigidity[159]. Additionally, the primary cilium is required for canonical Hh signaling[160]. Thus, we used qRT-PCR to measure mRNA levels of three genes associated with primary ciliogenesis, IFT88, BBS1, and KIF3A. Under serum-free conditions, BBS1 and KIF3A expression was significantly higher on rigid films as compared to compliant films, while IFT88

levels trended, but did not significantly change (Figure 20A). To determine if changes in mechanotransduction correlated with ciliogenesis, we used blebbistatin to inhibit myosin II, which led to significantly reduced numbers of primary cilium (Figure 20B). We then plated cells onto either rigid of compliant 2D films and used blebbistatin to inhibit myosin II before measuring changes in ciliogenesis gene expression to determine if mechanical signaling inhibition affected ciliogenesis. Surprisingly, on compliant films, myosin inhibition significantly increases KIF3A expression, while IFT88 and BBS1 were not significantly different (Figure 20C). On rigid films however, myosin inhibition significantly decreases IFT88, BBS1, and KIF3A mRNA expression (Figure 20D). These results demonstrate that the importance of mechanical signaling proteins on increasing ciliogenesis.

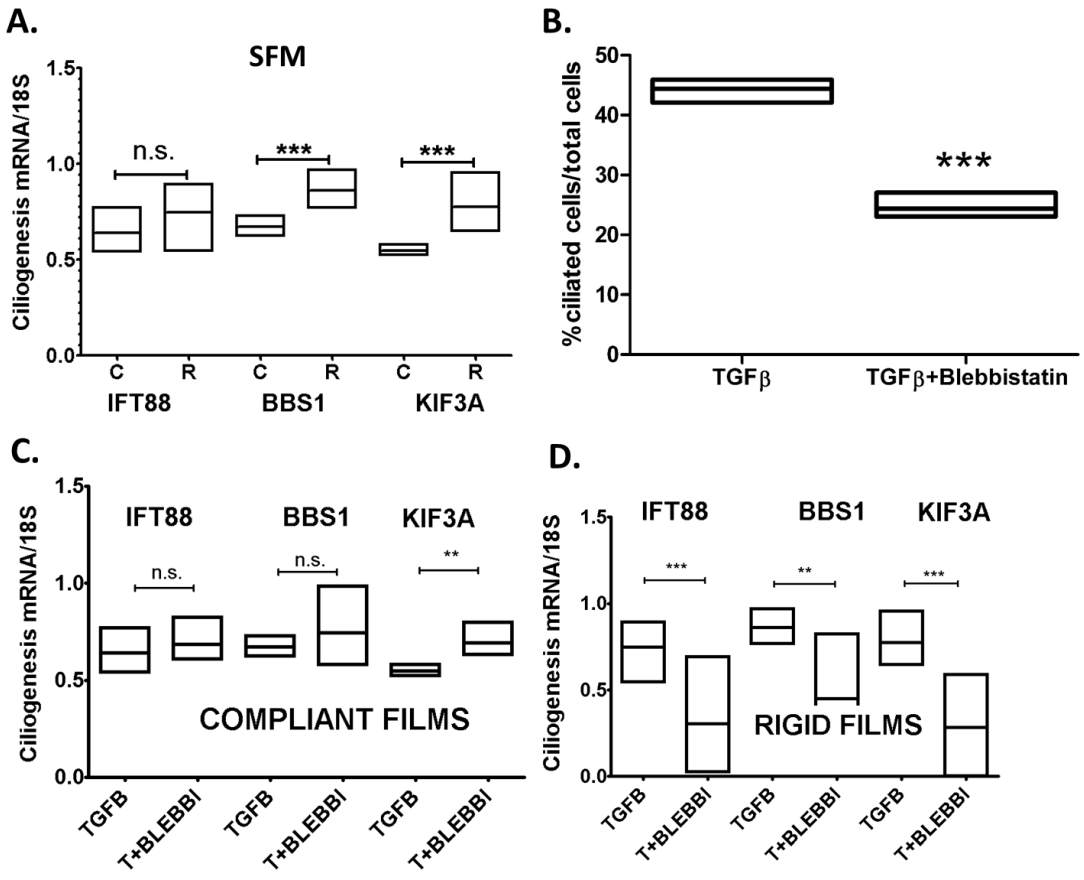


Figure 20. Rigid extracellular matrices increase expression of ciliogenesis genes and inhibition of mechanotransduction prevents stimulated ciliogenesis.

(A) OSCC cells significantly increase expression of ciliogenesis genes, BBS1 and KIF3A (but not IFT88) on rigid versus compliant films. **(B)** OSCC cells grown on compliant films increase levels of KIF3A, but not IFT88 or BBS1 when myosin activity is inhibited with blebbistatin. **(C)** OSCC cells grown on rigid films significantly decrease levels of KIF3A, IFT88 and BBS1 when myosin activity is inhibited with blebbistatin. **(D)** OSCC patient samples show an increase in ciliogenesis genes in tumors that have invaded the bone as compared to tumors that have only invaded soft tissue. **(E)** OSCC patient samples show an increase in levels of primary cilium and Gli2 in tumors that have invaded the bone as compared to tumors that have only invaded soft tissue. **(F)** OSCC cells grown on rigid films increase levels of primary cilium in response to rigidity, which facilitates Gli2 activation and PTHrP expression downstream.

Clinical OSCC samples with bony invasion show increased levels of focal adhesion kinases as compared to samples with no bony invasion. To determine the clinical relevance of these findings, we used the cBioPortal to determine if bony invasion correlates with mechanical signaling. In OSCC, we have found that the Hh-TGFβ/Gli2/PTHrP signaling axis is essential for bony invasion, and it is well known that activation of FAK, RhoA, and ROCK is important for mechanical signaling. Analyzing patient samples in cBioPortal, we found that Gli2 and PTHrP mRNA gene expression significantly correlates with ROCK2 and FAK respectively (Figure 21) in bony invasive OSCC. These results suggest that bony invasion in OSCC is correlated with the activation of mechanical signaling molecules in patient samples.

Gli2 inhibition, using GANT58-loaded microspheres significantly prevents osteolytic signaling and subsequent bone loss. Our *in vitro* findings suggest that mechontransduction inhibitors may be useful to prevent bony invasion in OSCC. However, existing inhibitors have poor pharmacokinetic profiles or quickly become ineffective[161]. Since Gli2 remains the essential modulating protein controlling bony invasion in OSCC, we chose to target Gli2 using GANT58 in a semi-orthotopic model of mandibular invasion. Targeting Gli2 has two main advantages. Since Gli2 activity is absent in many adult tissues[75], we anticipate fewer off-target effects. Additionally, because GANT58 directly targets Gli protein, there is a lower chance of acquired

Co-occurrence of Gene A and Gene B in 530 HNSCC samples (Modified from cBioPortal)		
Gene A	Gene B	p-value
PTK2	PTHLH	0.004

Figure 21. Clinical OSCC samples with bony invasion show increased levels of focal adhesion kinases as compared to samples with no bony invasion FAK (PTK2) significantly correlates with PTHrP (PTHLH) in a cohort of 530 HNSCC samples suggesting that FAK is important for bony invasion in OSCC.

resistance to Hh inhibition, unlike current Smo inhibitors[162, 163]. Unfortunately, GANT58 is difficult to administer in biologically relevant doses due to its hydrophobic nature. To address this limitation, GANT58 was loaded into poly-propylene sulfide (PPS) microspheres that degrade in the presence of cell-secreted reactive oxygen species[164, 165], which is commonly elevated at the tumor microenvironment. Incubation of GANT58 microspheres in 0.02 – 3% H_2O_2 resulted in a dose-responsive release of the drug (Figure 22A & 22B). OSCC cells were injected into the masseter muscle of male athymic mice and allowed to progress for ten days, at which time, GANT58 loaded or empty (control) microspheres were injected directly into the tumor site two to three times weekly for four weeks. High-resolution x-rays of dissected mandibles were used to quantify the area of bone loss. Mice treated with GANT58 microspheres had a significantly smaller total lesion area compared to mice treated with empty microspheres (Figure 23A). Additionally, μCT analyses of the mandibles showed that GANT58 treatment significantly prevented bone loss (Figure 23B). Histological analyses demonstrate a significant decrease in osteoclasts and PTHrP levels, demonstrating that Gli2 inhibition using GANT58 microspheres significantly prevents osteolytic signaling and subsequent bone loss (Figure 23C and 23D).

