Identification and Characterization of Neural-like Cancer Stem Cells
in Salivary Adenoid Cystic Carcinoma

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I. INTRODUCTION

I.1 Salivary gland structure and function

Salivary glands are simple exocrine glands of the oral cavity that assist in digestion through production of saliva, which is a mixture of electrolyte fluid, mucous, glycoproteins, and enzymes. Although the major function is to lubricate food and begin digestion, saliva also functions as a first line of organismal protection from microbes since it contains antibacterial compounds and secretory immunoglobulins. The three pairs of major salivary glands (the parotid, submandibular, and sublingual glands) are symmetrically oriented in the oral cavity (Figure 1). Although, the major salivary glands account for the vast majority (>95%) of saliva production, between 800 and 1,000 minor salivary glands are dispersed throughout the oral cavity.[1]
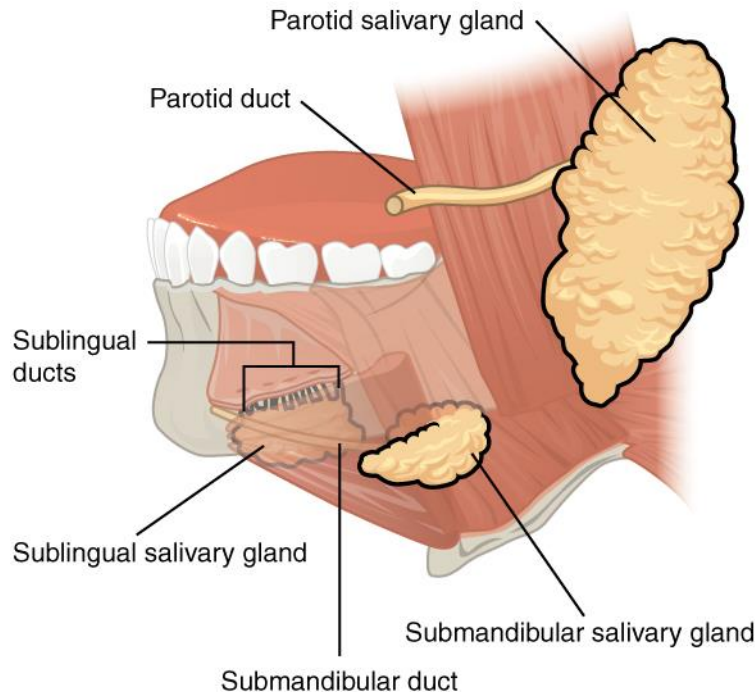
Figure 1: Anatomical locations of salivary glands.
Figure adapted with permission.[2]

I.2 Malignancies of the salivary glands

The World Health Organization recognizes 22 types of salivary cancer, and regardless of histological type, standard treatment is currently limited to surgery with or without post-operative radiation therapy.[3] Molecular defects in several tumor types are beginning to emerge, and there are early reports targeting some of these defects such as expression of androgen receptor, amplified HER2, or fused TrkC. Summarized below are the most common forms of salivary cancer.

The most prevalent cancer of the salivary glands is mucoepidermoid carcinoma (MEC), which occurs primarily in the parotid.[3] Mucoepidermoid tumors are graded based on the cystic component, presence of neural invasion, necrosis, mitotic cells per high power field, and anaplasia.[4-6] High grade tumors have an increased risk of metastasis and lower survival rates than low-grade tumors.[4-6] The most frequently, and sometimes only, molecular abnormality in MEC is a translocation of chromosomes 11q21 and 19p13, which results in activation of Notch target genes by fusing the mastermind-like 2 (MAML2) gene, a cofactor for NOTCH transcription, with a gene of unknown function.[7, 8] Ras pathway activation has also been implicated in MEC; mutations in H-ras correlate with high grade tumors and are observed in 18% of MECs.[9] Despite efforts to characterize molecular defects in MEC, neither the t(11:19)(q21;p13) gene fusion nor H-ras mutations are currently targetable, and treatment remains surgical resection followed by radiation for high grade tumors.

Acinic cell carcinomas are salivary tumors whose neoplastic cells have features of serous acinar cells. These tumors are thought to arise directly from serous acinar cells or from terminal duct cells with subsequent acinar differentiation.[3] Women are

slightly more likely to develop acinic cell carcinoma than men, but tumor incidence does not seem to relate to age or ethnicity.[10] While the majority of acinic cell carcinomas present as slow-growing, painless lesions, some patients present with pain or partial facial paralysis.[10-12] Molecularly, the majority (84% in one study) of acinic cell carcinomas exhibit loss of heterozygosity (LOH) in chromosomes 1, 4, 5, 6, or 17, but the loci affected vary between tumors.[13] Prognostically, ~35% of patients recur following surgical excision, with ~16% of patients ultimately succumbing to the disease.[11, 12, 14] For acinic cell carcinoma, tumor stage is a better predictor of outcome than histological grading, and regional and distal metastases associate with increasingly poor prognosis.[3]

Mammary analogue secretory carcinoma (MASC) was categorized as a subtype of acinic cell carcinoma until 2010.[15] Unlike acinic cell carcinoma, MASCs lack serous acinar differentiation and the microcystic and tubular spaces within the tumors are filled with a bubbly secretion reminiscent of breast secretory carcinoma.[15] Testing for the t(12;15)(p13;q25) translocation characteristic of breast secretory carcinoma revealed that >90% of MASC contain this translocation that is absent in acinic cell carcinomas with typical histology.[15] In MASC, this translocation fuses the ETV6 and NTRK3 genes creating a chimeric tyrosine kinase with transformative potential.[16, 17]

Salivary ductal carcinoma (SDC) represents ~9% of salivary malignancies that occurs nearly four-fold more frequently in men.[18, 19] SDC arises in each of the major and minor salivary glands, but is most frequently found in the parotid.[18-21] When compared to other salivary neoplasms, SDC presents at later stages due to its particularly aggressive growth and high rates of metastasis.[18] Histologically, SDC is

characterized by an infiltrative cribriform pattern, although patterns of pleomorphic adenoma may also be present.[3] Genetic alterations in SDC include frequent p53 mutations,[22, 23] amplification and overexpression of HER2/neu,[22, 24] and inactivation of CDKN2A/p16.[25] Androgen receptor is expressed in ~90% of SDCs, and these tumors can respond to androgen deprivation therapy.[26, 27] Despite molecular characterization of SDC, prognosis remains poor with ~65% of patients succumbing to disease, usually within four years of diagnosis.[3]

Adenoid cystic carcinoma (ACC) is the second most common salivary cancer, characterized by a slow-growing mass prone to perineural invasion and metastasis. While local disease is frequently controlled by surgical removal and post-operative radiation, this tumor is relentless resulting in very high rates of distant metastases.[3] This tumor subtype is the focus of this dissertation and will be discussed in detail below.

I.3 Adenoid cystic carcinoma (ACC)

I.3.1 Discovery of ACC

Cribiform morphology with characteristic tissue invasion and perineural spread of ACC was first described in 1853.[28] The tumor was originally described as "cylindroma" in 1856 and was not differentiated from the similarly-appearing benign cylindroma until 1930 when the name "adenoid cystic carcinoma" was coined by J. W. Spies.[29] The malignant nature of ACC wasn't established until 1942.[30]

Today, ACC has a yearly incidence of between 3 and 4.5 cases per million population, making it the second most common form of salivary cancer.[31] Outside of the parotid gland, ACC is the most frequent salivary gland malignancy.[32-34]

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I.3.2 Demographic and risk factors

Relative to many epithelial cancers, adenoid cystic carcinoma presents at a younger age, with the majority of cases occurring in the 5th to 6th decades of life.[31, 35-37] However, ACC can occur in all age groups and is more prevalent in women (55% to 60% of cases). To date, female gender remains the only known risk factor, as ACC incidence has not been associated with behaviors such as use of alcohol, tobacco, or illicit drugs.[31-34]

I.3.3 Diagnosis

ACC normally presents as a slow-growing mass in the oral cavity, salivary gland, or other sites of the upper aerodigestive tract, but more advanced tumors can cause hoarseness, difficulty swallowing, numbness (of the palate, face, or tongue), pain, and facial nerve paralysis. After identification of a mass in the salivary gland, computed tomography (CT) scan and/or magnetic resonance imaging (MRI) is used to better define the extent of the tumor involvement including bone and nerve invasion.[38]

Fine needle aspiration (FNA) of salivary masses is a quick, inexpensive procedure that uses a hollow thin needle to sample masses. Cytologically, ACC is characterized by large globules of extracellular matrix, partially surrounded by basaloid cells.[39] Cytology of salivary cancers can be challenging with a high rate of false negative evaluations previously reported.[40] At experienced centers, malignant and benign tumors can be distinguished with a sensitivity and specificity of 92.8% and 93.9%, respectively;[41] however, given the known uncertainty of cytology, histopathologic evaluation of excised tissue is the gold standard for diagnosis.

While histological analysis can easily differentiate ACC from the more frequent MEC, polymorphous low-grade adenocarcinoma (PLGA) is more difficult to differentiate based on morphology. Prognostically, ACC is much worse than PLGA, making differentiation of these tumor types important.[42] PLGA is extremely rare in the major salivary glands, so this distinction is mostly relevant to tumors of the minor salivary glands where small biopsies further complicate diagnostic difficulties.[43] Clinically, expression of genes characteristic of these tumors is assessed by measuring protein levels with immunohistochemistry (IHC).[42, 44-61] As summarized by Darling and colleagues, few proteins have been identified which can differentiate ACC from PLGA.[62] Although some markers have preferential expression patterns favoring either ACC or PLGA, the only marker with a clear diagnostic difference is vimentin, which is expressed in PLGA specimens, but not in ACC.[62]

I.3.4 Pathology and histology

Histologically, ACC consists of small non-luminal, basaloid cells with small to moderate amounts of cytoplasm and inconspicuous nuclei. Cells with myoepithelial differentiation predominate with the remaining tumor cells exhibiting luminal epithelial differentiation.[60, 63] The predominance of these cell types is not particularly surprising, since ACC originates from the intercalated duct where luminal epithelial and myoepithelial cells are prevalent.

Three distinct growth patterns are associated with ACC. The classic and most common is a cribriform pattern where cells are arranged into nests surrounding cell-free pseudocysts. ACC tumors that infiltrate into separate gland-like structures containing individual central lumens are described as tubular. This growth pattern is rarely seen

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alone; tumors with tubular growth often demonstrate cribriform patterning as well.[64, 65] Finally, some ACC tumors demonstrate a solid growth pattern where the cells grow in tightly-packed sheets without apparent lumen formation. This pattern is more frequently associated with high tumor grade, poor prognosis, and distant metastases.[64-66]

One hallmark of ACC is perineural invasion, which is frequently observed in early-stage tumors and has long been considered a marker of poor prognosis and distant metastasis.[35, 67] However, a recent study found no impact of perineural invasion on survival or formation of distal metastases. Rather, this study identified intraneural invasion as an independent predictor of poor prognosis.[68] Signaling pathways activated in ACC and in neural or neural-supporting cells may explain ACC neurotropism; however, mechanisms have not been well established.

I.3.5 Treatment and prognosis

Although ACC tumors are typically slow-growing, all grades of ACC are considered aggressive given high rates of recurrence. Standard treatment including radical resection, and postoperative radiotherapy provides a high rate of short-term local control; however, late recurrence at both local and distant sites frequently occur despite this aggressive treatment.[69-71] One cause of local recurrences is perineural invasion and associated skip lesions, which complicate the ability for surgeons and pathologists to ensure tumor-free margins. Nodal disease is not a cause of recurrence, because ACC rarely metastasizes to regional cervical nodal basins.[72] Unfortunately, distal metastases occur in 40% of cases, spreading primarily to the lungs, but also to the liver, kidney, bones, and brain.[72]

Although survival at five years post diagnosis is high (70-90%), survival rates dramatically decrease to only 35-40% and 10% at 15 and 20 years, respectively.[73-75] Diminished long-term survival is primarily associated with inability to control distant disease, and once distant metastases of ACC are identified, 5-year survival rate is approximately 20%.[76] Doubling time of pulmonary metastases in ACC have been estimated to average 393 days, suggesting that metastasis may have already occurred by the time the primary tumor presents clinically and that early detection and local therapy may not prevent distant disease.[77] The clinical course of ACC suggest that new therapies targeting widespread systemic metastases will be needed.

I.4 ACC research & clinical applications

I.4.1 Molecular profiling

I.4.1.1 Exome and genome sequencing

As with many tumor types, deep sequencing technology has been used to investigate ACC. Studies using exome and whole genome sequencing from a combined 84 ACC tumors show that the ACC genome is generally stable with a low level of somatic mutations per genome (range 1-36).[78, 79] Genetic changes identified in ACC were highly diverse, with mutations seen in chromatin regulators, genes associated with DNA damage, protein kinase A (PKA) signaling, MYB signaling, and PI3K signaling.[78, 79] Notably, the most recurrent aberrations identified in ACC are MYB-NFIB gene fusions detected in approximately 50% of ACC specimens.[80] These fusions result from the recurrent translocation t(6;9)(q22-23;p23-24) causing a pro-oncogenic MYB-NFIB fusion protein.[81] Transcriptional activation the MYB-NFIB fusion is induced

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by super-enhancers (regulatory DNA regions enriched with binding sites for transcriptional co-activators) brought to the MYB gene through the fusion.[82]

Although MYB-NFIB fusions occur in approximately 50% of tumors, MYB is highly expressed in up to 70% of ACC, suggesting alternative mechanisms of MYB activation in ACC. In addition, a MYB gene expression signature occurs in almost all ACCs, even those without detectable MYB expression.[80] Recently, whole genome sequencing identified a novel gene fusion between the MYB family member, MYBL1, and the terminal exons of NFIB in approximately one-third of ACC tumors that lack MYB-NFIB fusion. Structurally, MYBL1 is similar to MYB with a nearly identical DNA binding domain. Both MYB-NFIB and MYBL1-NFIB fusions produce C-terminal truncations of MYB or MYBL1; however, both fusions uniformly retain DNA binding and transcription activation domains. These findings strongly suggest that MYBL1 fusions serve a similar role as MYB fusions in ACC.[83]

I.4.1.2 Expression array

To identify genes that stimulate ACC tumorigenesis or behavior, microarray gene expression analyses was performed on15 ACCs and five normal salivary tissue specimens.[65] Consistent with myoepithelial differentiation of ACC, components of the extracellular matrix and basement membrane are among the most differentially expressed genes when comparing ACC and normal salivary gland. Sox 4, the highest scoring gene, is a transcription factor that functions in the heart, brain, reproductive system, and B-cell development; however, the function of SOX4 in cancer is relatively unknown. Additional transcription factors including AP-2α and AP-2γ, which regulate genes involved in the development of the neural crest and skin are also differentially

expressed. AP-2 targets include c-Kit, whose expression was previously associated with ACC. Other notable differentially expressed genes are genes associated with the Notch signaling pathways (ex: Notch3 and JAG1) and the WNT/β-catenin (ex: casein kinase 1, ε and Frizzled-7). Comparison of the top ranking genes in ACC to 175 non-ACC carcinomas from 10 biologic sites identified increased expression of versican; AP2-γ; casein kinase 1ε; and Frizzled-7 in ACC specimens.[65]

Adenoid cystic carcinoma also forms outside the salivary glands, including in the breast, lungs, skin, nasopharynx, trachea, and prostate. ACC of the breast is a rare form of triple-negative cancer accounting for <1% of breast cancers. Unlike most other breast cancer subtypes, breast ACC is primarily indolent, contains relatively quiescent genomes, and is largely resistant to radiation therapy. Comparison of ACC of different sites of origin reveals notably similar histological and genetic characteristics. Most notably, the recurrent MYB-NFIB fusion seen in salivary ACC is shared with ACC of the breast. Salivary and breast ACC tumors both harbor heterogeneous somatic mutations, including genes associated with chromatin remodeling or canonical signaling pathways such as FGFR2. There are, however, some key differences between these diseases, including breast ACC lacking the Notch pathway mutations seen in salivary ACC.[84]

I.4.1.3 MicroRNA profiling

A comparative study of 30 ACC specimens with four matched normal salivary tissues identified 55 microRNAs whose expression is higher (n = 19) or lower (n = 36) in ACC.[85] Among those whose expression is elevated in ACC, eight (~42%) represent the miR-17-92 cluster and its paralogs. Even within ACC specimens, this microRNA cluster is significantly associated with the clinically aggressive solid pattern of tumor growth and

reduced patient survival. There are no differences seen in microRNA expression patterns between tumors with and without the MYB-NFIB fusion,[85] possibly because most ACCs have expression patterns suggesting activation of MYB.[80, 83]

An alternative study on microRNA profiles of ACC screened microRNA profiles of ACC compared to the most common otolaryngologic malignancy-head and neck squamous cell carcinoma (HNSCC).[86]   Surprisingly, the resulting expression patterns differed by only a handful of microRNAs. Expression level of five microRNAs (miR-214, miR-125a-5p, miR-574-3p, miR-199a-3p/199b-3p and miR-199a-5p) being statistically higher in ACC, while only one (miR-452) is significantly increased in HNSCC. Although these miRNAs have been implicated in promoting metastatic niches, regulating tumor suppressor genes, TGF-β signaling, oncogenesis, stemness, and tumorigenicity in other cancers (including hepatocellular carcinoma, gastric cancer, colorectal cancer, and glioma), their function in ACC remains elusive.[86]

I.4.1.4 Epigenetic profiling

Epigenetic changes altering gene expression can relieve pressure for mutagenic activation of oncogenes or inactivation of tumor suppressors.[87] Rather, these chromatin changes modulate the ability of transcription factors, polymerases, and enhancer-binding elements to interact with DNA. Cytosine methylation of DNA within CpG islands (>200 bp regions with >60% G/C content) of gene promoters results in suppressed gene expression. A methylated CpG island amplification and microarray analysis identified seven hypomethylated CpG islands in ACC compared to normal salivary tissue, specifically located near the FBXO17, PHKG1, LOXL1, DOCK1, and PARVG genes. Hypermethylation in ACC was found in 32 CpG islands mapped primarily to

transcription factors (n = 19). The remaining 13 hypermethylated genes served a variety of functions including, but not limited to, stress response, detoxification, protein catabolism, and apoptosis. Deeper analysis of the hypermethylated transcription factor EN1 found that its expression correlates with histological grade, tumor location, and patient outcomes.[87]

I.4.2 Gene expression in primary tumors

I.4.2.1 MYB-NFIB fusions and signaling

As previously mentioned, the most frequent gene aberration in ACC is the translocation between MYB (6q22-q23) and NFIB (9p23-24) genes. Reported prevalence of this gene fusion in ACC ranges from 28-100%, but among tumors of the head and neck, it is exclusively seen in ACC.[15, 88, 89] While the location of the fusion in both MYB and NFIB varies between tumors, MYB sequences dominate the fusion product, with the most common breakpoint fusing exon 14 of MYB to the last coding exon of NFIB. The resulting fusion protein lacks exon 15 of MYB, as well as the 3'-untranslated region, containing several negative regulatory regions. Tumors harboring MYB-NFIB fusions demonstrate increased MYB expression compared to normal tissue controls.[90] The high frequency of MYB fusions and known MYB targets involved in apoptosis, cell cycle control, cell growth, and cell adhesion, suggest that MYB-NFIB fusions are the most prominent oncogenic driver in ACC. Recent identification of the MYBL1-NFIB gene fusions (8q13;9p23) further suggests that downstream signaling through these related transcription factors contribute to tumorigenesis in ACC.[83] While the exact function of MYB in ACC remains unclear, the predominance of activation of

this proto-oncogene and its family member suggests that it will be useful as a diagnostic marker, prognostic marker, and/or therapeutic target.

I.4.2.2 c-Kit

The receptor tyrosine kinase c-Kit (CD117) is a driver of several human tumors,[91-93] and it is expressed in 80-94% of ACC.[94-96] The ligand for c-Kit is stem cell factor (SCF), and c-Kit activation induces pathways involved in hematopoiesis, spermatogenesis, growth and migration of melanocytes, and maintenance of progenitor cells.[97, 98] c-Kit expression has previously been used to isolate progenitor cells from the submandibular gland, suggesting that c-Kit may be involved in maintaining cancer stem cells in ACC.

Somatic mutations of c-Kit have been described in gastrointestinal stromal tumors, mast cell neoplasms, and seminomas;[91-93] however, c-Kit mutations are rarely seen in ACC, suggesting an alternative method for activation. In ACC, c-Kit was initially correlated with tumor grade, but further analyses negated these conclusions.[95, 99-101] Disappointingly, c-Kit inhibitors have little-to-no effect against ACC in clinical trials.[102, 103]

I.4.2.3 Wnt/β-Catenin

Activation of the WNT/β-catenin pathway is implicated in many human diseases. Mutations of this pathway are found in ~35% of ACC, and expression array studies have identified aberrant expression of additional components of the pathway, including genes associated with malignancies: Wnt inhibitory factor (WIF1), Galectin-3, and cyclin D1.[104, 105] WIF1 functions as a Wnt receptor antagonist, physically preventing receptor-ligand interaction[57] and has been associated with a subset of salivary gland tumors, wherein a

chromosomal fusion between WIF1 and HMGA2 causes loss of function of the WIF1 gene.[106] Expression of galectin-3, a key regulator of the Wnt/β-catenin pathway, has been associated with increased aggressiveness in a variety of malignancies.[107, 108] In ACC, galectin-3 is linked to increased regional and distal metastases as well as poor prognosis.[109, 110] Cyclin D1, a critical cell cycle regulator, is the major downstream target of β-catenin signaling. While neither mutated nor amplified in ACC, cyclin D1 is overexpressed in ACC specimens, possibly signifying WNT/β-catenin pathway activation.[109, 111, 112]

Although studies have aimed to elucidate the connection between WIF1 loss and/or galectin-3 gain with induction of cyclin D1 expression in ACC, the mechanism appears to be more complicated, and understanding this pathway's role in ACC will require additional research.[109, 113]

I.4.2.4 FABP7

Fatty acid binding protein 7 (FABP7) is one of the five most differentially expressed genes between ACC and normal salivary gland, and expression of EN1, MYB, VCAN, and FABP7 distinguish ACC tumors from normal salivary tissue and benign salivary neoplasms. Amongst differentially expressed genes, FABP7 expression alone correlates with poor prognosis.[114]

FABP7 functions by binding fatty acids and other lipids and acts as a cytoplasmic chaperone for lipid metabolism. A member of a larger family of hydrophobic proteins, FABP7 is normally expressed in the brain, heart, testis, and adipose tissues.[115-117] Nuclear FABP7 is found in glioblastoma and melanoma, where it correlates with reduced survival.[118-121] In cribriform and tubular ACC histologies, FABP7 localizes

predominantly to the nucleus of myoepithelial cells.[114] A much lower level of staining also occurs in duct-like epithelial cells lining pseudocysts. In solid ACCs, nuclear FABP7 marks all malignant cells, consistent with the idea that nuclear FABP7 may identify aggressive tumor subtypes that demonstrate worse prognosis.[114]

The functional consequences of FABP7 expression in general, and especially nuclear localization of FABP7, have not been particularly well studied. However, FABP7 has been identified as a Notch target gene,[122, 123] and Notch signaling has been identified in ACC. Together, these data suggest that Notch may contribute to FABP7 expression, and raise the possibility that suppressing FABP7 within these tumors may have therapeutic benefit.

I.4.2.5 Other

Although MYB/NFIB fusion, c-Kit activation, and WNT/β-catenin have received the most attention, many other genes and pathways have been studied in ACC.

Epidermal growth factor receptor (EGFR) and EGFR family members are overexpressed in a variety of cancers. Expression of these genes assists cancer development by inhibiting apoptosis while stimulating angiogenesis. EGFR is overexpressed in ACC where its expression correlates with histological grade, but not prognosis.[124, 125] Although EGFR is expressed strongly in tumor myoepithelial cells with much weaker expression in ductal cells, tyrosine kinase inhibitors (TKIs), including those targeting EGFR, show little effect in clinical trials of ACC.[102, 103]

Vascular endothelial growth factor (VEGF), a signaling protein that induces angiogenesis, may play an important role in ACC. Tumors harboring increased VEGF unsurprisingly also demonstrated enhanced microvessel density.[126, 127] Although

inhibition of EGFR and c-Kit with TKIs failed to demonstrate significant effects, dual inhibition of EGFR and VEGF slowed tumor growth and lung metastases in mice.[128] While these data were initially very encouraging, a study published in 2009 revealed that six purported ACC cell lines, including the line used in this study, were misidentified and not derived from adenoid cystic carcinoma.[129]

Although the implications of perineural invasion in ACC are debated, it is a common characteristic of many ACC tumors. In addition to potential anti-apoptotic effects, perineural invasion complicates surgical resection by limiting the ability to ensure clear margins.[130] A potential mechanism underlying perineural invasion in ACC relates to shared neurotrophic signaling. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), both members of the neurotrophin family, are expressed in ACC. TrkA, the receptor for both of these proteins, is expressed in a subset of ACC tumors.[131, 132] While BDNF and NGF have been suggested to facilitate perineural invasion in ACC, more research is required to confirm this association.

Neuropilin-2 (Nrp2), a protein co-receptor, is overexpressed in greater than 90% of ACC specimens.[76] Nrp2 expression correlates with microvessel density, clinical stage, vascular invasion, and metastasis, consistent with its role seen in other cancers.[76] Given the use of contaminated cell lines for behavioral studies, the role of Nrp2 in ACC is unknown.

E-cadherin has long been associated with tumor progression and metastasis. A calcium-dependent transmembrane protein, E-cadherin assists with adherens junctions which bind cells together in tissues. In ACC, like other tumors, E-cadherin expression inversely correlates with tumor grade, size, infiltration, and metastasis.[133] Unfortunately,

E-cadherin is unlikely to be a driver of this cancer, as loss of E-cadherin seems to occur later in the progression of the tumor. Regardless, E-cadherin expression does appear to be a prognostic marker in ACC.

I.4.3 Tools for *in vitro* and *in vivo* studies

I.4.3.1 ACC cell lines

Research to advance molecular understanding of ACC has been hamstrung by the relative rarity of the disease and a complicated history resulting from difficulty establishing cell lines. Before 2009, studies examining behavior and therapeutic response of ACC extensively utilized six cell lines (ACC2, ACC3, ACCM, ACCS, ACCNS, and CAC2).[134-138] After being used for ACC research for more than three decades, short tandem repeat (STR) analyses in 2009 found that none of the ACC cell lines contained human ACC cells.[129] These unfortunate findings negate the validity of any research using these cell lines.

In the wake of this setback, many labs have attempted to create ACC cell lines. In 2013, a group from China produced a cell line, SACC-83, from an ACC of the minor salivary gland. Through a five-round selection of intravenous injections and lung collections, they derived a daughter cell line with increased metastatic potential (SACC-LM).[135, 139] Although these cell lines have not been confirmed to be from the original patient or the primary tumor, the group has done STR analysis which confirmed that these two cell lines have the same derivation and are distinct from HeLa cells. However, deeper comparison with publically available databases of a broad range of cell lines has not been performed.[140] While SACC-83 and its derivative line may represent true ACC cell lines, distribution of them outside of China has been limited.

Another cell line (MDA-ACC-01) was developed in 2014,[141] and while early passages grew well, population doubling times (PDT) increased at higher passage numbers making a long-term cell line impossible to establish. This cell line was immortalized with human telomerase reverse transcriptase (hTERT), which stabilized the PDT and allowed the culture to grow to 100 passages without senescence. Of concern, G-banding and Spectral Karyotyping (SKY) analyses identified a t(6q25;14q13) translocation in the primary tumor and early passages of the primary culture, which began to be lost as early as passage 5 and disappeared entirely by passage 10.[141] Chromosomal changes in cultured cells could be a sign of contamination, or indicate that this culture has lost some characteristics of the original tumor.

I.4.3.2 ACC xenografts

While creation of *in vitro* ACC models has been very challenging, efforts to create *in vivo* mouse xenograft models of ACC have been more successful with a reported 74% success rate for establishing subcutaneous xenografts from primary ACCs.[142] Passage into subsequent mice also succeeded for 15 of the 17 xenografts. Unsupervised analyses of expression array data revealed that xenografted tumors clustered most closely to their parental tumors with the only exception being a primary tumor that clustered more closely to a metastatic tumor, which formed later in the same patient. The resulting xenografts from these specimens formed an independent cluster closest to the tumors from which they were derived. Fluorescent *in situ* hybridization (FISH) analysis of the tumors identified 11 of 12 tested samples to have rearrangement

of the MYB locus with 10 of these 11 showing presence of a fusion with the NFIB gene that is commonly found in ACC (described above).[142]

While xenografts provide a powerful tool for ACC research, they are expensive to maintain, greatly amplifying the cost of experimentation. Distribution of xenografts also presents challenges since exchange of xenograft-bearing animals between institutions can be difficult. As an alternative, viable xenograft tissue can be shared, but viability can be decreased based on shipping time and conditions. Finally, xenografts do not allow the freedom to manipulate gene expression that exists with cell lines, limiting experimental options primarily to testing effects of established drugs.

I.4.4 Studies *in vitro* and *in vivo*

Many studies have been completed using presumed ACC cell lines ACC2, ACC3, ACCM, ACCS, ACCNS, and CAC2, both before and after their misidentification was reported. Since findings in these cell lines do not apply to ACC, these results will not be discussed. While SACC-83 and SACC-LM have not been extensively vetted, there is no data to disprove their validity at this time.

I.4.4.1 Genes contributing to migration, invasion, and perineural invasion

Using SACC-83 cells, depletion of microtubule-associated tumor suppressor gene (MTUS1), a tumor suppressor associated with many cancer types, alters ACC behavior.[143] Lower MTUS1 expression in ACC tumors correlates with increased distant metastases and reduced survival,[143] and MTUS1 expression is lower in the more aggressive, SACC-LM cells compared to the isogenic line SACC-83. Reintroduction of MTUS1 inhibits migration and invasion of SACC-LM cells and correlates with reduced

levels of Slug, vimentin, and activated extracellular-regulated kinase 1/2 (Erk1/2), while depletion of MTUS1 in SACC-83 cells has the opposite effects.[143] As vimentin expression is not characteristic of ACC, how these results translate to patient tumors remains to be seen.

Expression of PRL-3, a phosphatase correlating with poor prognosis in several tumor types,[144] is found in ACC tumors where it associated with tumor stage (p = 0.009), clinical stage, distant metastasis, tumor site, recurrence, and survival.[144] Comparison of the metastatic cell line, SACC-LM, to its parental line, SACC-83, revealed up-regulated PRL-3.[144] Reminiscent of MTUS1 depletion, overexpression of PRL-3 in SACC-83, demonstrated the same migratory and molecular changes.

