INVESTIGATING THE ROLE OF REACTIVE ALDEHYDES IN THE DEVELOPMENT OF HEAD AND NECK SQUAMOUS CELL CARCINOMA IN FANCONI ANEMIA

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Abstract

Investigating the Role of Reactive Aldehydes in the Development of Head and Neck Squamous Cell Carcinoma in Fanconi Anemia

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Fanconi anemia (FA) is a rare genetic disorder. Patients with FA are cancer-prone and commonly develop acute myeloid leukemia and several solid tumors, including head and neck squamous cell carcinoma (HNSCC). Typically, the FA DNA repair pathway, which is responsible for the repair of DNA interstrand crosslinks, is ubiquitously found in all tissues; however, FA patients lose the FA DNA repair pathway in all their tissues. Despite the absence of this pathway in all their tissues, the epithelium of the head and neck is disproportionately burdened with solid tumors, and little is known regarding why this epithelium is a major site for cancer in FA patients. The objective of my dissertation is to elucidate cellular mechanisms that explain why FA patients are highly susceptible to HNSCC. Formaldehyde and acetaldehyde are two aldehydes generated via normal cellular metabolic activity, and it has been proposed that both aldehydes are predominant contributors to DNA crosslinking events in humans. I measured endogenous aldehyde production, and I tested the effect of formaldehyde and acetaldehyde on human cell lines including oral keratinocyte cell lines and an epidermal keratinocyte cell line to understand more about the activity of the FA DNA repair pathway and the impact of these genotoxins

(i.e., formaldehyde and acetaldehyde) on the genome. Oral keratinocytes were compared to epidermal keratinocytes because there is not a high incidence of epidermal squamous cell carcinoma reported in FA patients. I found that endogenous aldehyde production is unlikely to be a contributing factor to HNSCC in FA patients based on data extrapolated from cell lines. I found that oral keratinocytes are more resistant to formaldehyde and acetaldehyde compared to the epidermal keratinocytes. Resistance to exogenous formaldehyde and acetaldehyde correlated with increased activation of the FA DNA repair pathway and γH2AX in the oral keratinocyte cell lines, which suggests that the oral keratinocyte cell lines may be more dependent on the FA DNA repair pathway for protection against exogenous sources of aldehydes. Conversely, the failure of the same concentration of exogenous aldehydes to activate the FA DNA repair pathway in the epidermal keratinocyte cell line suggests dependence on enzymes that catabolize aldehydes, and aldehyde dehydrogenase 2 has been reported to be expressed in the epidermis at higher levels compared to the oral mucosa. My research findings suggest that exogenous sources of aldehydes and the reliance on the FA DNA repair pathway for protection in the oral keratinocytes may explain the increased frequency of HNSCC in FA patients. This work is a leap towards understanding the complex intersection of genomic and genotoxic factors that contribute to and drive HNSCC development in FA patients, and findings could potentially lead to the development of therapeutic interventions.

DEDICATION

Dedicated to those like me who unapologetically walk to the beat of their own drum.

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TABLE OF CONTENTS

CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW

Head and neck squamous cell carcinoma (HNSCC) is a global public health burden [1]. HNSCC is cancer of the oral cavity and surrounding structures of the head and neck. HNSCC begins in the squamous cells that line mucosal surfaces of the head and neck. Globally, HNSCC is the sixth most common cancer [2]. Well-known risk factors for HNSCC include tobacco use and alcohol consumption [2-5]. Additional risk factors include infection with certain types of human papillomavirus (HPV) such as HPV type 16 and paan (betel quid) use [2, 6]. Some genetic disorders also predispose patients to cancer. Fanconi anemia (FA) is a genetic disorder, and patients affected by FA are predisposed to HNSCC [7, 8]. Little is known regarding the cellular etiology that explains why there is a disproportionate burden of cancer of the head and neck environment in FA patients. FA provides a unique lens and opportunity to investigate carcinogenesis of the head and neck squamous epithelia. If we can uncover factors that contribute to and drive HNSCC in FA patients, such findings may not only help FA patients, but may extend to the broader population of individuals who are affected by HNSCC but do not have FA. The purpose of this dissertation is to investigate cellular and molecular mechanisms that can help elucidate why FA patients are susceptible to developing HNSCC.

FA is a rare, multi-faceted genetic disorder that is characterized by a myriad of congenital and acquired anomalies that are hematologic and oncologic [9, 10]. FA was first described nearly 100 years ago by Dr. Guido Fanconi, a Swiss pediatrician and scientist [9]. In 1927, Dr. Fanconi described FA after observing peculiar hematologic and developmental phenotypes in his pediatric patients who were siblings [9]. FA is inherited

in an autosomal recessive or X-linked manner [11, 12]. FA affects males and females relatively equally with a male-to-female ratio of 1.2:1 [13].

FA is present in all races and ethnic groups; however, FA most commonly affects individuals of Ashkenazi Jewish ancestry, indigenous populations of South Africa, and the Roma population of Spain [13, 14]. FA is also seen in populations in North America. In 2010, it was estimated that there were approximately 550-975 persons with FA living in the United States [15]. The carrier frequency is a metric that describes the proportion of individuals who carry the recessive allele for one of the 22 FA genes, and in the United States, the carrier frequency for FA is 1 in 181 persons [15]. The incidence rate, which measures the chances that a child will be born with FA, is approximately 1 in 131,000 in the United States [15]. It is estimated that there are roughly 4 million births in the United States annually and of those 4 million, about 31 babies are born with FA [15, 16].

As the name implies, FA is primarily a blood disorder. Hematologic complications experienced by patients with FA include pancytopenia, anemia, leukopenia, thrombocytopenia, and macrocytosis [17]. Pancytopenia occurs when there is a deficit in the production of the three cellular components of blood: red blood cells, white blood cells, and platelets. Macrocytic anemia features enlargement of the red blood cells. Since FA is characterized by defects in all the blood lineages, the disorder involves disruption of the hematopoietic stem cells.

FA patients have numerous and variable clinical features which include progressive bone marrow failure (also known as bone marrow aplasia), short stature,

developmental abnormalities and delays, craniofacial defects, cardiac deficiencies, endocrine dysfunction, and an increased susceptibility to certain cancers including acute myeloid leukemia and squamous cell carcinoma [10]. FA patients have distinct oral clinical features, and specific oral findings have been reported in FA patients. Oral manifestations that have been observed in FA patients include gingival bleeding, melanin pigmentation in the oral cavity, dental biofilm, gingivitis, periodontitis, carious lesions (cavities), supernumerary teeth (extra teeth), alveolar bone resorption, microdontia (small teeth), and congenitally missing teeth [17-20]. Some of the oral conditions observed are attributed to poor dental hygiene, not necessarily the underlying disease; however, additional studies need to be completed to assess the oral state of FA patients so that a more comprehensive and accurate picture can be captured across a spectrum of patients [17].