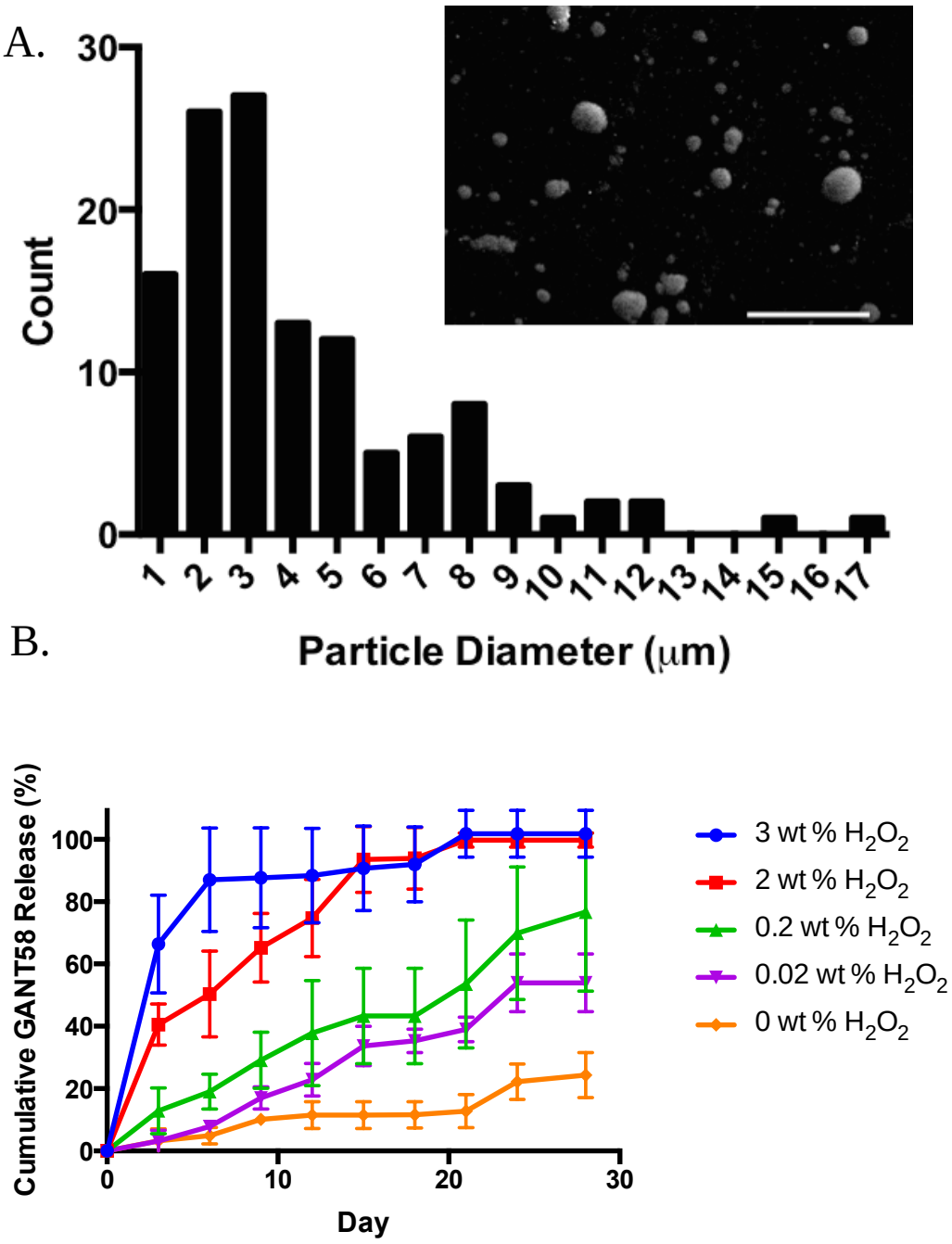


Figure 22. (A) Size distribution histogram of microsphere diameter of over 100 samples based on SEM images. Results indicated an average diameter of 4.2 μm with a standard deviation of 3 μm. Scale bar in SEM representative image is 50 μm. **(B)** *In vitro* ROS-dependent release kinetics of GANT58 from the PPS microspheres in the presence of H2O2 concentrations ranging from 0 to 3 wt%. Microspheres exhibit an H2O2 dose-dependent release over a 28-day period.

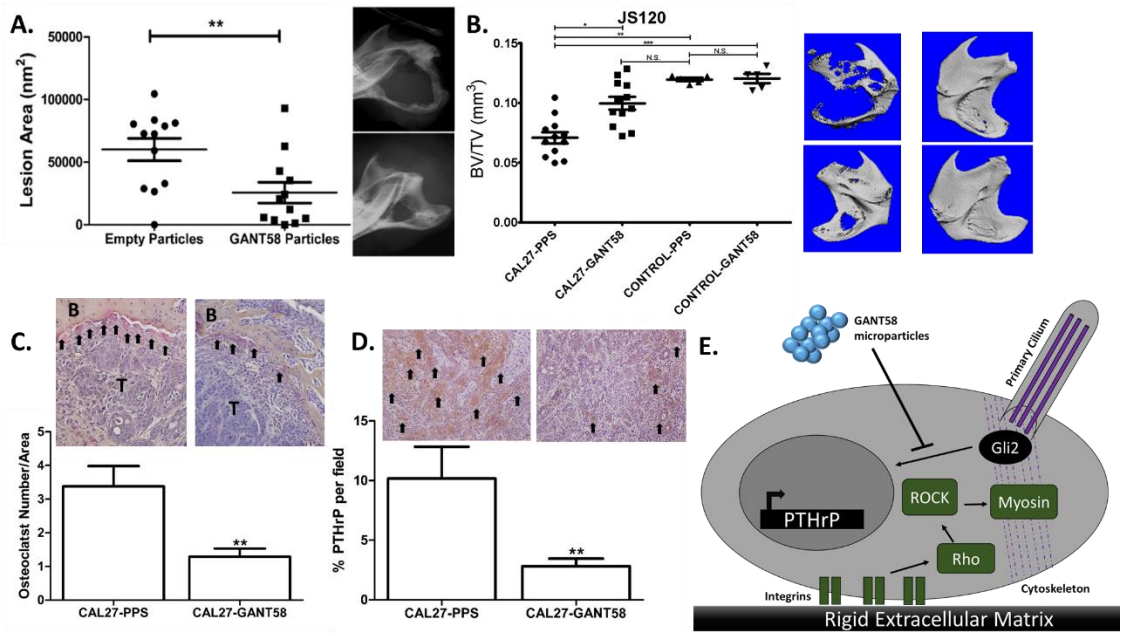


Figure 23. Gli2 inhibition using GANT58 decreases PTHrP expression and bony invasion *in vivo* **(A)** Male athymic mice treated with GANT58 loaded microparticles show a significant decrease in lesion area by high resolution x-ray. **(B)** Similarly, mice treated with GANT58 loaded microparticles have significantly more bone volume as compared to the control mice. Importantly, non-tumor bearing mice treated with GANT58 microparticles have bone volumes similar to that of mice treated with control particles. **(C)** Mice treated with GANT58 microparticles also show decreased levels of TRAP positive multi-nucleated cells as well decreased levels of PTHrP positive staining **(D)**. **(E)** Proposed mechanism of bone rigidity mediated OSCC invasion.

Discussion

Our studies have demonstrated that in OSCC, that bone-like rigidity can alter the expression of genes associated with bone destruction by activating the Hh-TGFβ/Gli2/PTHrP signaling axis. When OSCCs are grown on compliant matrices (basement membrane), they are unable to stimulate Gli2 expression. However, on rigid matrices (bone tissue), cells dramatically increase Gli2 and PTHrP levels. We demonstrate that this activation of Gli2 is driven by mechanical signaling, which can facilitate the formation of primary cilium, leading to an increase in Gli2 activation and subsequently PTHrP expression (Figure 23E).

The primary cilium is a complex organelle known to function as a mechanosensor and mechanotransducer[166-168]. While this is known to occur via calcium signaling, other pathways, such as Hh and PDGF signaling, also play a role[169, 170]. Additionally, primary cilium formation depends on, among other things, the organization and assembly of various cytoskeletal proteins and filaments. While tubulin is the main component of primary cilium, actin organization as well as myosin activity status also play important roles in ciliogenesis[171-173]. Furthermore, the activity and orientation of the cytoskeleton is itself dependent on the rigidity of the ECM[174, 175]. This suggests that primary cilium is not only important for cells to respond to ECM rigidity, but proper ciliogenesis may also dependent on it. This dynamic positive feedback loop integrates the role of ECM rigidity to increasing levels of primary cilium and thus canonical Hh signaling.

Currently, there are no published studies investigating the regulatory role of bone extracellular matrix rigidity on OSCC invasion. Recent reports have highlighted the contributions of ECM proteins such as fibronectin and thrombospondin, on increasing the invasive and metastatic potential of OSCC[22, 97]. In these studies, mechanical signaling via integrin and downstream Rho activation are responsible for initiating signaling cascades important for NK-kB signaling. In our studies, we specifically investigated the role of bone mimicking ECM on increasing bone-invasion potential. While the contribution of Rho/ROCK signaling was demonstrated to be important for increasing Gli2 and PTHrP levels, additional work is needed to understand how these signaling systems are activated upstream. In our system, cilengitide treatment to inhibit αv integrins was unable to decrease bony invasion, suggesting that other mechanotransducers are involved. Hippo network signaling, specifically at the level of YAP and TAZ, is the likely mediator converting mechanical cues into biochemical signaling cascades in bony invasive OSCC. In the last decade, significant strides have been made to understand canonical and non-canonical