Bmi-1 expression is associated with stem cell self-renewal and tumorigenesis in various malignancies.[145] Bmi-1 expression in ACC correlates with higher tumor stage, increased distal metastases, and reduced progression-free and overall survival. Bmi-1 inhibition reduced the migratory and invasive potential of SACC-LM cells while suppressing expression of cancer stem cell-associated genes.[145]

The chemokine CCL5 and its receptor CCR5 are implicated with tumor invasion and metastasis.[146, 147] In ACC tumors, expression of both genes is enhanced (CCL5: 35.9%; CCR5: 70.3%),[148] and expression of CCR5 is associated with perineural invasion. SACC-83 cells express CCR5, but not CCL5. Exogenous introduction of CCL5 in SACC-83 cells stimulated migration, invasion, and perineural invasion, and these effects were reversed by the CCR5 inhibitor, maraviroc.[148]

The most well-known and well-studied tumor suppressor, p53, is not mutated in ACC, but its inactivation by other mechanisms affects ACC behavior. Down-regulation

of p53 in SACC-83 cells inhibited apoptosis, increased proliferation, and induced expression of genes associated with epithelial-mesenchymal transition (EMT).[149] These molecular changes following depletion of p53 were accompanied by increased perinerual invasion of these cells, suggesting that p53 may function in perineural invasion of ACC by facilitating EMT.[149]

I.4.4.2 mTOR signaling

mTOR signaling is a characteristic of several human malignancies.[150] A downstream target of mTOR, phosphorylated-S6 (p-S6, S235/236), is significantly increased in ACC compared to pleomorphic adenoma or normal salivary gland.[151] Targeting of mTOR by rapamycin in SACC-83 cells reduces xenograft growth and suppresses expression of p-S6, p-Stat3 (T705), PAI, EGFR, and HIF-1α.[151]

PTEN loss, as a potential mechanism of mTOR activation, is associated with increased phosphorylation of S6 in ACC tumors,[152] and the more aggressive SACC-LM cells demonstrate reduced PTEN expression compared to parental SACC-83 cells. Inhibition of PTEN expression in SACC-83 cells increases proliferation, migration, and invasion *in vitro*, and stimulates tumor growth *in vivo*. Conversely, inhibition of PI3K/mTOR signaling with NVP-BEZ235 suppresses tumor growth and metastasis, cell proliferation, migration, and invasion in both cell lines.[152]

Together, these studies indicate that mTOR signaling may contribute to ACC proliferation, angiogenesis, and metastasis and may have therapeutic relevance in ACC.

I.4.5 Clinical trials

Despite years of research efforts identifying potential drivers and markers of ACC and many clinical trials, recommended treatment is limited to surgical resection and radiation. At the time of writing, efficacy of chemotherapy is not proven, and no targeted therapies are approved for treatment.[153]

I.4.3.1 Chemotherapy

Chemotherapy is reserved as a palliative measure for patients with metastatic and incurable ACC. A study combining cisplatin with 5-fluorouracil (5-FU) found the toxicity of this pairing to be manageable,[154] with only three of 11 (~27%) patients having any response (all partial responses), but with 64% of patients reporting symptom relief.[154] The median times to disease progression and survival was nine months and 12 months, respectively.

Similar results were shown in ACC patients treated with epirubicin, gemcitabine, and mitoxantrone. Of 20 patients treated with a low-dose regiment of epirubicin for eight weeks, two (10%) partial responses were observed, and another 10 (50%) demonstrated disease stabilization.[155] Despite this modest objective response rate, improvement of symptoms was seen in ~30% of patients within the 8-week treatment period.[155] No responses were seen in ACC patients treated with gemcitabine, but 6 month stable disease was reported in 48% of treated patients.[156] Mitoxantrone treatment resulted in a complete response rate in one patient, but no partial responses.[157] A subsequent phase II study examined effectiveness of mitoxantrone on 32 chemotherapy-naïve ACC patients revealing a partial response rate of 12%.[158]

Combined, these studies suggest that epirubicin gemcitabine, and mitoxantrone have little activity in treating recurrent or metastatic ACC.

I.4.3.2 Targeted therapy

The receptor tyrosine kinase c-Kit is expressed in normal salivary tissue and is upregulated in ACC compared to other head and neck tumors. Because of its expression in ACC, c-Kit was trialed in patients with recurrent or metastatic ACC. Imatinib (Gleevec) was used to treat 14 patients, including 10 patients whose tumors expressed c-Kit. No objective responses were seen. Rather, all but two tumors progressed on therapy.[103] Likewise, in a trial of Imatinib in c-Kit-positive ACC metastatic to the lungs found that imatinib may have accelerated disease progression with three of five patients having disease progression within 2-3 weeks of treatment and succumbing to disease within six months.[102] Despite dismal results of trials using imatinib as a single agent, combination of imatinib and cisplatin was trialed in patients with ACC.[159] c-Kit overexpression was required for enrollment, and treatment consisted of a two month run of imatinib followed by concomitant imatinib and cisplatin for six cycles, then a maintenance regiment of single agent imatinib until disease progression. A partial response, as measured by decreased fluorodeoxyglucose positron emission tomography (FDG-PET) was seen in 11%, while 68% of patients had stable disease. However, median progression-free and overall survival times were only 15 months and 35 months, respectively.[159] Taken together, these data suggest that as a single agent or in combination, imatinib is not effective in ACC.

EGFR is expressed in ACC[125] prompting a trial of lapatinib, a dual inhibitor of EGFR and ErbB2 (HER2) tyrosine kinase activity, that enrolled patients with ACC and

other malignant salivary gland tumors.[160] Amongst 19 ACC patients enrolled, there

were no objective responses despite expression of EGFR and/or ErbB2 in 88% of these

patients. Although lapatinib was well tolerated it is not useful for treatment of ACC.[160]

Likewise, a trial of the EGFR targeting antibody, Cetuximab revealed no responses; [161]

however, 47% (12/23) had stable disease for at least six months. In these small

studies, the time to progression with cetuximab treatment was nearly double that

observed with single agent lapatinib therapy.[161]

The less specific tyrosine kinase inhibitor, sunitinib (Sutent), which inhibits

vascular endothelial growth factor receptor (VRGFR), c-Kit, platelet-derived growth

factor receptor (PDGFR), ret proto-oncogene (RET), and FMS-like tyrosine kinase 3

(FLT3), was studied in patients with progressive, recurrent, or metastatic ACC.[162] No

responses were observed, and while 62% had stable disease for at least six months,

median time to progression and overall survival were 7.2 months and 18.7 months,

respectively.[162]

Bortezomib (Velcade), as an inhibitor of 26S proteasome and NF-κB, also failed

in a trial of ACC patients inducing no responses, but reporting stable disease in 71% of

patients.[163] Combination of bortezomib and doxorubicin, produced a single partial

response (10%) and a stable disease rate of 60%.[163]

A response in an ACC patient treated in a phase 1 trial of the mTOR inhibitor,

everolimus, prompted a phase II trial of patients with unresectable ACC.[164]

Disappointingly, no responses were observed, but 6 month stable disease was reported

in 38% of patients.[164]

Many other studies are underway attempting to identify an effective target in ACC. Ongoing clinical trials include dovitinib (FGFR3 inhibitor which may affect other RTKs), axitinib (TKI), pazopanib (TKI), vorinostat (HDAC inhibitor), nelfinavir (protease inhibitor with anti-retroviral capacity), HPPH (photosensitizer for photodynamic therapy), erlotinib (EGFR inhibitor), Trastuzumab (antibody inhibiting HER2/neu), pemetrexed (folate antimetabolite), and docetaxel (anti-mitotic chemotherapeutic). Results of these studies are pending.

The rate of failure for these clinical trials is remarkable and indicates that therapies were chosen without adequate preclinical data. The absence of cell lines or short-term cultures to evaluate drug efficacy likely contributed to the universal failure of these studies.

I.5 Cancer stem cells

I.5.1 Cancer stem cell hypothesis

The cancer stem cell (CSC) hypothesis professes that a subpopulation of tumorigenic cells with stem cell properties, including self-renewal and differentiation, are required for tumor maintenance. Cancer stem cells can regenerate to create all cellular subpopulations that may exist in a mixed tumor. As a mechanism of tumor resistance to therapy, CSC remain quiescent, allowing them to evade cell death induced by chemo- and radio-therapies. The first evidence supporting this hypothesis was published in 1994, when Lapidot et al. identified a population of stem-like cells within myeloid leukemia that effectively recapitulated patient tumors as well as demonstrated increased tumorigenic potential in non-obese diabetic severe combined immune-deficient (NOD/SCID) mice.[165] Conversely, the population of non-stem cells from the same

tumor were unable to form xenografted tumors. Cancer stem cells have since been

identified in a wide variety of tumors (e.g. brain, pancreatic, ovarian, colorectal, head

and neck, and liver cancers)[166-169] that are distinguished by a variety of markers

including: CD44+/CD24- cells in breast cancer,[169] CD44+ in head and neck cancer,[170, 171] and aldehyde dehydrogenase 1 (ALDH1) in breast and gastric cancers.[172, 173]

I.5.2 Cancer stem cells in ACC

Conflicting evidence regarding the presence of cancer stem cells in ACC has

been reported. Isolation of cells with high and low ALDH1 activity from ACC primary

tumors and xenografts revealed increased spheroid formation in culture, and xenograft

tumorigenicity and metastatic potential in cells with high ALDH1 activity.[174] On the other

hand, immunohistochemical analysis of ALDH1 expression in primary ACC revealed no

expression in epithelial cells in 89% of ACCs.[175] Further, ALDH1 expression in ACC did

not correlate with tumor size, perineural invasion, or overall survival.[175] Although it is

hard to reconcile these findings related to ALDH1 as a marker for ACC stem cells,

tumorigenicity assays are convincing that stem cells likely exist in ACC.[176]

I.6 Other tumors with neural crest origin

Expression profiling of salivary ACC reinforced histological interpretation that

ACC tumors include cells of a myoepithelial lineage. During development, progenitors

of myoepithelial cells migrate from the neural crest into tissues, and in adult life these

cells differentiate into myoepithelial cells, but maintain expression of neural crest stem

cell markers such as SOX10. Other tumors derived from the neural crest lineages

include melanoma, glioblastoma, and neuroblastoma. Additionally, the basal-like

subtype of breast cancer develops from the basal/myoepithelial cells from a gland that is very similar structurally to the salivary gland.

I.6.1 Melanoma

Melanoma is a highly aggressive malignancy that arises from pigment-producing melanocytes of the skin. Unfortunately, incidence of this disease, which accounts for ~60% of deaths from skin malignancies, is on the rise.[177] Risk factors for melanoma include family history, nevi formation, age, fair skin, and exposure to UV radiation.[178] Common drivers of melanoma include B-Raf (mutated in >60%), N-Ras (mutated in 18%), c-Kit (copy number increase in 36-39% of acral or mucosal melanomas), and PI3K/Akt/mTOR pathway members (mutated in 43-60%).[177]

The majority of melanomas contain cells with neural crest stem cell (NCSC)-like characteristics, including expression of SOX10. Cancer stem cells from melanomas establish xenograft tumors from very few cells, and these tumors support distant metastases mimicking behavior of the parent tumor.[179-181] Suppression of SOX10 expression in mice prevented giant congenital naevi and melanoma even when melanoma formation was driven by forced expression of mutant N-Ras in melanocytes. The critical role of SOX10 in melanoma extends to melanoma cell lines where SOX10 suppression reduced proliferation, increased apoptosis, and demonstrated aberrant differentiation.[182]

I.6.2 Neuroblastoma

In children, the most common extracranial tumor is neuroblastoma.[183] The origin of these tumors is believed to be NCSCs from which sympathetic neural ganglia and the

adrenal medulla are derived. Although neuroblastoma is a non-glandular solid tumor, molecular profiles of this tumor demonstrate similarities to ACC. This may relate to a common neural crest cellular origin. In addition to SOX10, which is expressed in neural crest stem cells, neurotrophin signaling is required for normal neuronal cell lineage distinction and is active in both neuroblastoma and ACC.[131, 132, 184, 185] In neuroblastoma,[186] expression of neurotrophin receptors TrkA and TrkC are associated with favorable prognosis possibly related to their role as death receptors in the absence of ligand.[187] Conversely, TrkB expression is involved in increased proliferation, stimulated migratory potential, chemoresistance, and is associated with the unfavorable MYCN amplification.[188] ACCs also express neurotrophin receptors, but the role of neurotrophin signaling in ACC remains to be fully explored.

I.6.3 Glioblastoma

Glioblastoma (GBM) is the most prevalent tumor of the central nervous system. Despite rarely metastasizing to distal organs, GBM is characterized by extensive invasion of surrounding brain tissue. Although targeted therapies are beginning to emerge, primary treatment remains maximal surgical resection followed by chemoradiotherapy and subsequent chemotherapy. While this has proven to extend survival with generally moderate side effects, cure remains elusive, and patients ultimately succumb to the disease.[189]

Cancer stem cell populations in glioblastoma have been well characterized and studied. The most common marker of CSCs within GBM is CD133 (Prominin-1), a cell surface glycoprotein that is characteristic of neural crest stem cells.[190] CD133-positive glioblstoma cells have increased self-renewal capabilities, proliferation, xenograft

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tumorigenesis, and neurosphere formation. Compared to control NCSCs, neurospheres

formed from CD133^{+} GBM cells proliferate more rapidly and form more secondary

spheroids. CD133^{+} cells injected into mice recreate all aspects of the original tumor by

forming oligodendrocytes, astrocytes, and neurons.

I.6.4 Basal-like breast cancer

Breast cancer remains the most common malignancy in women worldwide,

causing approximately half a million deaths each year. Microarray gene expression

analysis identified several subtypes of breast cancer including: luminal A, luminal B,

basal-like, and Her2/Neu.[191] The luminal subtypes were generally ER+ and/or PR+,

Her2/neu+ (luminal A, ~40%), or ER+ and/or PR+, Her2/neu- (luminal B, ~20%), and

the Her2/neu subtype is distinguished by expression of Her2/neu in the absence of ER

or PR expression.[191-194]

Gene expression in the basal-like subtype (~15-20% of all tumors) suggests that

the basal epithelial layer which, like in ACC, is made up of myoepithelial cells (Figure 2),

is the cell of origin.[191] Basal-like tumors usually lack expression of ER, PR and

Her2/neu (triple negative) explaining their resistance to hormone therapies and

contributing to their notoriety as having the poorest survival of any breast cancer cluster.

Arising from a similar environment and possibly similar cell of origin as ACC

(Figure 2), basal-like breast cancer remains one of the most insidious subtypes of

breast cancer.[195] As with ACC, the primary treatment modality for basal-like breast

cancer is surgery and radiation. Systemic chemotherapy is also used, but results are

disappointing.[196] Basal-like breast tumors remain a therapeutic challenge, and patients

with basal-like tumors have a high risk for early relapse, visceral and brain metastases, and eventual death.[197]
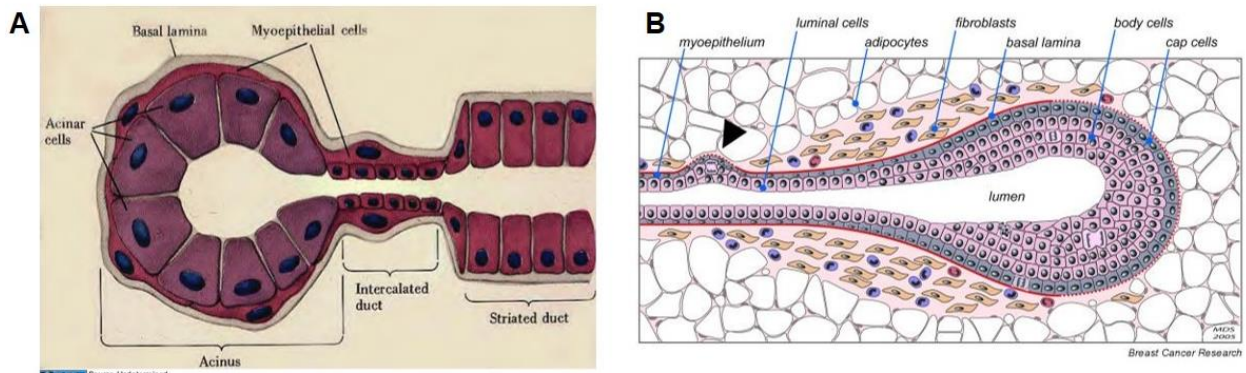
Figure 2: Comparative structures of (A) salivary and (B) mammary glands.
Images adapted with permission.[198, 199]

I.7 Introduction conclusions

ACC is an insidious malignancy with poor patient prognosis. Efforts to identify new options for therapy have been hampered by the low number of tumors, the paucity of funding, and the inability to establish cell cultures to accelerate preclinical studies. Clinical trials with chemotherapeutic or targeted agents against molecular markers of ACC have had disappointing results. To date, surgical resection with or without radiation remains the standard of care despite frequent failure at preventing recurrent disease. However, recent studies suggesting cancer stem cells in ACC may present a new direction for treatment of this disease.

II. RESULTS

II.1 TrkC Signaling is activated in adenoid cystic carcinoma and requires NT-3 to stimulate invasive behavior

Adapted from manuscript published in Oncogene.[200]

II.1.1 Abstract

Treatment options for adenoid cystic carcinoma (ACC) of the salivary gland, are limited to surgery and radiotherapy. Based on expression analysis performed on clinical specimens of salivary cancers, expression of the neurotrophin-3 receptor TrkC/NTRK3, neural crest marker SOX10, and other neurologic genes were identified in ACC. TrkC was highly expressed in 17 out of 18 ACC primary-tumor specimens, but not in mucoepidermoid salivary carcinomas or head and neck squamous cell carcinoma. Expression of the TrkC ligand, NT-3, and Tyr-phosphorylation of TrkC detected in this study suggested the existence of an autocrine signaling loop in ACC with potential therapeutic significance. NT-3 stimulation of U2OS cells with ectopic TrkC expression triggered TrkC phosphorylation and resulted in Ras, Erk1/2 and Akt activation, as well as VEGFR1 phosphorylation. Without NT-3, TrkC remained unphosphorylated, stimulated accumulation of phospho-p53, and decreased p-Akt and p-Erk1/2. NT-3 promoted motility, migration, invasion, soft-agar colony growth and cytoskeleton restructuring in TrkC-expressing U2OS cells. Immunohistochemical analysis demonstrated that TrkC-positive ACC specimens also expressed Bcl2, a Trk target regulated by Erk1/2. In normal salivary gland tissue, both TrkC and Bcl2 were expressed in myoepithelial cells, suggesting a principle role for this cell lineage in the ACC origin and progression. Sub-micromolar concentrations of a novel potent Trk inhibitor AZD7451 completely blocked TrkC activation and associated tumorigenic

behaviors in U2OS cells with ectopic expression of TrkC. Pre-clinical studies on ACC

mice xenografts revealed growth inhibition and low toxicity of AZD7451. In summary,

this study identified a previously unrecognized pro-survival autocrine neurotrophin

signaling pathway in ACC and linked it with cancer progression.

II.1.2 Introduction

ACC is one of the most frequent (22%) types of salivary gland cancers,[201] and is

among the most unpredictable and devastating tumors of the head and neck. ACC is

characterized by insidious local spread and propensity for perineural invasion and distal

metastases to lungs and bone.[202] Most ACCs are treated surgically with or without

postoperative radiotherapy. Insufficient molecular insights into signaling that drives

tumorigenesis and neural tropism in ACC combined with lack of reliable cell lines[129] are

major impediments to the development of better treatment for this insidious cancer.

With the goal to characterize signaling pathways involved in tumor growth and invasion

and to enhance therapeutic insight, expression analysis of macro-dissected primary

ACC specimens was performed. From this study, TrkC/NTRK3, a receptor tyrosine

kinase that binds neurotrophin-3 (NT-3), was identified in ACC. Interest in TrkC was

further stimulated by its overexpression in other cancer types (e.g., neuroblastoma,[203]

melanoma,[204] and breast cancer[205]), trophic stimulation by NT-3, and by the availability

of small-molecule Trk inhibitors.[206] Trk receptors and neurotrophins are involved in

almost all stages of neural development,[207-210] where TrkC conveys NT-3-dependent

pro-survival effects but triggers apoptosis in the absence of ligand.[211] Inactivating

mutations in TrkC/NTRK3 have been recently associated with Hirschsprung disease, a

developmental disorder characterized by the absence of ganglion cells in the

mysenteric and submucosal plexi likely attributable to defects in neural crest migration, differentiation, or survival.[212, 213] In cancers, where TrkC activities are not fully understood, TrkC pro-survival signaling was found to prevail over apoptotic signaling in the presence of NT-3,[214, 215] or when ligand requirement is bypassed by activating mutations[216] or fusions.[217] Potentially activating somatic mutations in the TrkC kinase domain have been reported in breast, lung, colon, and pancreatic cancers.[216, 218-220] Rearrangements of NTRK3 and ETV6 genes (an ETV6–NTRK3 fusion) have been detected in congenital fibrosarcomas, secretory breast carcinomas, and mammary analog of secretory carcinoma of salivary glands.[15, 17, 221] Overexpression of wild-type TrkC and/or other neurotrophin receptors (TrkA, TrkB and p75/NGFR) has been detected in cancers, including medulloblastoma, neuroblastoma, and melanoma. The developmental cell of origin for all of these tumors is derived from the neural crest.[222]

Recent evidence suggests that TrkC/NT-3 signaling is linked with invasion in medulloblastoma,[223, 224] melanoma,[204] and breast cancer.[205, 225] In addition, TrkC expression has been correlated with the invasion into venous and nervous tissues in pancreatic ductal carcinoma;[226] however, the molecular and cellular mechanisms of such activity remain unclear. To explore the cellular and molecular consequences of TrkC signaling, TrkC was ectopically expressed in cancer cells lacking expression of any neurotrophin receptors or their ligands. Herein, TrkC signaling was shown to be activated in human ACC and a model of TrkC in U2OS cells revealed that activated TrkC stimulated cell motility, chemotaxis, invasion and colony formation in soft agar, as well as fillopodia formation. It was further shown that NT-3-induced TrkC

phosphorylation and pro-invasive cell behavior was effectively blocked by sub-micromolar concentrations of AZD7451, a small molecule pan-Trk inhibitor.[227]

II.1.3 Materials and methods

II.1.3.1 Head and neck cancer patients and tumor specimens. This study included three independent cohorts of head and neck cancer patients treated at Vanderbilt Ingram Cancer Center (VICC), the University of Virginia (UVA) Hospital, and MD Anderson Cancer Center. Primary salivary cancer and HNSCC specimens were obtained from 36 patients: adenoid cystic carcinoma (*n*=28), mucoepidermoid carcinoma (*n*=6), adenocarcinoma (*n*=2), HNSCC (*n*=10). Patients who were treated between 2003 and 2008 provided written consent to have tissue collected for research purposes from the Institutional Review Board (IRB#030062, WGY). The UVA ACC specimens used in the study (*n*=11) were grown as murine xenografts, as previously described.[228]

II.1.3.2 RNA isolation and expression array analyses. Total RNA was either isolated from snap-frozen clinical macro-dissected specimens, as described before,[229] or from cell lines using miRNeasy or RNeasy kits (Qiagen, Valencia, CA, USA). RNA quality and quantity were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Nanodrop ND-1000 (NanoDrop Products, Wilmington, DE, USA). Complementary RNA was prepared from 100 ng of total RNA following the Affymetrix 3′IVT Express kit labeling protocol and standardized array processing procedures recommended by Affymetrix, including hybridization, fluidics processing, and scanning of the Affymetrix HG-U133 Plus 2.0 arrays. The raw data (Affymetrix CEL

files) were normalized using RMA (Robust Multi-array Analysis). Gene Ontology (GO) analyses of differentially expressed genes were performed using WebGestalt,[230] GSEA,[231] and g:Profiler[232] portals.

II.1.3.3 RT-PCR and sequencing. To produce NTRK3 cDNA for sequencing from clinical RNA specimens, the following primers were designed and used: NTRK3F1 (pos. 357-376 on NM_002530): 5′-GCTGGGAAGCGTCTGGCTGG-3′; NTRK3R1 (pos. 1269-1250): 5′-GATGCAGTGCTCCAGGCGCA-3′; NTRK3F2 (pos. 1249-1268 on NM_002530): 5′-CTGCGCCTGGAGCACTGCAT-3′; NTRK3R2 (pos. 2811-2786): 5′-GTATGAATTCATGACCACCAGCCACC-3′. To specifically amplify isoform d, the following primers were used: TrkCFdel (pos. 1497-1515): 5′-GGAGCCCTTTCCAGTTGAC-3′; TrkCRdel (pos. 1523-1501): 5′-CTCACTTCGTCAACTGGAAAGGG-3′. To amplify both isoforms for gel-separation, the following primers were used: TrkCisoF (pos. 1426-1446 onNM_002530): 5′-GGCAACTATACCCTCATTGCC-3′ and TrkCisoR (pos. 1559-1540): 5′-GGAGGTGTGGGACTCACTTC-3′. To amplify NTRK3 and NTF3 cDNAs for validation by RT–PCR and sequencing, NTRK3 primers (5′-CTGCGCCTGGAGCACTGCAT-3′; 5′-GAGCAGCTCGGCCTCCCTCT-3′, pos. 1249-1268 and 2082-2063 on NM_002530, respectively) and NTF3F primers (5′-GGGCCCGCCAAGTCAGCATT-3′; 5′-TATCCACCGCCAGCCCACG-3′; pos. 309-328 and 803-785 on NM_002527, respectively) were used.

II.1.3.4 Cell lines and transgenic expression. The human osteosarcoma cell line U2OS was transfected with NTRK3 expression construct (Myc-DDK-tagged ORF clone,

NM_002530) purchased from OriGene (Rockville, MD, USA) and validated by sequencing. U2OS clones with constitutive expression of TrkC were produced via G418 selection.

II.1.3.5 Antibodies for western analysis, TrkC phosphorylation assay, and immunohistochemical staining. TrkC/NTRK3, TrkB/NTRK2, p75/NGFR, TGFBR3, phospho-Akt(Ser473), and phospho-Erk1/2 rabbit monoclonal antibodies were from Cell Signaling (Danvers, MA, USA). Phospho-Tyr antibody was from BD Transduction Laboratories (San Jose, CA, USA). Polyclonal Ephrin B3 antibody was from Novus Biologicals (Littleton, CO, USA), and DO1 p53 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For IHC, Cell Signaling TrkC antibody (1:100 dilution), NT-3 antibody from Santa Cruz Biotechnology (N-20, 1:300), and Bcl2 antibody from Leica Microsystems (PA0117, Buffalo Grove, IL, USA) were used with Novocasra Bond Polymer Refine Detection system, as recommended by the manufacturer.

II.1.3.6 Ras activation and protein phosphorylation assays. To measure Ras activity, Ras Activation Assay kit produced by Cytoskeleton (Denver, CO, USA) was used. Protein phosphorylation was detected using the Human Phospho-Kinase and Phospho-RTK Antibody Arrays (R&D, Minneapolis, MN, USA).

II.1.3.7 AZD7451. AZD7451 is a potent small-molecule inhibitor of Trk kinases with a high degree of specificity and selectivity demonstrated as compared with other kinases.[227] AZD7451 was found to inhibit the *in vitro* activity of all three Trk isoforms, with an IC_{50}=0.2 nM for TrkA in cells and inhibition of phosphorylation of TrkB and TrkC

at 3 nM. AZD7451 also exhibited slow-off rate kinetics for Trk kinases, suggesting for increased residence time on the target. *In vitro* kinase selectivity assays involving 83 kinases tested, demonstrated that only 15 (18%) showed <100-fold window versus TrkA. Of these, only FGFR1 and CaMKII showed <10-fold window for AZD7451 as compared with TrkA. AZD7451 has also shown potent cellular activity against all three TrkA, B, and C receptor isoforms in established cell-based systems overexpressing wild-type, full-length forms of human TrkA, TrkB or TrkC at concentrations of <5 nM.

II.1.3.8 Cell motility, migration, invasion and colony formation in soft agar.
Scratch (wound healing) assay was performed as described,[233] following the inhibition of proliferation using mitomycin C at 5 µg/ml. Migration and invasion assays used 8.0 µm polycarbonate membrane inserts for 24-well plates (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, 2×10^4 of serum-starved cells were loaded into each insert in serum-free DMEM media and incubated in a CO_2 incubator for 22 h with or without NT-3 (PeproTech, Rocky Hill, NJ, USA) added at 100 ng/ml to bottom media supplemented with serum. Colony formation was determined with or without NT-3 (100 ng/ml) in 0.3% agar/complete DMEM. In all these experiments, Trk inhibitor AZ7451 was used at 100 nM.

II.1.3.9 Immunoprecipitation and mass spectrometry. Proteins that bind TrkC were determined following immunoprecipitation with Cell Signaling TrkC antibodies and Dynabeads (Carlsbad, CA, USA). Protein bands were separated using polyacrylamide gel electrophoresis (PAGE) and bands isolated after staining with coomassie blue.

Protein identification was determined via mass spectrometry by ProtTech (Norristown, PA, USA).

II.1.3.10 Cytoskeleton visualization. Mouse monoclonal anti-TUBB2B (Abnova, Walnut, CA, USA) with anti-mouse Alexa 555 (Invitrogen, Carlsbad, CA, USA) and FITC-phalloidin (Sigma, St Louis, MO, USA) were used to stain cells according to manufacturer's instructions. Nuclei were counterstained with DAPI.

II.1.3.11 AZD7451 studies on ACC xenografts. The *in vivo* efficacy on AZD7451 was evaluated in a series of primary ACC tumor xenograft models[228] through the South Texas Accelerated Research Therapeutics (San Antonio, TX, USA) program. Animals were maintained in accordance with the Institutional Animal Care and Use Committee guidelines. Female athymic *nu/nu* mice (Harlan Sprague–Dawley) between 4–6 weeks of age were housed on irradiated corncob bedding (Teklad bedding) in individually ventilated cages (Sealsafe Plus, Tecniplast, Exton, PA, USA) on a 12-h light–dark cycle at 70–74 °F (21–23 °C) and 40–60% humidity. Mice were fed water *ad libitum* (reverse osmosis, 2 ppm Cl_2) and an irradiated standard rodent diet (Teklad 2919) consisting of 18% protein, 5% fat and 4% fiber. Fragments of human ACC tumor specimens, ACCX6 and ACCX9 were subcutaneously implanted and grown to produce cohorts averaging n=8 per group. Tumor-bearing mice were randomized into control and treatment groups when tumors reached ~0.2 cm^3 in size. AZD7451 treatment was given by oral administration at 50 mg/kg, following a daily schedule for 35 days total dosing alongside vehicle (0.5 HPMC/0.1% Tween 80) controls. To assess AZD7451 toxicity, animals were observed daily and weighed twice weekly using a digital scale;

data including individual and mean gram weights (mean We±s.d.), mean percent weight change versus Day 0 (%vD$_0$), and mean percent weight change versus prior measurement (%vD$_{-x}$) were recorded for each group and plotted at study completion. To assess AZD7451 efficacy, tumor dimensions were measured twice weekly by a digital caliper, and data including individual and mean estimated tumor volumes (Mean TV±s.e.m.) were recorded for each group; tumor volume was calculated using the formula TV=width^2 × length × 0.52. At the end point, percent (%TGI) values were calculated and reported for each treatment group (T) versus control (C) using initial (i) and final (f) tumor measurements by the formula: %TGI=$1-T_f-T_i/C_i-C$; single agent or combination therapies resulting in a TGI >58% at study completion were considered active in the tested model at the evaluated treatment regimen according to NCI guidelines. Statistical differences in tumor volume were determined using a one-tailed *t*-test. BrdU incorporation studies were performed upon intraperitoneal injection with 1 mg of BrdU (BD Biosciences, San Jose, CA, USA) in 200 mkl PBS 2 h before killing the mice. Tumor cells positively stained with BrdU-antibody (Cell Signaling) were counted from 10 high-power fields (× 40) (>300 cells per field) and proliferative index determined as BrdU-positive cells divided by total cell count.

II.1.4 Results

II.1.4.1 ACC gene expression signature identifies expression of neurologic stem cell markers and elements of the Wnt and Notch pathways

Microarray expression analyses comparing global expression patterns of human salivary ACC (n =7) to normal tissue and other cancers of the head and neck revealed that the ACC expression profile differed from salivary mucoepidermoid carcinoma

(MEC, n = 6), and head and neck squamous cell carcinoma (HNSCC, n = 10).