The clinical features observed in FA patients stem from sensitivity to DNA crosslinking agents. FA patients are extremely hypersensitive to DNA crosslinking agents [21]. DNA crosslinking agents chemically modify the DNA to its detriment [22]. A DNA interstrand crosslink forms when a covalent bond is created between the two opposing strands of DNA. This cytotoxic bond forms when crosslinking agents react with the nucleotides of the two opposing DNA strands, resulting in the formation of a covalent bond between the two strands. The covalent bond is detrimental because it distorts the DNA double helix, and such distortion is conducive for chromosomal breakage. Furthermore, chromosomal breakage leads to genomic instability, a hallmark of cancer. Genomic instability is favorable for cancer initiation and progression. Additionally, DNA crosslinks also hinder DNA replication and transcription [23]. DNA crosslink inducers

can be endogenous or exogenous. Endogenous DNA crosslinking agents are produced intracellularly via normal metabolic activity while exogenous crosslinking agents come from the extracellular environment. Aldehydes produced via normal metabolic activity are examples of endogenous crosslinkers, and FA patients are sensitive to crosslink-inducing aldehydes [24]. Chemotherapeutic drugs and UV radiation are exogenous DNA crosslinkers [25-27]. Many chemotherapeutic drugs work by intentionally inducing DNA crosslinks in cells; in turn, the crosslinks interrupt normal cellular processes, and eventually the cells undergo apoptosis [26, 27]. DNA crosslinking agents are used to diagnose FA in the clinical setting via the chromosome break test. In the chromosome break test, a crosslinking agent such as mitomycin C (a chemotherapeutic agent) or diepoxybutane is added to the cells of a potential FA patient; such cells can be peripheral blood T lymphocytes or skin fibroblasts [10, 28-31]. If the patient has FA, the chromosomes will break upon exposure to the genotoxic crosslinker due to extreme genotoxic sensitivity [10]. Fortunately for FA patients, the DNA repair system that remedies UV-induced DNA damage is intact. UV-induced DNA damage is repaired via nucleotide excision repair [32]. Unfortunately for FA patients, they are susceptible to a host of crosslinking agents that induce DNA damage that goes unrepaired due to the absence of a functional FA DNA repair pathway.

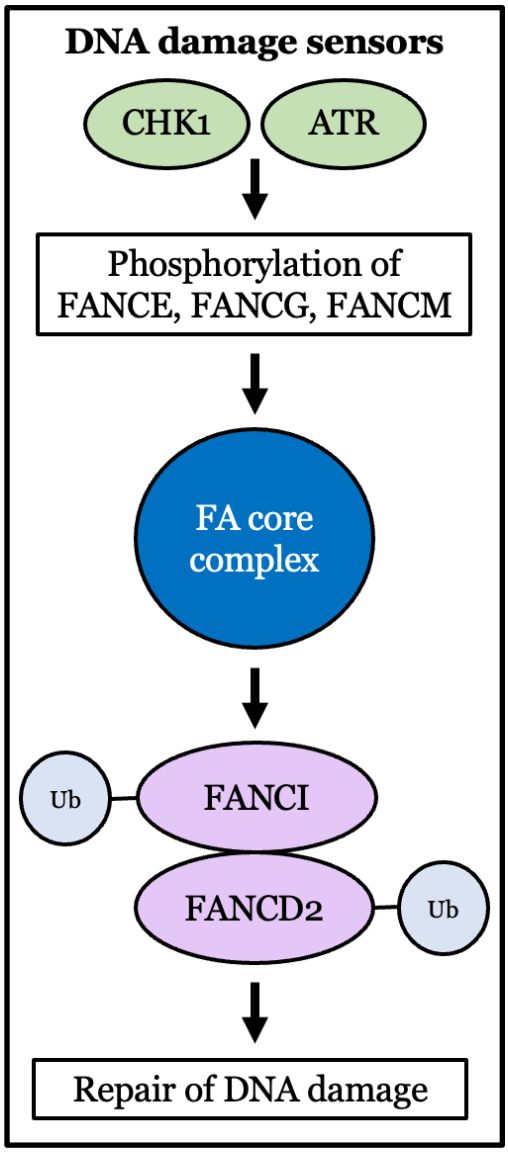

Figure 1.1: Adapted from (Nalepa G, Clapp DW. *Nat Rev Cancer*. 2018.)[10] At a molecular level, FA is characterized by faulty DNA repair. The FA DNA repair pathway functions as a repair system that maintains genomic stability. The pathway preserves the integrity of the genome by repairing DNA interstrand crosslinks. FA patients do not have a functional FA DNA repair pathway, hence their hypersensitivity to DNA crosslinking agents. In the FA DNA repair pathway, DNA damage sensors, CHK1 and ATR, detect DNA damage. Upon recognizing DNA damage, CHK1 phosphorylates FANCE and ATR phosphorylates FANCG and FANCM. The phosphorylation of FANCE, FANCG, and FANCM stimulates the assembly of the multiprotein FA core complex. The core complex is critical because the core complex aids in the mono-ubiquitination of the FANCD2/FANCI heterodimer. The mono-ubiquitinated FANCD2/FANCI heterodimer binds to chromatin at the site of the interstrand crosslink and orchestrates the repair of the DNA damage. Once the DNA damage is resolved, the FANCD2/FANCI complex is deubiquitinated, rendering the FA pathway inactive, and the heterodimer is removed from chromatin.

In the cells of healthy individuals who do not have FA, the FA pathway functions as a DNA repair system that maintains genomic stability [33]. The FA pathway is also referred to as a tumor-suppressive pathway that guards the human genome [34]. The primary function of the FA pathway is to repair DNA damage, specifically DNA interstrand crosslinks [23]. FA develops when there are germline mutations in any of the genes that code for the FA proteins that makeup the FA DNA repair pathway. Currently, there are 22 known genes in the FA DNA repair pathway, and a mutation in any one of these can lead to the development of FA [10]. The three genes commonly mutated in FA patients include FANCA, FANCC, and FANCG which are a part of the FA core complex; mutations in FANCA, FANCC, and FANCG account for approximately 80-90% of all FA cases worldwide [28]. The gene most commonly mutated in FA patients in the United States is FANCA [28].