YAP/TAZ regulation. In fact, several groups have identified YAP/TAZ nuclear localization when cells are grown on rigid 2D or 3D substrates[176, 177]. On rigid substrates, YAP/TAZ is shuttled into the nucleus, where it activates gene expression. On compliant substrates, however, YAP/TAZ remains trapped in the cytosol, where it is inactive[178]. While we did not directly investigate Hippo network signaling, its role as a mechanosensing and mechanotransducing signaling system suggests that it is likely playing a role in our system as well[179, 180]. Existing research investigating mandibular invasion of OSCC has yet to translate into actionable targets to prevent bony invasion. However, our findings support a targeted and viable approach to prevent bony invasion by using newer local delivery systems. Engineered particles, on the micro and nano scale, are useful to increase drug retention and uptake[181]. Additionally, the use of these particles allows for solubilization of drugs that would otherwise be difficult to administer in physiologically relevant doses. GANT58 for example, though efficacious *in vitro*, is too non-polar to be used in physiological doses. We show, for the first time, that in a relevant pre-clinical animal model of mandibular invasion, encapsulated GANT58 can be used to prevent bony invasion of OSCC tumor cells. This key experiment extends the application of Gli2 inhibition to all subtypes of OSCC that express Gli2 and PTHrP using both canonical and non-canonical Hh signaling. These results can also be extended beyond OSCC. Several other tumor types that use Hh signaling as a driver of tumorigenesis, such as medulloblastoma and osteosarcoma, can be targeted using Gli2 inhibitors[182]. Additionally, unlike current Hh inhibitors that target Smo, GANT58 inhibits the final Hh effector protein Gli which is much less likely to lead to acquired resistance. Collectively, these findings demonstrate the feasibility of using locally delivered Gli2 inhibitors to prevent rigidity-mediated tumor-induced bone destruction in OSCC patients and should be evaluated further as a possible adjuvant targeted treatment.

Materials and Methods

Cell lines

The OSCC cell lines SCC4 and CAL27 were used as described. Cell lines were cultured in 50% Dulbecco's modification of Eagle's medium and 50% Nutrient Mixture F12 (DMEM/F12) (ThermoFisher Scientific), supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories) and 1% penicillin/streptomycin (Mediatech). Cells were maintained at 37°C with 5% CO2.

Drug Treatments

All drug treatments were carried out in serum free DMEM/F12 for 24 hours. TGFβ was used at 10ng/mL. TGFβ buffer (5%BSA in 4mmol HCl) was used as a control. GANT58 (Sigma-Aldrich) was used at 10μM. Ihh and SIS3 (EMD Millipore) were used at 25ng/mL and 10μM respectively. Cyclopamine (LC Labs) was used at 12nM. SB202190 (Tocris) was used at 10μM. Y-27632 (Sigma) was used at 20μM. Cilengitide (SelleckChem) was used at 100nM. Rhosin (Calbiochem) was used at (30 μM). Blebbistatin (Tocris) was used at 2μM.

Transfections/ Luciferase Assays

Cells were transfected per manufacturer's instructions using Lipofectamine 2000 (ThermoFisher Scientific). Briefly, cells were incubated overnight in Opti-Mem (ThermoFisher Scientific) before being transfected at a ratio of 1.25 μg DNA to 1 μl Lipofectamine 2000. All cells were transfected with the Gli reporter plasmid and the pRL Renilla Luciferase control reporter plasmid (Promega) at a ratio of 3:1. Fresh media containing drug treatments and antibiotics was added to cells the following morning. Cells were harvested 24 hours later and luciferase activity measured using the Dual-Luciferase Assay System (Promega) and the 20/20n Luminometer (Turner Biosystems).

Quantitative real-time PCR

Cells were harvested by direct lysis and total RNA extracted using the RNeasy Mini Kit (Qiagen). The qScript cDNA synthesis kit (Quanta, VWR) was used to synthesize cDNA from 1ug RNA. Validated Taq-Man primers from (ThermoFischer Scientific) were used to measure gene expression in triplicate (from a minimum sample number of n=3) using the 7500 Real-Time PCR System from Applied BioSciences (ThermoFisher Scientific). Absolute gene expression was quantified using a standard curve and 18S was used as an internal control.

Antibodies

Primary Antibodies: Gli2 (Novus Biologicals) 1:500, Histone H3 (Abcam) 1:200, EGFR (Abcam) 1:5000, GAPDH (Cell Signaling) 1:10 000, PTHrP (Jack Martin) 1:4, acetylated tubulin (Sigma) 50ng/ml. Unlabeled goat IgG and rabbit IgG from Santa Cruz (1:400) were used as control primary antibodies.

Secondary Antibodies for Western Blots and IHC: Anti-mouse IgG HRP (Advansta) 1:25 000, Anti-goat IgG HRP (Santa Cruz) 1:5000, Anti-rabbit IgG HRP (Santa Cruz) 1:5000

Secondary Antibodies for IF: Alexa Flour 488 anti-rabbit IgG (Life Technologies) 1:2000, Alexa Flour 594 anti-mouse (Life Technologies) 2 drops/ml.

Western Blots

Briefly, cell lysate was harvested using RIPA Buffer (Sigma) supplemented with protease and phosphatase inhibitors (Roche). Nuclear and cytoplasmic extracts were separated using NE-PER (Pierce) and lysates were quantified using the BCA Protein Assay Kit (Pierce). 10-15ug of protein were loaded per well and gels were run using Nu Page supplies (Novex by Life Technologies) before being transferred to PVDF membranes. Membranes were blocked in TBS with .1% Tween-20 and 5% BSA or 5% non-fat dry milk for 2 hours. Primary antibody incubations were done overnight at four degrees under gentle agitation, and secondary antibodies were incubated for one

hour at room temperature under gentle agitation. Membranes were exposed using Western Lightening Plus-ECL (Perkin Elmer).

Clinical OSCC Samples

Dr. Kim Ely reviewed patient charts to identify OSCC patients that underwent a mandibulectomy as compared to those that underwent soft tissue removal which was used to purchase 16 matching cyrosections from the Vanderbilt-Baker Head and Neck Bio-repository.

Immunofluorescence Staining

Cells were fixed for 20 minutes in 4% formaldehyde, then permeabilized for five minutes in .1% Triton X-100 (Sigma) before blocking in 5% BSA. Primary antibodies were used overnight at four degrees. Secondary antibodies were used for one hour at room temperature. Cells were stained with DAPI (100ng/ml) or TO-PRO®-3 (1 μM) to distinguish nuclei before being cover-slipped for imaging.

Microsphere characterization

Microspheres were generated as described [31]. GANT58-loaded microspheres were size characterized using SEM and had an average molecular diameter of 4.2 ± 3 μm. An average diameter greater than 4 μm was imperative to discourage macrophage phagocytosis of the microsphere. A sufficient microsphere size was achieved by using a 0.5% PVA solution as a stabilizer during the microsphere fabrication process as well as adjusting the homogenization time to 45 seconds. The loading and encapsulation efficiency were determined using a fluorescence plate reader and the fluorescence of GANT58 after being dissolved in DMSO for a 24-hour period. The loading and encapsulation efficiency was determined to be 14% and 25%, respectively, using established formulas as described.

Animal Studies

All animal studies were carried out in compliance with the Vanderbilt University Institutional Animal Care and Use Committee and the National Institutes of Health guidelines.

Masseter muscle injections: 1x106 cells were injected into the right and left masseter muscle (parallel to the mandible) of 4-6-week-old athymic male mice from Harlan Laboratories. Tumor control mice received PBS injections. Drug treatments began once tumors were palpable (~10 days) which consisted of 50-75 µl injections of ~5mg/kg GANT58 loaded microparticles or empty-PPS microparticles as controls. Mice were weighed weekly to assess tumor burden and provided with soft food (Diet Gel, Clear H20) ad libitum. All mice were sacrificed after 28 days and mandibles were dissected for ex vivo analyses. The XR-60 digital radiography system from Faxitron was used to acquire high-resolution images from dissected mandibles.

Immunohistochemical Staining

Mandible specimens were dissected and fixed in 10% neutral-buffered formalin (Fisher Scientific) for 48 hours at four degrees. Mandibles were then decalcified in 10% EDTA for 10 days at room temperature under gentle agitation and then embedded in paraffin. Mandible sections (5-7µm thickness) were stained with hematoxylin & eosin, orange G, and phloxine to measure tumor burden. Antibody staining against Gli2/PTHrP was used to measure Gli2/PTHrP protein expression. HRP linked rabbit anti-goat or goat anti-rabbit (Santa Cruz) and ImmPACT NOVA RED from Vector Laboratories were used to visualize staining. TRAP staining was used to measure osteoclast numbers. All slides were examined under an Olympus microscope at 20X and 40X and images (taken using Olympus DP71 camera and software) were quantified using Metamorph software (Molecular Devices, Inc.) for thresholding and region of interest analysis.

Immunohistochemistry/Immunofluorescence Analyses

Histological images were uploaded into Metamorph. For each image, the bottom incisor was used as a landmark. The area of tumor was traced using region of interest analyses, then, positive staining was quantified for each slide using a representative threshold (based on the positive control). For PTHrP, positive staining is marked by tumor specific brown staining. For Gli2, positive staining is marked by tumor specific nuclear brown staining. Non-specific staining from the IgG control was used measure background staining. This value was subtracted from each slide to normalize values. The resulting value represents positive staining, which is then divided by the total area of the image yielding percent positive staining.

Statistical analyses

All *in vitro* experiments were done in triplicate with a minimum n =3 samples. All *in vivo* experiments were done with a minimum of n=8 mice per group. All statistical analyses were done using InStat v3.03 software from GraphPad Software. Significance is presented as asterisks, where * denotes $p<.05$, ** denotes $p<.01$ and *** denotes $p<.001$.