Specimens in this study were collected at the time of biopsy or resection at the

Vanderbilt Ingram Cancer Center (Table 1). ACC and MEC were more similar, with

3345 differentially expressed genes (p < 0.01), compared to HNSCC and the salivary

tumors, with 7127 (ACC, p < 0.01) and 8137 (MEC, p < 0.01) differentially expressed

genes.

Unsupervised clustering of all specimens using the 3345 genes that

discriminated ACC from MEC identified a hierarchical association that specifically

segregated all but one of the ACC specimens from all other tumor types (Figure 3A).

Likewise, MEC and HNSCC clustered together in distinct branches from ACC.

Cluster analyses revealed that ACC expressed 210 genes at markedly higher

levels compared to either MEC or HNSCC. One of the most immediately notable

characteristics of this gene set was a large number of probes identifying TRKC as

expressed only in ACC, which prompted reference to these genes as the TrkC-centered

signature (TCCS). GO analyses of the TCCS demonstrated significant enrichment in

genes associated with the Notch and Wnt pathways (Table 2) that have previously been

associated with neural differentiation and cancer.[234-236] Other genes associated with the

TCCS included: neural crest stem cell markers SOX10,[237] MAP2,[238] SALL2,[239] and

SLITRK6;[240] melanoma markers SHC438 and MUM139; and surface receptors FGFR1

and EFNB3.

To further validate the ability of the TCCS to identify ACC specimens, expression

patterns of murine xenografts derived from primary ACC specimens (n = 11) were

analyzed.[228] When expression data from the xenografted ACCs were combined with

data from primary tumors and re-normalized, unsupervised clustering using the

previously identified differentially expressed genes clustered 10 xenografts with all but

two of the primary ACC specimens (Figure 3B). Consistent with clustering of primary

ACC (Figure 3A), genes highly expressed in ACC centered on TrkC and confirmed that

this gene cluster was relatively overexpressed in xenografted and primary ACCs.

TCCS components, such as EFNB3, GPM6B, SOX10, and TCF7L2 were again

identified as highly expressed in ACC from the combined analysis. The TGF-β receptor

TGFBR3 was also found adjacent to the NTRK3 cluster; however, its expression was

not limited to ACC, but seen also in MEC, and to a lesser extent in HNSCC.

ID	Gender	Age	Site	Diagnosis	Stage
ACC-1	F	42	Oral cavity/buccal	ACC	T1N0M0
ACC-2	F	86	Oral cavity/hard palate	ACC	T4N0M0
ACC-3	F	65	Parotid	ACC	T2N0M0
ACC-4	F	75	Submandibular	ACC	T3N0M0
ACC-5	M	78	Nasal cavity	ACC	T2N0M0
ACC-6	F	61	Nasopharynx	ACC	T3N0M0
ACC-7	F	75	Nasopharynx	ACC	T2N0M0
AD-1	M	57	Nasopharynx	AD	T4N0M0
AD-2	M	60	Nasal cavity	AD	rT4bN0M0
MEC-1	M	56	Parotid	MEC	T1N0M0
MEC-2	M	65	Parotid	MEC	T4N0M0
MEC-3	F	55	Oropharynx	MEC	T1N0M0
MEC-4A	F	63	Oropharynx	MEC	T1N0M0
MEC-4B	F	63	Oropharynx	MEC	T1N0M0
MEC-5	F	75	Oral cavity/tongue	MEC	T2N0M0
MEC-6	F	31	Parotid	MEC	T1N0M0
HNSCC-1	M	60	Oropharynx	HNSCC	T2N1M0
HNSCC-2	M	74	Oropharynx	HNSCC	T2N2cM0
HNSCC-3	M	53	Oropharynx	HNSCC	T4N2cM0
HNSCC-4	M	44	Oropharynx	HNSCC	T1N2cM0
HNSCC-5	M	49	Oropharynx	HNSCC	T2N2bM0
HNSCC-6	M	42	Oropharynx	HNSCC	T1N2aM0
HNSCC-7	F	44	Oropharynx	HNSCC	T1N1M0
HNSCC-8	M	57	Oropharynx	HNSCC	T2N2cM0
HNSCC-9	M	53	Oropharynx	HNSCC	T3N2bM0
HNSCC-10	F	79	Oropharynx	HNSCC	T1N1M0

Table 1: Primary tumor specimens of ACC, MEC, AD and HNSCC used in the study.
Abbreviations: ACC, adenoid cystic carcinoma; MEC, mucoepidermoid carcinoma.
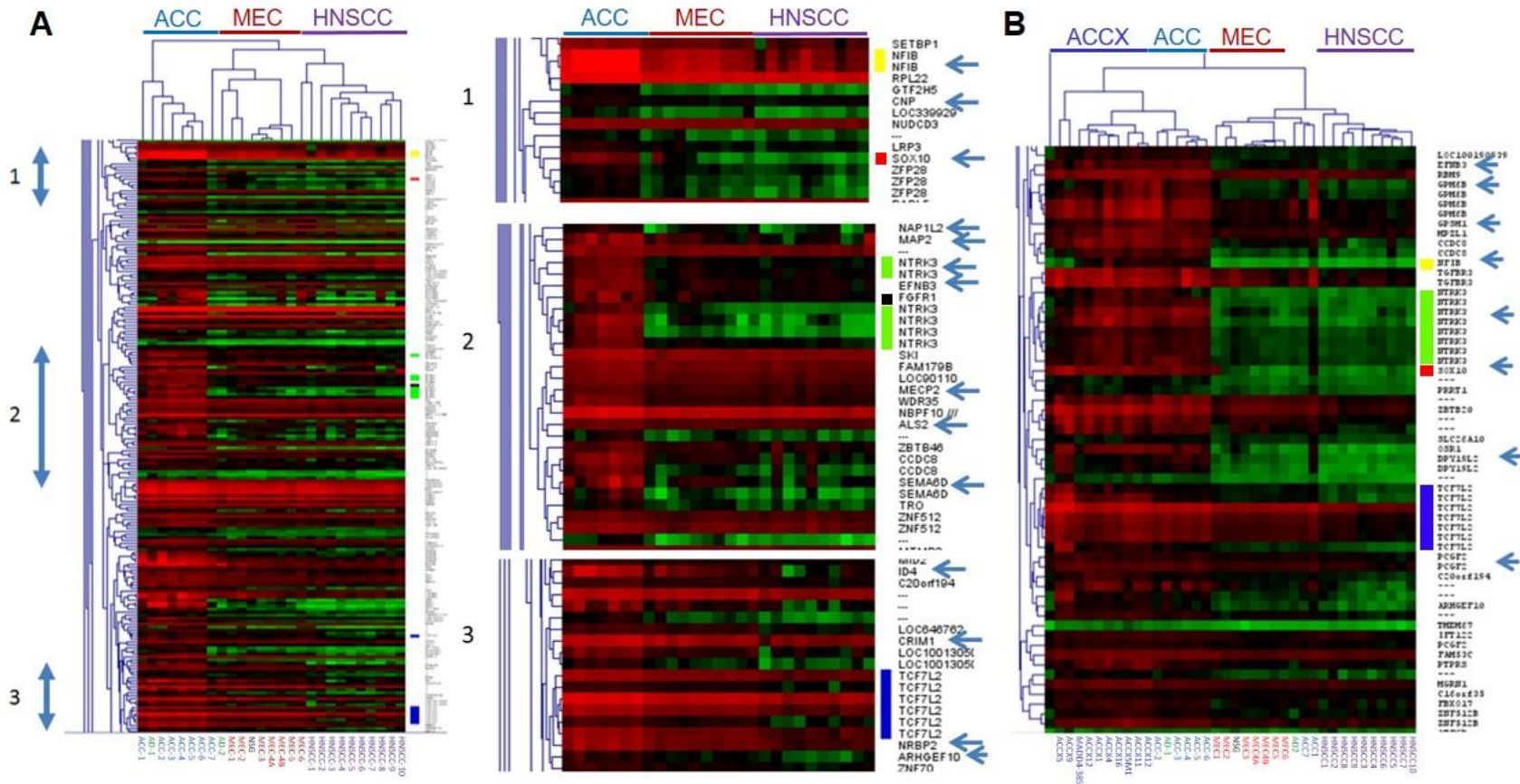
Figure 3: TrkC-centered gene signature (TCCS) distinguishes ACC from MEC and HNSCC.
(A) Expression profiling of clinical specimens derived from 25 primary tumors (left) and close up of clusters 1, 2, and 3 (right). Colored boxes and arrows along heatmap indicate overexpression of TrkC/NTRK3 (green boxes), SOX10 (red box), NFIB (yellow box), TCF7L2 (blue box), and multiple genes associated with neurogenesis (blue arrows). FGFR1 (black box) and EFNB3 (orange box) are clustered with NTRK3. Individual specimen codes are shown along heatmap bottom. (B) Validation of TCCS on 11 subcutaneously grown ACC xenografts derived from an independent patient cohort.[228] When added to the previous clinical set, 11 independently collected and xenografted ACC specimens (ACCX set) showed similar gene signature and clustered with 5 out of 7 primary ACC specimens. Core TCCS elements are shown with colored boxes as above. All ACCX specimens are labeled with letter X at the bottom of heatmap with XMA designating MADO4-385.

Wnt	Notch	Nervous system development	Neurologic stem cell markers
CTBP1	CTBP1	ATXN3	MAP2
CTBP2	CTBP2	CRIM1	SALL2
CXXC4	KAT2A	EFNB3	SOX10
FBXW11	NOTCH1	GPM6B	SLITRK6
LRP6		GPSM1	
SMAD4		NDRG2	
TBL1X		NTRK3	
TCF7L1		SEMA6D	
TCF7L2		SMARCC2	
WNT6		SMARCE1	

Table 2: Enrichment of TrkC-centered gene signature with genes involved in the Wnt and Notch pathways, neurologic development and with neurologic stem cell markers

II.1.4.2 TrkC as a marker of ACC

TrkC was of particular interest as it had is overexpressed in select cancers (e.g., neuroblastoma,[203] melanoma,[204] and breast cancer[205]) and stimulated by a neural-associated gene, NT-3, which could play a role in the characteristic perineural invasiveness of ACC, and potent small-molecule Trk inhibitors have been developed. However, detecting of TrkC might have been evidence of a broader network of neurotrophic signaling in ACC. Indeed, expression array identified expression of neurotrophic receptors p75/NGFR and TrkB/NTRK2 in a subset of ACC. Expression of p75/NGFR and TrkB/NTRK2 was not as ubiquitous as TrkC among ACC specimens, and TrkA/NTRK1 expression was not identified in ACC. Thus, TrkC appeared to be the major effector of this family in ACC demonstrating 100-430-fold up-regulation in 17 of the 18 studied primary ACC specimens and xenografts compared to normal salivary tissue (Figure 4A). To confirm the expression array data, TrkC protein expression was measured by immunoblot in clinical ACC, MEC, and normal salivary specimens, revealing markedly increased levels of TrkC in all primary ACC specimens and

xenografts studied, whereas expression was at the limit of detection in the remaining tissues (Figure 4B). Expression of other neurotrophic (p75/NGFR and TrkB/NTRKB) and membrane (Efnb3 and Tgfbr3) receptors was consistent with microarray results (Figure 4B). Examination of ACC xenografts confirmed high expression of TrkC was maintained in this model system (Figure 4C).
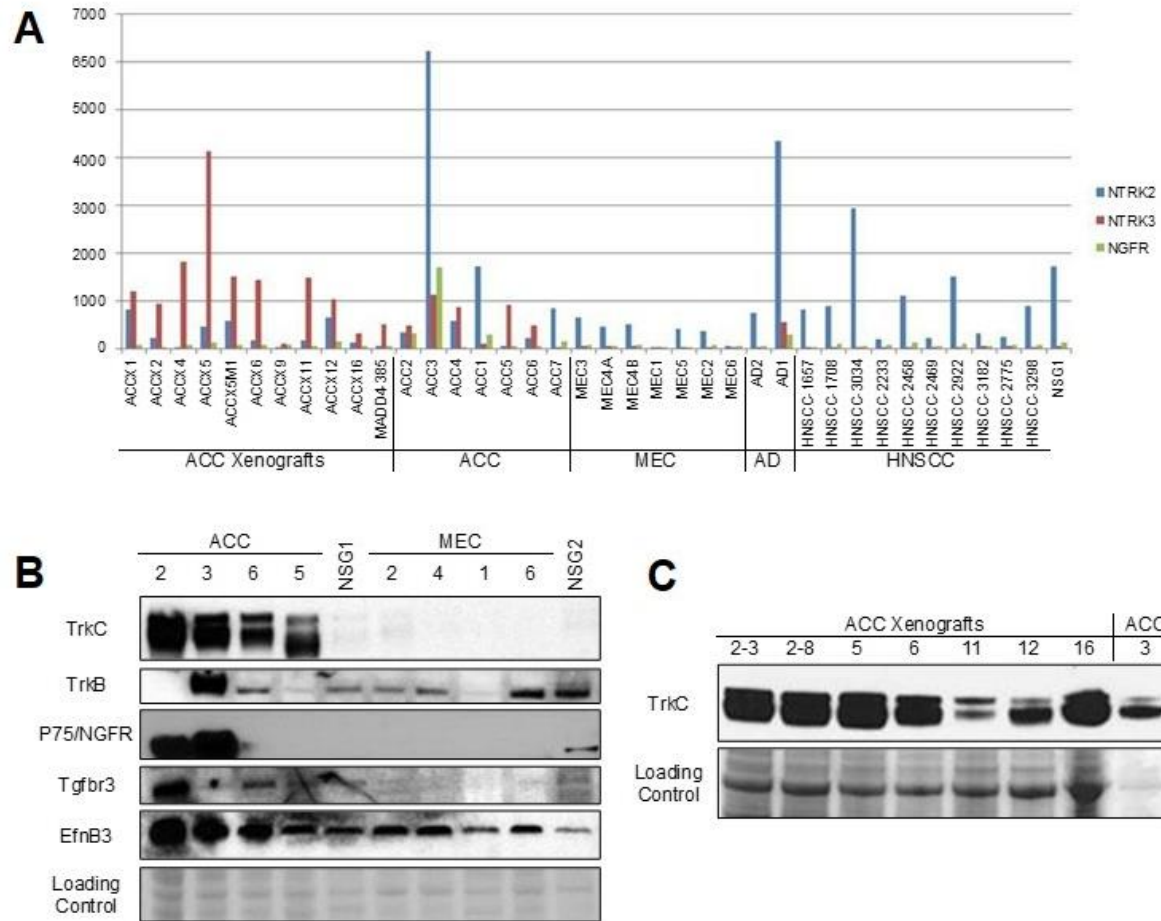
Figure 4: TrkC is expressed in ACC.

(A) Expression of neurotrophic receptors in clinical and subcutaneously grown ACC xenograft specimens. Expression values of TrkB/NTRK2, TrkC/NTRK3, and p75/NGFR were derived from combined microarray study and shown in arbitrary units. Expression of TrkA was not detected. (B) Immunoblot validation of TrkC, TrkB, NGFR, and other receptor expression in clinical ACC and MEC specimens compared to normal salivary gland tissue (NSG). In agreement with microarray data, ACC specimens also expressed EfnB3 and Tgfbr3. (C) Expression of total TrkC protein in subcutaneously grown ACC xenografts as compared with clinical specimen ACC-3. Numbers of passages for ACCX2 (either 3 or 8) are indicated. Loading control was assessed by Ponceau staining.

II.1.4.3 ACC tumors express full-length TrkC and its ligand NT-3

As activating mutations and fusions of TrkC have been described in diverse tumor types, it was important to determine whether ACC specimens contained such mutations. TrkC transcripts were amplified from primary ACC tumors and sequenced, revealing that the open reading frames (ORFs) of TrkC from all six TrkC-positive ACC tumors were full-length and wild-type. Remarkably, all of these specimens (ACC1-6) expressed the canonical isoform b that encodes a fully active protein (NM_002530), but also contained splicoform d (NM_001243101) that lacks exon 10, producing an 8-amino-acid (aa) in-frame deletion in the juxtamembrane TrkC domain. This isoform, which was expressed in ACC at levels similar to isoform b (data not shown), was previously described as a polymorphism,[241] but its biological significance remains unclear.

Although TrkC was expressed in the majority of ACC specimens, biological effects of TrkC activity in these tumors were unknown. In other tumor types such as neuroblastoma, TrkC expression is a positive prognosticator, likely related to the function of TrkC function as death receptor when its ligand is not present. In order to determine if TrkC was active, as a surrogate for ligand presence, phosphorylation of the receptor was analyzed. TrkC immunoprecipitation from lysates of ACC xenografts followed by immunoblotting for phosphotyrosine indicated that TrkC is activated in ACC (Figure 5A). To determine if an autocrine loop between NT-3 and TrkC was possible in ACC, NT-3 expression was determined in primary (n = 7) and xenografted ACC specimens (n = 11). NT-3 expression was detected and verified by sequencing in both primary and xenografted ACC specimens, suggesting that an autocrine survival

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pathway may be active in ACC (Figure 5B&C). Levels of NT-3 transcripts showed

substantial variation from specimen to specimen with no obvious correlation with

expression of TrkC (R = 0.43). Taken together, these results demonstrate that ACC

tumors contain high levels of TrkC, and that TrkC signaling is persistently active in these

tumors, likely owing to autocrine NT-3 production.
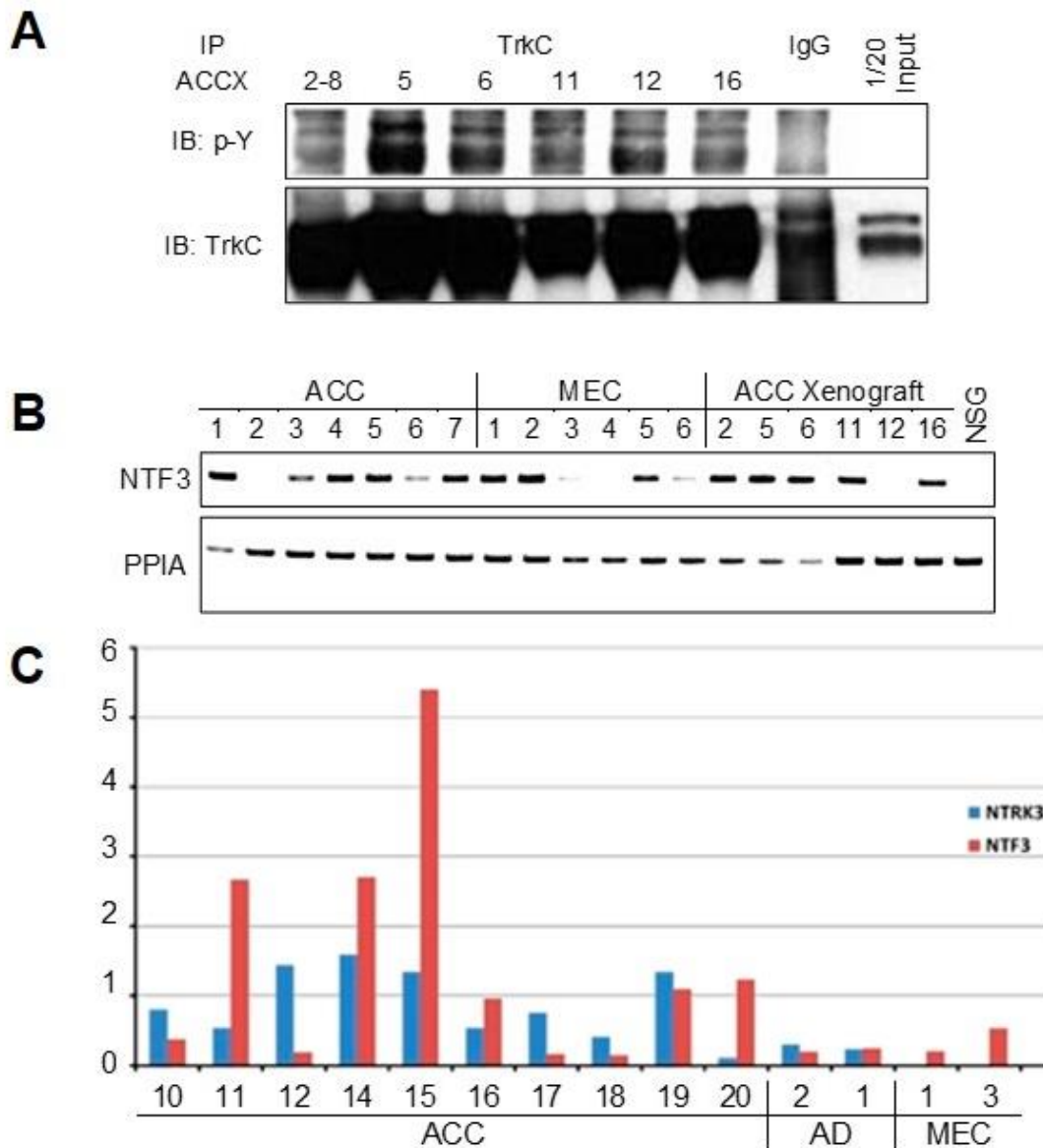
Figure 5: Activation of TrkC in ACC.
(A) Tyrosine phosphorylation of TrkC in ACC xenografts assessed via TrkC-immunoprecipitation. Rabbit IgG was used as a negative control and 1/20th of the protein amount used in immunoprecipitation was loaded for Western analysis. Semi-quantitative detection of NTF3 expression by end-point RT–PCR (B) and comparison between NTF3 and NTRK3 levels in clinical specimens of ACC (C). Equal amounts (50 ng) of total RNA isolated from clinical (ACC) and subcutaneous xenograft (ACC Xenograft), MEC and AD specimens, and normal salivary tissue (NSG) were subjected to 30 cycles of RT–PCR with primers for human NTF3, NTRK3, and PPIA (last used as a loading control). Graph shows PPIA-normalized expression values in arbitrary units.

II.1.4.4 Creation and validation of a TrkC-expressing surrogate cell line

Although a link between TrkC/NT-3 expression and aggressive cancer behavior has been established in some tumor types,[205, 242] the molecular and cellular consequences of TrkC overexpression are not fully understood. TrkC expression in primary and xenografted tumor specimens did not correlate with other neurotrophin receptors such as TrkA, TrkB or p75/NGFR (Figures 4A&B), suggesting a special role for TrkC in this tumor type. In order to explore the consequences of TrkC expression and signaling, we wanted to disrupt TrkC signaling *in vitro*; however, due to a lack of reliable cell lines in ACC,[129] a surrogate cell line with forced expression of TrkC was utilized. For this purpose, human osteosarcoma cells (U2OS) were chosen as they lack endogenous expression of neurotrophic receptors or their ligands. While this model may not perfectly replicate the activities of TrkC in ACC, this cell line enabled identification of potential molecular mediators and behavioral consequences of TrkC signaling.

TrkC-expressing U2OS cells tolerated high levels of TrkC expression, enabling the study of TrkC signaling in the presence or absence of NT-3. In the absence of NT-3, TrkC remained unphosphorylated, but stimulation with its ligand induced rapid and marked TrkC phosphorylation at tyrosine residues (Figure 6A). The pan-Trk inhibitor, AZD7451 (AstraZeneca) completely prevented/reverted TrkC phosphorylation when added at nanomolar concentrations regardless of the presence of NT-3 ligand (Figure 6A). As ligand-activated Trk signaling is associated with receptor clustering and internalization,[243] cellular localization of TrkC with and without NT-3 was assessed by immunofluorescence. Cellular localization was consistent with TrkC receptor

clustering/internalization following NT-3 stimulation, as diffuse membrane staining

changed to a pattern of distinct bright puncta (Figure 6B).
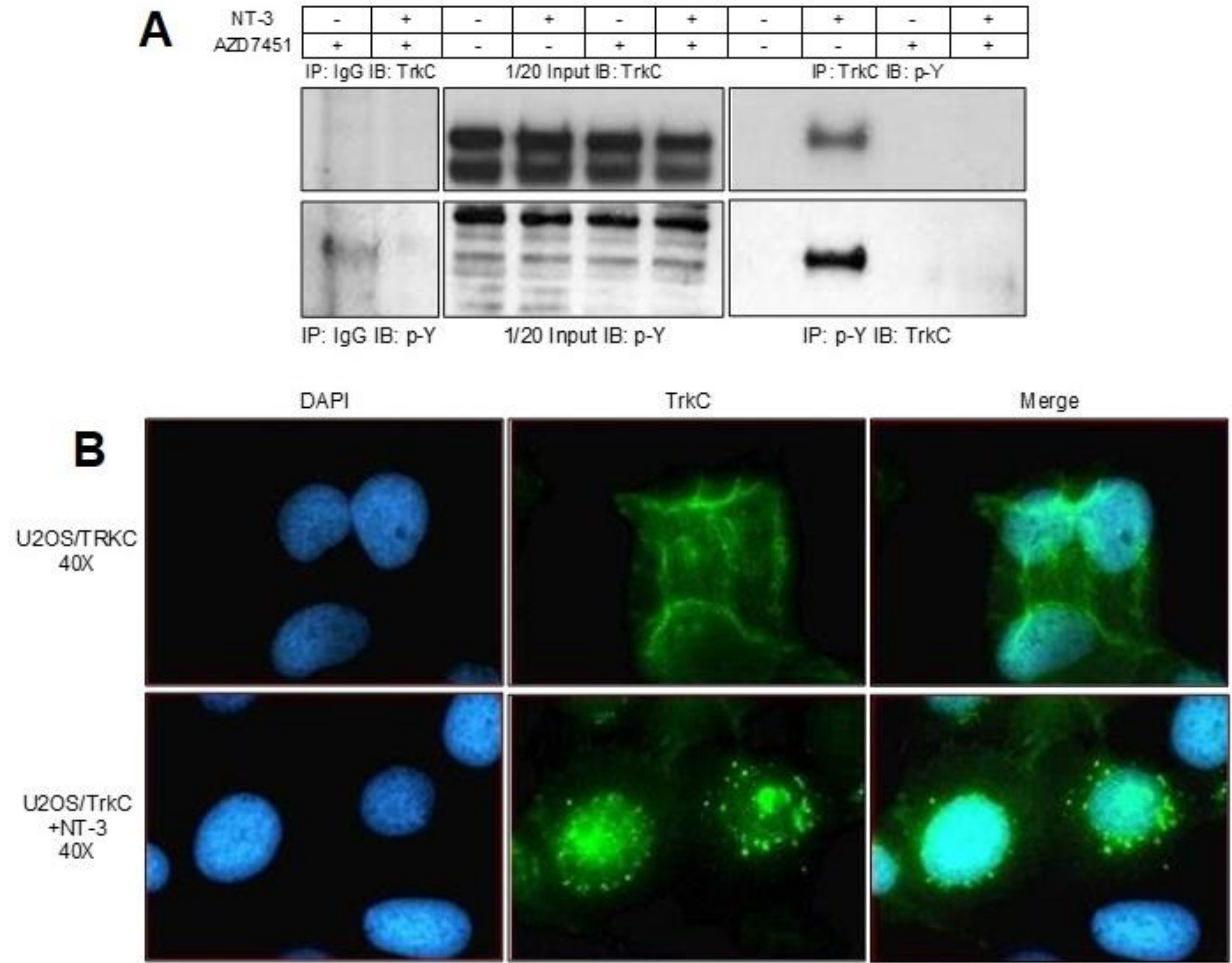
Figure 6: TrkC activation in U2OS surrogate cell line.
(A) NT-3-dependent TrkC tyrosine phosphorylation assessed in TrkC-expressing U2OS cells. (B) Immunofluorescence staining of TrkC in TrkC-expressing cells with (bottom row) and without (upper row) NT-3.

II.1.4.5 TrkC activation induces proliferative and anti-apoptotic pathways

In the presence of its ligand, TrkC signaling in non-tumorigenic neural tissue promotes survival and proliferation, at least partially, through activation of Ras and Akt as its downstream mediators (http://www.genome.jp/kegg/pathway/hsa/hsa04722.html). To identify additional signaling pathways activated by TrkC expression in the presence or absence of the NT-3 ligand, stably transfected U2OS cells (vector control and TrkC) with and without addition of NT-3 were analyzed using phospho-protein antibody arrays (Figure 7A). The most notable difference following TrkC expression was the activation of p53 indicated by increased phosphorylation at multiple sites, including the critical S15 residue (Figure 7A). Additionally, TrkC expression had a moderate suppressive effect on Akt phosphorylation and induced phosphorylation of β-catenin. Stimulation of TrkC-expressing cells with NT-3 resulted in a very different profile of pathway activation compared with cells expressing TrkC in the absence of ligand. Upon NT-3 stimulation, Erk and Akt were markedly activated in cells expressing TrkC, whereas phosphorylation of β-catenin was reduced (Figure 7A). Phosphorylation of p53 was decreased upon NT-3 treatment in cells expressing TrkC (Figure 7A), consistent with TrkC acting as a death receptor in the absence of its ligand. Despite TrkC being the only described receptor for NT-3, p53 phosphorylation increased in cells lacking TrkC (Figure 7A) when exposed to NT-3. Immunoblot experiments confirmed the effects of TrkC and NT-3 on the expression of phosphorylated Akt, Erk1/2, and p53 seen in the arrays (Figure 7B). Interestingly, co-immunoprecipitation experiments indicated that TrkC exists in complex with p53, and that this association is not dependent upon the presence of NT-3 (Figure 7C).

A similar phospho-receptor tyrosine kinase (RTK) antibody array was employed to assess changes in tyrosine phosphorylation following TrkC expression or NT-3 stimulation. As expected, TrkA and TrkB phosphorylation were not detected in these cells, and TrkC tyrosine phosphorylation was confirmed upon NT-3 stimulation only in cells expressing TrkC (Figure 7A). In addition to stimulating TrkC phosphorylation, TrkC expression in the presence of NT-3 triggered VEGFR1 tyrosine phosphorylation, suggesting that NT-3 may promote angiogenesis in a TrkC-dependent manner.