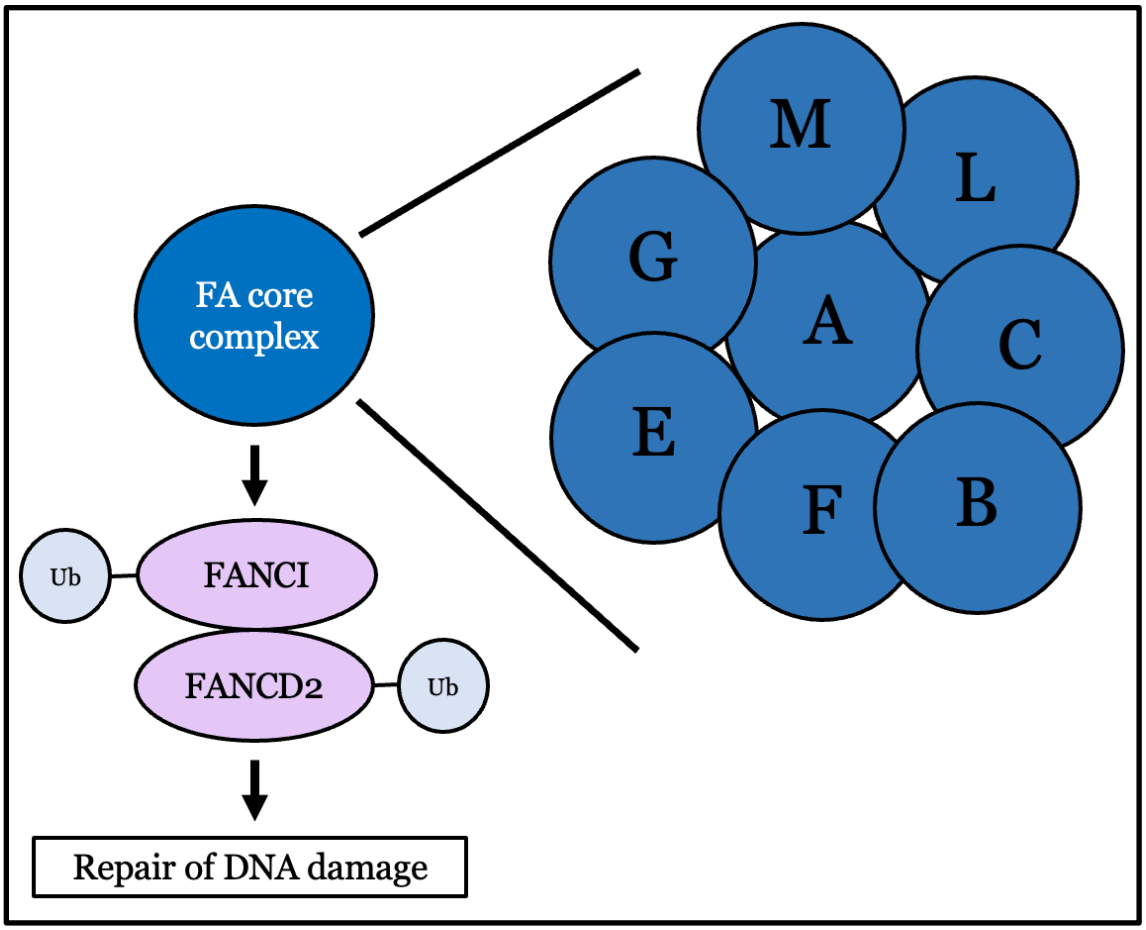

Figure 1.2: Adapted from (Nalepa G, Clapp DW. *Nat Rev Cancer*. 2018.)[10] and (Green AM, Kupfer GM. *Hematol Oncol Clin North Am*. 2009.)[11] The FA core complex consists of eight FANC proteins; this multiprotein complex is essential for the mono-ubiquitination of FANCD2. Mono-ubiquitinated FANCD2 is the final step in a functional FA DNA repair pathway. Upon mono-ubiquitination of FANCD2, DNA damage (i.e., DNA interstrand crosslinks) can be repaired. Resolution of DNA damage is critical in maintaining cellular health and viability.

A key player in the FA DNA repair pathway is the protein FANCD2. FANCD2 forms a heterodimer with FANCI, and the FANCD2/FANCI dimer is essential for the repair of DNA interstrand crosslinks upon mono-ubiquitination of FANCD2 and FANCI [35, 36]. Upon mono-ubiquitination, the FANCD2/FANCI heterodimer binds to the chromatin at the site of the interstrand crosslink to initiate DNA repair. Hölzel et al. (2003) showed that in normal human tissues, FANCD2 expression is observed in proliferative tissue

types. These tissues are then predisposed to cancer in FA [37]. For example, Hölzel et al. (2003) showed FANCD2 expression in the head and neck epithelia [37]. While FANCD2 may be present in cells at baseline, the protein is only active and therefore capable of fulfilling DNA repair responsibilities upon mono-ubiquitination. When mono-ubiquitination occurs, a ubiquitin tag is added to FANCD2, thus rendering it active. Monoubiquitinated-FANCD2 serves as a reporter for activity of the FA DNA repair pathway because it is among the final proteins that within the pathway cascade that is activated following the assembly of the FA core complex.

FA patients have a high risk of developing aggressive HNSCC at a relatively young age, and unfortunately the patients have few treatment options; the primary treatment for the HNSCC in FA patients is surgery to remove the malignant tumors [7, 38, 39]. The incidence of HNSCC in FA patients is approximately 600-fold higher than in the general population [28]. Of the FA patients who survive to age 40, about 1 in 7 (14%) will be diagnosed with HNSCC [7]. In a literature review that reported cancer in FA patients from 1927-2012, of all solid tumor cancers reported, approximately 40% were HNSCC [28]. The 40% of solid tumors of the head and neck consisted of patients who sporadically developed HNSCC and those who developed HNSCC post bone marrow transplant [28].