CHAPTER IV

CONCLUSIONS AND DISCUSSION

OSCC remains a significant clinical problem globally. While understanding factors that cause OSCC has led to improved prevention methods (such as dental screening and smoking cessation programs), patients afflicted with the disease continue to have limited treatment options and poor prognoses[6]. EGFR inhibition in OSCC, using Cetuximab, was a highly anticipated targeted treatment option and is in fact the first FDA approved targeted treatment for HNSCC. Unfortunately, like may targeted treatments, drug tolerance and tumor relapse develops in many OSCC patients. In fact, although 75% of HNSCC over-express EGFR, clinical trials have demonstrated that Cetuximab alone is mostly beneficial in cases of metastatic disease, and in some staging groups does not increase progression free survival[28, 30]. Recently, HPV related increases in carcinoma of the head and neck (particularly in men ages 30 -40) has garnered much attention in the media and has increased awareness of the disease. However, HPV induced HNSCC mostly develops in the oropharynx (which is not included in the oral cavity) and actually show better response rates to treatments, increasing overall survival. Unfortunately, this is not the case with OSCC, as many patients show tumor relapse and decreased overall survival. While EGFR is often overexpressed in OSCC, it is now generally accepted that EGFR amplification is not a common driver of tumorigenesis in OSCC patients, though it certainly contributes to tumor progression. Current clinical trials in OSCC seek to investigate possible synergistic treatment effects with Cetuximab in combination with different classes of chemotherapy/radiation and/or another immunotherapy, namely PD-1. Research in OSCC has identified other genes important for OSCC, with a few (such as the p53 superfamily), being implicated as a driver mutation. OSCC sequencing studies however, do not show consistent mutated/upregulated genes that could also

serve as reliable targets. Additionally, many OSCC tumors demonstrate signaling plasticity as opposed to oncogene addition, where many patients fail to respond to Cetuximab in an appreciable amount even though tumor biopsies stain positive for EGFR. The field of OSCC research has seemed to acknowledged that OSCC initiation and progression is multifactorial and several groups have demonstrated the importance of personalized therapy based on the gross genetic differences not only between different OSCC patient tumors (derived from sequencing data), but also within tumors themselves. Without improvements in targeted treatment options however, improvements in survival for OSCC seem unlikely.

Hh/Gli Signaling Axis

The work presented in this text demonstrates some of the signaling plasticity observed in other OSCC tumors. Importantly however, it contributes to the body of knowledge surrounding OSCC signaling mechanisms. For OSCC invasion into the bone, this is especially important as there have been very few studies investigating this subset of OSCC. In Appendix C, we have shown that bony invasion and bone destruction is controlled by Hh and TGFβ signaling, which are required to increase Gli2 expression and protein activity and PTHrP expression and protein levels in OSCC. Additionally, beta-catenin has the potential to regulate Gli2 and PTHrP, but Wnt signaling is dysregulated in OSCC. This signaling system requires functional mechanical signaling, where rigid matrices increase ciliogenesis in a Rho/Rock/myosin dependent manner. Increased ciliogenesis leads to an increase in canonical Hh signaling which is at the apex of Gli protein regulation in OSCC. Bony invasion and bone destruction can be significantly decreased by targeting Gli protein, the effector molecule of this osteolytic signaling system, using GANT58 loaded microparticles. This work demonstrates the central role of Hh signaling, specifically Gli2 on regulating PTHrP, an essential osteolytic signaling molecule in OSCC.

Gli role in invasion. Gli2 in OSCC is known to correlate with tumor progression and poor patient prognoses[75]. Our studies indicate that targeting Gli2 would be beneficial to reduce bony invasion and bone destruction in patients. Patients without mandibular involvement may also benefit from Gli2 inhibition. In the infiltrative OSCC line, CAL27, we used shRNA to stably knock-down Gli2 expression. Different clones of CAL27 cells with repressed Gli2 expression were used in an MTT assay to measure cell viability and no significant differences in cell viability between any clones and the transfection control CAL27 cells were observed. However, when shGli2 CAL27 cells are injected into the masseter muscle of athymic male mice, a distinct phenotype was observed. CAL27 cells with repressed Gli2 expression had significantly lower levels of soft tissue invasion into the muscle, which was the site of tumor injection. In some mice, there was no observable tumor take or the tumors were very small, not well vascularized and not attached to the muscle tissue. (Appendix D) We have also found the opposite to be true, where Gli2 protein overexpression in SCC4 significantly increases PTHrP expression. In vivo however, SCC4 cells overexpressing Gli2 present as mixed (osteoblastic and osteoclastic) lesions, making analyses difficult to interpret. Appendix E shows no significant difference in the degree of anisotropy, or organization, in the long bones of tumor bearing mice. Additionally, although we observed greater bone loss in the Gli2 over expressing group, there was also increased new bone formation denoted by the starburst pattern of bone by x-ray (Appendix F). These findings highlight the role of Gli2 on mediating invasion and bone destruction in OSCC. We have found similar results in vitro using the Gli antagonist GANT58. CAL27 cells were used in migration and invasion assays, where CAL27 cells are seeded in serum-free medium in chambers on top of a porous membrane with serum-containing medium in the chambers below. Under these conditions cells migrate downward through the pores into the serum- containing medium. When GANT58 is added to the cells seeded

in serum-free medium, cells are still able to migrate in similar numbers as control cells, suggesting that GANT58 does not play a major role in mediating tumor cell migration. However, to model invasion, a thin layer of serum reduced Matrigel was coated onto the porous membrane, requiring cells to first invade through the Matrigel layer before migration through the pores. This invasion is an active process, as cells have to secrete factors, such as MMP's (Appendix G) to degrade the crosslinked proteins of the Matrigel layer. Cells grown on Matrigel in serum-free medium invade as small clusters of cells to the bottom chamber. However, when transfected to express shRNA against Gli2, cells are unable to invade through the Matrigel (Appendix H). These findings demonstrate the importance of Gli signaling for invasion *in vitro* and can be used to extrapolate mechanisms behind similar observation *in vivo*.

Implications of Gli targeting. The functional consequence of Gli in OSCC invasion (both bony and non-bony) suggests that Gli inhibition would be beneficial in OSCC patients. We propose the use of GANT58 to inhibit Gli activity to prevent bony invasion, though there are clear applications for its use in soft tissue invasion as well. Gli expression is silenced in most adult tissues, although can be found in basal/stem-cell compartments, making Gli2 a better target due to less anticipated side effects. There are currently no direct Gli inhibitors on the market, though inhibitors to its upstream regulator Smo have been, and continue to be, developed. Unlike Smo, which develops several drug resistant mutations before and after treatment, Gli is highly conserved and is not expected to develop actionable mutations. One caveat of Gli inhibition using GANT58 centers around unintended targets. Gli2 is one of three Gli proteins (Gli1, Gli2 and Gli3). While Gli3 is structurally distinct and functions as a transcriptional repressor, Gli1 and Gli2 are very similar and are both targets of GANT58. This may be beneficial, as Gli2 inhibition alone may lead to a compensatory upregulation of Gli1contributing to drug resistance and treatment failure. In this

case, Gli1 and Gli2 inhibition ensures that downstream Gli targets are effectively silenced. However, targeting both proteins also means that cell compartments that actively use Hh signaling, such as in the stem cell niche of the hair follicle[183], would be deregulated leading to possible negative side effects. In OSCC however, Gli1 mRNA is not detectable in several cell lines. We postulate that in these cells, Gli1 remains silenced as it should be in normal adult tissues and has not been induced oncogenically. OSCC cell lines transfected to express Gli1 also do not show an increase in PTHrP expression, supporting that in OSCC, Gli2 is the regulator of PTHrP expression. Another major caveat of GANT58 treatment to inhibit Gli is drug delivery. GANT58 is highly polar and is unable to be delivered to the tumor in appreciable quantities. Our group has overcome this limitation by loading free GANT58 drug into microparticles that disassemble in hypoxic environments, similar to what is seen at the tumor microenvironment. In our system, microparticles are delivered directly to the tumor site, where they serve as a drug depo and release GANT58 as needed. This delivery mechanism works well for clearly visible and/or more superficial tumors. Invasive tumors that are not easily seen however, would be harder to treat, like those found on the floor of the mouth.

Gli inhibition using GANT58 to prevent bony invasion would not be done as a single agent. In athymic male mice bearing CAL27 tumors treated with GANT58, tumors grew to be similar in size as those treated with the control microparticles. To this end, GANT58 should be paired with an antitumor treatment to decrease tumor burden while also preventing bony invasion. Based on our findings, there may be synergistic applications of inhibiting Gli *in vivo*. Several groups have attempted to understand which genes are mutated in OSCC to determine if there exists a genetic signature in OSCC that can be used to stratify tumor drivers, tumor types, treatment options, and prognoses. While these studies have not directly identified Hh/Gli signaling molecules, genes that

have the potential to regulate Gli have been identified. This includes Ras signaling and beta-catenin. Both of these studies investigated gene mutations, and included Smo in their gene panels. It is important to note however that wild-type Smo can function as a potent oncogene and demonstrates mutational resistance after drug treatment, which was not controlled for in these studies. Additionally, using cBioPortal, we observe that from a 528-sample cohort, 15% of samples show Gli2 alteration. Strikingly, only three of the 15% demonstrated mutations, with the resulting 12% being upregulation and/or amplification of the Gli2 gene. These results highlight the limitation of investigating a mutagenic phenotype in a system where the presence/absence of a gene is just as, if not more, important.