Although TrkC expression has been associated with Ras activation,[205, 225] this was not assessed by the antibody arrays. Activation of Erk and Akt downstream of TrkC could indicate Ras pathway activation, and to further explore this possibility control- (vector) and TrkC-expressing U20S cells were stimulated with NT-3 for increasing time periods before measuring activated Ras by determining the amount that bound to a peptide of the Ras binding domain (amino acids 51-149) of human Raf1. NT-3 stimulation resulted in rapid and sustained Ras activation only in TrkC-expressing cells (Figure 7D). These effects were consistent with previous reports demonstrating that Ras is a mediator of TrkC signaling,[205, 225] and further suggested that proliferative and survival pathways are activated in the absence of additional neurotrophin components such as TrkA, TrkB, NGFR, or their ligands.
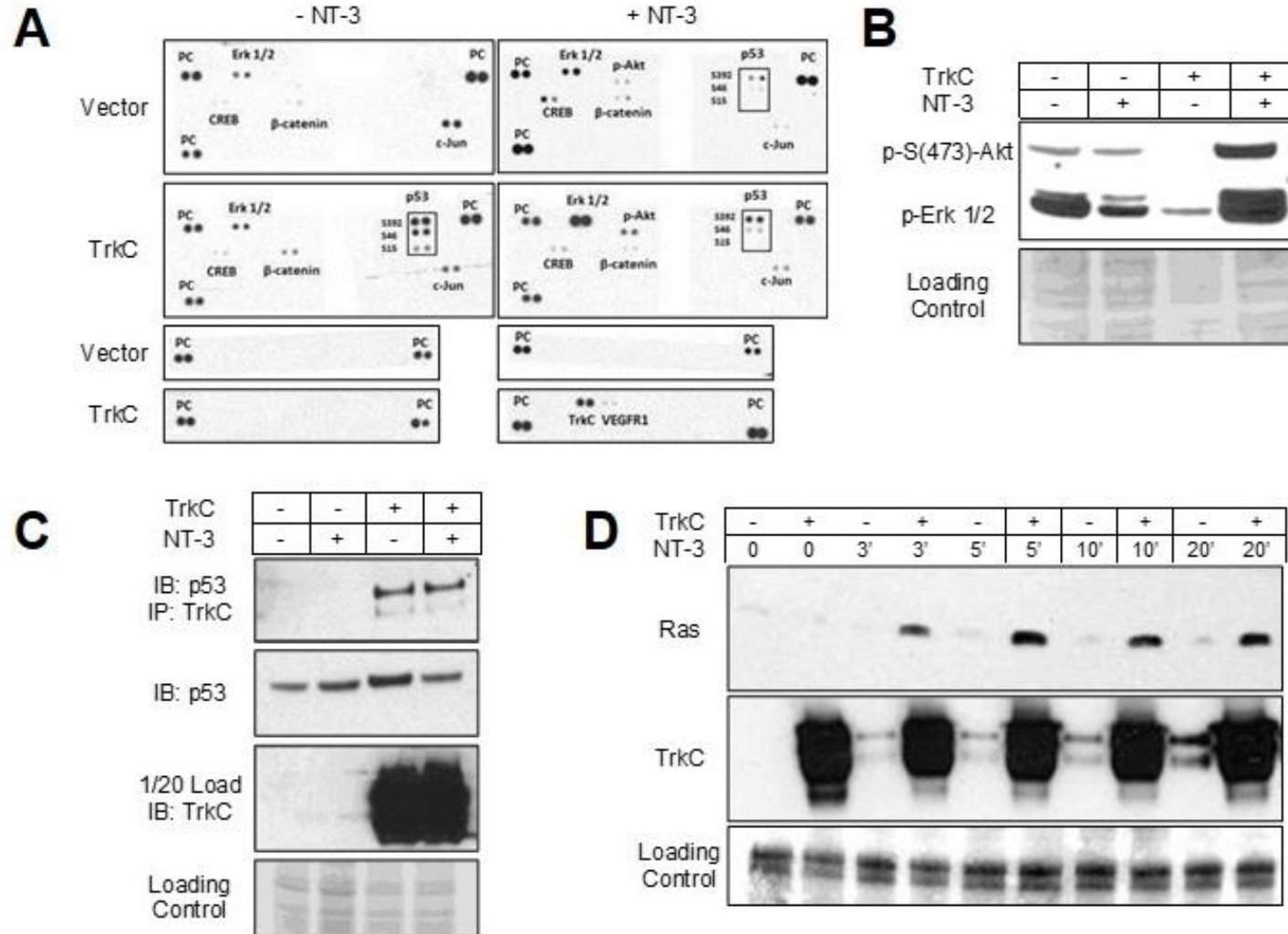
Figure 7: NT-3 induces pro-survival signaling in TrkC-expressing U2OS cells.
(A) TrkC- and NT-3-dependent phosphorylation of Erk1/2, Akt, β-catenin, and VEGFR1. Tyrosine phosphorylation of TrkC induced by NT-3 is shown. PC, positive controls provided with membranes. (B-C) Validation of TrkC and NT-3 effects on Akt, Erk1/2 (B), and association with p53 (C). (D) Rapid and sustained NT-3-induced Ras activation in cells expressing TrkC. Loading control assessed by Ponceau staining.

II.1.4.6 Phenotypic effects of TrkC activation and signaling

Ras activation is associated with cellular motility, migration, invasion, proliferation and survival.[244] To determine the role of TrkC in cellular migration, a scratch assay was performed on U2OS cells with and without TrkC expression in the presence or absence of NT-3 or the pan-Trk inhibitor AZD7451. Control cells had no change in migration following addition of NT-3, but the gap closure was greatly stimulated by NT-3 in cells expressing TrkC (Figure 8A, ~1.8 fold, $p<10^{-5}$). Addition of AZD7451 did not significantly alter migration of control U2OS cells ($P = 0.17$) but inhibited the migration of TrkC-expressing cells (Figure 8A, ~2.3-fold, $p < 3.9 \times 10^{-8}$).

To further confirm the results of scratch analyses which suggested that activated TrkC stimulated migration, a Boyden transmembrane migration assay was performed with and without NT-3 addition. Consistent with the scratch assay, NT-3 stimulated migration of TrkC-expressing U2OS cells (Figure 8B). NT-3 induced migration of TrkC-expressing cells was inhibited by addition of AZD7451 (Figure 8B). Neither NT-3 or AZD7451 altered migration of control cells. A modified Boyden chamber assay using matrigel coating as a basement membrane surrogate was used to determine if TrkC altered cellular invasion. Similar to results of the migration assays, the addition of NT-3 promoted invasion of TrkC-expressing cells (~2.5-fold), but not control cells (Figure 8C). Once again, the Trk inhibitor, AZD7451, blocked the increased invasion observed in the presence of NT-3.

To further explore the tumorigenic potential of TrkC-expressing U2OS cells, anchorage-independent growth in soft agar was measured. Although U2OS cells are transformed, they inefficiently grow in soft agar, forming very few and relatively small

colonies. Addition of NT-3 to TrkC-negative cells or expression of TrkC in the absence

of NT-3 did not enhance the ability of U2OS cells to grow in this assay. Remarkably,

stimulation of TrkC-expressing cells with NT-3 dramatically increased both number

(~10-fold, $p < 10^{-9}$) and size/calculated volume (~9-fold) of colonies that formed in soft

agar (Figure 8D&E). Treatment of cells with AZD7451 prevented the stimulatory effect

of NT-3 on colony formation seen in TrkC-expressing cells. Together, these data

suggest that TrkC activation by NT-3 in the absence of additional neurotrophin

stimulation or co-operation with other Trk receptors increases tumorigenic behavior and

may drive tumor growth, migration, and invasion.
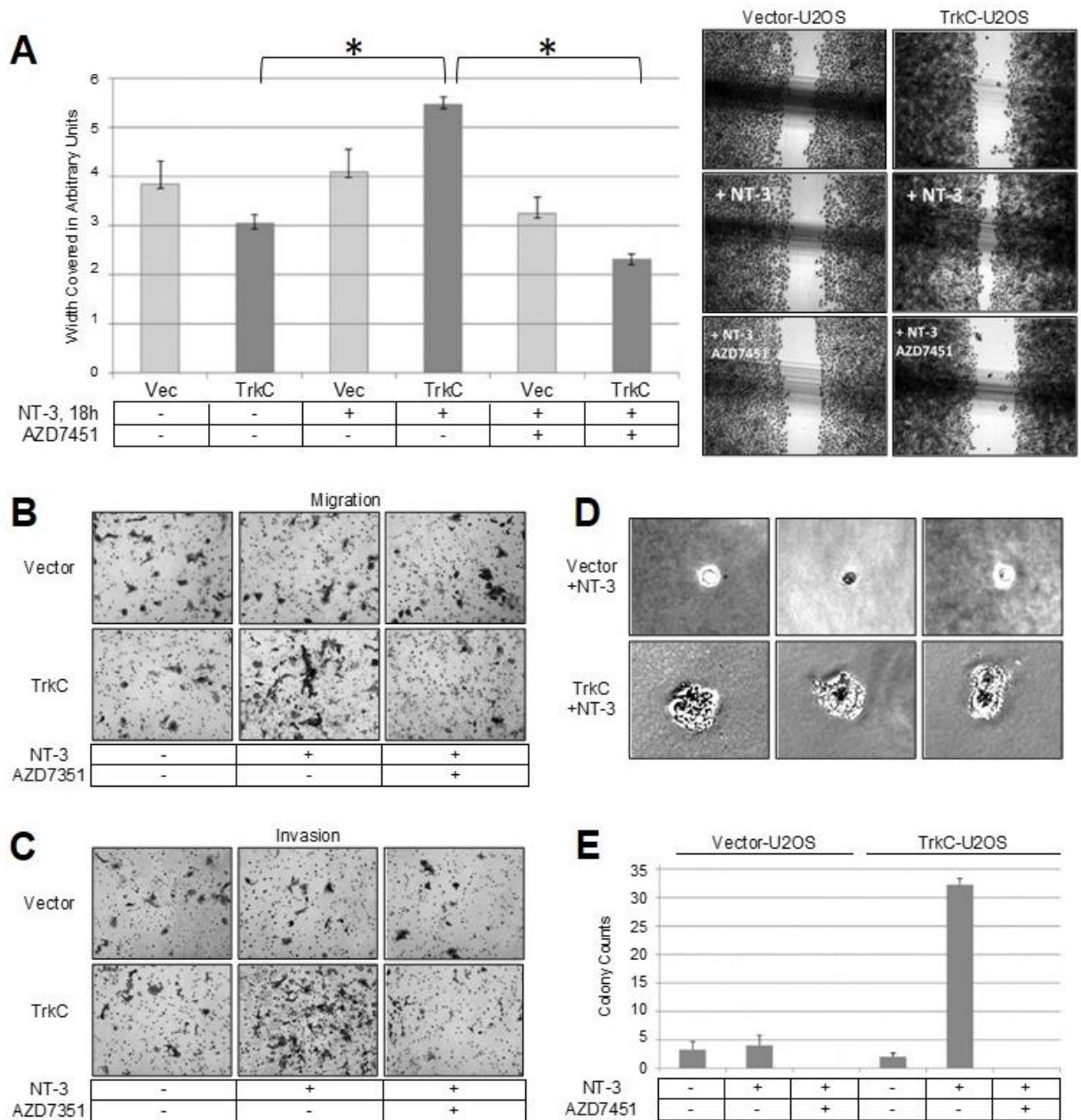
Figure 8: NT-3 promotes motility and chemotaxic migration/invasion in TrkC-expressing cells.
(A) NT-3 promotes TrkC-dependent motility of U2OS cells in scratch assays with mitomycin C. Scratch closure is shown in arbitrary units. Asterisks indicate statistically significant differences. NT-3 stimulates (B) migration through Boyden chamber membranes and (C) invasion through matrigel-covered membranes in TrkC expressing cells. (D-E) NT-3 promotes colony formation and growth in soft agar in TrkC expressing cells. (D) Examples of colony size in the presence or absence of ligand-stimulated TrkC. (E) Average soft agar colony count per visual field in the presence or absence of TrkC, NT-3 or the Trk inhibitor, AZD7451.

II.1.4.7 Effects of TrkC and NT-3 on cytoskeleton components

Migration and invasion require coordinated restructuring of the cytoskeleton, and Ras activation is involved in this process.[245] To examine the extent of cytoskeletal rearrangement resulting from TrkC activation, F-actin was stained with phalloidin-FITC in U2OS cells. Following NT-3 stimulation, TrkC-expressing cells had actin patterns indicative of cytoskeleton rearrangement including F-actin condensation (puncta) and F-actin enriched protrusions (Figure 9A). These effects were similar to the NT-3-triggered changes in the morphology of neuroblastoma cells described previously.[246]

TUBB2B is a class II b-tubulin described as being exclusively expressed in neural tissue (http://biogps.org/) and critically involved in neuronal migration.[247] Expression array analyses revealed that this gene is expressed in the majority of ACC specimens (Figure 9B), and overexpression of TrkC in U2OS cells resulted in increased TUBB2B expression as assessed by immunofluorescence (Figure 9B). These preliminary studies suggested that TrkC may affect restructuring of the cytoskeleton. Taken together, these observations suggested an important role for the TrkC/NT-3 interaction in cytoskeleton regulation resulting in tumor migration and invasion.
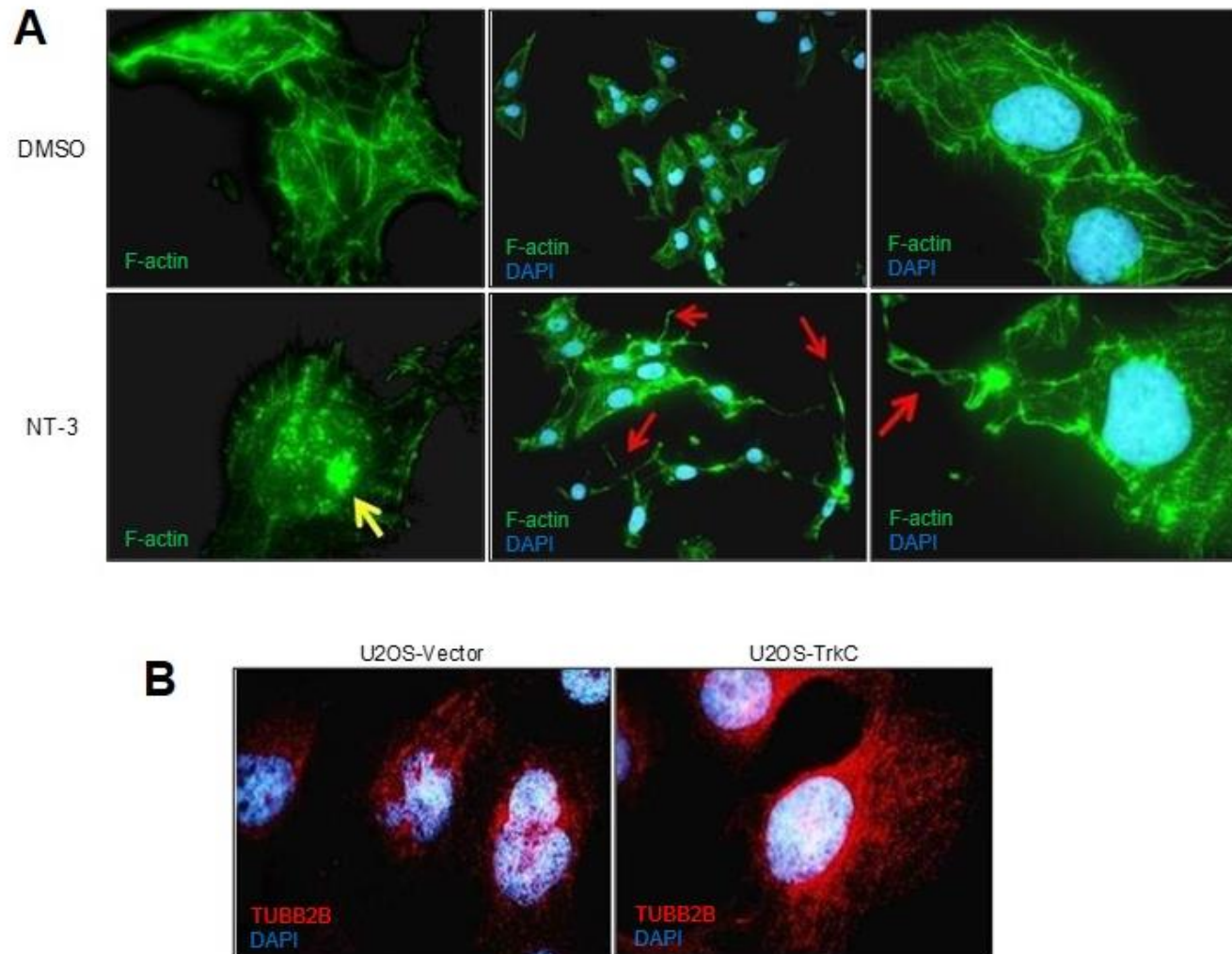
Figure 9: **Association of TrkC with cytoskeletal regulation.**
(A) NT-3 stimulates development of F-actin puncta (yellow arrow, 400x) and F-actin-rich fillopodia (red arrows, middle, 100x; right, 400x). (B) TrkC stimulates expression of class II β-tubulin TUBB2B.

II.1.4.8 Up-regulation of p-Erk1/2 and Bcl2 in TrkC-positive ACC cells

Given that there were no ACC cultures for behavioral or biological study, ACC tissues were analyzed to determine if they expressed proteins observed in the U2OS model system or that had been previously reported. In agreement with activation of Erk1/2 in TrkC-expressing U2OS cells (Figure 7A&B), TrkC has been associated with Erk1/2 and Bcl2 up-regulation in cylindromas,[248] and up-regulation of Erk1/2 by neurotrophins has been linked with Bcl2 up-regulation (http://www.genome.jp/kegg-bin/show_pathway? hsa04722). In order to assess whether these finding are also seen in ACC, clinical ACC specimens were immunostained to determine expression of Erk1/2, Bcl2 and TrkC. As seen in cylindroma, ACC specimens expressed TrkC, p-Erk1/2 and Bcl2 in their neoplastic myoepithelial layer (Figure 10). The patterns of expression of Erk and TrkC in ACC tumors varied amongst cells of the basal layer, while Bcl-2 was expressed more uniformly; however, expression of these genes overlapped at least partially. Expression of these genes was not detected in MEC in agreement with lack of TrkC expression in this cancer. Interestingly, normal salivary gland tissue displayed TrkC and Bcl2-positive signals in myoepithelial cells (data not shown). This finding, however, was not entirely unexpected given the important roles that myoepithelial cells have in ACC development.[249]
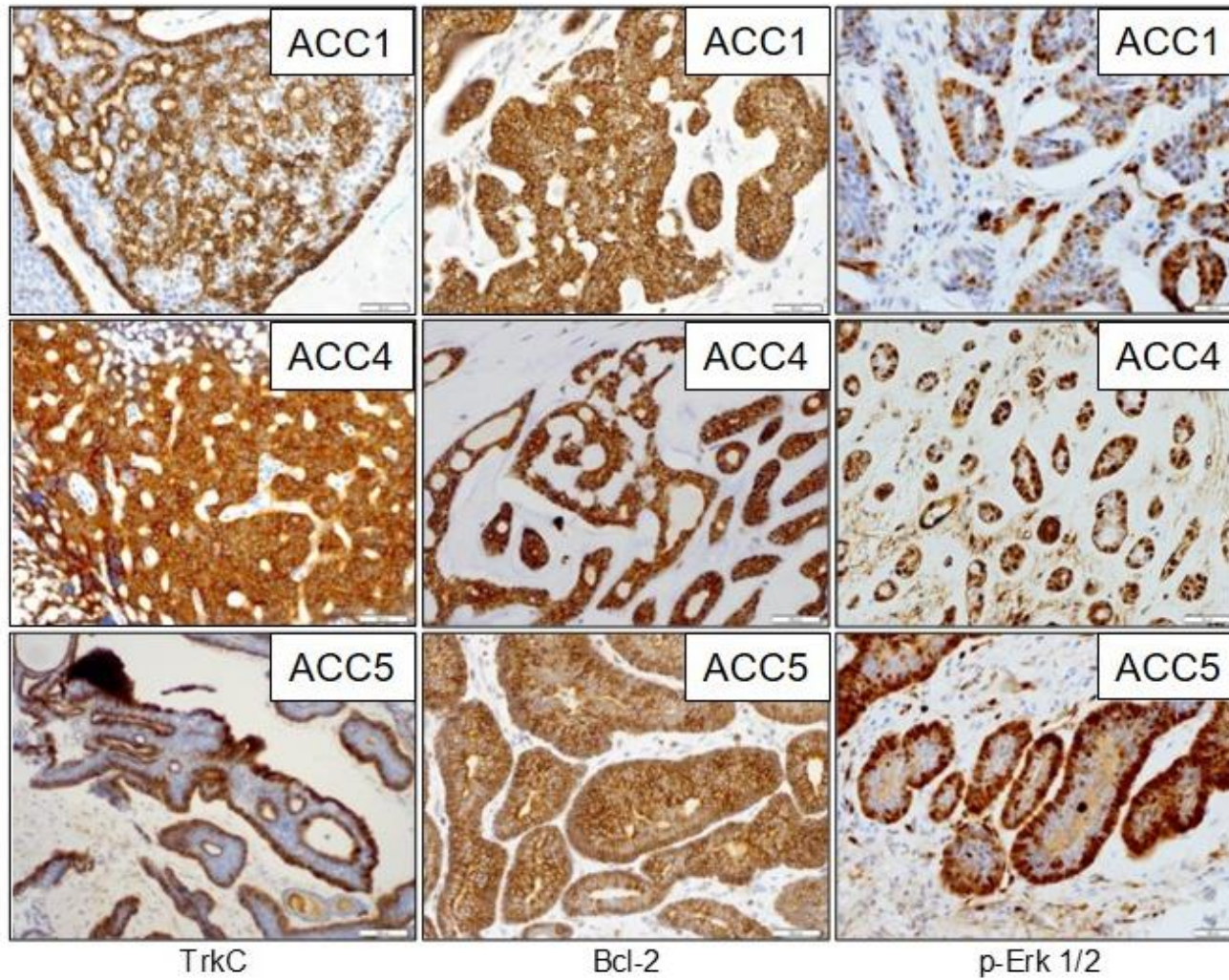
Figure 10: Expression of TrkC, Bcl2, and phospho-Erk in ACC.
High levels of TrkC. Bcl2 and Erk1/2 activation in ACC.

II.1.4.9 Inhibition of TrkC signaling suppresses ACC tumor growth in vivo

The effect of TrkC inhibition on ACC xenograft growth was determined using two xenograft models of human ACC (1) ACCX6, a xenograft created from a metastatic tumor, and (2) ACCX9, a xenograft from a primary tumor.[228] AZD7451 was administered orally at 50 mg/kg once daily. In ACCX6, AZD7451 demonstrated a marked anti-tumor effect as a single agent (Figure 11A, tumor growth inhibition (TGI) = 64%, $p < 0.05$). However, ACCX9 did not see a significant effect at the 35-day experimental course (Figure 11B, TGI = 43%, $p > 0.05$). Nevertheless, analysis of BrdU-incorporation performed on tumors generated from ACCX9 showed a robust reduction in the proliferation index (Figure 11C, PI = % BrdU-positive cells) from 17% to 5% ($p < 10^{-5}$), suggesting that AZD7451 inhibited cellular proliferation in ACCX9. This observed difference between anti-tumor effects of AZD7451 in ACCX6 and ACCX9 could, in part, be due to ACCX9 displaying a sluggish growth profile from days 1-21, thus leaving insufficient time in this experiment for drug effects to become significant. The observed changes in tumor growth are not likely due to toxicity, as AZD7451 administration was not accompanied by weight loss of the animals. These preclinical data support further investigation of single agent Trk kinase inhibitors as a potential therapeutic option in ACC.
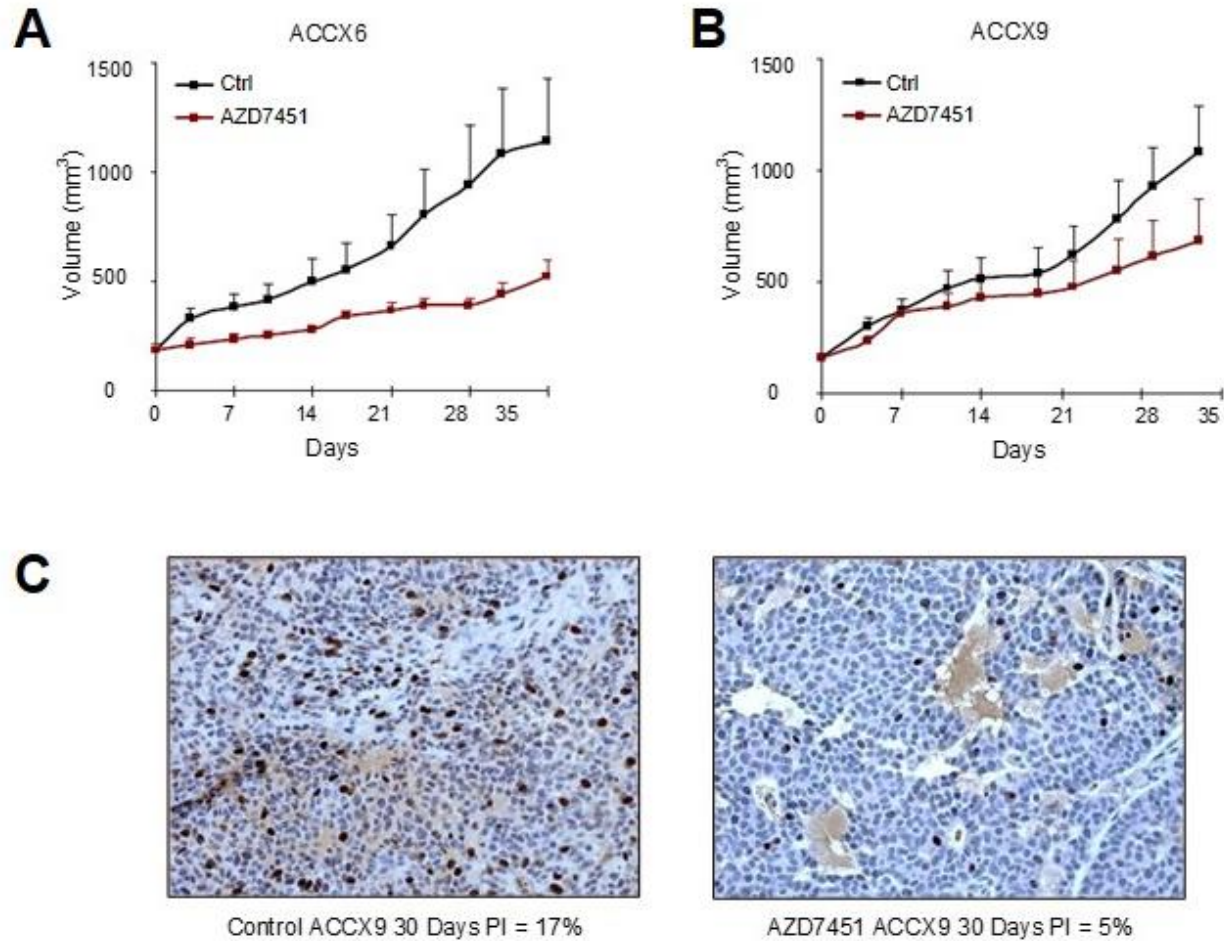
Figure 11: Assessment of AZD7451 efficacy *in vivo*.
(A-B) ACC tumor growth in the ACCX models with or without Trk inhibitor. From day 3 for ACCX6 p<0.05. (C) Reduction in the proliferative index (PI) in the ACCX9 model as assessed by BrdU staining.

II.1.5 Discussion

In this study, a novel ACC marker, receptor tyrosine kinase TrkC/NTRK3 was characterized as a potential therapeutic target. Unusually high TrkC expression levels were found in ACC (up to ~100-fold as compared with the normal salivary tissue), raising an important question if this RTK may serve as a driver of ACC. Lack of activating mutations suggested that malignant ACC cells may benefit from increased activity of wild-type TrkC signaling, possibly achieved either via spontaneous TrkC dimerization and activation due to overexpression[250] or through interaction with its only known ligand NT-3. Given that other neurotrophin receptors did not correlate with TrkC expression, TrkC signaling alone was explored for its ability to stimulate pro-invasive signaling and cell behavior. TrkC-expressing U2OS cells in response to NT-3 stimulated: (1) TrkC tyrosine phosphorylation, (2) Ras, Erk1/2, Akt, and VEGFR1 activation, (3) cell motility and invasion, (4) chemotaxis toward NT-3, (5) adherence-independent growth, and (6) outgrowth of F-actin-rich processes typical for migrating cells (Figures 7-9). Without NT-3, TrkC remained unphosphorylated, did not stimulate pro-tumorigenic qualities, and had anti-tumorigenic signaling effects including increased phosphorylation of p53, in agreement with the dependence receptor paradigm.[251] In agreement with previous studies, NT-3 was the only neurotrophin capable of activating TrkC, as NGF, BDNF and NT-4 failed to induce TrkC phosphorylation (data not shown). These observations suggest that highly overexpressed TrkC is oncogenic when NT-3 is available, stimulating further research into the autocrine TrkC/NT-3 signaling that was discovered in ACC.

Without NT-3, TrkC induces apoptosis and thus may suppress tumor progression.[214] These studies revealed a potential novel molecular mechanism of this NT-3-independent mode of TrkC function. In the absence of ligand, TrkC stimulated TUBB2 expression (Figure 9) while inhibiting Akt and Erk1/2 activation (Figure 7A&B). TrkC in the absence of ligand stimulated the expression of total and serine-phosphorylated p53, and this effect was alleviated by NT-3. Interestingly, immunoprecipitation suggested that TrkC is in a complex with p53 (Figure 7C). Of note, TrkA, which is also considered a dependence receptor with a pro-apoptotic function similar to TrkC in the absence of ligand,[211, 251] binds p53, which mediates TrkA activities.[252-255] Tumor-suppressive TrkC activities suggest that association of TrkC overexpression with good prognosis observed in medulloblastoma[4] may be explained by lack of NT-3 stimulation. Indeed, autocrine TrkC/NT-3 loops detected recently in neuroblastomas were linked with poor prognosis.[215]

Up-regulation of Bcl2 along with TrkC in ACC, which is also observed in CYLD-deficient cylindromas that overexpress TrkC,[248] is an intriguing discovery, given the important role that Bcl2 has in cell survival and, particularly, the resistance to radio- and chemotherapies.[256] High Bcl2 levels detected in ACC tumors (Figure 10) are consistent with results reported on four ACC specimens[257] and may contribute to the relative resistance of this cancer to cytotoxic therapies.

Expression of TrkC in normal myoepithelial cells of the salivary gland provides an interesting insight into the potential cell origin of ACC. ACC is thought to arise from the intercalated salivary duct where myoepithelial cells are abundant,[258, 259] and is histologically and clinically distinct from MEC, whose origin is linked with the excretory

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duct.[259, 260] Accordingly, expression array data reliably discriminated between these two cancers and identified TrkC as one of the most distinguishing markers of ACC. As myoepithelial cells have important roles in normal salivary gland functions and the pathogenesis of adenoid cystic carcinoma,[261, 262] characterization of TrkC as a previously unknown marker of these cells sheds more light onto their function, origin, and role in ACC.

Overall, these findings reveal a previously unknown autocrine NT-3/TrkC signaling pathway in ACC that can be targeted with small-molecule inhibitors. A novel pan-Trk inhibitor AZD7451 was characterized in this study as a potent TrkC inhibitor, which blocks its tyrosine phosphorylation in a nanomolar range and suppresses TrkC/NT-3-mediated cell migration, motility, invasion, and growth in soft agar (Figure 8). It is reasonable, therefore, to suggest that this drug may be used to block ACC neuroinvasion and growth, and pre-clinical assessment of its activity on murine xenografts that models invasive ACC supports its tumor-suppressive properties (ACCX6, Figure 11). In conclusion, distorted neurologic signaling detected in ACC advances understanding of its molecular etiology, suggests similarity of its molecular pathology with neural crest cancers, and stimulates development of multi-drug treatment modalities that target TrkC/NT-3, Ras, Erk 1/2, Akt and Bcl2.