FA patients have three primary hurdles that they encounter: progressive bone marrow failure, acute myeloid leukemia, and HNSCC. To overcome the first two hurdles, progressive bone marrow failure and acute myeloid leukemia, the most common and effective treatment is hematopoietic stem cell transplantation (HSCT) [40]. HSCT is a common treatment for patients with FA and is effective at restoring hematopoietic

function in patients; however, HSCT does not cure the entire disease. One of the side effects of HSCT is the development of oral chronic graft-versus-host disease (cGVHD) which is an additional risk factor for malignancies, including oral cancer and other cancers of the head and neck [41-48]. Likewise, Rosenberg et al. (2005) reported that the risk of developing SCC of the head, neck, and the esophagus was approximately 4.4-fold higher in FA patients who received a transplant compared to FA patients who did not receive a transplant [49]. It is predicted that FA patients are prone to developing cGVHD due to the conditioning regimen that is received pre-transplant. The conditioning regimen, which can include the use of irradiation, suppresses the recipient's immune system so that the new donor cells are not attacked and rejected by the host [40, 50]. After transplantation, it is estimated that FA patients are about 500- to 700-fold more likely to develop head and neck cancer, compared to the population that is unaffected by FA [51]. Other malignancies that often develop in FA patients include cancers of the genitourinary and gynecological tracts [52]. In addition to HSCT, gene therapy is a promising research avenue that focuses on remedying the FANC mutations in the hematopoietic stem cells to restore the function of the FA DNA repair pathway in the hemopoietic lineage in FA patients [53-55]. However, the use of gene therapy to decrease the risk for HNSCC in FA patients remains unexplored.

Genomic instability is the underlying basis and a driver of cancer [56]. Cancer is a disease that is associated with recurrent damage to the cellular genome, and in FA patients, their ability to repair DNA damage in the form of DNA interstrand crosslinks is non-existent. DNA damage comes from exposure to endogenous or exogenous agents [57]. It is known that FA patients are highly sensitive to DNA crosslinking agents such as

mitomycin C, diepoxybutane, and reactive aldehydes such as formaldehyde and acetaldehyde [21]. Without sufficient DNA repair capabilities, the cells of FA patients are susceptible to genotoxic metabolites that are produced endogenously. Examples of endogenous metabolites include reactive oxygen species (ROS) and aldehydes. While ROS do not induce DNA crosslinks, reactive aldehydes induce crosslinks.

Formaldehyde is the simplest type of aldehyde and is a known carcinogen. Formaldehyde is a product of methanol metabolism (**Figure 1.3**). Humans may be exposed to methanol via the consumption of fruits and vegetables [58]. Methanol is catabolized to formaldehyde by alcohol dehydrogenase, and formaldehyde is broken down to formic acid by formaldehyde dehydrogenase. The ability to metabolize formaldehyde is essential in cells that lack the FA DNA repair pathway [59]. Mono-ubiquitinated FANCD2 is an important protein that is responsible for mediating the repair of DNA interstrand crosslinks induced by formaldehyde [60].

Following formaldehyde, acetaldehyde is the next simplest aldehyde and is a product of ethanol metabolism (**Figure 1.4**). Like formaldehyde, acetaldehyde is also classified as a carcinogen due to its ability of wreak havoc on the genome [61, 62]. It is recommended that FA patients avoid consuming alcoholic beverages and mouthwashes that contain alcohol because the ethanol present in these products could pose a danger to FA patients [63, 64]. When ethanol is metabolized, it is broken down into acetaldehyde by the enzyme alcohol dehydrogenase. Acetaldehyde is further broken down into acetic acid which is less toxic to the body compared to the toxic nature of acetaldehyde. Disulfiram is a drug that inhibits the activity of aldehyde dehydrogenase 2 (ALDH2), and

in the clinical setting disulfiram is used as a medication to treat chronic alcoholism because it results in a buildup of acetaldehyde in the body which produces unpleasant physical effects for the patient [65]. The unpleasant physical effects that result from the accumulation of acetaldehyde in the body mirror hangover symptoms and include fatigue, thirst, drowsiness, headache, dry mouth, nausea, and reduced alertness [66]. More research needs to be done investigating ALDH2 activity in FA patients.

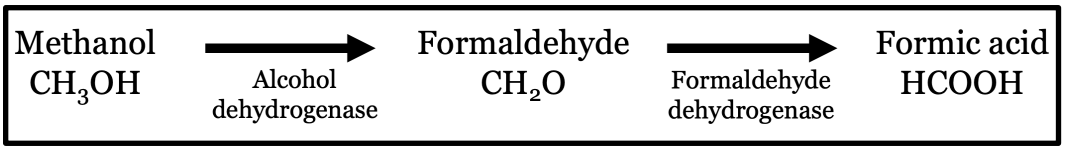

Figure 1.3: Methanol metabolism generates formaldehyde. Methanol is converted to formaldehyde by alcohol dehydrogenase. Formaldehyde is then converted to formic acid by formaldehyde dehydrogenase.

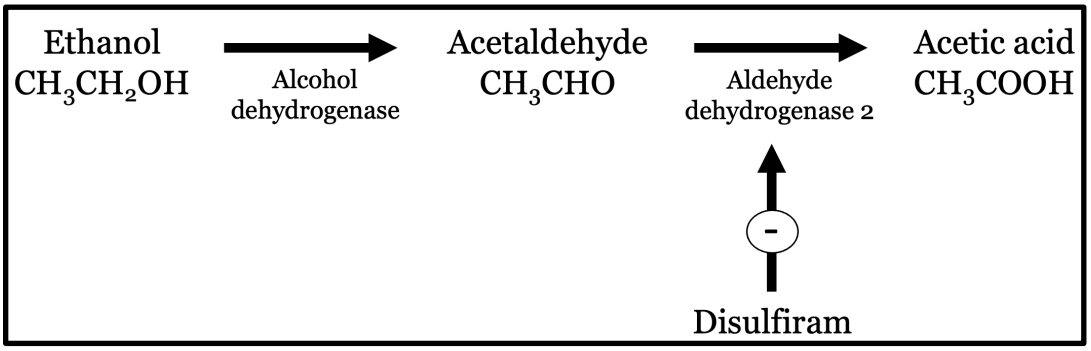

Figure 1.4: Ethanol metabolism generates acetaldehyde. When ethanol is consumed, it is broken down into acetaldehyde by alcohol dehydrogenase. Acetaldehyde is metabolized to acetic acid, which is less toxic than acetaldehyde, by aldehyde dehydrogenase 2. Disulfiram is a drug that inhibits the activity of aldehyde dehydrogenase 2.

The method by which aldehydes lead to carcinogenesis is not completely understood. As mentioned above, DNA adduct formation may play a pivotal role in DNA damage induced by acetaldehyde, and it is known that acetaldehyde and formaldehyde induce DNA crosslink formation [67-69]. Unrepaired DNA crosslinks resulting from

formaldehyde and acetaldehyde production pose a threat by destabilizing the genome. Two types of genetic lesions that compromise genomic integrity have been observed in FA patients: DNA rearrangements caused by double-strand breaks and mutations. Both can be caused by unrepaired interstrand crosslinks [70].