TGFβ Signaling in Bony Invasion.

In our work, we specifically investigated other signaling systems that have the ability to regulate Gli2, at the level of transcriptional control as well as protein, independently of canonical Hh signaling. TGFβ signaling, which is known to regulate PTHrP expression via Smad/MapK activation is also active in OSCC[66]. Our work demonstrates the contribution of Smad2/3 overexpression on increasing PTHrP expression quite dramatically. We have observed functional Smad signaling is also important for cell viability (Appendix I), where Smad3 inhibition decreases cell viability 50% after just 24 hours. MapK is essential as well, since p38/MapK inhibition using SB202190 significantly decreased stimulated PTHrP expression. The question remains, however, if Smad2/3 regulates PTHrP via Gli2. Existing work demonstrates functional Smad binding sites on the promoter region of PTHrP, suggesting that TGFB may be able to regulate PTHrP expression independently of Gli2. However, it is also known that there are functional Smad2/3 binding sites on the Gli2 promoter as well. We have observed, using luciferase reporter assays that Gli2 promoter activity is significantly increased when TGFβ signaling is active. This suggests that

TGFβ may directly increase Gli2 expression and may also directly and indirectly increase PTHrP expression. Based on existing information and because of the sequential and connected roles of this signaling system, the extensive experiments required to delineate precisely if and how TGFβ signaling activates Gli2 and PTHrP are not warranted.

Wnt Signaling in Bony Invasion.

Hh signaling and Wnt signaling have been known to cross-talk in development, and in our system, a similar observation has been made. While Wnt signaling in OSCC appears to be dysregulated, downstream activation of beta-catenin and Wnt related targets have been shown. In OSCC it is not clear if upstream Wnt receptors Frizzled and Lrp are functional. Endpoint PCR for LRP5/6 expression showed the presence of LRP5/6 mRNA in erosive SCC4 cells. Treatment with lithium chloride however did not have a measurable effect on PTHrP mRNA expression. By qRT-PCR lithium chloride treatment also did not increase DKK1, a well-known Wnt target gene that should be transcriptionally activated in response to active canonical Wnt signaling. Interestingly, TGFβ treatment in SCC4 cells led to a threefold increase in DKK1 mRNA expression. Although Wnt activation using lithium chloride had no measurable effect, one possible explanation for this observation may be the concentration of lithium chloride needed may have been different from that given to the SCC4 cells. This possibility is unlikely though, as SCC4 cells were treated with one, five, and ten micro molar concentrations of lithium chloride to measure PTHrP expression using luciferase reporter assays which showed no significant differences in PTHrP promoter activity with or without lithium chloride treatment. An additional experiment was used to test 20 micro molar and 40 micro molar concentrations of lithium chloride on increasing PTHrP mRNA by qRT-PCR (which also showed similar results). While it is unlikely that higher dosages are required to activate Wnt signaling, there are several possibilities for these observations that can be

investigated. Firstly, although SCC4 cells express LRP5/6 mRNA, it is not known whether the LRP5/6 receptors are actually present on the cell surface, and if they are whether they are functional/mutated or not. Additionally, the presence and/or levels of the receptor Frizzled was not investigated. Wnt signaling in OSCC is probably mutated/dysregulated, which is in line with reports of Wnt signaling molecules in patient samples. Evidence for this is seen in SCC4 cells treated with Sclerostin, which is a Wnt inhibitor that binds directly to the Wnt receptor LRP. SCC4 cells treated with Sclerostin significantly increase PTHrP expression, more so than TGFβ stimulation, which suggests that Wnt signaling is active but abnormal. These findings do not rule out the possibility of Wnt signaling silencing downstream of the receptor, since we have not yet been able to measure increased beta-catenin levels/activation with upstream Wnt modulation. Additional experiments investigating whether Wnt signaling can be activated with receptor based stimulation are still needed to understand the status of Wnt signaling in OSCC. One plausible explanation is that Wnt signaling is inactive in OSCC. Our experiments modulating beta-catenin represent exogenous expression of a form of beta-catenin that is not easily degraded. Thus, beta-catenin regulation of Gli2 and PTHrP that we have observed may represent a possible signaling avenue for OSCC as compared to an existing one.

Mechanical Signaling in Bony Invasion.

Finally, the regulation potential of bone rigidity demonstrates a novel role of ECM rigidity on increasing Hh signaling in OSCC. We have found that rigid matrices increase the osteolytic potential of OSCC by supporting ciliogeneis and thus, Gli activation. Based on our previous work investigating osteolytic signaling in triple negative breast cancer, we reasoned that in OSCC, extracellular signaling proteins that respond to changes in the ECM would be indispensable for our observed phenotype. Integrins are one of the main responders to ECM rigidity and the OSCC

cell lines used express several classes of integrins. However, OSCC cells treated with cilengitide to inhibit αv integrins remained responsive to TGFβ and significantly increased PTHrP expression. Integrin regulation however, makes it difficult to determine the status of integrin activity when cells are treated with cilengitide. Integrin inhibition can have profound effects on integrin protein levels, protein activity, and even gene expression, and it has been previously demonstrated that these events are not always correlative. For example, in glioblastoma (where cilengetide was used in clinical trials) data from basic research demonstrated that cilengitide could greatly reduce levels on integrins in the cell. Unfortunately, patients on cilengitide had poor responses and progressed rapidly, even though cilengitde levels were indeed lower. Later it was revealed that although protein levels were lower in cilengitide treated patients/cells, protein activity was similar to that of the untreated controls. This finding was essential to understanding the mechanism of resistance but is also context specific. In a bone-metastatic clone of the triple negative breast cancer cell line, MDA-MB-231, cilengitide treatment significant decreases integrin β3 expression. In OSCC treated with cilengitide, downstream activation of ROCK 1 was not significantly different from the control group. One plausible explanation for this observation is that in the integrin expression/activity profile in OSCC has not yet been explored. What is known though, is that another integrin receptor, integrin α5β1 is also capable of binding to the ECM of the rigidity models used in our experiments and importantly, is not a target of the αv inhibitor, cilengitde. Additional experiments investigating expression/activity levels of this integrin would be required to better understand the findings we observed. Similarly, to cilengitde treatment, RhoA inhibition using Rhosin did not prevent TGFβ induced expression of PTHrP. Here however, Rhosin treatment decreased levels of the downstream target ROCK1 by Western blot, suggesting that RhoA was inhibited as expected. We reason that the downstream activation of mechanical signaling that we

observed is probably not through the expected signaling cascade of integrins and RhoA. We speculate that other mechanosensitive signaling mechanisms, such as Hippo signaling with effector molecules YAP and TAZ, could be playing a role and should be investigated further[176, 177]. Regardless of the upstream activators, we expect an increase in activation of focal adhesion kinases once mechanical signaling is active. Data from the cBioPortal demonstrates a significant correlation between FAK gene expression and PTHrP in a large cohort of HNSCC patients. While this data suggests an important correlation between matrix rigidity and osteolytic signaling, gene expression does not always correlate with protein levels and more importantly, protein activity. To investigate this, we measured levels of FAK and active, phosphorylated FAK in a small cohort of OSCC patients. While the data trended, due to small sample size (n=12 total) and staining localization variability, FAK/pFAK was not found to correlate with bony invasion. Interestingly, some patients showed intense staining at the periphery of the tumor, while others showed diffuse staining throughout and still others showed intense staining in the adjacent stroma. These results were unexpected and confounded data analysis. Literature on this observation suggests that differential FAK/pFAK activation serves as a prognostic tool for tumor progression, especially in the context of invasion[184-186]. While we were unable to study this phenotype in greater depth, FAK/pFAK localization may prove to be informative to characterize different invasion patterns of OSCC in the future.