II.2 Diagnostic SOX10 gene signatures in salivary adenoid cystic and breast basal-like carcinomas

Adapted from manuscript published in British Journal of Cancer.[263]

II.2.1 Abstract

Salivary adenoid cystic carcinoma (ACC) is an insidious slow-growing cancer with the propensity to recur and metastasize to distant sites. Basal-like breast carcinoma (BBC) is a molecular subtype that constitutes 15–20% of breast cancers, shares histological similarities and basal cell markers with ACC, lacks expression of ER (estrogen receptor), PR (progesterone receptor), and HER2 (human epidermal growth factor receptor 2), and, similar to ACC, metastasizes predominantly to the lung and brain. Both cancers lack targeted therapies and have incomplete understanding of their molecular drivers contributing to the absence of targetable defects. Gene expression profiling, immunohistochemical staining, western blot, RT-PCR, and *in silico* analysis of cancer expression data sets were used to identify novel markers and potential therapeutic targets for ACC and BBC. For detection and comparison of gene signatures, co-expression analysis was performed using a recently developed web-based multi-experiment matrix tool for visualization and rank aggregation. Characteristic and overlapping *SOX10* gene signatures that contained a large set of novel potential molecular markers were identified in ACC and BBC. SOX10 was validated as a sensitive diagnostic marker for both cancers, and its expression was linked to normal and malignant myoepithelial/basal cells. In ACC, BBC, and melanoma (MEL), SOX10 expression strongly co-segregated with the expression of ROPN1B, GPM6B, COL9A3, and MIA. In ACC and breast cancers, SOX10 expression negatively correlated with FOXA1, a cell identity marker and major regulator of the luminal breast

subtype. Diagnostic significance of conserved elements of the SOX10 signature (MIA, TRIM2, ROPN1, and ROPN1B) was confirmed in BBC cell lines. SOX10 expression in ACC and BBC appears to be part of a highly coordinated transcriptional program characteristic of cancers with basal/myoepithelial features. Comparison between ACC/BBC and other cancers, such as neuroblastoma and melanoma (MEL), reveals potential molecular markers specific for these cancers that are likely linked to their cell identity. SOX10 as a novel diagnostic marker for ACC and BBC provides important molecular insight into their molecular etiology and cell origin. Given that SOX10 was recently described as a principal driver of MEL, identification of conserved elements of the SOX10 signatures may help in better understanding of SOX10-related signaling and development of novel diagnostic and therapeutic tools.

II.2.2 Introduction

Adenoid cystic carcinoma (ACC) of the salivary gland, the second most frequent salivary cancer, is notorious for neural or perineural invasion and late recurrence.[264] When compared with salivary mucoepidermoid and head and neck squamous cell carcinomas, ACC overexpressed a large cluster of neuronal genes grouped around TrkC/NTRK3, a tyrosine kinase neurotrophic receptor associated with neurogenesis and cancer.[200] This observation suggested, for the first time, that ACC aberrantly expresses genes involved in neural stem cell differentiation. ACC was also found to express neurotrophin-3 (NT-3/NTF3), the TrkC ligand, suggesting that activation of TrkC through an autocrine signaling loop may contribute to tumor growth and dissemination.

Ectopic expression of TrkC revealed that NT-3/TrkC signaling activates Ras, Akt, and Erk1/2 and promotes tumorigenic and aggressive behaviors, including increased

motility, chemotaxis, invasion, and growth in soft agar.[200] To further characterize the activation of the gene expression program identified in ACC, focus was given to SOX10. SOX10 was of particular interest because of its roles as a marker of neural crest stem cells (NCSCs) and in the maintenance and migration of NCSCs.[265-267] Remarkably, TrkC and SOX10 may be functionally linked, as inactivating mutations in NTRK3, NTF3, and SOX10 were identified as independent drivers of Hirschsprung disease,[212, 213, 268, 269] a genetic condition linked to the inability of NCSCs to migrate, differentiate, and develop into the enteric nervous system.[270]

In addition to its role in neural crest development, SOX10 has also been identified as a driver of melanoma (MEL) progression, a cancer that develops from melanocytes that are neural crest derivatives.[182] In this study, overexpression of SOX10 in ACC was identified and SOX10 was established as a sensitive ACC marker. Using available public data sets, a SOX10 gene signature was identified in basal-like breast carcinoma (BBC) and compared to the SOX10-centric signature in ACC. BBC is perhaps the least understood breast cancer subtype that largely overlaps with triple-negative breast cancers (TNBCs), lacks obvious molecular markers, and has no effective targeted therapeutic approach.[271, 272] Together, these data suggest that a large portion of ACC and BBC may share neurologic signaling pathways associated with SOX10, which is also activated in MEL, and that these molecular similarities are of potential therapeutic importance.

II.2.3 Materials and methods

II.2.3.1 Head and neck cancer specimens. Original expression array data were obtained on clinical specimens from 25 patients treated at Vanderbilt Ingram University

Medical Center: ACC (*n*=7), mucoepidermoid carcinoma (MEC, *n*=6), adenocarcinoma (*n*=2), and head and neck squamous carcinoma (*n*=10) (for clinical details, see (Ivanov *et al,* 2012)). The validation set of ACC specimens (*n*=13) was obtained from the Salivary Gland Tumor Biorepository (MD Anderson Cancer Center, Houston, TX, USA).

II.2.3.2 Cell lines. A375, HCC38, HCC1569, MCF7, and T47D were obtained directly from ATCC (Manassas, VA, USA). MX-1 cells were purchased from the NCI tumor repository (Frederick, MD, USA).

II.2.3.3 Expression array analyses. Collection and processing of expression array data has been described previously.[200] Analysis of data sets from public domains available from the ArrayExpress Archive (http://www.ebi.ac.uk/arrayexpress/) was performed using MEM (http://biit.cs.ut.ee/mem/index.cgi).

II.2.3.4 Western blot analysis and antibodies. Anti-human SOX10 antibodies (NBP1-68983; Novus Biologicals, Littleton, CO, USA) and cell lysates produced from snap-frozen VUMC and UVA specimens were used, as well as 13 additional specimens from MD Anderson Cancer Center specimens as described.[200]

II.2.3.5 Immunohistochemical studies. The salivary cancer tissue microarray (TMA, 45 cores, 1mm diameter, 14 cases in triplicates) was assembled in the laboratory of Dr. Yarbrough. Additional salivary cancer specimens (myoepithelial carcinoma, epimyoepithelial carcinoma, and basal cell adenoma) were obtained from the Department of Pathology, Yale School of Medicine. The breast cancer TMA that

included triple-negative cases (YTMA-49-10, 0.6 mm core, *n*>300) was produced by the

Yale Department of Pathology. Mouse embryo slides (stage E15) were obtained from

Zyagen (San Diego, CA, USA). Staining with Sox10 antibodies (goat polyclonal, N-20;

Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed as described.[273]

II.2.4 Results

II.2.4.1 SOX10 is a novel and sensitive biological marker for ACC and other

salivary cancers that originate from the acinar and intercalated duct regions

Analyses of expression array data of grossly dissected ACC, MEC, and HNSCC

tumors, revealed that SOX10 was specifically expressed in 17 of the 18 ACCs

(including 11 ACC xenografts). When compared to normal salivary gland tissue,

SOX10 expression in primary ACC specimens was markedly increased (Figure 12A, ~5-

fold, ACC1; ~25-fold, ACC3). Xenografted ACC tumors expressed a higher median

level of SOX10 and a maximum of ~46-fold increase of SOX10 expression compared to

normal salivary tissue (Figure 12A). This high level of SOX10 expression was

confirmed in a subset of ACC specimens at the protein level by immunoblotting (Figure

12B). SOX10 expression was determined in an independent collection of 13 clinical

ACC specimens (gift of Adel El-Naggar, MD, Anderson Cancer Center) wherein SOX10

was detected in all but one ACC, but was below the limit of detection in normal salivary

specimens (Figure 12C). IHC performed on tumor tissues revealed SOX10 staining in

the majority of ACC tumor cells (Figure 12D). Of note, specimens that demonstrated

the lowest level of SOX10 expression also contained the lowest percentage of tumor

cells in the specimen. By IHC, SOX10 demonstrated intense nuclear staining as well as

detectable cytoplasmic staining in ACC. Conversely, only one MEC contained

72

detectable SOX10 expression (MEC1, Figure 12C), but the staining pattern for this tumor was quite different than seen in the ACC specimens, with only moderate nuclear/cytoplasmic staining (Figure 12E&F). In addition to SOX10 expression in MEC1, this specimen was characterized as an outlier from other MECs in previous expression array analyses, and re-evaluation of the pathology (Dr. Manju Prasad, Yale University) for MEC1 classified this specimen as a carcinoma not otherwise specified (NOS) rather than a MEC.

As SOX10 is expressed in other tumors, such as melanoma, the potential value of SOX10 as a diagnostic marker beyond ACC was assessed. Two cases of myoepithelial carcinoma, three cases of epithelial-myoepithelial carcinoma, and one basal cell adenoma were immunostained for SOX10. In all cases, SOX10 staining was observed in >80% of cancer cells. This staining was limited to the myoepithelial cells as confirmed in epithelial-myoepithelial carcinomas by expression of p63, calponin, and CK7 (data not shown).
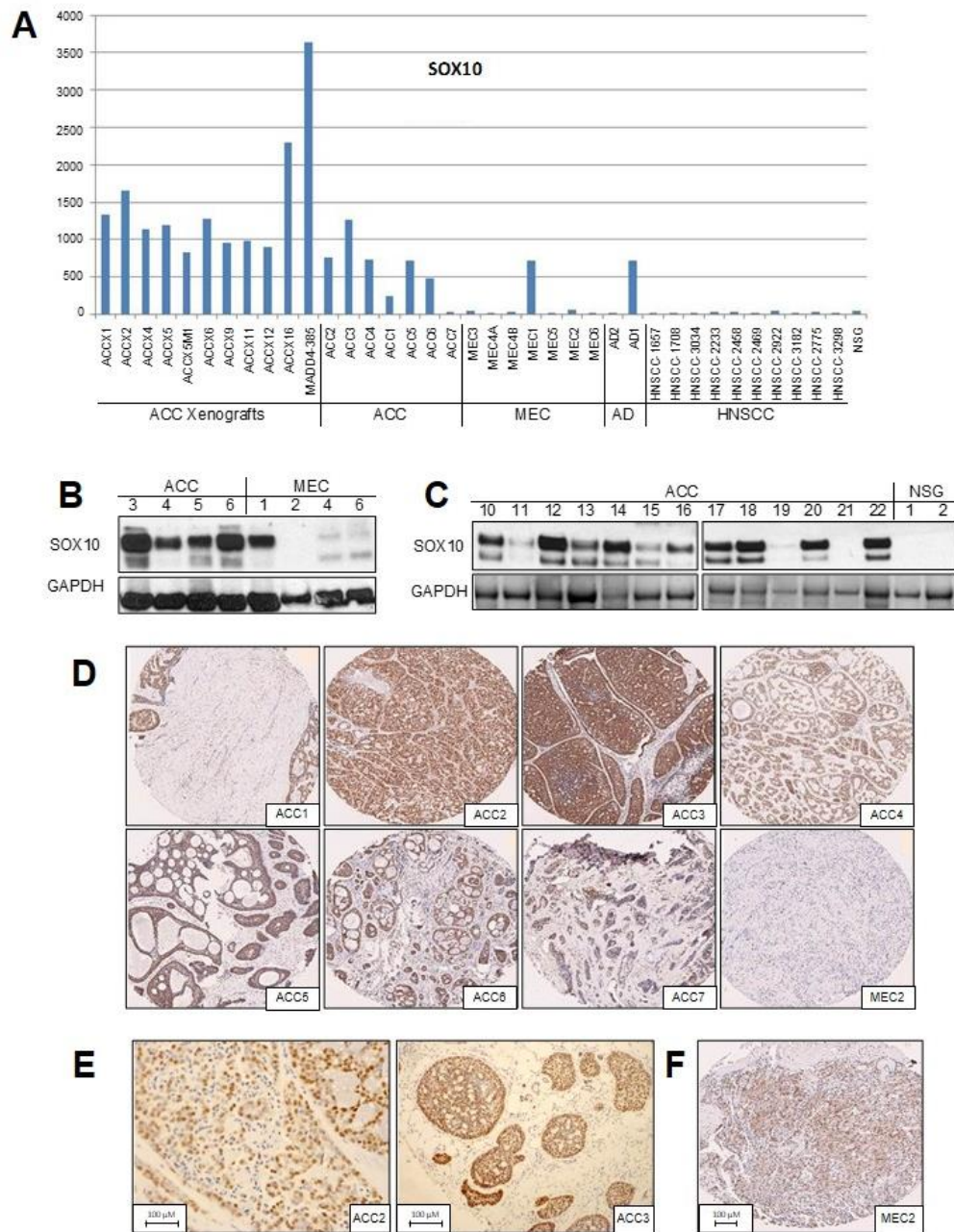
Figure 12: **SOX10 expression in ACC.**
(A) Expression array data show high SOX10 levels in most primary and xenografted ACC specimens compared to MEC, AD, HNSCC, and NSG tissues. (B) Validation of expression array data by western blot. (C) Expression of the SOX10 protein in 13 additional primary ACC specimens. (D) Immunohistochemical localization of SOX10 expression in ACC1-7. MEC2 stains negatively for SOX10. (E) Nuclear expression of SOX10 in ACC cells. (F) Nuclear-cytoplasmic SOX10 expression detected in one out of six MEC specimens studied.

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II.2.4.2 SOX10 is expressed in embryonic and differentiated salivary tissues

Outside of cancer, SOX10 is recognized as a marker and principal regulator of neural crest stem cells (NCSC).[273-275] To determine if SOX10 is expressed in developing and mature salivary gland tissue, SOX10 immunostaining of mouse E15 embryonic tissue and human adult salivary tissue was performed. In mouse embryos, SOX10 was expressed in presumptive acinar cells, but not the ductal epithelium, suggesting that SOX10 may be involved in the development of differentiation of acinar structures (Figure 13A). In line with this observation, SOX10 was observed staining the nuclei of acinar and myoepithelial cells of the human adult salivary tissue (Figure 13B). As expected, Sox10 antibodies similarly stained the nuclei of melanocytes of normal skin and cutaneous melanoma (Figure 13C). Together, these data indicate that SOX10 has a role in the development and maintenance of salivary tissues, particularly those derived from neural crest.
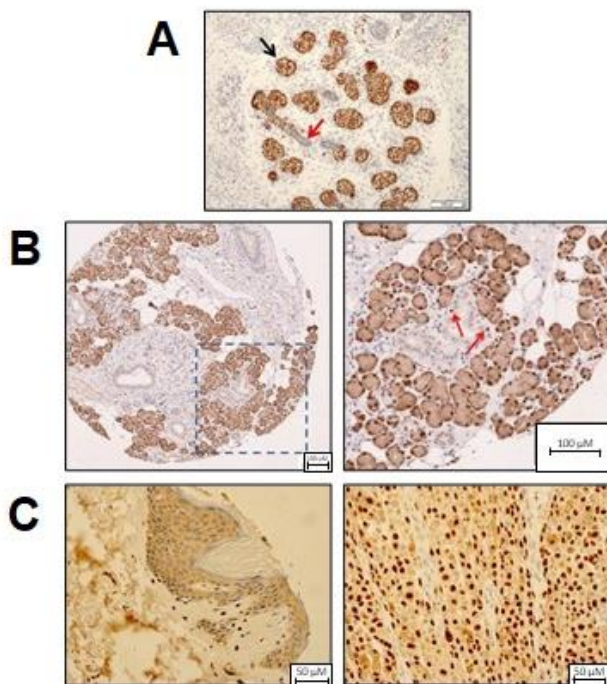
Figure 13: Immunohistochemical analysis of SOX10 expression.
SOX10 Expression in (A) mouse embryonic and (B) human adult salivary glands. The red arrow points to the developing duct, whereas the black arrow shows the acinus. (C) Validation of SOX10 on sections of normal skin (left) and cutaneous MEL (right).

II.2.4.3 SOX10 expression in basal-type breast carcinoma

To better understand the significance of SOX10 expression in human cancer, its

expression was examined in 1764 publicly available cancer datasets using the

Affymetrix U133 Plus 2.0 platform (http:// biit.cs.ut.ee/mem/). This database utilizes a

novel, noise-resistant rank aggregation and visualization algorithm developed by Adler

et al.[276] and Kolde et al.[277] allowing for simultaneous comparison of gene expression

across massive data sets to identify genes co-expressed with SOX10 (Figure 14A).

Notably, breast cancer (22 studies, $10^{-80} < p < 10^{-30}$), melanoma, neuroblastoma, and

glioma, demonstrate strong patterns of SOX10 co-expressed genes, but SOX10 and the

associated gene signature were not seen in other cancers. Delving into studies in

which breast cancers were identified by molecular subtype, SOX10 expression was

restricted to the basal-like subtype (Figure 14B). Analysis of SOX10 expression in the

basal subtype from two studies (E-GEOD-21653 and E-GEOD- 20711, n = 163)

demonstrated that SOX10 was expressed in 72% of basal-like specimens (Figure 14B

and data not shown). When compared with luminal subtypes, the basal subtype

expressed at least 16-fold higher levels of SOX10 (Figure 14C, $p < 10^{-10}$). The results

of expression array analyses were confirmed by immunostaining of a TMA containing

normal and malignant breast tissue (Figure 14D). As seen in salivary tissue, normal

breast tissue showed SOX10 immunostaining in the nuclei of basal/myoepithelial cells,

although some luminal breast cells showed low expression of SOX10. In the triple

negative breast cancer (TNBC) specimens, which largely overlap with BBC, nuclear

SOX10 expression was seen in >60% of malignant cells. Taken together, these data

suggest that SOX10 is expressed in normal breast tissue primarily in the

basal/myoepithelial cells as well as in basal like breast cancers.
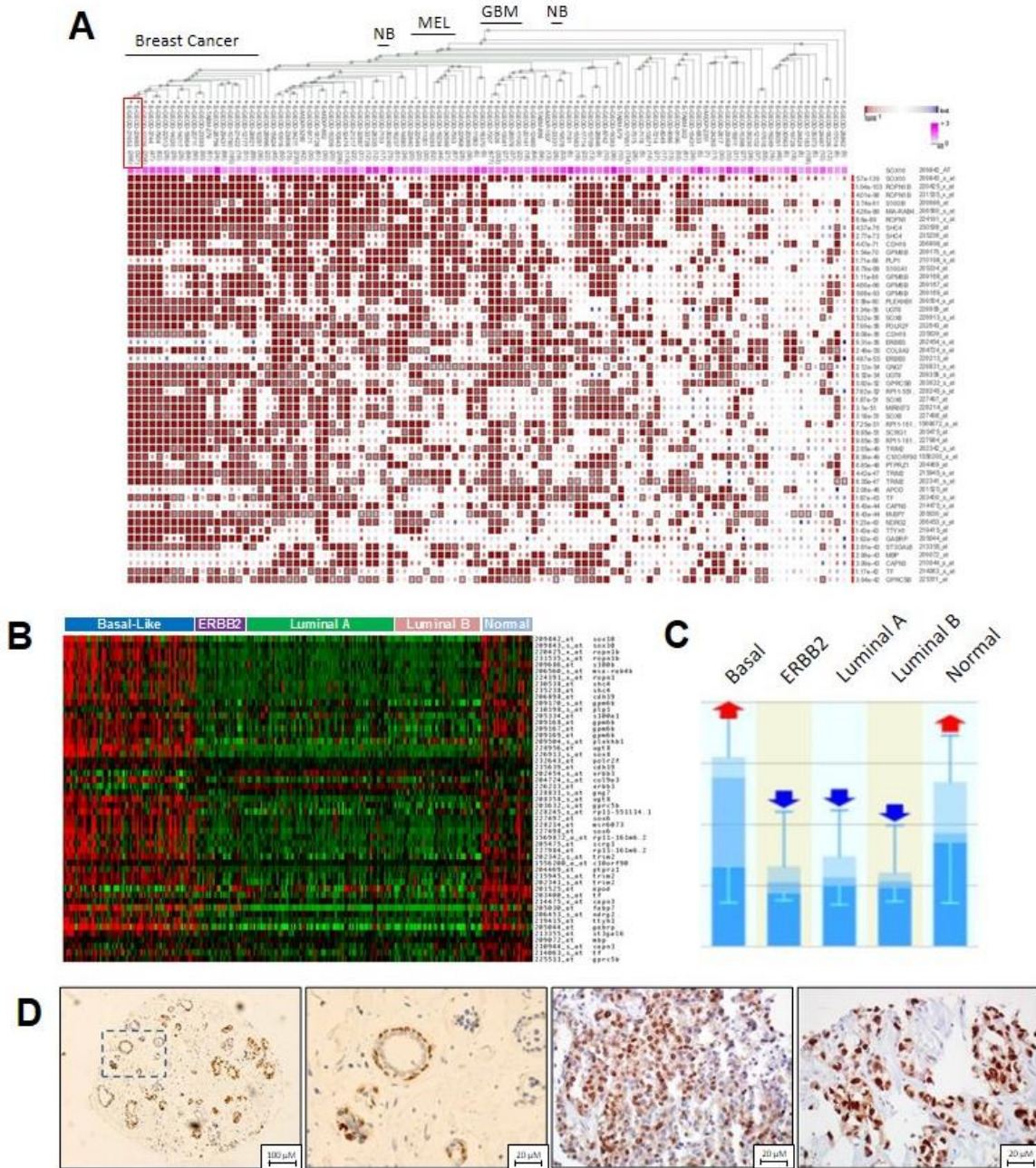
Figure 14: Characterization of SOX10 signature in BBC.
(A) Rank aggregation analysis identifies genes whose activity co-segregates with that of SOX10 in basal-like breast cancer (BBC), melanoma (MEL), and neuroblastoma (NB). Two breast cancer studies that stratify specimens by molecular subtypes are marked in a red frame. (B) SOX10 overexpression in BBC. (C) The heat map for the E-GEOD-21653 study shows SOX10 signature expression in a great majority of basal-like specimens but not in other breast cancer subtypes. (D) Validation of SOX10 expression in normal (Left and left-center) and malignant (YTMA-49-10 TNBC cases 1840 and 1843; right-center and right, respectively) breast tissues.

II.2.4.4 Genes commonly co-expressed with SOX10 in ACC, BBC, and MEL

In order to identify genes co-expressed with SOX10 across multiple tumor types, comparative analysis of the SOX10 gene signatures of ACC, BBC, and melanoma was performed. For each cancer, the 160 genes most strongly co-segregating with SOX10 were selected (Figure 15). Comparing these lists revealed substantial overlap with ACC and BBC having 24 common genes (15%), BBC and melanoma had 17 common genes (~11%), and ACC and melanoma had six common genes (~4%). The lower overlap between SOX10 gene signatures in ACC and melanoma may, in part, be due to SOX10 in melanoma functioning primarily through MITF, which is not associated with ACC. Some of the genes co-expressed with SOX10 are markers of poor prognosis in melanoma (MIA,[278] S100A1,[279, 280] S100B,[278, 280] and SHC4/RaLP[281]), BBC (FABP7,[282] FZD7,[283] and MFGE8[284]) and ACC (FABP7)[114] suggesting that SOX10 may be driving gene expression that correlates with aggressive or resistant phenotypes; however, the mechanistic and clinical significance of many of the signature genes remains to be explored including ROPN1B, GPM6B, and COL9A3, which co-segregated with SOX10 in all three tumor types.

ACC/BBC
ART3
CHRM3
CHST3
CYP39A1
EN1
FABP7
FZD7
ITGA9
LGR6
MFGE8
NDRG2
NFIX
NRTN
OSR1
PDE9A
PLEKHB1
SOX8
TTYH1
ZNF462

BBC/MEL
C100RF90
CDH19
GPRC5B
PLP1
ROPN1
RP11-551L14.1
S100A1
S100B
SHC4
SOX6
ST3GAL6
TRIM2

ACC

BBC

19

SOX10
ROPN1
ROPN1B
COL9A3
MIA

MYEF2

12

Melanoma
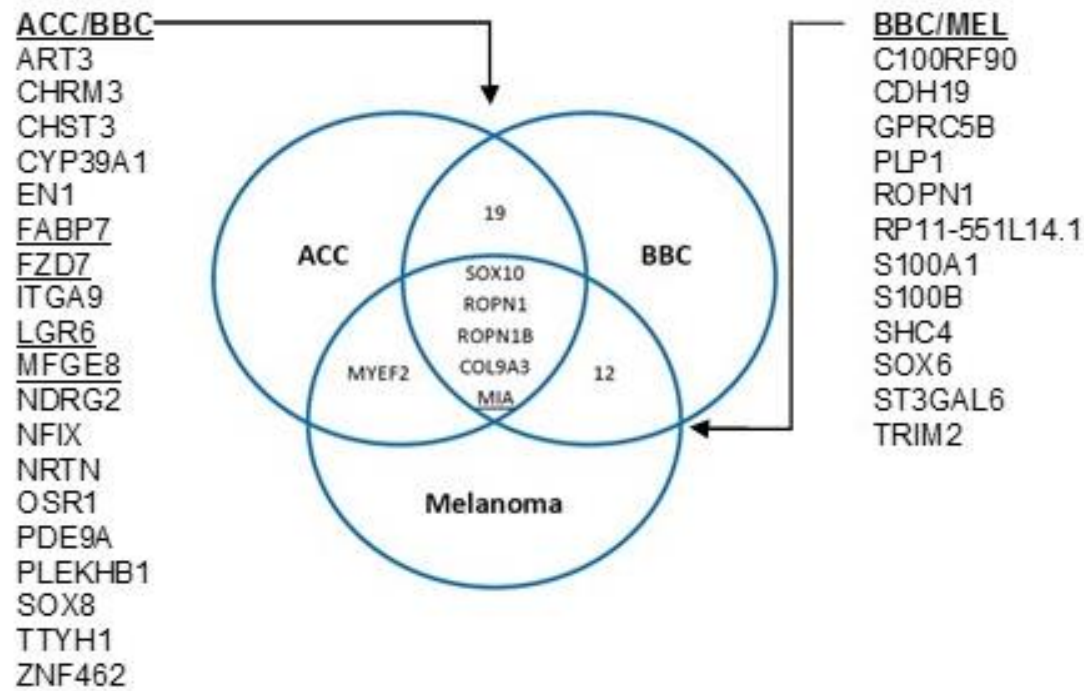
Figure 15: Common elements of SOX10 signatures in ACC, BBC, and MEL.
ROPN1B, COL9A3, GPM6B, and MIA are strongly co-expressed with SOX10 in all three cancers. This and other overlaps contain prospective clinical targets, several of which have been identified as clinically significant (underlined).

II.2.4.5 SOX10 signature is recapitulated in BBC cell lines

To validate *in silico* findings, expression of SOX10 signature elements was examined in A375 melanoma, luminal breast, (MCF, T47D) and basal-like (HCC38, HCC1569, and MX-1) breast cancer cell lines. Melanoma and BBC cells expressed SOX10 and several of its previously identified co-expression partners (MIA, TRIM2, ROPN1, and ROPN1B), whereas estrogen receptor (ESR1)-positive luminal MCF7 and T47D cell lines expressed only limited amounts of TRIM2 and ROPN1B with little or no detectable expression of SOX10 and MIA (Figure 16).
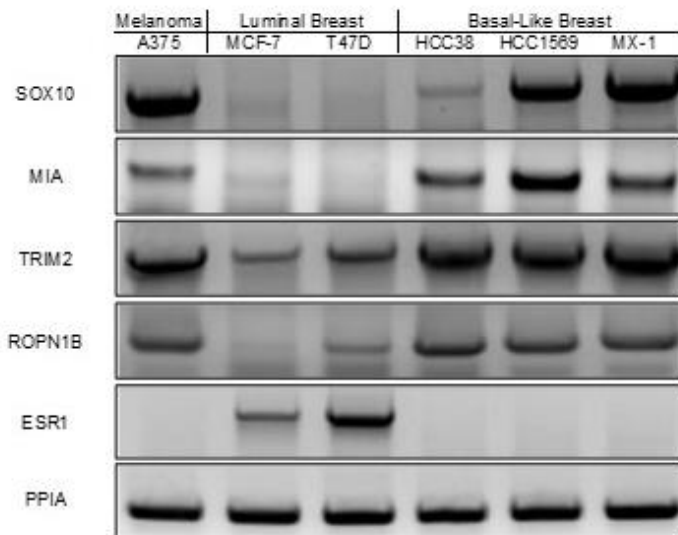
Figure 16: Expression of SOX10 signature components in MEL and BBC cell lines.
End-point RT-PCR shows that MEL and BBC cell lines recapitulate the expression of SOX10 and elements of its signature, whereas ESR1-positive luminal breast cancer cell lines are negative for these genes.

II.2.4.6 Genes whose expression negatively correlates with SOX10 expression in breast and salivary cancers

To further explore SOX10 in breast cancer, correlation analysis was performed on the TCGA Invasive Breast Carcinoma dataset (Agilent mRNA expression microarrays, n = 547).[285] This analyses identified FOXA1, ESR1, GATA3, XBP1, and CA12 as top-rank genes whose expression negatively correlated with SOX10 (data not

shown). Expression of FOXA1 had the strongest negative correlation with SOX10, which was confirmed through analysis of E-GEOD-21653 BBC data set (Figure 17A), as well as analyses of the ACC expression array[200] data set (Figure 17B). The opposing expression of FOXA1 and SOX10 was consistent with reports that FOXA1 supports luminal breast cancer morphology[286] and suppresses the basal-like phenotype.[287] Additionally, FOXA1 cooperates with ESR1 as a pioneer factor that maintains luminal identity in breast cancer and is required for ~50% of ESR1's activity.[288] Pioneer factors are chromatin remodelers with the capacity to modulate cellular identity by defining the genomic regions accessible for other transcription factors.[289] In addition to FOXA1, three other genes identified as negatively correlating with SOX10: GATA3, XBP1, and CA12 are each linked to the ESR1 and FOXA1 activities.[286, 290-292] Taken together, these data indicate that expression of SOX10 and FOXA1 are largely mutually exclusive in breast and salivary cancers and that their expression is linked with distinct molecular subtypes.
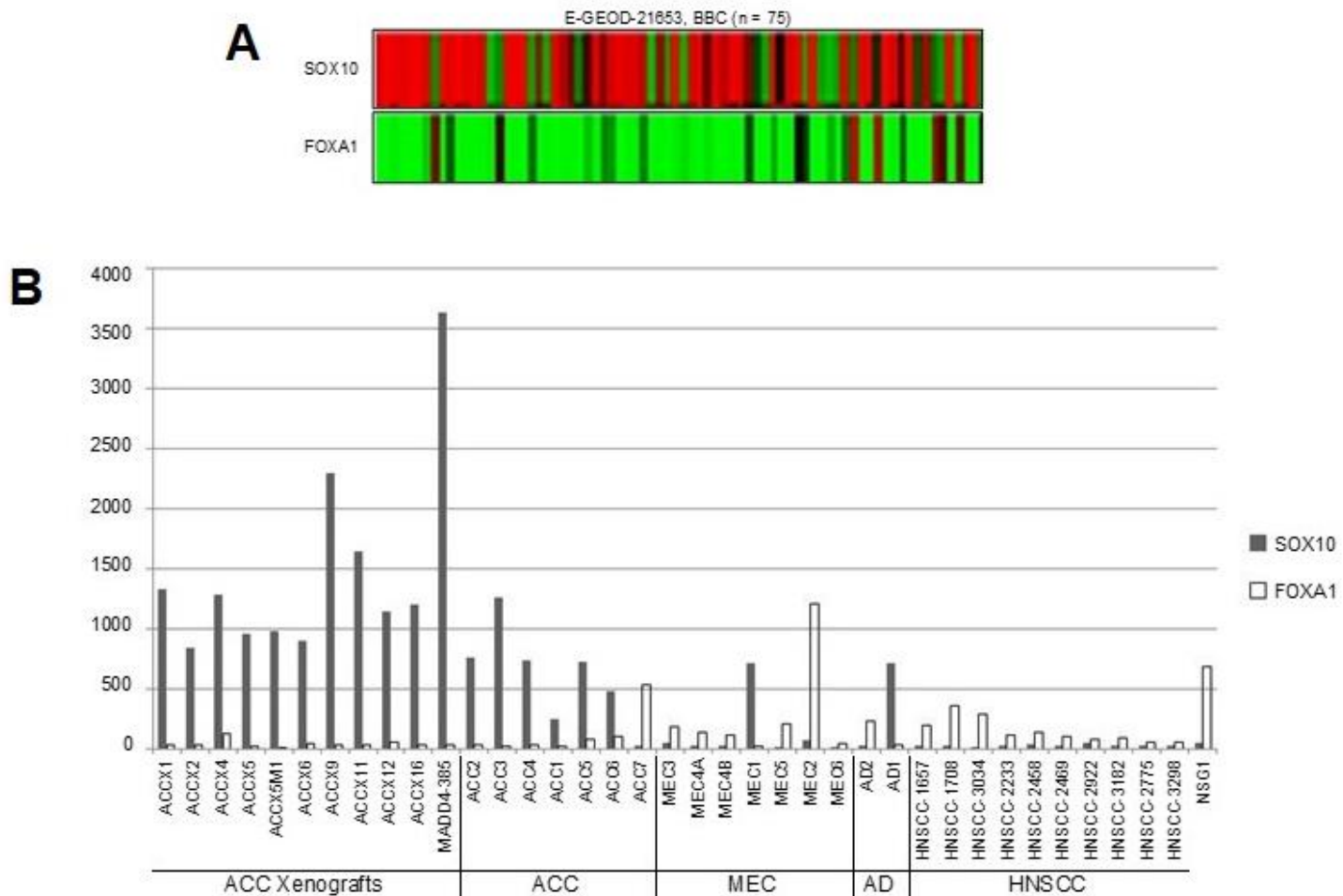
Figure 17: Mutually exclusive expression of SOX10 and FOXA1 in breast and salivary cancers.
(A) Heat map for the E-GEOD-21653 study and (B) expression array data on head and neck cancers show inverse SOX10 and FOXA1 expression in ACC and breast cancer, respectively.