Garaycoechea et al. (2012) showed that endogenous aldehydes play a key role in the pathogenesis of FA [71]. In the study, mice that lacked the ability to catabolize acetaldehyde ($Aldh2^{-/-}$) and that lacked a functional FA DNA repair pathway ($Fancd2^{-/-}$), experienced bone marrow failure, a clinical feature that affects most FA patients (see **Figure 1.5**). As shown in the blood film in **Figure 1.5**, in the doubly mutant mice, there was a decrease in the number of blood cells compared to WT mice and compared to mice that either lacked the ability to catabolize acetaldehyde or that lacked a functional FA DNA repair pathway singly. A decrease in blood cells is evidence of bone marrow failure due to the bone marrow's inability to produce an adequate amount of blood cells. Additionally, **Figure 1.5** shows histological evidence of bone marrow failure and of an increase in DNA double-strand breaks (γH2AX) in the mice that are deficient in both acetaldehyde catabolism and the FA DNA repair pathway. This study demonstrated that in mice and presumably humans, the inability to catabolize acetaldehyde contributes to the pathogenesis of FA.

Shen et al. (2020) also showed that aldehydes promote the pathogenesis of FA [72]. Shen et al. (2020) illustrated that when hematopoietic stem cells differentiate, an excess of formaldehyde is generated endogenously, which then causes a deficit in the cells that stem from the hematopoietic lineage [72]. This finding aligns with physical

manifestations of FA that are seen in the clinic (e.g., bone marrow failure). In contrast to the formaldehyde accumulation observed when hemopoietic stem cells were induced to differentiate in vitro, Shen et al. (2020) showed that when stem cells were induced to differentiate into neurons, adipocytes, and osteocytes, no increase in formaldehyde was observed [72]. The formaldehyde generation was specific to cells of the hematopoietic lineage, a lineage of cells that are highly affected and impaired in FA patients. Shen et al. (2020) demonstrated that absence of the FA DNA repair pathway rendered the hematopoietic stem cells vulnerable to assault from endogenous formaldehyde generation.

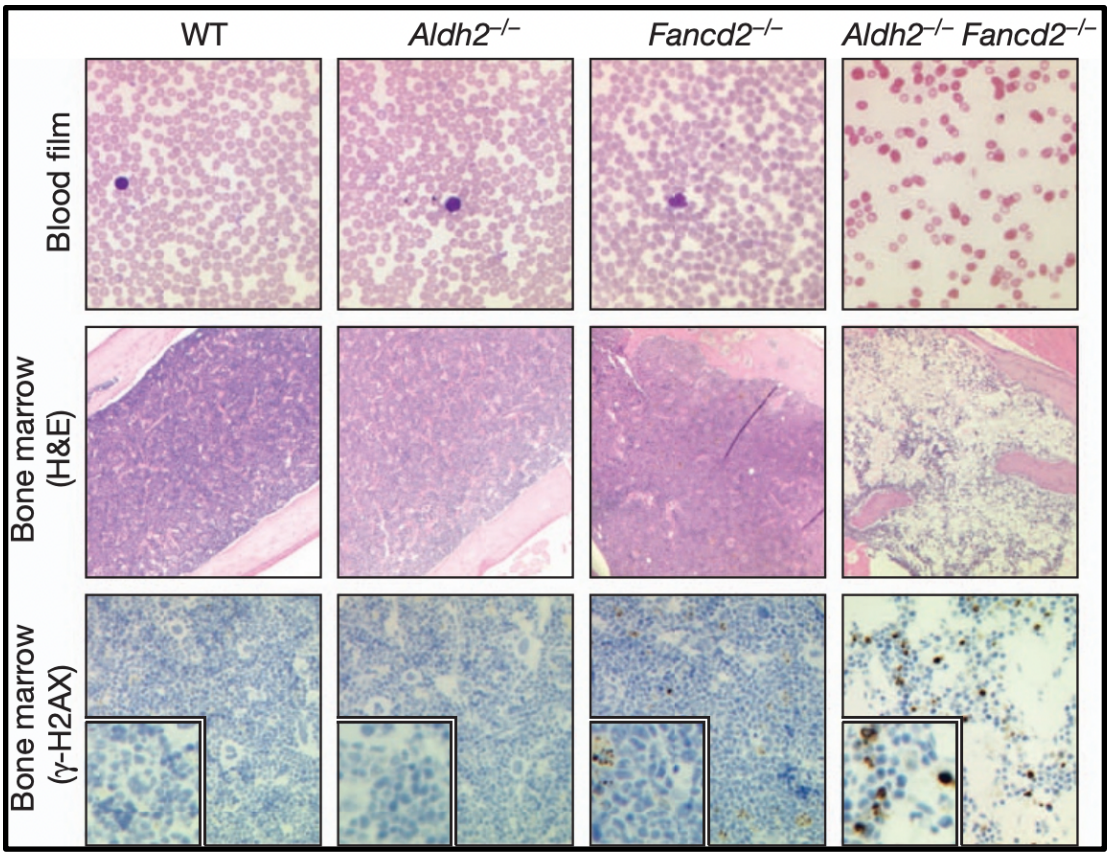

Figure 1.5: This figure is excerpted from a larger figure previously published in (Garaycoechea JI, et al. *Nature*. 2012.) [71] Endogenous aldehydes play a role in the pathogenesis of FA. Mice that lack the ability to catabolize acetaldehyde and that lack a functional FA DNA repair pathway experience bone marrow failure. Bone marrow failure is demonstrated in the blood film with the decreased number of blood cells compared to WT mice, mice that lack the ability to catabolize acetaldehyde alone, and mice that lack a functional FA DNA repair pathway alone. Additionally, the bone marrow histology shows the failure of bone marrow and an increase in DNA damage in the mice that are deficient in both acetaldehyde catabolism and the FA DNA repair pathway.