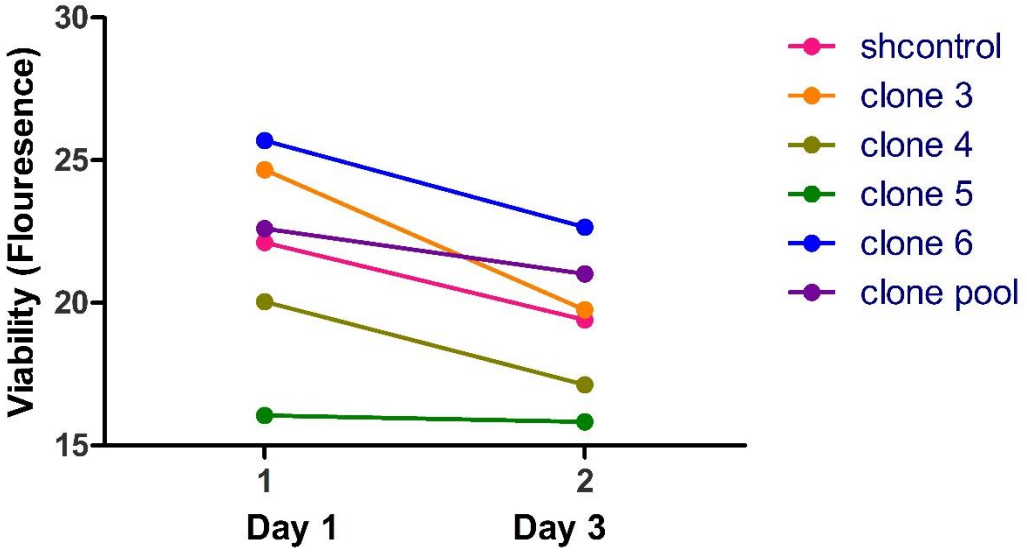
Ciliogenesis gene expression and numbers of primary cilia, were increased on bone-like matrices and dependent on mechanical signaling, respectively. The primary cilium is essential for canonical Hh signaling and though it is well studied in development and physiological settings, it is much less understood in cancer. In OSCC, primary cilium seems to be regulated as expected, since use of HPI-4 to inhibit ciliogenesis led to smaller numbers of primary cilia by

immunofluorescence and decreased expression of Gli targets by an EMT qRT-PCR array (Appendix J). In our system, it is not known what regulators are important for ciliogenesis gene expression, but we surmise that they are downstream or secondary targets of mechanical signaling. The osteolytic signaling system observed in OSSC grown on matrices with bone-like rigidities is dependent on engineered two-dimensional films of synthetic material, which presents a distinct set of challenges. Cells on compliant matrices take much longer (>3x longer) to adhere to the matrix when plated, as compared to cells grown on rigid films. In addition, cells grown on rigid matrices are phenotypically different, appearing larger, more uniform, and more "connected" with other cells in appearance. These clear phenotypic differences suggest that signaling systems between these groups may also be different. One bonus of using engineered 2D matrices is that the two rigidities are identical, in composition, but are tunable over a wide range of rigidities. Additionally, while we adsorb fibronectin to the 2D films to facilitate cell adhesion when plating, the ECM priotein can easily be replaced to match a range of cell specific needs. The rigidity dependent phenotype observed in these studies parallels the observation that once OSCC invades into the mandible, tumors progress, excess bone is resorbed and patients suffer from poor quality of life and shorter survival times. Importantly, this work demonstrates the feasibility of targeting the central regulator of OSCC invasion into bone, Gli2. These finding contribute substantially to the current knowledge of mechanisms controlling bony invasion. Before this work, these signaling systems were not shown to play a role in OSCC invasion. With this added body of knowledge, additional research, investigating the efficacy of Gli2 targeted treatment to prevent bony invasion, is warranted.

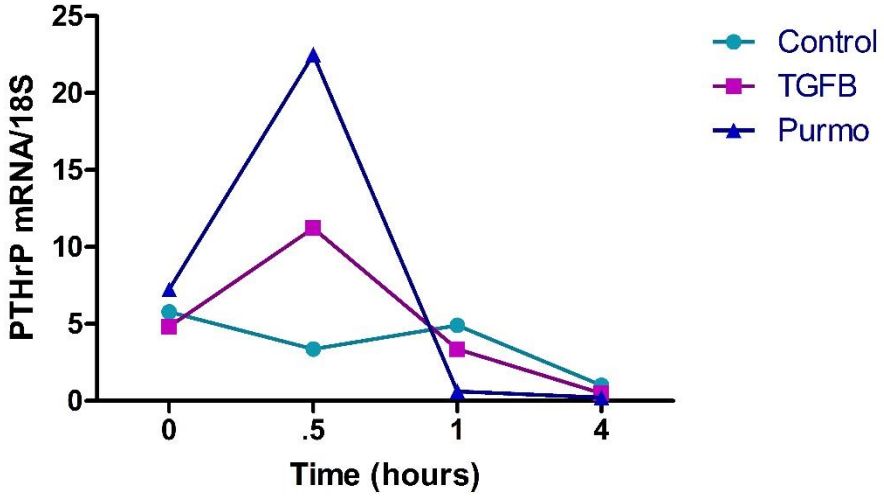
Future Directions

GANT58 loaded defect supports. The long-term goal of this project is to use the elucidated signaling mechanisms controlling bony invasion and bone destruction in OSCC as a basis for targeted treatments for patients at risk for recurrence of mandibular involvement. To do this, we propose a two-step approach to structurally support the mandible as well as prevent bony invasion. We have used 2D polyurethane films *in vitro* to modulate ECM rigidities, but we can also generate polyurethane matrices in 3D, using a 3D printer. Using uCT imaging, these scaffolds are almost perfect replicas of the normal resected bone and can be constructed in near micron scale. Additionally, by changing the composition of the starting materials to include polylactic acid, we can make 3D scaffolds that can be resorbed by osteoclasts. This becomes especially important when exogenous factors are embedded into the 3D scaffolds. We plan to fabricate 3D scaffolds containing lyophilized GANT58 to be used *in vivo.* We plan to experiment with also embedding growth factors to aid in healing and anti-tumor drugs to prevent tumor growth. Our preliminary experiments suggest that this plan is feasible (Appendix K), and warrants investigation. If successful, these findings can be used for the first targeted therapy to prevent bony invasion and bone destruction in OSCC patients.

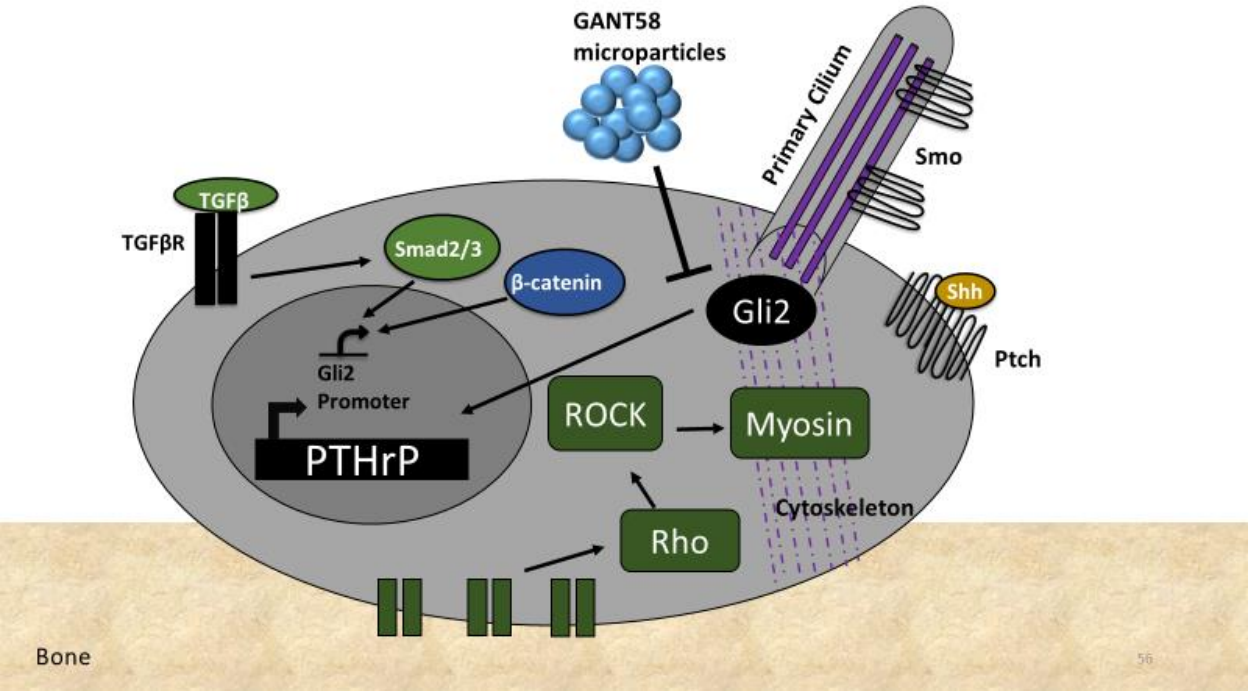
APPENDIX



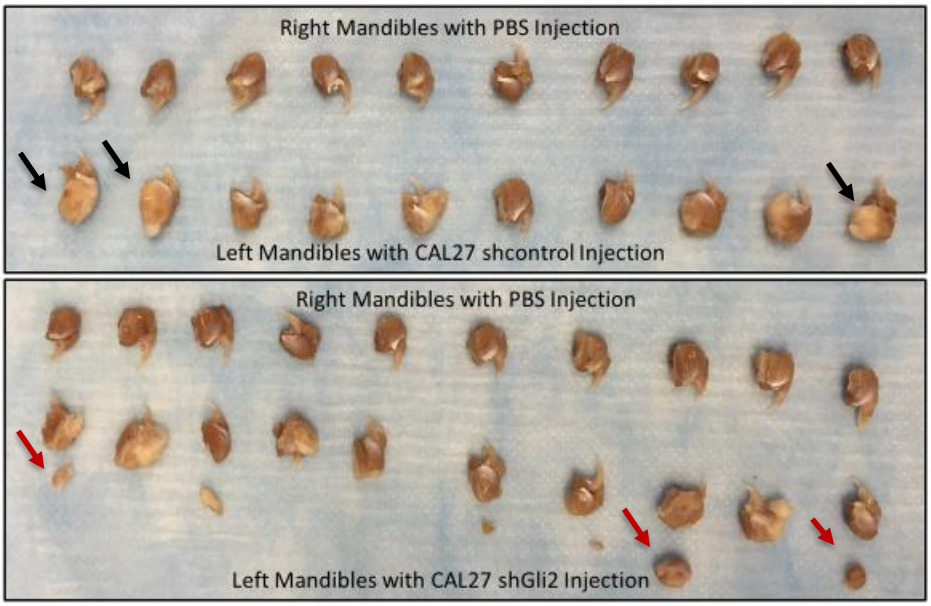
Appendix A: Gli2 loss in CAL27 cells does not significantly affect viability. CAL27 cells transfected to stably express shRNA against Gli2 show varied, although not significant, differences in viability by MTT assay.