II.2.5 Discussion

The transcriptional factor SOX10 appears to support stem-like properties in normal tissues and cancer cells. In normal tissue, SOX10 maintains stem cells in their undifferentiated state by preventing differentiation,[237, 293, 294] whereas in MEL it serves as a marker of the stem-like CD271-positive cells.[180] In ACC, as was previously demonstrated,[200] SOX10 expression correlates with the neural stem markers TrkC, MAP2, SALL2, and SLITRK6. In this study, SOX10 was established as a novel and sensitive ACC marker, which is expressed normally during salivary gland differentiation and is markedly up-regulated in a great majority of ACC cells. This is similar to the trend seen in differentiating melanocytes and melanoma suggesting that SOX10 may function similarly in development of these diseases. These studies also characterize SOX10 as a marker of BBC, a molecular subtype of breast cancer that lacks expression of estrogen, progesterone, and HER2 receptors (human epidermal growth factor receptor 2).[295] As with ACC, BBC express basal cytokeratins[296] and other genes linked to myoepithelial cells.[297] The diagnostic value of SOX10 in BBC was confirmed by others.[298] Unlike TrkC, which is highly specific for the myoepithelial cells/cancers of salivary gland and myoepithelial cells of breast tissue, SOX10 expression in salivary tissue was not restricted to the myoepithelial cells and tumors that show myoepithelial differentiation, but was also expressed in acinar cells, acinic tumors, and, occasionally, in the basal cells of the intercalated duct (data not shown). Thus, SOX10 has a broader specificity than TrkC and may be helpful for the diagnosis of salivary cancers that originate from the acinar and intercalated duct areas of the salivary gland.

Characterization of SOX10 as a marker of both ACC and BBC supported the hypothesis that cancer cells hijack the inherent plasticity of normal stem cells.[299] These studies may stimulate examination of the therapeutic and biological importance of SOX10 expression. These novel potential markers and targets within SOX10 co-expressed genes, once validated, may significantly increase the accuracy of FNA diagnosis in ACC and BBC. Importantly, some SOX10 co-expressed genes have been validated as diagnostic and prognostic markers in cancer.

Although SOX10 activity in MEL is essential for cell survival and growth,[182] targeting of transcription factors is challenging. As SOX10 expression in each of three cancers appeared to be part of a highly coordinated expression profile containing hundreds of genes, a better understanding of molecular mechanisms, signaling pathways, and critical drivers that orchestrate such expression may provide a more efficient and broader means for tumor suppression. As was shown, the overlaps between SOX10 gene signatures can identify common elements of the SOX10 network. Remarkably, two out of four genes that were consistently co-expressed with SOX10, GPM6B, and COL9A3 (Figure 15) have been previously reported to bind EGFR,[300] a commonly recognized BBC marker and regulator.[301] Thus, it would be interesting to explore the possible involvement of this receptor in SOX10 signaling. Two of the closest SOX10 co-expression partners are ROPN1B and MIA. Although little is known about ROPN1B, its function is most likely mediated through its R2D2 motif, which is implicated in cAMP-dependent PKA signaling,[302] and PKA activity is implicated in melanocyte proliferation.[303] It is essential to investigate the ROPN1B role in ACC and BBC. Unlike ROPN1, the melanoma inhibitory activity protein (MIA) is a well-

established diagnostic and prognostic serum marker and therapeutic target in melanoma;[304-307] however, its link with SOX10 has not been previously established. Studies on serum derived from ACC and BBC patients are warranted in order to assess the clinical value of MIA in these cancers.

Overall, these findings highlight previously unrecognized transcriptional networks and signaling pathways related to SOX10 expression in various cancers. This work may stimulate further studies to identify common and cancer type-specific biomarkers and prospective therapeutic targets whose expression strongly co-segregated with SOX10.

II.3 Co-activation of NOTCH1 and SOX10 in Adenoid Cystic Carcinoma Stimulates CD133+ Cells with Neural Stem Properties

Adapted from manuscript published in Cell Signaling.[308]

II.3.1 Abstract

While existence of cancer stem cells (CSC) in adenoid cystic carcinoma (ACC) has been hypothesized, lack of assays for their propagation and uncertainty about molecular identify or markers prevented their characterization. Previous studies supported expression of neural stem cell markers in ACC.[309] Here, we identified a subpopulation of cells in ACC with properties of CSC, characterized their behavior and identified critical signaling pathways that support their propagation. In order to isolate CSC from ACC and characterize them, ROCK inhibitor-supplemented cell culture techniques were optimized. Immunomagnetic cell sorting and *in vitro/in vivo* assays were used to determine CSC viability and tumorigenicity. CD133 was identified as a marker of a subpopulation of ACC cells that preferentially expressed NOTCH1 and SOX10, formed spheroids, and initiated tumors in nude mice. NOTCH1 was activated only in CD133-positive (CD133+) cells, and these cells had the ability to generate CD133-negative (CD133-) cells. On the other hand, CD133- cells preferentially expressed JAG1 and neural differentiation factors: NR2F1, NR2F2, and $p27^{Kip1}$. Single depletion of NOTCH1, SOX10, or their common effector FABP7, resulted in decreased expression of all three proteins. Loss of NOTCH1, SOX10, or FABP7 inhibited spheroidogenesis and induced cell death in CD133+ cells, suggesting that they were essential for CSC maintenance. Downstream effects of FABP7 knockdown included suppression of a broad spectrum of genes involved in proliferation, ribosome biogenesis, and metabolism. Among proliferation-linked NOTCH1/FABP7 targets SKP2

and its substrate p27^{Kip1} were identified. NOTCH inhibition using a γ-secretase inhibitor, DAPT, selectively depleted CD133$^+$ cells, suppressed SKP2, induced p27^{Kip1}, inhibited ACC growth *in vivo*, and sensitized CD133$^+$ ACC cells to radiation. These results established that ACC contains a previously uncharacterized population of CD133$^+$ cells with neural stem properties that are dependent on SOX10, NOTCH1, and FABP7. Sensitivity of these cells to Notch inhibition and their dependence on SKP2 offer new opportunities for targeted ACC therapies.

II.3.2 Introduction

Adenoid cystic carcinoma (ACC) accounts for nearly one quarter of malignant neoplasms of the salivary gland and is a slow-growing yet unpredictable tumor with a propensity for insidious growth, perineural invasion, and distant metastases. Three major histological subtypes of ACC are distinguished in morphological features: tubular, cribriform, and solid. Although variation in clinical presentation of these types has been reported, all three patterns are neuroinvasive and show extremely high rates of recurrence. Treatment for ACC is currently limited to surgery with or without radiation, consistent with limited insight into its molecular drivers.[33] ACC has a high recurrence rate and dismal survival in part because of its intrinsic resistance to radio- and chemotherapies.[310] Existence of cancer stem cells (CSC) in ACC has been proposed,[309] but their molecular identity has remained elusive due to difficulties with ACC culture and the inability to identify stemness markers in these tumors. Development of targeted therapies for ACC has been further complicated by lack of reliable *in vitro* models, as there are currently no ACC cell lines available from centralized resources. Six previously created and shared cell lines were proven to be

grossly contaminated or misidentified, invalidating years of work and publications that used these lines.[129]

Recently, characterization of differentially expressed genes in primary salivary tumor specimens, ACC patient-derived mouse xenografts (PDX), head and neck squamous cell carcinoma, and normal salivary gland tissue[228] revealed a gene profile in ACC indicating neural stem signaling was active. ACC are rare tumors; therefore, subcutaneous PDX models that recapitulate basic ACC features, such as histologic appearance of the original tumor, characteristic t(6;9) translocations, and gene expression patterns were used as a reliable source of tumor tissue for study.[200, 228] While there are drawbacks of PDX models including relatively high maintenance costs and lack of interactions with the immune system, advantages of PDX models include preservation of tumor cell heterogeneity within a three-dimensional tumor. Many stem cell-centric cancer studies heavily rely on PDX models to maintain CSC subpopulations.[311-314] Analysis of clinical and PDX data revealed expression of neuronal genes and stem cell markers intrinsic to ACC, suggesting aberrant activation of a transcriptional program that controls neural stem cells (NSC). This finding was supported by the association of ACC with high expression of SOX10, a major transcriptional regulator and molecular marker of normal and malignant cells that originate from the neural crest.[182, 266] Expression profiles of many tumor types revealed that SOX10 gene signatures were present in basal-like breast carcinoma, melanoma, neuroblastoma, and glioma.[263]

Here, a ROCK inhibitor-based approach was optimized to support propagation of stem cells[315, 316] and produce sustainable ACC cell cultures that maintained cell lineage

identity. Using this new approach, a previously unknown population of tumorigenic CD133+ cells that expressed SOX10, NOTCH1, and activated intracellular NOTCH1 domain (N1ICD) was identified and characterized. NOTCH1 activity in CD133+ ACC cells was supported by expression of NOTCH1 targets including SKP2, an E3 ubiquitin ligase that targets p27^Kip1 for degradation and stimulates proliferation of CSC.[317, 318] On the other hand, CD133- cells expressed JAG1 (a Notch ligand), p27^Kip1 (a cyclin-dependent kinase inhibitor), and neural differentiation genes NR2F1 and NR2F2. As Notch signaling is linked to cell proliferation and radiation resistance,[319, 320] the effect of NOTCH1 inhibition on CD133+ cell survival and radiation sensitivity was investigated.[321] This work identified a population of stem-like cells and delineated principal signaling pathways in ACC that may be used in the near future for ACC treatment.

II.3.3 Materials and Methods

II.3.3.1 PDX and primary tumor specimen. PDXs of ACC in nude mice have been previously described.[200, 228] One clinical ACC specimen was collected from the Smilow Cancer Center at Yale New Haven Hospital (HIC# 1206010419).

II.3.3.2 Tissue processing. 5-10 mg of fresh or cryopreserved (90% FBS and 10% DMSO) tumor tissue was rinsed once with PBS, 70% EtOH, 100X Anti-Anti (GIBCO), twice with PBS containing 1:500 ceftazidime, and minced. Digestion was performed at 37°C for 1-2 h with occasional agitation in 3 mL of DMEM media (10% FBS, 1x Pen/Strep, 1x L-Glutamine) supplemented with 1 mL of Dispase (BD Biosciences, San Jose, CA), 30-150 μL hyaluronidase (Sigma, St. Louis, MO), and 30-150 μL collagenase (Roche, Indianapolis, IN). Digested tissue was collected at 1,500 rpm for 3

min., rinsed with PBS, re-centrifuged, transferred into 3 mL of F+Y media (see below), and filtered using a 100 µm cell strainer. Tumor cells were cultured in a CO_2 incubator with irradiated 3T3-J2 cells or conditioned media derived from these cells (see below).

II.3.3.3 Cell culture. 3T3-J2 feeder cells were grown as described.[316] To create conditioned media, irradiated 3T3-J2 cells were incubated in a T-150 flask supplemented with 30 mL of DMEM media for 4 days. Media was filter-sterilized and then mixed in a 1:4 ratio with F+Y media.

II.3.3.4 Real-time RT-PCR. RNA was isolated from frozen cell pellets of cells using RNeasy kit (Qiagen, Valencia, CA). cDNA was generated using Bio-Rad iScript Reverse Transcriptase kit. Real-time PCR was performed using iQ SYBR Green Supermix and CFX96 real-time detection system (Bio-Rad, Hercules, CA).

II.3.3.5 Western blot analysis. The following antibodies were used: SOX10 (Abcam, ab155279), NOTCH1 (Cell Signaling, #3608), FABP7 (Cell Signaling, #13347), cleaved NOTCH1 (Cell Signaling, #4147), p27^{Kip1} (Cell Signaling, #2552), SKP2 (Cell Signaling, #4313), β-actin (Santa Cruz, sc-47778), and GAPDH (Santa Cruz, sc-25778). Pre-cast gels, Rapid Transfer Turbo-blot System, and gel imaging software were from Bio-Rad.

II.3.3.6 Microsatellite analysis. For cell line authentication, Short Tandem Repeat (STR) analysis as recommended by ATCC https://www.atcc.org/~/media/PDFs/ Technical%20Bulletins/tb08.ashx was performed using Promega GenePrint 10 STR analysis PCR kit with fluorescent tagging. PCR products were analyzed on Applied Biosystems 3730xL DNA Analyzer at the Yale Keck Facility and data processed using

GeneMapper 3.7 (Applied Biosystems) software. The results were compared to STR databases (http://www.cstl.nist.gov/strbase/, http://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html).

II.3.3.7 Flow cytometry. Cells were collected and incubated with CD133-PE antibody (Miltenyi) for 10 minutes. Fluorescence-activated cell sorting (FACS) was performed to determine percentage of CD133$^+$ cells using a FACS Caliber BD machine. Data analysis performed using Flowing Software version 2.5.0.

II.3.3.8 Cell sorting. For separation of CD133$^+$ and CD133$^-$ cells from cultured cells and grafted tissue, CD133 Tumor Tissue MicroBead Kit from Miltenyi (San Diego, CA) was used according to the manufacturer's protocol.

II.3.3.9 Tumorigenicity assays. All mouse experiments were performed in accordance with NIH national guidelines and were approved by Yale University IACUC. Athymic NCr-*nu*/*nu* mice purchased from NCI-Frederick (Frederick, MD) were used for subcutaneous injections in the flanks with a specified number of viable tumor cells.

II.3.3.10 Spheroidogenesis and spheroid viability assays. For spheroid studies, approaches as previously described were performed.[322] Briefly, spheroid formation and disintegration were studied in six-well plates with or without DAPT (Eli Lilly, GSI-IX) from Selleckchem, Houston, TX, or DMSO control. At 24 hour intervals, three separate representative digital images per well at 4X magnification were used to quantify spheroid number by direct visual counting.

II.3.3.11 Immunofluorescence staining. Cells were plated onto glass slides at a density of 25,000-30,000, incubated for 24 h, washed twice with PBS, and fixed with 3-4% paraformaldehyde at RT for 30 min. Cells were permeabilized with 0.15% Triton X-100 in PBS for five minutes, washed twice, and blocked with 3% BSA for 30 minutes. Incubation with primary antibodies diluted in 3% BSA was done at 4^0C overnight with subsequent washing once with PBS and once with 3% BSA. The following antibodies were used: SOX10 (Abcam, ab155279), FABP7 (D8N3N), NOTCH1 (D1E11), and SKP2 (#4313) from Cell Signaling, JAG1 (C-20) and NR2F1 (sc-74561) from Santa Cruz, NR2F2 (H7147) from Parsons Proteomics, and AlexaFluor secondary antibodies from Life Tech.

II.3.3.12 Immunohistochemical staining. The following antibodies were used: cleaved NOTCH1 (Val1744) antibodies from Cell Signaling (Danvers, MA), SOX10 antibodies from Cell Marque (Rocklin, CA), and FABP7 antibodies from Abcam (Ab32423, Cambridge, MA).

II.3.3.13 Proliferation assays. Cells were cultured in 96-well black clear bottom plates and treated with DAPT versus control (DMSO). After cells were grown for 24-96 hours, viable cells were assayed using the Cell Titer Glo system (Promega).

II.3.3.14 Cell cycle analysis. Cells were fixed in 70% cold ethanol and stained with propidium iodide. A FACS Caliber BD machine was used and data analysis performed using Flowing Software version 2.5.0.

II.3.3.15 Cell death assay. Cells were incubated for 10 minutes with CD133-FITC antibody (Miltenyi) as well as Annexin V antibody and 7-AAD DNA stain (Annexin V:PE apoptosis detection kit I, BD Biosciences). A FACS Caliber BD platform was used and data analysis was performed using Flowing Software version 2.5.0.

II.3.3.16 Radiosensitivity assay. Cells plated in T-25 flasks were irradiated using Mark I Cesium-137 irradiator, incubated for 48 hours in the presence or absence of DAPT, and stained with CD133-PE antibody. FACS was performed to determine percentage of CD133$^+$ cells. Statistical differences in tumor volume were determined using a two-tailed *t*-test.

II.3.3.17 DAPT studies on ACC xenografts. *In vivo* efficacy of DAPT was evaluated in an Accx11 xenograft model generated via subcutaneous injection of 10^6 cultured Accx11 cells. Tumor cell-injected mice were randomized into control (vehicle-injected) and experimental (DAPT in corn oil) groups 15 days after cell injection. DAPT was given via intraperitoneal (i.p.) injection at 50 mg/kg, following a 3/4 injection schedule (three days of treatment/four days rest) for 35 days. To assess DAPT toxicity, animals were observed daily and weighed weekly using a digital scale. To assess DAPT efficacy, tumor dimensions were measured weekly by a digital caliper and data including individual and mean estimated tumor volumes (mean TV ± s.e.m.) were recorded for each group. Tumor volume was calculated using the formula TV = width^2 × length × 0.52. Statistical differences in tumor volume were determined using a two-tailed *t*-test.

II.3.4 Results

II.3.4.1 Generation and validation of primary cell cultures from ACC tissue

As indicated by the absence of available ACC cell lines, ACC cells are difficult to culture using standard techniques (our unpublished observations). Validated PDX models were used as a source of ACC tissue to develop new culture techniques, since primary tumor tissue is scarce.[200, 228] A recently described conditional reprogrammed cell culture (CRC) protocol[316] was optimized to produce intermediate and long-term cell cultures from five ACC xenografts and one primary ACC tumor (Fig. 18A&B). Short-tandem repeat DNA profiling as recommended by ATCC,[323] was performed to determine provenance of newly created cell cultures (Table 3).

Previous expression analyses of ACC identified markers associated with neural stemness including TrkC/NTRK3, SOX10, NOTCH1, and FABP7.[200, 263] Reassuringly, all ACC cell cultures expressed all four neural stem genes (Figure 18C), recapitulating expression of these genes in clinical ACC specimens and the parental PDXs (Figure 19). However, cultures of normal salivary gland cells did not express FABP7 and had levels of SOX10 and NTRK3 that were at the limit of detection. Thus, the optimized ROCK inhibitor and cell feeder-based protocol proved to be an effective solution to the ACC cell culture problem. Continued use and optimization of this technique may significantly advance translational ACC research.

Figure 18: Clinical, cytological, and molecular properties of ACC cell cultures.
(A) Features of ACC cell cultures as well as PDXs (Accx) or tumor (Acc33) from which cultures were derived. m, metastases. (B) Low and high magnification brightfield images of cultured ACC cells at indicated passages for Accx5m1, Accx11, Accx19, and Acc33. (C) Real-time PCR quantification (qRT-PCR) of gene expression for SOX10, NOTCH1, FABP7, NTRK3/TrkC, and PROM1/CD133 in cultured ACC cells compared to cultured cells isolated from normal salivary gland (NSG). In all qRT-PCR experiments, expression is normalized to β-actin and standard error bars representative of at least two independent experiments.
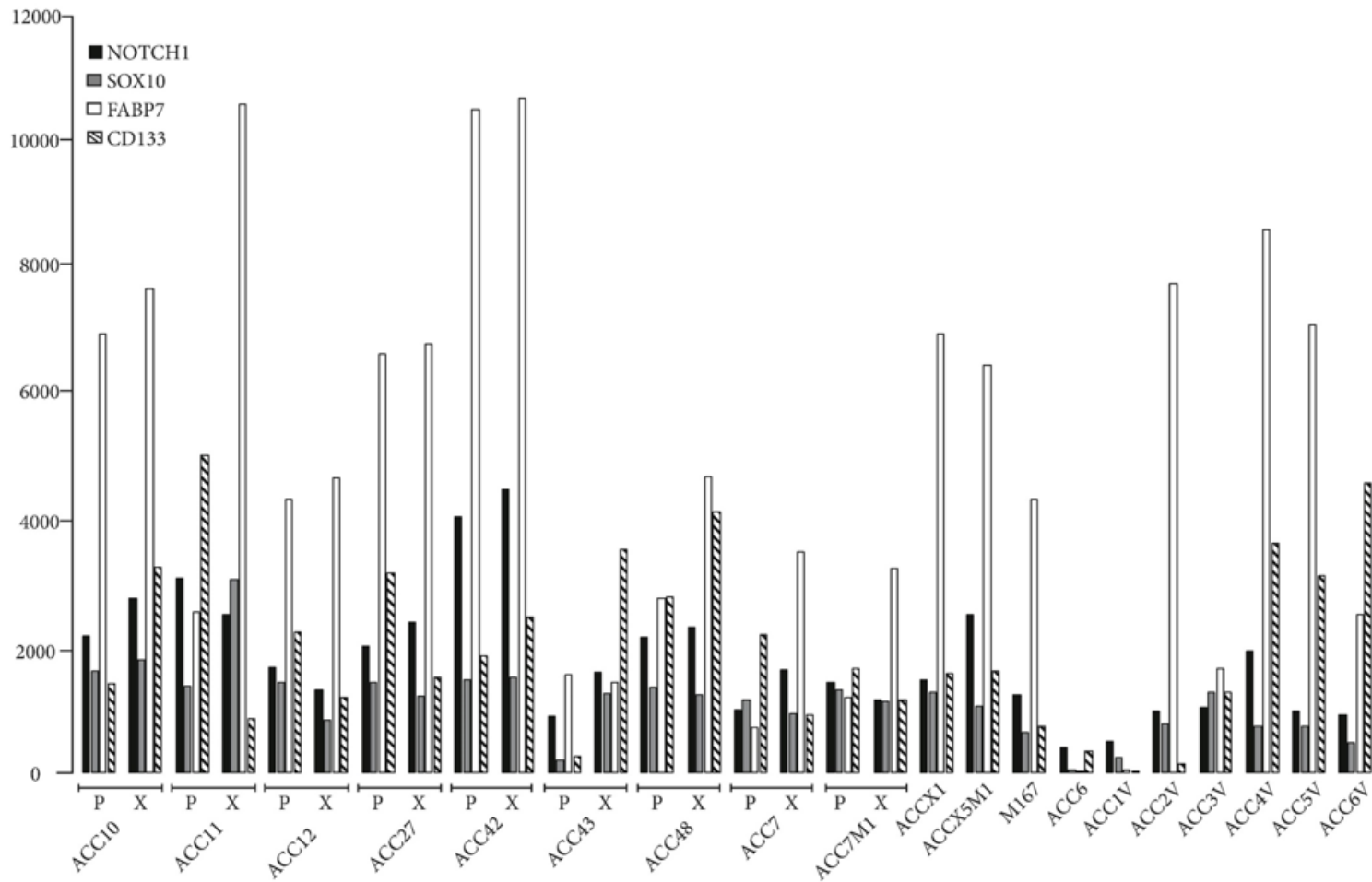
Figure 19: Expression of SOX10, NOTCH1, FABP7, and CD133 in ACC tumor specimens from patients (P) and PDX (X).
Comparison is based on previously produced Affymetrix data.[200]

Sample	Source of cells/tumors	TH01	D21S11	D5S818	D13S317	D7S820	D16S539	CSF1PO	Amelogenin
ACCX2	Xenograft	10	28.3, 31, 32	10.1, 11.1	11, 12, 15	11	11, 12.1	11, 12	-
Accx2	Culture	10	27.3, 28.3, 30.3, 31.2	10.1, 11.1, 12.3	11, 13, 14	8, 11	10, 11, 12.1	6.3, 11, 12	X
ACCX5M1	Xenograft	9.1, 10	28, 29.3, 32, 32.1	12.3	10, 14	7, 11	9, 11	9.3, 11	X, Y
Accx5M1	Culture	9.1, 10	29.3, 32.1	12.3	11, 14	7, 11	9, 13.1	9.3, 10.3	X, Y
ACCX11	Xenograft	6.1, 10	28.3, 31.1	11.1	13, 14,	8, 9.1	11, 12.1,	10, 12	X
Accx11:9	Culture	6.1, 10	28.3, 31.1	11.1	13, 14,	8, 9.1	11, 12.1,	10, 12	X
ACCX11:9p:0	Xenograft from Cx	6.1, 10	28.3, 31.1	-	13, 14,	8, 9.1	11, 12.1,	10, 12	X
ACCX11 CD133-	Xenograft from Cx	6.1, 10	28.3, 31.1	11.1	13, 14	8, 9	11, 13	10, 12	X
ACCX11 CD133+	Xenograft from Cx	6.1, 10	28.3, 30, 31.1	11.1	13, 14	8, 9	11, 13	10, 12	X
ACCX14:9	Xenograft	8, 10, 13.3	28.3	10.1, 11	15	9, 10	12.1	11, 12	X
Accx14:9	Culture	8.3, 10	28.3	10.1, 11.1	14	9.1, 10	11, 12.1	11, 12	X
ACCX19	Xenograft	7.1, 8	27.3	12.3	13, 15	8, 11	10, 11	6.3, 12	-
Accx19	Culture	7.1, 8	27.3	12.3	13, 15	8, 11	10, 11	6.3, 12	-
ACCX29	Xenograft	6.1, 7.1	29.3, 31	10.1, 11	13, 14	10	9, 11	12	X
Accx29	Culture	6.1, 7.1	29.3, 31.1	10.1, 11.1	13, 15	9.3,	9, 11	12,	X
ACC33	Patient tumor	9.1, 10	28.3, 29.3	9	11, 15	9.3, 11	11, 12.1	11, 14	X
Acc33	Culture	9.1, 10	28.3, 29.3	9	10, 14	10, 11	11, 12.1	11, 14	X

Table 3: Short-tandem repeat pattern of ACC cell cultures and parental xenografts.

II.3.4.2 Isolation and characterization of previously unrecognized CD133+/ SOX10+/NOTCH1+ ACC cells

Expression of the key neural stem cell marker, SOX10, in ACC and other cancer types that originate from neural crest[200, 263] suggested that either all ACC cells may express this stem cell marker, or that a subpopulation of ACC cells with neural stem properties may exist. To begin distinguishing these possibilities, cell surface markers co-expressed with SOX10 in ACC were identified. CD133/PROM1 (hereafter, CD133), a CSC cell surface marker used in neural cancer stem cell isolation,[324] was expressed in nearly all clinical ACC specimens and PDXs (Figure 19). While CD133 expression was recently reported in ACC,[325] there have been no published attempts to isolate and characterize CD133+ ACC cells. Interestingly, CD133 was expressed in all ACC cell cultures that were generated but not in cultured normal salivary epithelial cells (Figure 18C), suggesting that it may be used as a tool for CSC isolation.

To begin analyzing CD133-expressing ACC cells, a robustly proliferating and spheroid-forming culture, Accx11 (Figure 18A&B), was the initial focus. Accx11 was derived from a xenograft of a grade 3 ACC that is distinguished by solid histology and poor prognosis.[326] FACS analysis of Accx11 cells revealed high CD133 expression in a mean of 12% of cells (Figure 20). CD133+ and CD133- fractions enriched by magnetic activated cell sorting (MACS) with CD133 antibody coupled beads produced more than 50-fold enrichment of CD133 expression (Figure 21A). Excitingly, CD133+ cells expressed at least 25-fold higher levels of SOX10, NOTCH1, and FABP7 as compared to CD133- cells, suggesting that expression of these genes co-segregated with cell surface expression of CD133 (Figure 21B).
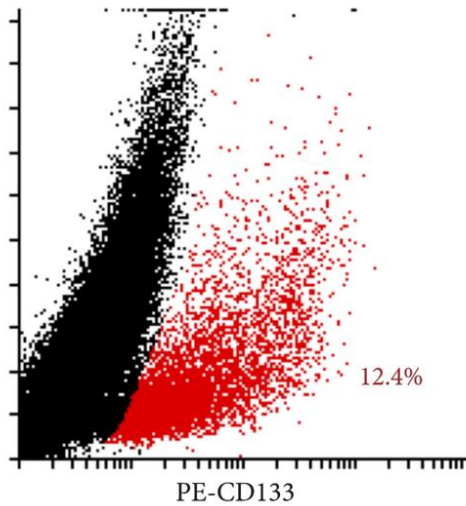
Figure 20: FACS analysis of Accx11 cells stained with PE-conjugated CD133 antibody. CD133$^+$ cells (red) comprise ~12.4% of the bulk population.

After separation, CD133$^+$ cell cultures generated both spheroid-forming and tightly adherent cells. Cultured CD133$^-$ Accx11 cells, however, remained adherent and were not able to produce either CD133$^+$ cells or spheroids (data not shown). These data suggest that CD133$^+$, but not CD133$^-$ cells, express key stem cell markers and are capable of asymmetric division recreating both CD133$^+$ and CD133$^-$ cell populations. To further explore signaling in CD133$^+$ cells, expression of SCRG1, PLP1, MIA, SHC4, and TTYH1 previously linked to the SOX10 neural gene signature in ACC[263] was investigated. Remarkably, without exception these genes were more highly expressed in CD133$^+$ ACC cells compared to bulk or CD133$^-$ cells (Figure 21B). In light of the association of these genes with cancers of neural stem cell origin,[281, 327-329] their selective expression in CD133$^+$ ACC supported the existence of a stem cell subpopulation in ACC.

Activation of Notch is a common feature of non-cancer neural stem cells and CSC of neural origin.[330] Immunoblotting revealed high expression of NOTCH1 and cleaved/activated NOTCH (N1ICD) in CD133$^+$, but not in CD133$^-$ or unsorted cells, and

confirmed high expression levels of SOX10 and FABP7 in CD133$^+$ cells (Figure 21C). In line with Notch activation, its canonical targets HEY1, HEY2, and MYC were predominantly expressed in CD133$^+$ cells (Figure 22D).

In order to determine if other ACC cultures harbored similar cell populations, Accx19 cells (Figure 19A-C) were examined. In agreement with lower expression levels of SOX10, NOTCH1, and FABP7, the percentage of CD133$^+$ cells in Accx19 determined by FACS analysis was lower than in Accx11 comprising <8% of total cells (data not shown). MACS separation of Accx19 cells produced 2.5-3-fold enrichment of CD133 expression in CD133$^+$ cells compared to CD133$^-$ cells. In CD133$^+$ Accx19 cells, expression of NOTCH1, SOX10, and FABP7 was similarly enriched (data not shown). Overall, these data supported the existence in ACC of a previously unknown population of SOX10-positive CD133$^+$ cells with activated NOTCH1.