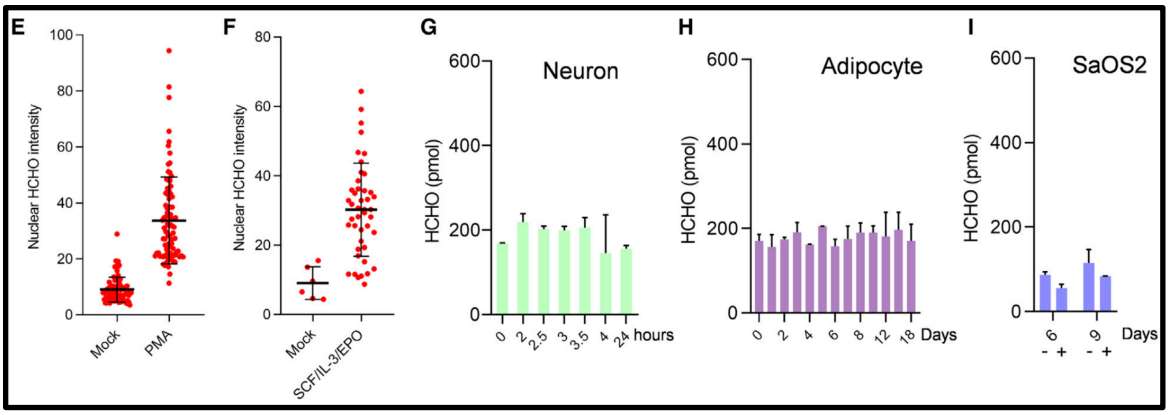

Figure 1.6: This figure is excerpted from a larger figure previously published in (Shen X, et al. *Mol Cell.* 2020) [72] Aldehydes play a role in the pathogenesis of FA. When hemopoietic stem cells are induced to differentiate in vitro, there is an increase in formaldehyde (E and F). When stem cells are induced to differentiate into neurons (G), adipocytes (H), and osteocytes (I), no increase in formaldehyde is observed.

The **primary question** that frames my dissertation is as follows: why are FA patients predisposed to HNSCC at a higher rate compared to other non-hematopoietic malignancies? A reasonable and widely believed explanation is that the head and neck epithelia are exposed to higher levels of reactive aldehydes in comparison to epithelia at other regions of the body [73]. Studies show that acetaldehyde levels in the saliva are 10-20 times greater than the levels found in the blood [21]. The literature also shows that oral microbes can produce acetaldehyde [74]. While formaldehyde and acetaldehyde have also been found in a variety of foods and beverages [75-77], it is also possible that the elevated levels of reactive aldehydes are produced endogenously.

My **favored hypothesis** is that FA patients are predisposed to HNSCC at a higher rate compared to other non-hematopoietic malignancies such as skin carcinoma because the mouth encounters higher levels of environmental (exogenous) genotoxins (e.g., reactive aldehydes). This hypothesis is in alignment with evidence that epithelia in the

mouth and pharynx are exposed to foods and beverages consumed by the patient [75-77]. An **alternative hypothesis** is that FA patients are predisposed to HNSCC at a higher rate compared to other non-hematopoietic malignancies such as skin carcinoma because oral keratinocytes are inherently different and have greater endogenous genotoxin production than epidermal keratinocytes. This model is consistent with the study alluded to previously that found that during normal differentiation of the hematopoietic lineage, a high production of an endogenous reactive aldehyde was observed in the hematopoietic cells but not the other cell types (i.e., neurons, adipocytes, and osteocytes) [72].

This study was designed to uncover the molecular and cellular mechanisms that explain why FA patients are more susceptible to developing HNSCC compared to other types of SCC such as skin carcinoma. Gathering information about the response to aldehyde assault in normal and FA pathway null cells is a logical step toward understanding the underlying mechanisms of cancer induction in FA. Using cell culture models, I measured the endogenous production of aldehydes, assessed cellular viability in response to aldehydes, and investigated activity of the FA DNA repair pathway in normal epidermal keratinocytes, in normal oral keratinocytes, and in malignant oral cells.

This project is significant because I aim to understand how formaldehyde and acetaldehyde contribute to DNA crosslinking events which would consequently lead to genomic instability and HNSCC. Understanding the mechanisms that drive the development of HNSCC in FA patients can lead to prevention strategies. It is beneficial to approach the issue of HNSCC in FA patients from a prevention angle. Treatment options are severely limited since normal tissues of FA patients are highly sensitive to

chemotherapies used to treat metastatic and unresectable HNSCC. However, if a regimen that can mitigate the development of HNSCC can be found, it will make life better for FA patients and for non-FA patients who are at risk for HNSCC. Exposing and understanding the molecular and cellular underpinnings of head and neck carcinogenesis in FA patients will be novel and a vital contribution to the field of FA research that can potentially extend to the clinical arena.

CHAPTER 2:
SURVEYING ENDOGENOUS LEVELS OF REACTIVE ALDEHYDES IN ORAL AND NON-ORAL EPITHELIA

2.1 Overview

In FA, patients are predisposed to HNSCC at a higher rate compared to other non-hematopoietic malignancies. It is known that reactive aldehydes induce DNA damage (i.e., DNA interstrand crosslinks). It is likely that reactive aldehydes play a role in the pathogenesis of HNSCC in FA patients by inducing DNA damage in the head and neck epithelia which contributes to genomic instability, a hallmark of cancer. Exposure to reactive aldehydes can explain the higher incidence of HNSCC in FA patients. There are two possible sources of the reactive aldehydes: endogenous and exogenous. Endogenous reactive aldehydes are produced intracellularly via normal metabolic processes [72]. Exogenous reactive aldehydes can be found in the extracellular environment as a by-product of oral microbe metabolism as well as in foods and beverages [74-77].

Between these two possibilities regarding the source of aldehyde exposure (i.e., endogenous or exogenous), I favor the possibility that the primary source of aldehyde damage that contributes to head and neck carcinogenesis in FA is exogenous. In this chapter, to test the two possibilities, I measured the levels of endogenous aldehyde production across human cell lines using two assays: the Aldehyde Assay and headspace gas chromatography/mass spectrometry (HS GC/MS). The Aldehyde Assay was performed on the epidermal keratinocyte (HaCat), oral keratinocyte (TIGK, OKF4), and oral squamous cell carcinoma (SCC) (FANCA$^{+/+}$, FANCA$^{-/-}$) cell lines. HS GC/MS analysis was performed on the epidermal keratinocyte (HaCat) and oral keratinocyte (TIGK,

OKF4) cell lines. Keratinocytes were used because keratinocytes undergo genetic changes (e.g., mutations) and become squamous cell carcinomas. The Aldehyde Assay is a pan-aldehyde assay and detected all aldehydes present in cell culture media. In contrast, HS GC/MS is specific and made it possible to detect formaldehyde and acetaldehyde in cell culture media. The first assay yielded clear-cut results while the HS GC/MS method lacked the necessary sensitivity for the intended purposes. Nevertheless, the results were in alignment with the favored hypothesis which suggests that an explanation for the higher incidence of HNSCC that is observed in FA patients is due to exposure to exogenous aldehydes as there was no evidence of endogenous aldehyde production in the normal cell lines assessed (i.e., the epidermal (HaCat) and oral (TIGK, OKF4) keratinocytes).