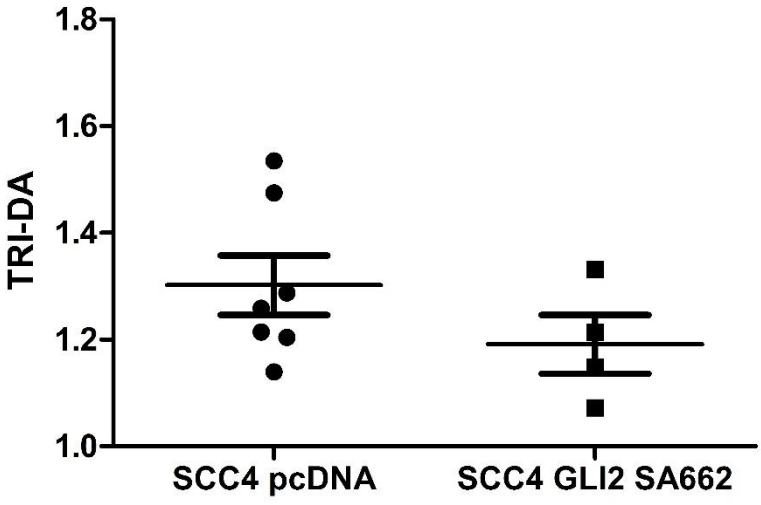
Appendix B: Purmorphamine treatment transiently increases PTHrP expression. SCC4 cells were treated with Gli activators TGFβ or purmorphamine along with Actinomyosin D to prevent transcription. PTHrP mRNA were measured using qRT-PCR and showed a potent, but transient increase of after stimulation. TGFβ stimulation is expected to increase PTHrP mRNA expression at later time points.



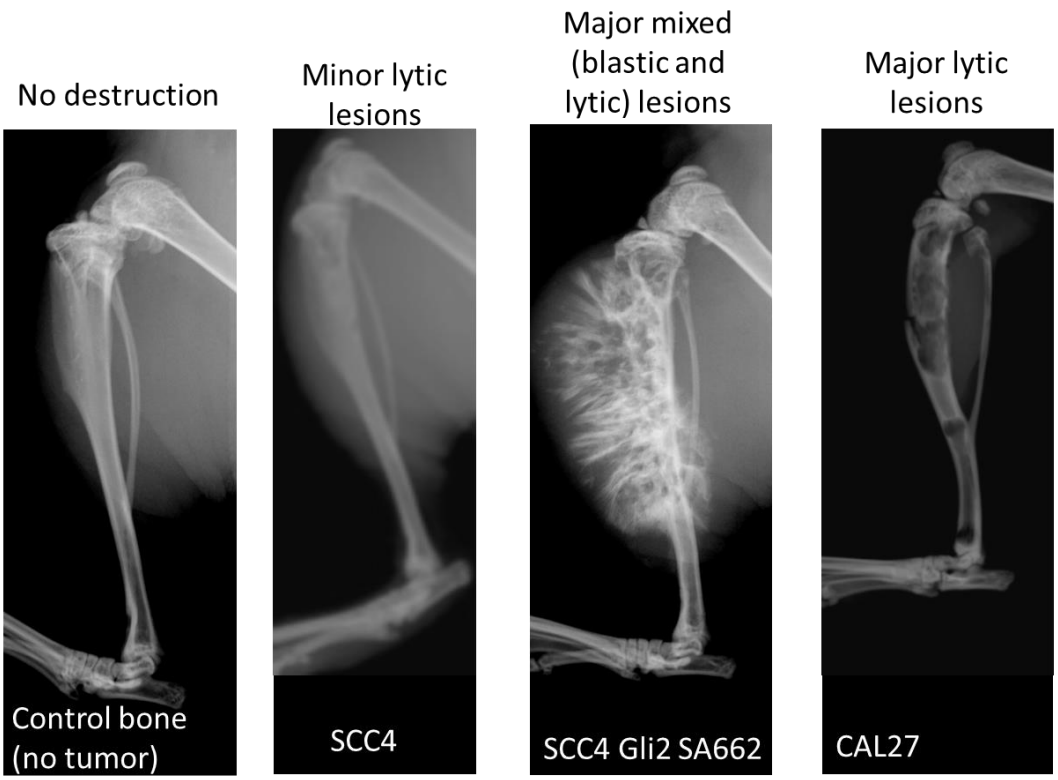
Appendix C: Mechanism of bony invasion and bone destruction in OSCC. Bony invasion and bone destruction is controlled by Hh and TGFβ signaling, which are required to increase Gli2 expression and protein activity and PTHrP expression and protein levels in OSCC. Additionally, beta-catenin has the potential to regulate Gli2 and PTHrP, but Wnt signaling is dysregulated in OSCC. This signaling system requires functional mechanical signaling, where rigid matrices increase ciliogenesis in a Rho/Rock/myosin dependent manner. Increased ciliogenesis leads to an increase in canonical Hh signaling which is at the apex of Gli protein regulation in OSCC. Bony invasion and bone destructed can be significantly decreased by targeting Gli protein, the effector molecule of this osteolytic signaling system, using GANT58 loaded microparticles.



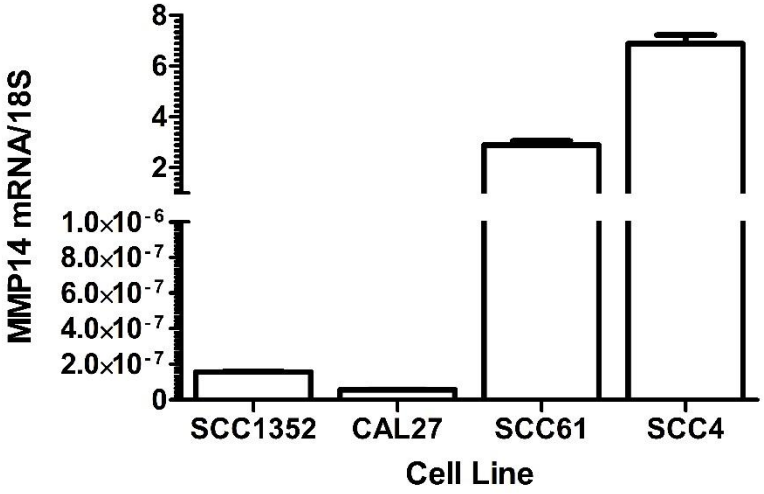
Appendix D: Loss of Gli2 in CAL27 cells reduces bony invasion and tumor size *in vivo*. CAL27 cells stably transfected to express shRNA against Gli2 were injected into the masseter muscle of male athymic mic and allowed to progress. Control tumors are larger and have invaded into the muscle tissue (black arrows) while tumors in the shGli2 group are much smaller and non-invasive (red arrows).



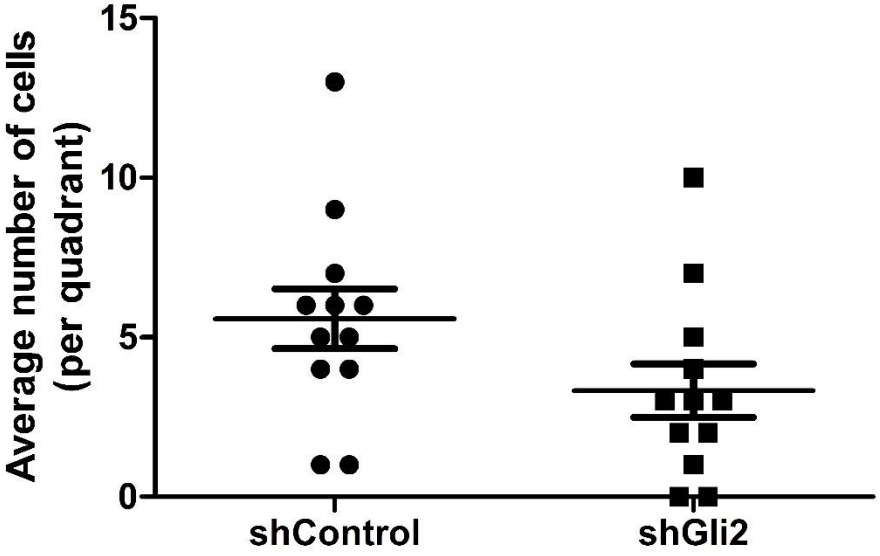
Appendix E: SCC4 control and Gli2 SA662 cells have similar degrees of anisotropy. SCC4 cells stably transfected to express Gli2 SA662 were injected into the tibiae of male athymic mic and allowed to progress. While Gli2 SA662 tumors showed greater tumor progression, uCT analysis demonstrate similar levels of bone disorganization.