II.3.4.3 CD133$^-$ ACC cells express neuronal differentiation genes NR2F1 and NR2F2, p27^{Kip1}, and a Notch ligand, JAG1

CD133-sorted cells were analyzed for expression of two orphan nuclear receptors whose expression is required for NSC differentiation, NR2F1 and NR2F2.[331] This comparison revealed markedly increased expression of both genes in CD133$^-$ cells (Figure 21E), suggesting that these cells may be more differentiated than CD133$^+$ cells.

Jagged-1 (JAG1), a canonical Notch ligand, is an essential component of Notch signaling, and in many cancers it is expressed not in stem cells but in supporting niches.[332, 333] Interestingly, JAG1 expression was markedly higher (~11-fold) in CD133$^-$ Accx11 cells (Figure 21E). Enhanced expression of NR2F2 and JAG1 was also observed in CD133$^-$ cells isolated from Accx19 cultures (data not shown).

p27[Kip1] is a critical effector of neural differentiation, and in NSC NOTCH1 blocks p27[Kip1] expression.[334, 335] Given that NOTCH1 is preferentially activated in CD133^{+} cells and that CD133^{-} cells express markers of neural differentiation, p27[Kip1] expression in both fractions was analyzed. Immunoblotting of unsorted and CD133^{-} cells revealed significantly higher p27[Kip1] levels compared to the CD133^{+} population (Figure 21F).

Selective expression of NOTCH1 and its targets in CD133^{+} cells and JAG1 and NR2F1/2 in CD133^{-} cells indicated that these ACC cell subpopulations may communicate via NOTCH1/JAG1 interaction (Figure 21G). To begin dissecting differences and interaction between CD133^{+} and CD133^{-} cells, cultures of mixed cell subpopulations, as indicated by loosely adherent cells/spheroids versus adherent Accx11 cells, were stained by immunofluorescence. In agreement with RT-PCR and immunoblot data (Figure 21B&C), membrane CD133 and NOTCH1 staining was detected in cells associated with loosely adherent cells/spheroids, but not in the firmly adherent cell population (Figure 21H&I). Similarly, SOX10 and FABP7 stained cells in loosely adherent cells/spheroids, while differentiation markers NR2F1 or NR2F2 were seen in adherent surrounding cells (Figure 21I-K). Remarkably, NOTCH1-expressing cells within loosely adherent cells/spheroids were closely surrounded by adherent cells expressing membrane-localized Jagged-1 (Figure 21L). Z stack images revealed that JAG1 and NOTCH1 were co-localized on the surface of these adjacent cells (Figure 21L, see arrows) supporting NOTCH1/JAG1 communication and a signal-sending supportive role of JAG1-producing CD133^{-} cells.
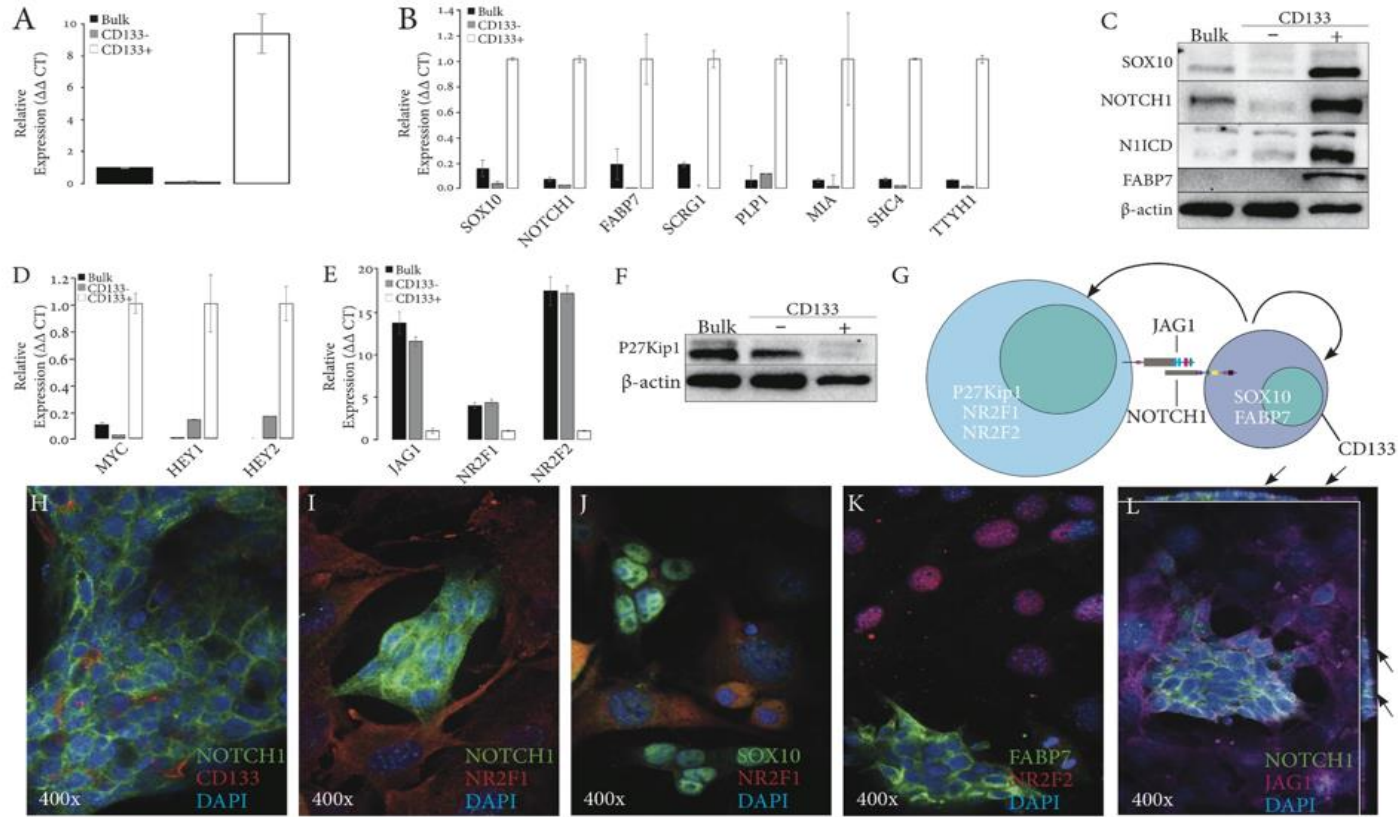
Figure 21: Isolation and characterization of CD133+ and CD133- cells in Accx11 culture.
(A) qRT-PCR of CD133 expression in Accx11 cell fractions following magnetic-activated cell sorting (MACS). (B) Expression analysis by qRT-PCR of ACC-associated (SOX10 & FABP7) and NSC-associated (NOTCH1, SCRG1, PLP1, MIA, SHC4, & TTYH1) genes in MACS-sorted Accx11 cell fractions. (C) Immunoblot analysis shows selective expression of SOX10, NOTCH1, activated intracellular domain of Notch-1 (N1ICD), and FABP7 in CD133+ cells. β-actin serves as a loading control. (D) Expression analysis by qRT-PCR of Notch targets MYC, HEY1, and HEY2 in MACS-sorted Accx11 cell fractions. (E) Expression analysis by qRT-PCR of JAG1 and neural differentiation markers NR2F1 and NR2F2 in MACS-sorted Accx11 cell fractions. (F) Immunoblot analysis of CD133 fractions demonstrates CD133- cell-specific p27Kip1 expression. β-actin serves as a loading control. (G) Molecular determinants of stem-like CD133+ cells and more differentiated CD133- cells. (H-L) Immunofluorescence (IF) staining demonstrates great selectivity of CD133+ and CD133- cell markers and confirms spheroid-forming property of CD133+ cells. Z-stack images (L) show NOTCH1 and JAG1 co-localization at sites of spheroidal and non-spheroidal cell contacts (arrows).

II.3.4.4 CD133+ and CD133- populations identified in grafted ACC tissue

In order to diminish the possibility that findings were the result of cell culture artifacts, the existence of CD133+ and CD133- cell populations was validated using PDX tissue derived from 5 ACC patients. To this end, fresh PDX tumors were dissociated into cells, and CD133+ and CD133- populations immediately magnetically separated. CD133+ cells comprised from 21 to 65% of viable cells derived from xenografts (Figure 22A) and real-time PCR revealed that these cells expressed higher levels of SOX10, NOTCH1, and FABP7, with a few exceptions (Figure 22B-E). On the other hand, CD133- cells universally expressed higher levels of JAG1, while NR2F1 expression was more variable (Figure 22F&G). These results mirrored the findings in cultured cells derived from ACCX11 and ACCX19 (Figure 21 and data not shown). Collectively, these data confirmed the existence of two distinct cell populations in ACC marked by expression of either SOX10+/NOTCH1+/FABP7+/CD133+ or JAG1+/CD133- in ACC.
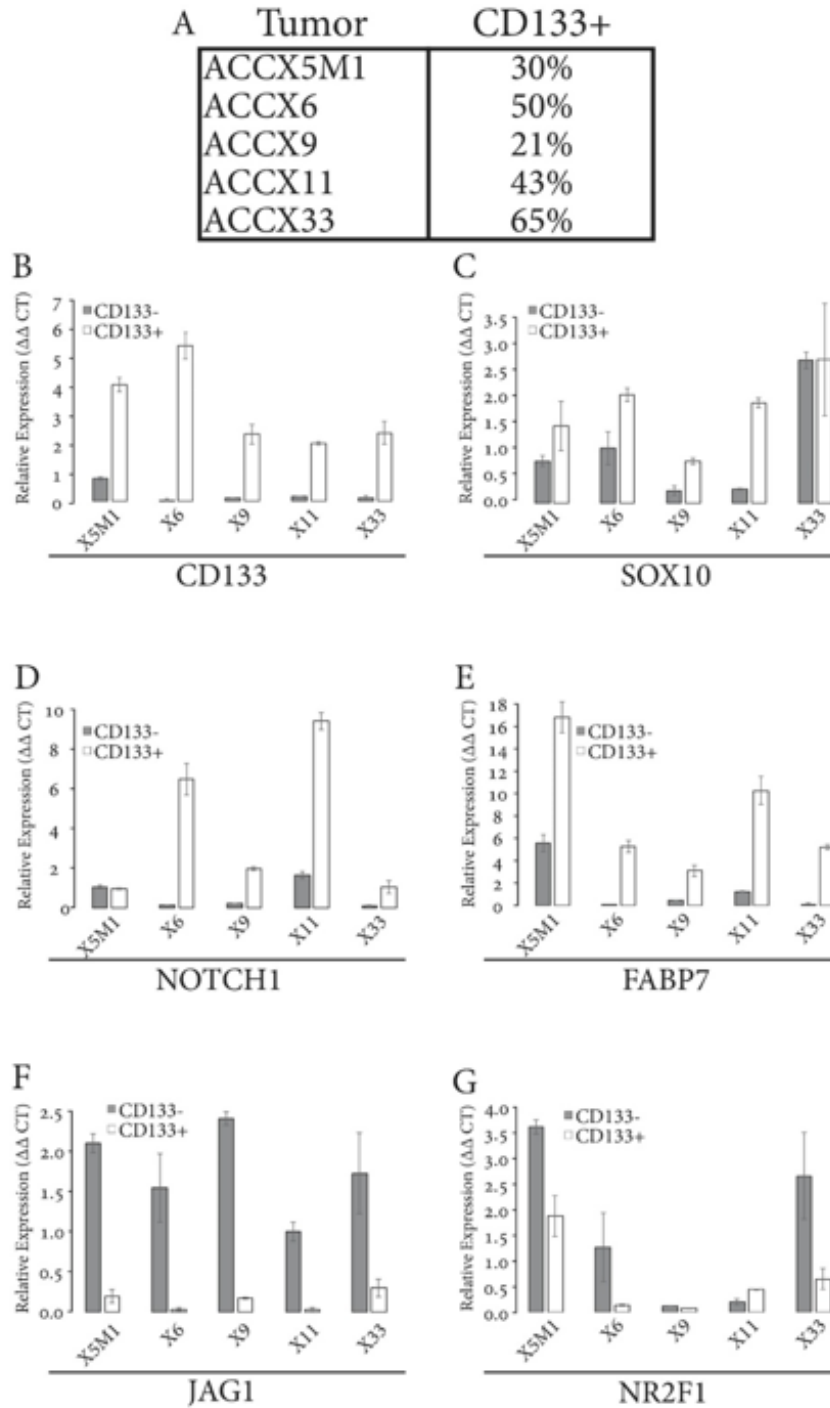
Figure 22: Isolation and characterization of CD133 fractions from ACC xenografts.
(A) Variation in the percentage of CD133+ cells across ACC xenografts. (B) qRT-PCR analysis of CD133 expression confirms adequate separation of CD133+ and CD133- cells isolated from ACC xenografts. (C-E) Selectivity of SOX10, NOTCH1, and FABP7 to CD133+ cells isolated directly from ACC xenografts as confirmed by qRT-PCR. (F-G) Selectivity of JAG1 and NR2F1 to CD133- cells isolated directly from ACC xenografts as confirmed by qRT-PCR.

II.3.4.5 Tumorigenicity of ACC cultures and dependency on CD133 status

The ability of cultured ACC cells to initiate tumors was investigated via subcutaneous injections of 10^6 cells into flanks of nude mice. Among ACC cultures tested to date, only Accx11 cells were tumorigenic in nude mice creating tumors with solid histology and an intervening stromal component similar to the parental patient tumor and PDX (Figure 23A). Relative expression of CD133, SOX10, NOTCH1, and FABP7 was similar in the original PDX and in the tumor produced from injection of cultured cells (Figure 23B&C).

In various cancers, CD133 expression defines populations of CSC with enhanced tumor-initiating properties.[336] To investigate CSC properties of CD133-expressing ACC cells, bulk Accx11 cells were magnetically sorted based on CD133 expression and 10^4 cells injected subcutaneously into right (CD133$^+$) and left (CD133$^-$) flanks of three nude mice. After 22 weeks, tumors were observed in three of three sites injected with CD133$^+$ cells, but in none of three sites injected with CD133$^-$ cells. At 32 weeks, a single tumor formed at a site injected with CD133$^-$ cells. Histologic evaluation of the parental ACCX11 tumor, tumors derived from CD133$^+$ cell injections, and the single tumor from a CD133$^-$ cell injection revealed similar histologic appearance (data not shown). Expression analyses of CD133, SOX10, NOTCH1, and FABP7 by qRT-PCR and immunoblotting revealed expression of all markers in tumors derived from both CD133$^+$ and CD133$^-$ cells (data not shown), suggesting that small amounts of CD133$^+$ are trapped in the CD133$^-$ fraction or that injected CD133$^-$ cells can convert to CD133$^+$ cells.

As experience with CD133 cell separation increased, it became apparent that a single round of separation using CD133 antibody conjugated to beads left detectable traces of CD133$^+$ cells in the CD133$^-$ population reflecting technical limitations of this procedure. Also, separation decreased viability of CD133$^+$ cells more than CD133$^-$ cells, possibly due to the damage inflicted by elution of column-bound cells (data not shown). To more adequately compare tumorigenicity of CD133$^+$ and CD133$^-$ cells, a second round of magnetic separation was performed on the unbound cells to isolate a more pure CD133$^-$ population. Following this double separation, 10^5 viable cells of each population were injected into the flanks of nude mice. Tumors began to form at the sites of CD133$^+$ injections as early as 6.5 weeks after injection. By 13 weeks post-injection, four of five CD133$^+$ injection sites had distinct tumors while none of five CD133$^-$ injections showed signs of tumor formation (Figure 23D). With a marked delay, two of five injection sites of CD133$^-$ cell injections formed small tumors with the earliest tumor developing at 19 weeks. At 27.5 weeks, all of the five CD133$^+$ injection sites formed tumors, while no additional tumors grew in CD133$^-$ injection sites. Overall, these experiments demonstrated enhanced tumorigenicity of CD133$^+$ cells compared to CD133$^-$ cells.
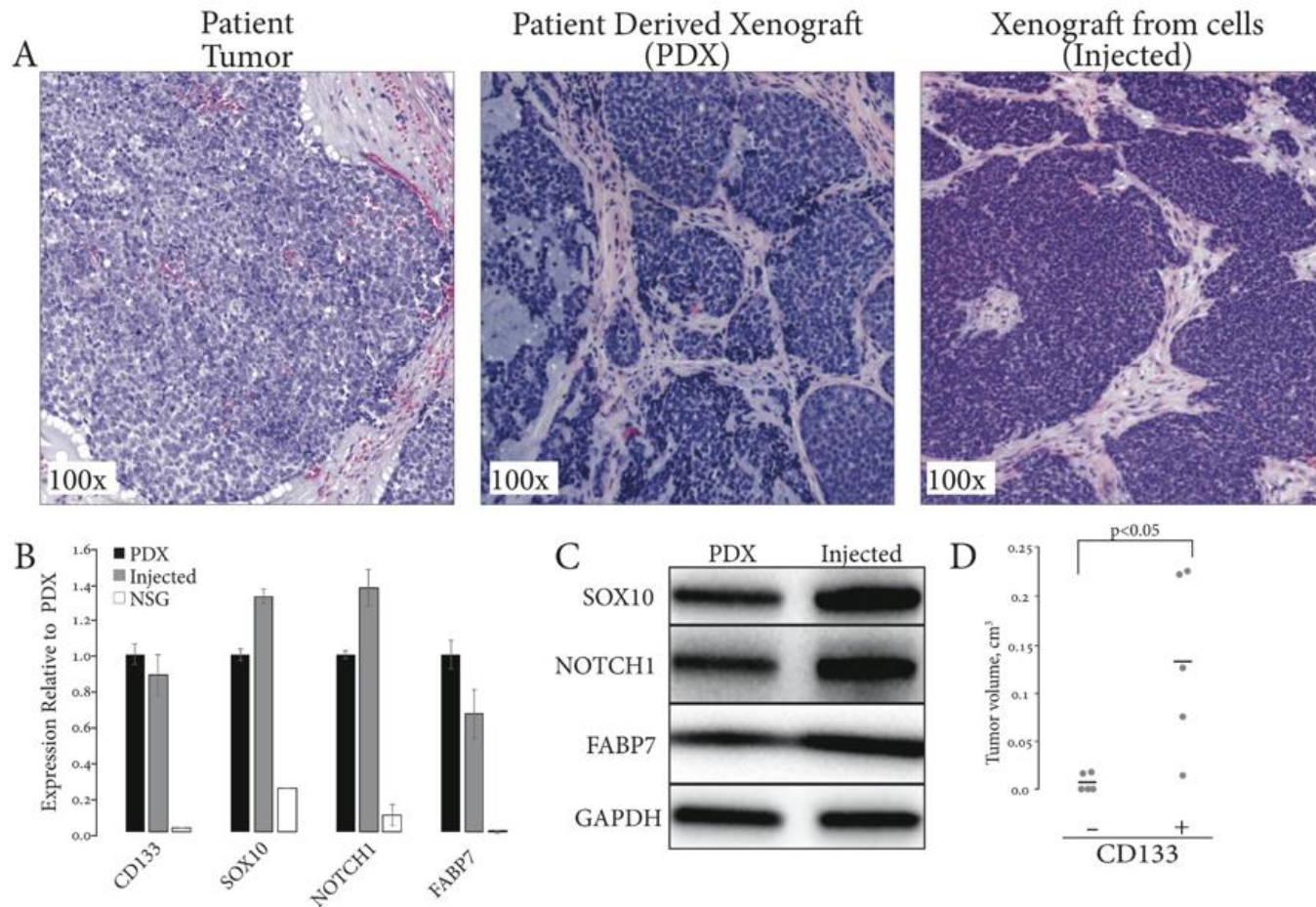
Figure 23: Tumorigenic properties of bulk and CD133-fractionated Accx11 cells.
(A) Cultured bulk Accx11 subcutaneously injected in athymic nude mice produce tumors with histology similar to patient tumor and parental xenograft (H&E staining). (B) CD133, SOX10, NOTCH1, and FABP7 expression by qRT-PCR confirms ACC identity of PDX and tumors generated from injection of bulk Accx11 cells. (C) Immunoblot analysis confirms similar expression of SOX10, NOTCH1, and FABP7 in PDX and tumor formed from injected bulk Accx11 cells. GAPDH serves as a loading control. (D) Dot plot of tumor volumes 90 days after injection of 10^5 CD133- or CD133+ MACS-sorted Accx11 cells.

II.3.4.6 Interdependence between NOTCH1, SOX10, and their common effector FABP7

Essential individual roles for SOX10 and NOTCH1 in neural stem cell maintenance are well established, and loss of Notch signaling decreases SOX10 expression in neural progenitors.[337, 338] FABP7 has recently been described as critical for proliferation and to prevent differentiation in neural progenitors.[339] Given that SOX10, NOTCH1, and FABP7 are each implicated in neural stemness, their interdependence in ACC cells was explored. Following individual depletions, expression of all three genes was measured by qRT-PCR and immunoblotting. Knockdown of SOX10 markedly decreased NOTCH1 expression, and similarly, depletion of NOTCH1 was associated with decreased SOX10 both at the mRNA and protein levels (Figure 24A&B), suggesting interdependence and functional cooperation. As expected, since FABP7 is a common NOTCH1/SOX10 target,[122, 340] depletion of either NOTCH1 or SOX10 markedly suppressed FABP7 mRNA and protein levels. Depletion of FABP7 had less suppressive effects on NOTCH1 and SOX10 mRNA than did either NOTCH1 or SOX10 knockdowns. At the protein level, FABP7 had no obvious effect on NOTCH1 expression while modestly decreasing SOX10 levels (Figure 24A&B).

Individual roles of NOTCH1 and SOX10 in CSC and NSC survival are well established, but their co-operation via FABP7 has not yet been studied. To begin exploring the role of FABP7 in adenoid cystic carcinoma signaling, it was depleted in Accx11 cells followed by gene expression profiling. Analyses revealed that 1158 genes were downregulated (Table 4, 2-fold threshold), and KEGG pathway analysis

(http://bioinfo.vanderbilt.edu/webgestalt/) implicated these genes in cell cycle regulation, ribosome biogenesis, cell metabolism, and signaling pathways involved in these processes (Table 4 and data not shown). Among these genes, 11 belonged to the Notch signaling network and 26 genes overlapped with the ACC SOX10 gene signature previously characterized.[263] This observation further supported the feedback signaling from FABP7 to NOTCH1 and SOX10. In addition, SKP2, a major NOTCH1 effector involved in the regulation of cell cycle and proliferation,[341] was among 31 cell cycle progression and 11 NOTCH1-regulated genes downregulated by FABP7 knockdown (Table 4). In summary, these data suggested that NOTCH1, SOX10, and FABP7 may be co-regulated in ACC, and that FABP7 may serve as a pro-survival NOTCH1 and SOX10 effector.

Pathway/Gene Signature	# Genes	Gene Name	Statistics, p value
Cell cycle	31	ANAPC7, BUB1, BUB1B, CCNA2, CCNB2, CCND1, CCNE2, CDC20, CDC6, CDK1, CDK4, CDK6, CDKN2C, CHEK1, DBF4, MAD2L1, MCM2, MCM3, MCM4, MCM6, MYC, ORC5, PRKDC, PTTG1, **SKP2**, TFDP1, TFDP2, TGFB2, TTK	2.19E-22
Ribosome	27	RPL0, RPL3, RPL4, RPL5, RPL6, RPL7A, RPL10, RPL10A, RPL13, RPL13A, RPL14, RPL15, RPL22, RPL23, RPL27A, RPL31, RPL35A, RPL37, RPS2, RPS3, RPS6, RPS7, RPS8, RPS15A, RPS20, PS21, RPS23	1.08E-21
Metabolic pathways	82	ACAT1, ACO1, ACOX1, ACY1, ADI1, ADSL, GPS, AHCY, ALDH5A1, ALDH6A1, ALDH7A1, ALG10B, ALG13, ALG9, ARG2, ATP5G2, ATP6V0E2, BCAT2, COX15, COX17, CPS1, CTH, DBT, DHFR, DPM3, DTYMK, DUT, ENO1, EPT1, ETNK1, FECH, FH, GFPT1, GLCE, GLS, GOT1, GPAM, GPAT2, HADH, HADHA, HSD17B1, HSD17B2, IDH2, IMPDH2, KDSR, LTA4H, MGAT4A, MTHFD1, MTHFD2, MTHFD2L, MTR, ODC1, PAICS, PFAS, PGM2, PHGDH, PIGK, PIGN, PLA2G12A, POLA1, POLD2, POLE3, POLE4, POLR2J, PRIM1, PRIM2, PTDSS1, PTGES, PTGIS, PYCR2, QARS, RRM1, RRM2, SEPHS1, TK1, TKT, TUSC3, TYMS, UQCRB, UQCRH, UQCRQ, ZNRD1	8.47E-18
NOTCH1	11	CCND1, ENO1, HES6, HEY1, HEY2, JAG1, MIB1, MYC, NOTCH1, **SKP2**, YY1	2.30E-05
SOX10 ACC signature	26	ABI2, ACTR3B, CADM1, CCNB1IP1, CHDH, DPY19L2, EPHA7, FGFR2, FRMD4A, GPM6B, ITGA9, LGR6, MTL5, MYEF2, NLN, NRCAM, NRTN, PPP1R1B, RAP2A, RPL3, SEPT4, SHANK2, SLC35F3, TEX261, TMEM63A, TTC3	1.40E-12

Table 4: Suppressive effects of FABP7 depletion on gene expression in Accx11 cells.

II.3.4.7 SOX10, NOTCH1, and FABP7 stimulate spheroidogenesis of Accx11 cells

NSC and CSC have the propensity to form spheroids in culture.[342] Using

spheroid formation as a surrogate of stem cell survival and proliferation, functional

consequences of SOX10, NOTCH1, and FABP7 knockdowns were determined.

Individual depletion of these genes significantly suppressed spheroid formation in

Accx11 cells (Figure 24C&D). These data suggest that SOX10, NOTCH1, and FABP7

are vital for spheroid formation, which could reflect an effect on CSC survival and

growth. Flow cytometry analyses of Accx11 were performed to assess the effects of

SOX10, NOTCH1, and FABP7 depletion on cell cycle progression and cell survival. A

marked (~4-6-fold) increase in the subG1 populations, decrease in G1, and increase in

S phases were observed following NOTCH1, SOX10, or FABP7 depletion (Figure 24E).

Altogether, these results suggested that NOTCH1, SOX10, and FABP7 are important

for survival of ACC cells, cell cycle progression, and maintenance of stem-like

phenotype.
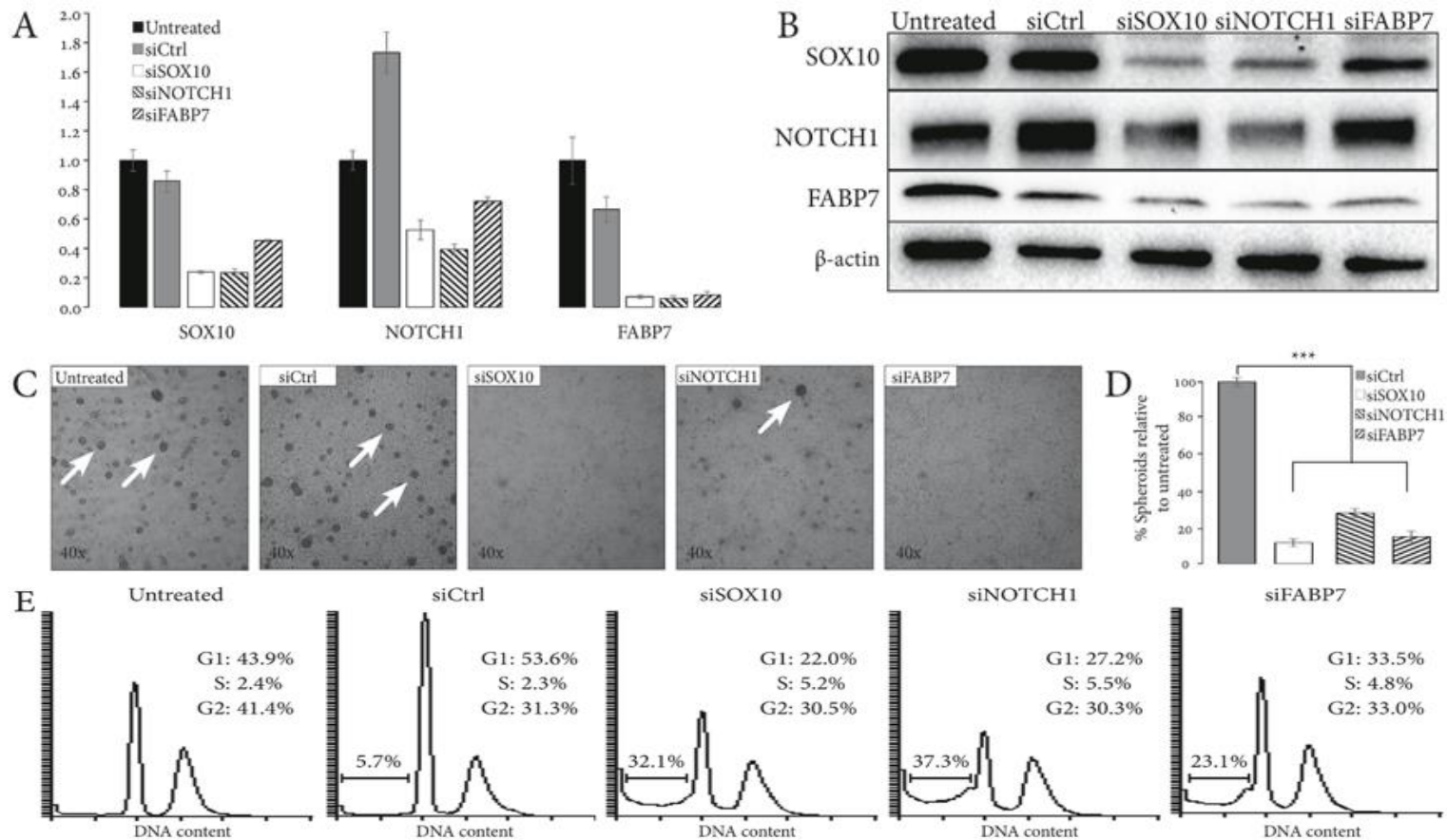
Figure 24: Interrelated stimulatory effects of SOX10, NOTCH1, and FABP7 on ACC cells.
(A) qRT-PCR analysis of SOX10, NOTCH1, and FABP7 expression following siRNA knockdowns reveals their regulatory interdependence. (B) Validation of SOX10, NOTCH1, and FABP7 interdependence by Western blot. β-actin serves as a loading control. (C) Depletion of SOX10, NOTCH1, and FABP7 by siRNA suppresses spheroidogenesis of Accx11 cells at 72 h. Arrows: brightfield images of spheroids. (D) Quantification of suppressive SOX10, NOTCH1, and FABP7 knockdown effects on spheroids compared to untreated Accx11 cells. Error bars represent standard errors and are representative of at least two independent experiments. In each case, siRNA knockdown resulted in a significant decrease in spheroid formation (p<0.001, Student's t-test). (E) Depletion of SOX10, NOTCH1, and FABP7 by siRNA increases sub-G1 cell population indicative of cell death. Histograms represent cell counts based on propidium iodide (PI) staining of Accx11 cells. Percentages of live cells in different cell cycle phases are shown.