2.2 Methods

2.2.1 Cell lines and culture conditions

Name of Cell Line	Source	Tissue Type
HaCat	Dr. Masaoki Kawasumi (University of Washington) and Dr. Paul Nghiem (University of Washington)	Epidermis
TIGK	Dr. Richard Darveau (University of Washington School of Dentistry)	Gingiva (gums)
OKF4	Dr. Bruce Clurman (Fred Hutchinson Cancer Research Center)	Floor of the mouth
UM-SCC-01 FANCA$^{+/+}$	Fanconi Anemia Research Materials	Floor of the mouth
UM-SCC-01 FANCA$^{-/-}$	Fanconi Anemia Research Materials	Floor of the mouth
JHU-SCC-FaDu FANCA$^{+/+}$	Fanconi Anemia Research Materials	Pharynx

Table 2.1: List of cell lines used for experiments. Throughout this dissertation, the HaCat cell line will also be referred to as epidermal keratinocytes, the TIGK and OKF4 cell lines will also be referred to as oral keratinocytes, the UM-SCC-01 FANCA$^{+/+}$ and UM-SCC-FANCA$^{-/-}$ cell lines will be referred to as FANCA$^{+/+}$ and FANCA$^{-/-}$ (oral SCC), and the JHU-SCC-FaDu cell line will be referred to as FaDu (oral SCC).

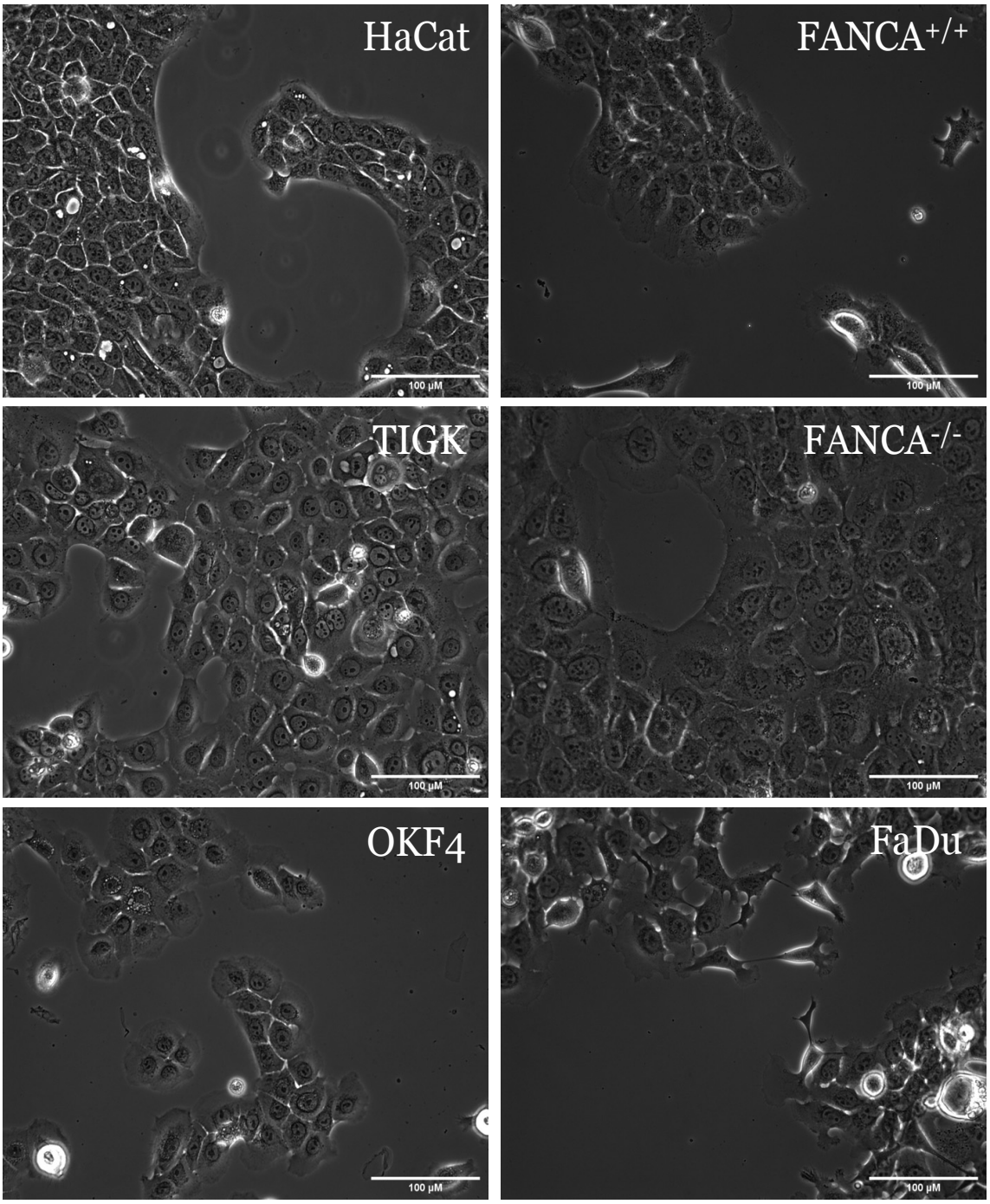

Figure 2.1: Phase contrast images at 20X objective of cell lines in culture.

The HaCat cell line was a gift from labs of Dr. Masaoki Kawasumi (University of Washington) and Dr. Paul Nghiem (University of Washington). The HaCat cell line [78] was derived from adult skin and was grown in Dulbecco's Modified Eagle Medium (DMEM; #11995-073, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; #10438-026, Invitrogen; FBS; #SH30071.03, Hyclone Laboratories) and 1% Penicillin-Streptomycin (#15140-122, Gibco). Cells were harvested using TrypLE, a cell dissociation reagent, (#12604-021, Invitrogen) for passaging and experiments.

The TIGK cell line was a gift from the lab of Dr. Richard Darveau (University of Washington School of Dentistry). The TIGK cell line [79] was derived from adult gingival tissue and was grown in Keratinocyte Serum-Free Media (KSFM; #17005042, Gibco) supplemented with 25 μg/mL Bovine Pituitary Extract (BPE; #13028-014, Gibco), 0.2 ng/mL EGF Human Recombinant (EGF; #10450-013, Gibco), 1% Penicillin-Streptomycin (#15140-122, Gibco), and 0.4 mM $CaCl_2$. Cells were harvested using 0.25% Trypsin-EDTA (1x) (#25200-056, Gibco) for passaging and experiments.