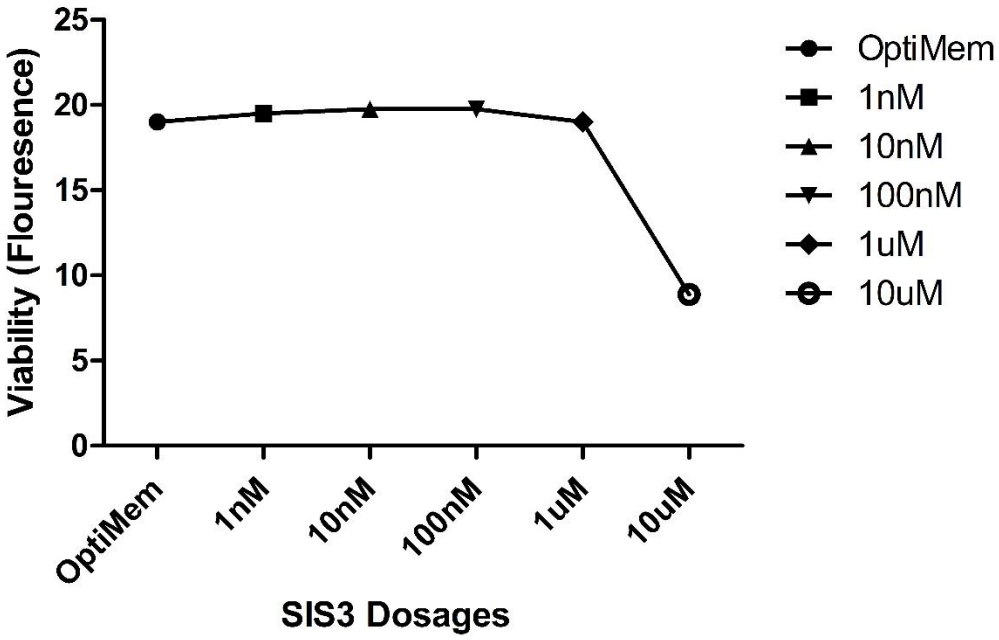
Appendix F: SCC4 Gli2 SA662 tumors cause mixed lesions *in vivo*. SCC4 cells stably transfected to express Gli2 SA662 were injected into the tibiae of male athymic mic and allowed to progress. When compared to non-transfected SCC4 and CAL27 tumors, SCC4 Gli2 SA662 mice show both bone destruction and new bone formation, which resembles starburst patterning.



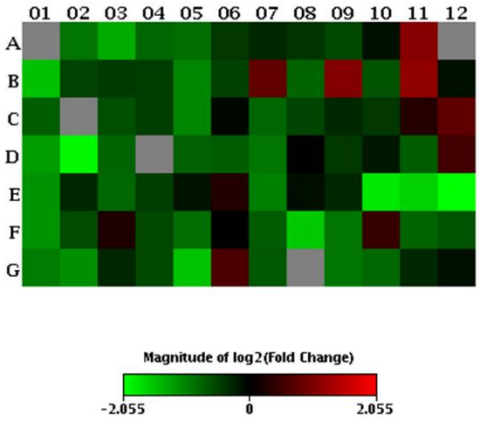
Appendix G: MMP14 Levels in OSCC. qRT-PCR shows that SCC61 and SCC4 express significantly more MMP14 than SCC1352 and CAL27 at basal levels.



Appendix H: CAL27 shGli2 invasion. Using Matri-Gel coated transwells, less CAL27 cells, stably transfected to express shRNA against Gli2, are able to invade, as compared to control transfected cells.

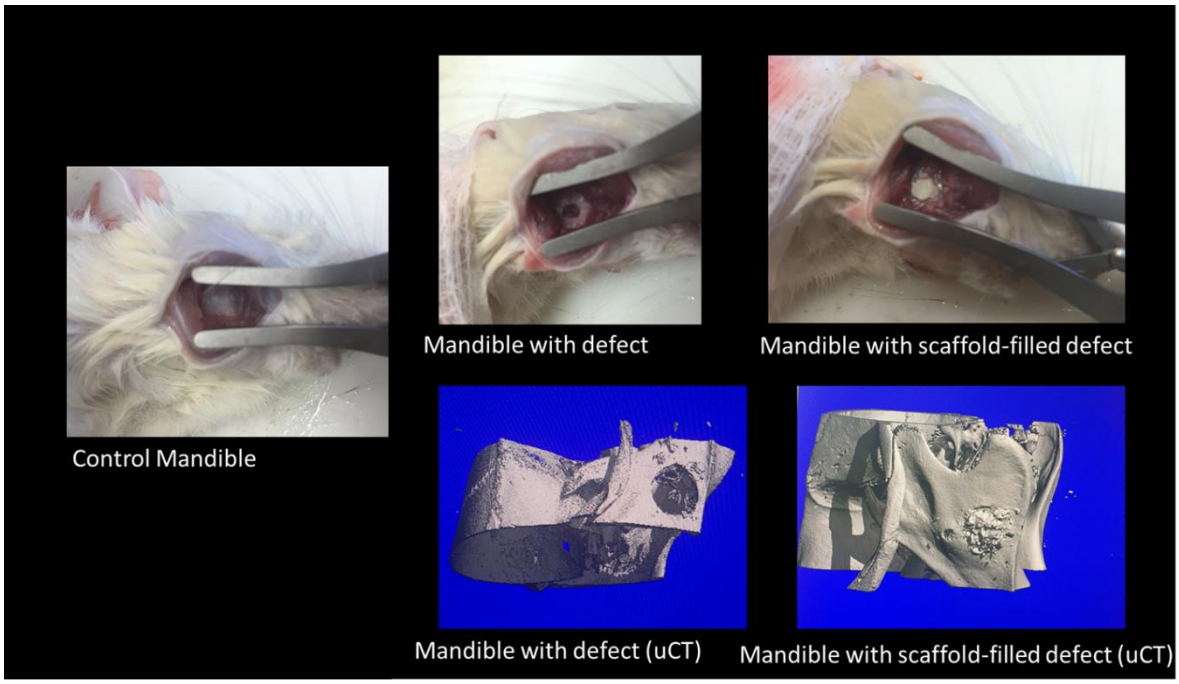


Appendix I: CAL27 MTT Assay with SIS3 treatment. CAL27 cells treated with a selective inhibitor of Smad3 for 24hours show 50% less viability at the indicated treatment concentrations.



Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AHNAK 1.03 C	AKT1 -1.93	BMP1 -2.61	BMP2 -1.78	BMP7 -1.86 A	CALD1 -1.36	CAMK2N1 -1.25	CAV2 -1.33	CDH1 -1.50	CDH2 -1.09	COL1A2 2.13	COL3A1 1.03 C
B	COL5A2 -2.95 A	CTNNB1 -1.46	DSC2 -1.39	DSP -1.41	EGFR -2.11	ERBB3 -1.45	ESR1 1.73 B	F11R -1.75	FGFBP1 2.08	FN1 -1.60	FOXC2 2.25 B	FZD7 -1.10
C	GNG11 -1.70	GSC 1.03 C	GSK3B -1.56	IGFBP4 -1.41	IL1RN -2.12	ILK -1.04	ITGA5 -1.77	ITGAV -1.48	ITGB1 -1.26	JAG1 -1.37	KRT14 1.23	KRT19 1.69 A
D	KRT7 -2.41 B	MAP1B -4.04	MMP2 -1.74 B	MMP3 1.03 C	MMP9 -1.72 B	MSN -1.67	MST1R -1.93	NODAL -1.01 B	NOTCH1 -1.36	NUDT13 -1.14	OCLN -1.69	PDGFRB 1.45 B
E	PLEK2 -2.29	DESI1 -1.26	PTK2 -1.78	PTP4A1 -1.41	RAC1 -1.12	RGS2 1.21	SERPINE1 -2.05	GEMIN2 -1.08	SMAD2 -1.25	SNAI1 -3.70 B	SNAI2 -3.25	SNAI3 -4.16 B
F	SOX10 -2.29 B	SPARC -1.52	SPP1 1.19 B	STAT3 -1.50	STEAP1 -1.85	TCF3 -1.01	TCF4 -1.67	TFPI2 -3.08 B	TGFB1 -1.94	TGFB2 1.36	TGFB3 -1.74 A	TIMP1 -1.60
G	TMEFF1 -2.00	TMEM132A -2.24	TSPAN13 -1.23	TWIST1 -1.50	VCAN -3.00 B	VIM 1.53	VPS13A -1.64	WNT11 1.03 C	WNT5A -1.93	WNT5B -1.79 B	ZEB1 -1.25 B	ZEB2 -1.09

Appendix J: CAL27 PCR Array with ciliogenesis inhibition. CAL27 cells treated with the ciliogenesis inhibitor HPI-4 for 24hours decreased EMT gene expression. Several of the downregulated targets belong to the Hh/Gli signaling family (i.e. SNAI and ZEB)



Appendix K: Mandibular defect repair in rats. In a non-survival feasibility study, full thickness defects were drilled through rat mandibles before being filled with an injectable, settable polyurethane composite. Cross sectional uCT images are also shown.

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