II.3.4.8 NOTCH1 depletion and γ-secretase inhibition decrease the proportion of CD133+, suppress spheroidogenesis, inhibit tumor growth in nude mice, and sensitize CD133+ to radiation

Inhibitors of SOX10 and FABP7 are not available for clinical use at this time, but Notch is targeted through γ-secretase inhibitors (GSI) that have advanced to clinical trials.[343] The suppressive effects on spheroid formation and cell viability seen with NOTCH1 loss (Figure 24C-E) suggested that NOTCH1 is required for survival of CD133$^+$ ACC cells. To test this hypothesis, NOTCH1 was depleted in bulk Accx11 cells and the percentage of CD133$^+$ cells was measured. NOTCH1 depletion resulted in a ~2.5-fold decrease in the proportion of CD133$^+$ cells (Figure 26A). Bulk Accx11 cells treated with DAPT (a GSI developed by Eli Lilly) showed suppression of spheroid formation in a concentration-dependent manner (Figure 26B&C). To determine if γ-secretase activity is also required for maintenance of pre-formed spheroids, Accx11 cells were grown to confluency to allow spheroid formation as shown in Fig. 1B. Over a 9-day time course, a dose-dependent loss of pre-formed spheroids was observed in DAPT-treated Accx11 cells compared to controls (Figure 26D); however, the effect of γ-secretase inhibition on maintenance of preformed spheroids was not as dramatic as its effect on spheroidogenesis and plateaued after six days of treatment.

Effects of NOTCH1 inhibition on spheroids in Accx11 coupled with analyses showing that spheroids are enriched in CD133$^+$ cells suggested that Notch may be critical for maintenance and survival of CSC. To validate DAPT selectivity towards CD133$^+$ cells, ACC cells were CD133-sorted and treated with DAPT for 72 hours. DAPT treatment suppressed proliferation of CD133$^+$ cells with differences noted at all

doses (1, 5, and 10um), while proliferation of CD133⁻ cells was not affected (Figure

26E). FACS analysis confirmed selective DAPT effect demonstrating a ~2.5-fold

increase in sub-G1 population in CD133⁺ cells without obvious effects on the G1, S, and

G2/M ratios. Neither sub-G1 nor cell cycle progression was altered in CD133⁻ cells

(Figure 26F). Annexin and propidium iodide co-staining confirmed that DAPT treatment

selectively killed CD133⁺ cells, but did not reveal a pattern typical of apoptosis (Figure

25). Finally, to perform pre-clinical assessment of DAPT as a single agent, nude mice

were subcutaneously injected with Accx11 cells. In this study, DAPT had a statistically

significant tumor-suppressive effect starting at week 2 (Figure 26G) but exhibited no

obvious toxicity. Overall, these *in vitro* and *in vivo* data suggest that GSI effects as a

monotherapy should be evaluated in clinical trials with ACC patients.
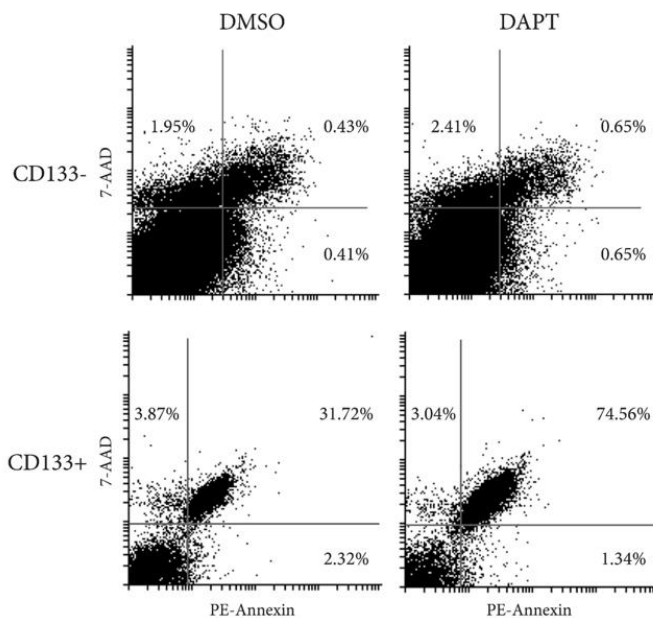
Figure 25: DAPT selectively increases cell death in CD133⁺ Accx11 cells.
PE-annexin and 7-AAD double staining of CD133⁻ and CD133⁺ Accx11 cells treated with DAPT. DMSO treatment serves as a control.
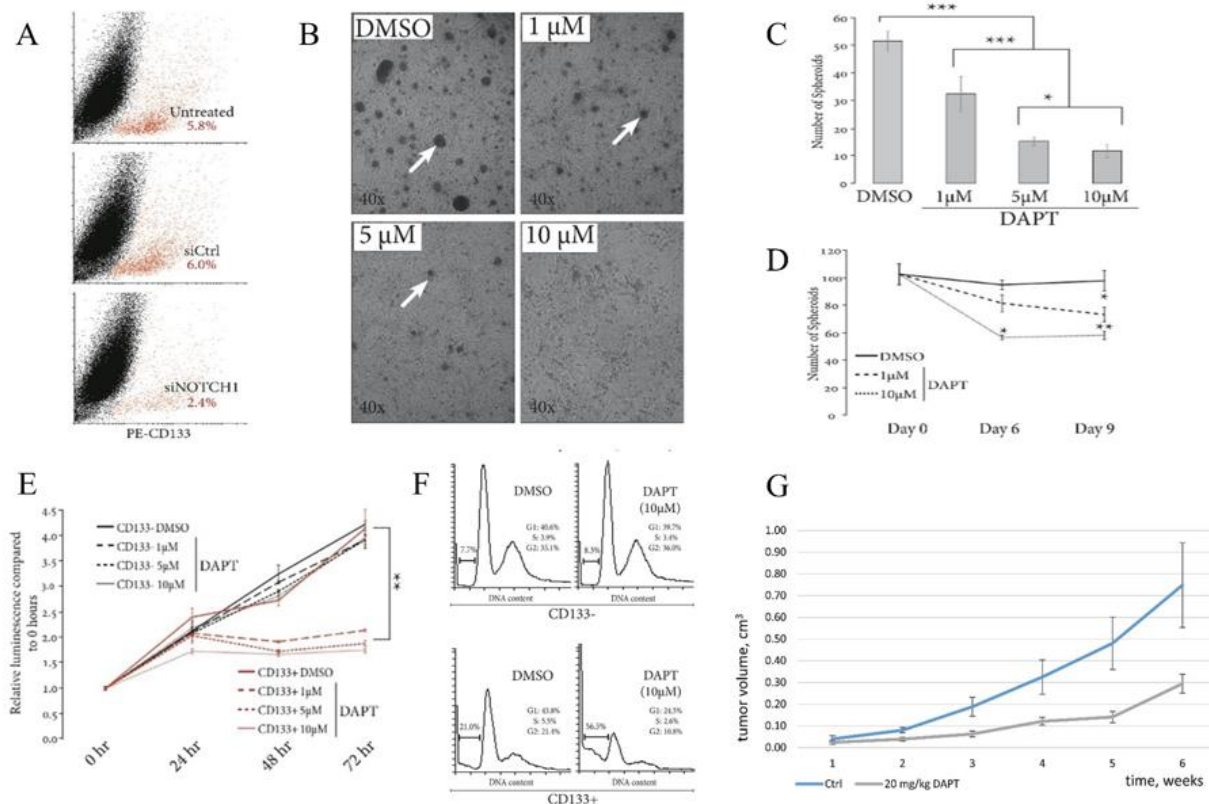
Figure 26: Inhibition of Notch signaling selectively depletes CD133⁺ cells.

(A) Suppression of NOTCH1 signaling by siRNA decreases the CD133⁺ cell fraction (red) as demonstrated by FACS analysis using PE-conjugated CD133 antibody. (B) Inhibition of Notch signaling with γ-secretase inhibitor DAPT demonstrates dose-dependent suppressive effects on spheroid formation (arrows) in Accx11 culture at 48 h. (C) Quantification of DAPT effects on spheroid formation compared to control (DMSO-treated) Accx11 cells, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), Student's t-test. (D) Breakdown of pre-formed spheroids induced by DAPT as shown by quantification of remaining spheroids following DAPT treatment versus control (DMSO-treated) Accx11 cells, $p<0.05$ (*), $p<0.01$ (**). (E) Inhibitory DAPT effect on cell proliferation is highly CD133⁺-selective and dose-dependent. At 72 h, there is a statistically significant decrease ($p<0.01$) in luminescence (Cell Titer Glo assay), $p<0.01$ (**). (F) DAPT selectively induces cell death in the CD133⁺ population as demonstrated by increase in sub-G1 cell population. Histograms represent cell counts based on PI-staining of separated Accx11 cells. (G) Suppressive effect of DAPT on tumor growth in a nude mouse model subcutaneously injected with Accx11. Starting at week2, $p<0.01$.

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Because CSC have been implicated in radiation resistance in many tumor types,[336] the effect of targeting CD133$^+$ cells through Notch inhibition in combination with radiation was determined by treating with DAPT for 24 hours and then exposing cells to increasing doses of radiation. Radiation alone had minimal effect on the amount of CD133$^+$ cells: even 6 Gy of radiation decreased this fraction only moderately, from 2.7 to 2.3 %, which translates into a ~15% depletion (Figure 27). However, DAPT as a single agent showed a two-fold (~56%) decrease in the proportion of CD133$^+$ cells. Remarkably, when a single 3 Gy or 6 Gy dose of radiation was added to DAPT treatment, the combined effect resulted in an almost 2-fold enhancement of CD133$^+$ cell depletion as compared with DAPT alone (compare 0.7% with 1.2% in Fig. 5H, $p<0.05$). The enhanced loss of CD133$^+$ ACC cells following radiation in the presence of DAPT suggested that combination of radiation with GSI therapy may be worth exploring in this radiation-resistant tumor.
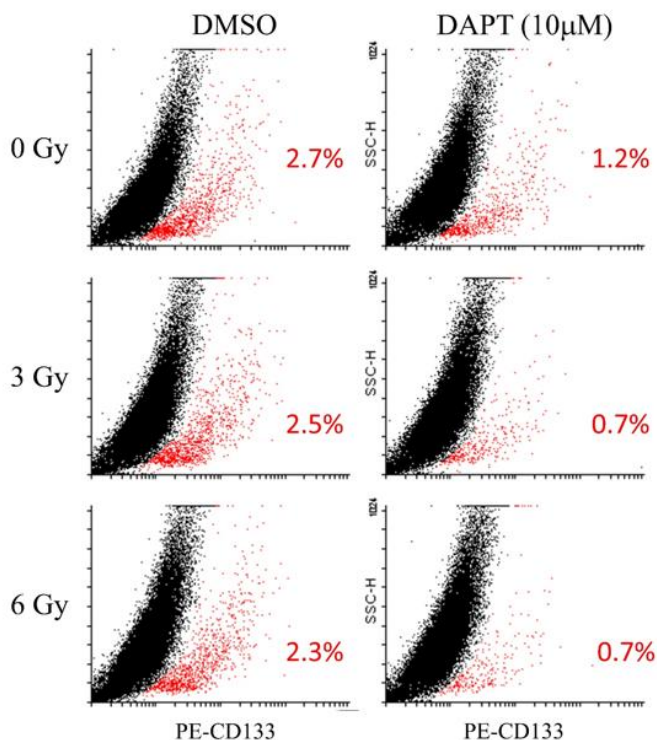
Figure 27: Combination of DAPT with radiation is more detrimental to CD133$^+$ cells (red) than DAPT or radiation alone.
Representatives of 3 replicates are shown ($p<0.05$ in all comparisons except 3 Gy vs 6 Gy, radiation only).

II.3.4.9 NOTCH1 depletion down-regulates SKP2, an E3 ubiquitin ligase that targets p27^{Kip1}

Activated NOTCH1 reduces p27^{Kip1} levels by stimulating expression of the p27^{Kip1} E3 ubiquitin-ligase SKP2.[344] Identification of SKP2 downstream from a NOTCH1 effector, FABP7 (Table 4), suggested that SKP2 and p27^{Kip1} are engaged in the pro-survival NOTCH1 signaling in stem-like ACC cells. Indeed, decreased expression of p27^{Kip1} was noted in CD133$^+$ Accx11 cells, where NOTCH1 was activated (Figure 22C&F). Assessment of SKP2 expression by qRT-PCR and immunoblotting showed up-regulation of SKP2 in CD133$^+$ compared to CD133$^-$ cells (Figure 28A&B). Immunofluorescent staining of Accx11 cultures revealed that SKP2 was detected primarily in NR2F1-negative spheroidal cells, and was localized to the cytoplasm, where it has been associated with oncogenic activity (Figure 28C).[345] Since SKP2 is a transcriptional NOTCH1 target, inhibition of NOTCH1 activity is expected to suppress SKP2 expression and, in turn, increase p27^{Kip1} protein stability and levels. In agreement with this expectation, siRNA-mediated NOTCH1 knockdown dramatically decreased SKP2 mRNA levels in Accx11 cells, as did DAPT treatment in a dose-dependent manner (Figure 28D&E). In order to determine if Notch effects on SKP2 and p27^{Kip1} are seen predominantly in CD133$^+$ cells, bulk Accx11 cells, CD133$^+$, and CD133$^-$ populations were treated with DAPT for 48 hours, and then cell lysates immunoblotted. As expected, CD133$^+$ cells expressed higher levels of N1ICD than bulk or CD133$^-$ cells, and DAPT effectively inhibited NOTCH1 activation as marked by decreased N1ICD in these cells (Figure 28F). SKP2 was detected at very low levels in bulk and in CD133$^-$ Accx11 cells (long exposure, data not shown), but was readily detected in CD133$^+$ cells,

whereas p27^{Kip1} was detected in bulk cells and in CD133^{-} cells at much higher levels

than in CD133^{+} cells. Treatment with DAPT did not markedly alter expression of p27^{Kip1}

in bulk or CD133^{-} cells, but dramatically enhanced its expression in CD133^{+} cells

(Figure 28F). Together, these data suggested that NOTCH1 inhibition up-regulates

p27^{Kip1} selectively in CD133^{+} cells.
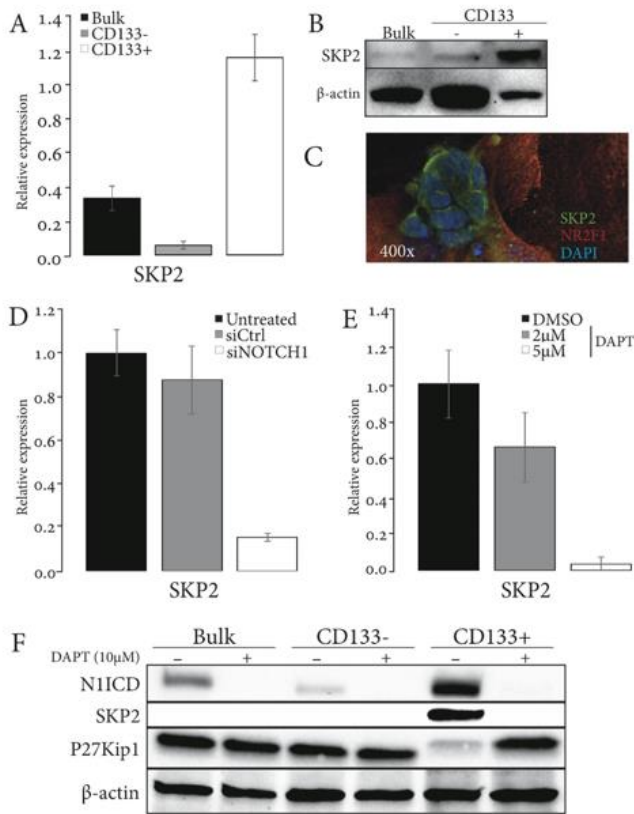
Figure 28: CD133^{+}-cell-selective and NOTCH1-dependent SKP2 expression in ACC cells.

(A-C) SKP2 is selectively expressed in CD133^{+} Accx11 cells as demonstrated by (A) qRT-PCR and (B) immunoblot. β-actin serves as a control. (C) Spheroid-forming CD133^{+} cells show cytoplasmic localization as demonstrated by IF. (D-E) Inhibition of Notch signaling by (D) siRNA and (E) DAPT decreases expression of SKP2 in bulk Accx11 cells (qRT-PCR). (F) DAPT blocks expression of activated NOTCH1 (N1ICD) and its target SKP2 in CD133^{+} ACC cells and up-regulates p27Kip1, a SKP2 substrate.   β-actin serves as a loading control.

II.3.5 Discussion

CSCs isolated from various malignancies possess many features of embryonic or tissue stem cells and can be targeted via highly conserved Notch, Hedgehog (HH), and Wnt pathways activated in these cells.[346] While previous studies confirmed the existence of CSC in ACC using ALDH as a marker,[309] difficulties with ACC culture and lack of reliable stem cell markers prevented their characterization. Our recent studies provided a set of novel ACC markers (NOTCH1, SOX10, FABP7) and other genes whose expression is associated with propagation and prevention of differentiation of NSCs,[200, 263] suggesting that isolation of CSC from ACC may be possible. This study developed and implemented a novel technology and an additional set of stem cell markers for isolation, propagation, reliable validation, and characterization of these cells. For the first time, ACC cells with CSC properties were isolated directly from tumor tissue or propagated and purified from primary ACC cultures. Using these new approaches, a subpopulation of ACC cells expressing SOX10, NOTCH1, and FABP7 was identified that also expressed CD133, a cell surface marker. As demonstrated, ACC cells that express CD133 have basic characteristics of CSC including the ability to generate both CD133$^+$ and CD133$^-$ populations, form spheroids in culture, and initiate tumors in nude mice.

Gene knockdown studies highlighted the essential roles and potential functional cooperation between NOTCH1 and SOX10 in maintenance of CD133$^+$ ACC cells. An important insight into this cooperation, which has not been previously appreciated, was provided by the co-expression of FABP7 that relied on both NOTCH1 and SOX10, and the role of FABP7 in regulating expression of genes critical for cell survival. FABP7 has

been recently described as a novel diagnostic and prognostic ACC marker[114] and was also implicated in glioblastoma, melanoma, and basal-like breast cancer; however, its downstream targets and molecular mechanisms of activation have not been studied. The current data associate FABP7 with the expression of SKP2 and multiple other genes involved in survival, proliferation, and metabolic pathways pointing at a novel signaling pathway that stimulates CSC in ACC.

In this study, SOX10 was one of the most consistent markers of CD133^{+} stem-like ACC cells. Expression of SOX10 is also seen in other cancers with neural crest lineage suggesting that they may contain similar stem-like cells. SOX10 expression has been reported in basal-like breast carcinomas, a histologically and clinically distinct subtype of breast cancer that lacks targeted therapy and expresses NOTCH1 and FABP7.[263, 298] It would be interesting to determine if basal-like breast carcinoma is SOX10- and NOTCH1-dependent similar to ACC and other neural crest-derived cancers such as melanoma, neuroblastoma, and glioblastoma.

ACC is a radioresistant tumor, and currently there are no curative treatment options for patients with unresectable disease. Targeting CSC via Notch has been linked with sensitization of glioblastoma to radiation.[319] Thus, Notch-targeting therapies offer a new opportunity for ACC, and while there are no available drugs to inhibit SOX10 or FABP7, agents targeting Notch are currently in clinical trials.[330] This study demonstrated that activated NOTCH1 stimulates proliferation and survival of ACC cells with neural stem properties. In line with this scenario, NOTCH1 targeting selectively inhibited proliferation of CD133^{+} ACC cells and triggered cell death resulting in depletion of CD133^{+} cells. Notch inhibitors have strong antineoplastic activity in numerous

preclinical models when combined with DNA damaging therapies,[346] and here DAPT

had single agent efficacy in pre-clinical ACC models and also sensitized CD133$^+$ ACC

cells to γ-irradiation. Overall, these experiments provided preliminary data that stimulate

interest in Notch inhibitors for ACC therapy either as a single agent or in combination

with radiation.

Mutations in the NOTCH1 gene are detected in 5% of ACCs,[78, 79, 347] The low

incidence of these mutations, however, suggests the existence of other mechanisms

that trigger Notch activation in ACC. Intrinsically high NOTCH1 expression in ACC is an

important pre-requisite for its activation, achieved via ligand-binding. Hence,

association of NOTCH1 with JAG1, a NOTCH1 ligand produced by CD133$^-$ cells,

provides a potential mechanism for oncogenic NOTCH1 activation and aggressive

tumor growth in these tumors. Indeed, up-regulation of JAG1 has been previously

linked to NOTCH1 activation and poor survival in breast, pancreatic, and ovarian

cancers.[332]

In search of additional actionable targets associated with NOTCH1 signaling in

ACC, p27^{Kip1} and its ubiquitin ligase SKP2 were identified. SKP2 and p27^{Kip1} were

differentially expressed in CD133$^+$ and CD133$^-$ cells, with CD133$^+$ cells expressing

SKP2 and CD133$^-$ cells expressing p27^{Kip1}. Inhibition of NOTCH1 markedly decreased

SKP2 levels in CD133$^+$ cells and increased protein levels of p27^{Kip1}. In addition to its

central roles in the regulation of proliferation, apoptosis, and senescence, which are

mediated at least partially through degradation of p27^{Kip1},[348] SKP2 is a tumor survival

factor during energy stress.[349] Multifaceted SKP2 activities at a crossroad of many

oncogenic signaling axes suggest that SKP2 may be an additional therapeutic target in ACC.

Overall, characterization of neural stem-like cells in ACC and delineating signaling events essential for their maintenance provide a new concept for ACC research and treatment that focuses on cancer cells with neural stem properties. Notch inhibitors that show promise in neuroblastoma and brain tumors[330] may be used as therapy for ACC in combination with Trk inhibition that was previously tested on PDX[200] and cytotoxic modalities. Future combination strategies for ACC may also include SKP2 inhibitors that are currently in development.[317]

III. SUMMARY AND FUTURE DIRECTIONS

III.1 Summary of studies

Some objectives of this work were to: 1) identify molecular markers distinguishing ACC from normal salivary gland and other common malignancies of the head and neck; 2) test the role of identified markers in ACC development and progression; 3) create and validate one or more sustainable ACC cell cultures, and; 4) identify potential targets for ACC therapy. Presented here are data which support progress made toward accomplishment of these objectives.

Using microarray datasets from ACC, MEC, HNSCC, AD, and NSG specimens, a signature of genes centered around a strong cluster of TrkC probes was identified. This signature suggested characteristics of neural crest stem cells (NCSCs), as well as the Notch and Wnt/β-catenin pathways. While at the time, cell lines were not available for ACC, an exogenous expression model of TrkC in U2OS cells devoid of endogenous expression of neurotrophin ligands or receptors allowed further investigation of how TrkC might be functioning in ACC. Activation of TrkC signaling by addition of its ligand induced TrkC phosphorylation, stimulated proliferation and anti-apoptotic pathways, cell motility, migration, invasion, and anchorage independent cell growth. All NT-3-induced effects were reversible by pan-TrkC inhibitor AZD7451. Additionally, *in vitro* studies using ACC xenografts suggest that TrkC inhibition may be useful for inhibiting tumor growth. While these results suggest a role of TrkC in ACC, further studies are needed in ACC cell lines to elucidate the role it is serving within this tumor.

Without cell lines available to extend the studies on TrkC, reanalysis of expression array data landed focus on SOX10, a gene well described as a marker of

NCSCs. Additionally, SOX10 had been identified as a critical factor for melanoma (melanocytes are derived from neural crest) formation and progression. Immunostaining of a head and neck TMA confirmed strong staining within ACC specimens and clear absence of SOX10 in the vast majority of MEC and HNSCC. Analysis of publicly available datasets found strong SOX10 gene signatures within tumors of neurologic origin (melanoma, glioblastoma, and neuroblastoma), as well as the basal-like subtype of breast cancer. Comparison of the identified gene signatures with the one generated from our own ACC datasets revealed substantial overlap, with the greatest similarity existing between ACC and basal-like breast cancer. Interestingly, these tumors are both believed to arise from myoepithelial cell-containing areas of structurally similar glands. In addition to being a sensitive biomarker of these tumors, basal-like breast cancer cell lines harboring SOX10 expression maintained characteristics of both positively- and negatively-associating SOX10 signature genes in culture, suggesting that this network may be important for maintenance of these cells.

In order to further analyze the role of SOX10 in ACC, tumor cells from ACC xenografts were successfully cultured by utilizing a new technique for cell culture. A robust culture, Accx11, continued to grow in culture beyond 30 passages and maintained expression of ACC markers including SOX10. In culture, Accx11 cells displayed characteristics of stem-like cells including spheroidogenesis. Cell sorting based on expression of CD133 of Accx11 cells and dissociated xenografts generated a CD133$^+$ cell population enriched with markers of ACC including SOX10, NOTCH1, and FABP7, as well as downstream targets of Notch signaling. Conversely, CD133$^-$ cells expressed markers of neural differentiation and the Notch ligand JAG1, suggesting that

CD133$^-$ cells may differentiate from CD133$^+$ cells and function to support the CD133$^+$ cell population. Mouse injections of sorted cells confirmed CD133$^+$ cells as being more tumorigenic than their CD133$^-$ counterparts. Knockdown experiments using siRNA demonstrated interrelated signaling between SOX10, NOTCH1, and FABP7, and that CD133$^+$ cells were dependent upon each of these proteins. Although pharmacological agents are not available for inhibition of SOX10 and FABP7, Notch pathway inhibition using the γ-secretase inhibitor, DAPT, reduced the CD133$^+$ cell population, suppressed spheroidogenesis, and sensitized CD133$^+$ cells to radiation. These data open up new avenues to be explored in the treatment of ACC.

III.2 Future directions

The findings in these studies have provided new insights into the molecular landscapes and signaling networks involved in ACC. In particular, generation of the short- and long-term ACC cultures creates an experimental platform for testing new ideas. In addition, isolation and characterization of CSC in ACC creates a new concept of CSC targeting in this tumor type.

III.2.1 SOX10 in breast cancer

While ACC remains an insidious tumor with poor patient outcomes, it is an orphan tumor affecting approximately 1200 people annually in the United States. Some of the most exciting findings presented here are the conserved expression patterns seen in distinct tumor types that share their neural crest origin. A conserved signaling pathway in neural crest-associated tumors suggests that targeting this pathway may have far-reaching effects on a myriad of tumors. Amongst tumors examined, basal-like breast cancer demonstrated the highest similarity to the SOX10 co-expression gene

profile found in ACC. Basal-like breast cancer is a common malignancy with severely limited treatment options. Importantly, SOX10 is critical for melanoma tumorigenesis, maintenance, and progression, and data presented here suggests that SOX10 plays a critical role in the maintenance of stem-like cells within ACC.

The most strongly negatively correlating gene to SOX10 in both ACC and basal-like breast cancer is FOXA1. While the implications of FOXA1 expression in ACC remain unknown, in breast cancer, it can have profound implications. In luminal breast cancers, FOXA1 is required for ~50% of the function of ESR1, suggesting that the lack of FOXA1 in basal-like tumors may, in part, contribute to the lack of ESR1 expression in these tumors. Moving forward, studies inhibiting SOX10 expression in basal-like breast cancer cell lines MX-1 and HCC1569 will help to identify how SOX10 is contributing to the behavior of these tumors. Potentially, even if SOX10 inhibition alone is incapable of killing tumor cells, this may reverse FOXA1 suppression allowing for ESR1 function. While SOX10 is not currently targetable by clinically available therapeutics, SOX10 targeting could allow for new avenues for pharmaceutical intervention.

III.2.2 Generation of ACC cultures from primary tumors

The history of cell culture in ACC is a sordid affair. From contamination to unexplained losses of DNA mutations central to the parent tumor, ACC cultures have been notoriously unreliable. Now that a reliable culture system for ACC cells dissociated from tissues has been optimized, it should be utilized to create additional cultures. In addition to providing tools for ACC research, additional cultures will allow for validation of findings and avoid limitations and biases that limited numbers of specimens or cultures can produce. While Accx11 displays markers characteristic of

ACC, it is derived from xenografted tissue, which will have changed due to the selection induced by growing in mice. As experience culturing with the CRC system has increased, the ability to successfully generate cultures from small tissue specimens has improved. This is important as, moving forward, the goal is to produce cultures from primary ACC tumors without the need for xenograft expansion.

III.2.3 TrkC function in ACC cell culture

Accx11 maintains a large variety of ACC markers, which can be manipulated for *in vitro* and *in vivo* studies. In particular, TrkC results, generated primarily in U2OS cells, will be reassessed. Currently planned are proliferation, migration, invasion, and expression studies on Accx11 cells with or without stimulation of TrkC with its ligand, NT-3. Additionally, having already identified CD133^{+} stem-like cells in this culture, future studies will focus on assessing TrkC expression in these cell subpopulations. Should TrkC expression be associated with any of the phenotypes seen previously in U2OS cells *in vitro*, injections of Accx11 cells for mouse xenograft experiments using AZD7451 or other Trk-family inhibitors will be performed to see how pharmacological intervention may affect these phenotypes *in vivo*.

III.2.4 MYB-NFIB and MYBL1-NFIB gene fusions in ACC

Until now, studies have only been able to confirm the existence of the MYB-NFIB and MYBL1-NFIB gene fusions or analyze the expression of MYB in ACC tumors. The ability to culture ACC cell from xenografts and primary tumors provides a unique opportunity for studying these fusions and their roles in ACC. Break-apart FISH studies demonstrated MYB-NFIB gene fusions in seven of the nine PDX models described and

used in our lab, including ACCX11. Preliminary studies on current ACC cultures

suggest the presence of at least two distinct MYB-NFIB fusions which have been

previously described. Using si/shRNA and CRISPR targeting sequences within the

MYB open reading frame, MYB expression will be suppressed in culture in order to

determine expression and cell behavior changes *in vitro*. Further studies are planned to

determine the role MYB serves in tumorigenicity *in vivo* and to determine if MYB

expression is limited to CSCs or alters CSC behavior. Particular interest will be focused

on inducible loss of MYB in order to determine if established tumors maintain

dependence on MYB.

III.2.5 NOTCH inhibitors in development

The presented studies demonstrate that inhibition of NOTCH selectively kills

ACC cells with stem-like properties *in vitro* resulting in reduced tumorigenicity.

However, clinically, GSIs have primarily performed poorly as a single agent, largely due

to gastrointestinal side effects induced by even low doses. Fortunately, efforts are

being made to create small molecules and antibodies to inhibit NOTCH that do not

function through γ-secretase. These inhibitors intend to specifically target NOTCH

isoforms, which in turn will reduce side effects in noncancerous cells. As these drugs

become available, their efficacy will be tested on ACC cultures.

III.2.6 Combinatorial treatment of ACC

As with most tumors, it is unlikely that a single agent will be successful in treating

ACC, due to the development of resistance. Identification of additional pathways

employed in ACC for tumorigenic purposes will hopefully allow a combination of

therapies for control, palliation, or cure of this disease. These studies have

demonstrated that TrkC, SOX10, and NOTCH1 all play important roles in the

development and maintenance of ACC. Moving forward, effects of combinatorial

inhibition of NOTCH1 and TrkC on tumorigenesis of injected cells and xenografts will be

studied. Additionally, the presented *in vitro* studies suggest that NOTCH inhibition with

GSI can sensitize ACC cells with stem-like properties to radiation, something important

to confirm *in vivo*. Other potential pathways for therapy include MYB (as discussed

above) and WNT signaling, both of which are topics for further study using the tools and

cultures that we have created.

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