The OKF4 cell line was a gift from the lab of Dr. Bruce Clurman (Fred Hutchinson Cancer Center). The OKF4 cell line was derived from the floor of the mouth and was grown in Keratinocyte Serum-Free Media (KSFM; #17005042, Gibco) supplemented with 25 μg/mL Bovine Pituitary Extract (BPE; #13028-014, Gibco), 0.2 ng/mL EGF Human Recombinant (EGF; #10450-013, Gibco), 1% Penicillin-Streptomycin (#15140-122, Gibco), and 0.4 mM $CaCl_2$. Cells were harvested using 0.25% Trypsin-EDTA (1x) (#25200-056, Gibco) for passaging and experiments.

The UM-SCC-01 FANCA$^{+/+}$ and UM-SCC-01 FANCA$^{-/-}$ cell lines were a gift from the Fanconi Anemia Research Materials resource, located at Oregon Health & Science University. The UM-SCC-01 cell lines were derived from the floor of the mouth in of a non-FA patient with HNSCC (mutations in TP53, CASP8, and EP300) and were grown in Dulbecco's Modified Eagle Medium – High Glucose (DMEM; #D5796, Sigma) supplemented with 10% Fetal Bovine Serum (FBS; #SH30071.03, Hyclone Laboratories), 1% Non-Essential Amino Acids (#11140050, Gibco), and 1% Penicillin-Streptomycin (#10378016, Gibco). Cells were harvested using 0.25% Trypsin-EDTA (1x) (#25200-056, Gibco) for passaging and experiments. *Note: Throughout the rest of the dissertation, UM-SCC-01 FANCA$^{+/+}$ and UM-SCC-01 FANCA$^{-/-}$ will be referred to as FANCA$^{+/+}$ and FANCA$^{-/-}$, respectively.

The JHU-SCC-FaDu FANCA$^{+/+}$ cell line was a gift from the Fanconi Anemia Research Materials (located at Oregon Health & Science University). The JHU-SCC-FaDu FANCA$^{+/+}$ cell line was derived from the pharynx of a non-FA patient with HNSCC (mutations in TP53, FAT1, and CDKN2A) and was grown in Eagle's Minimum Essential Media (MEM; #10-010-CV, Corning) supplemented with 10% Fetal Bovine Serum (FBS; #SH30071.03, Hyclone Laboratories), and 1% Penicillin-Streptomycin (#10378016, Gibco). Cells were harvested using 0.25% Trypsin-EDTA (1x) (#25200-056, Gibco) for passaging and experiments. Each cell line was cultured at 37°C in a humid atmosphere with 5% CO_2 level.

2.2.2 Aldehyde measurements

Aldehyde content in cell culture media was measured to yield quantitative data regarding the endogenous production of aldehydes across the cell lines and to determine whether differential endogenous production of formaldehyde and acetaldehyde exists across diverse cell types.

Two methods were employed to measure endogenous aldehyde production: the Aldehyde Assay Kit (Colorimetric) (ab112113) and HS GC/MS. The Aldehyde Assay Kit is less specific and detected all aldehydes present in the cell culture media inclusive of the two aldehydes of interest (formaldehyde and acetaldehyde). The chemical analysis technique, HS GC/MS, is more specific and was used to measure only the endogenous formaldehyde and acetaldehyde production in cell culture media.

In the Aldehyde Assay a chromogenic product is generated when an aldehyde reacts with the assay dye. Two 6-well plates were used for each cell type. This was an independent experiment done in quadruplicate. In the first 6-well plate, 3 mL of media were added to four wells (quadruplicate). This represented the “unspent” media which was the control condition (media that was not exposed to cells). In the second plate, 3 mL of media were added to four wells, and 300,000 cells were seeded per well. This represented the “spent” media (media that was exposed to cells). After 72 hours in the incubator, 50 microliters of media were harvested from each well and added to a 96-well plate along with 50 microliters of each aldehyde standard. The aldehyde standards were prepared per the kit instructions, and the concentrations were as follows: 0, 1, 3, 10, 30,

100, 300, 1000 μM. After adding the 50 microliters of standards and samples to the 96-well plate, 50 microliters of the reaction mixture were added to each standard and sample well and incubated at room temperature for 30 – 60 minutes. The plate was covered with aluminum foil to shield it from light during the incubation period. Using a microplate reader (Biotek Synergy 2, Winooski, VT), the final signal was read at an emission wavelength of 405 nm.

Measurements of endogenous production of formaldehyde and acetaldehyde were performed using HS GC/MS. I completed HS GC/MS at the University of Washington Chemistry Mass Spectrometry Facility in collaboration with Dr. Martin Sadilek. The results for this assay represent an independent experiment done in triplicate (for DMEM media and HaCat cell line) or duplicate (for KSFM media and TIGK/OKF4 cell lines). Two 6-well plates were used for each cell type. In the first 6-well plate, 3 mL of media were added to three wells (triplicate) or two wells (duplicate). This represented the "unspent" media which was the control condition (media that was not exposed to cells). In the second plate, 3 mL of media were added to three wells (or two wells) and 300,000 cells were seeded per well. This represented the "spent" media (media that was exposed to cells). After 48 hours, 1 mL of unspent and spent media was harvested from each well. In addition to the unspent and spent media samples, formaldehyde and acetaldehyde standards were prepared using the following concentrations: 0, 0.25, 0.5, 0.75, 1 mM. Standards were prepared for each media type (DMEM and KSFM).

Derivatization is essential in preparing the samples for HS GC/MS. Derivatization is a process that occurs when a compound is chemically altered, producing a new

compound with a similar chemical structure. In the context of my experiment, derivatization of the formaldehyde and acetaldehyde was necessary due to the volatile nature of the two compounds, and the process produced more stable versions of the aldehydes upon reaction with *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBOA). For the derivatization protocol, 300 mg NaCl, 40 μL of 10 mg/mL PFBOA, and internal standard at a final concentration of 100 μM were added to glass vials (National Scientific Amber 4mL vials T/S septa (100 / box, CAT #: C4015-88AW)). The capped vials were incubated at 60°C for 60 minutes and then cooled to room temperature. After cooling, the vials were spun in a centrifuge at 2500 RPM for 7 minutes. The aldehyde derivatives entered the "headspace" of the glass vial, from which the sample was collected. Per sample, 50 microliters of gas were injected into the 6890 Gas Chromatograph with 5973 Mass Selective Detector (Agilent Technologies, Santa Clara, CA). See Appendix D for the HS GC/MS protocol and instrument parameters. The average intensity of ions in chromatographic peaks was quantified using the Enhanced Data Analysis software (version E.02.02) (Agilent Technologies, Santa Clara, CA).

2.3 Results

Aldehyde Assay:

To perform the Aldehyde Assay, a standard curve was created (**Figure 2.2**) which was used to calculate the aldehyde concentrations of the unspent media and spent media. Unspent media refers to media that was not exposed to cells and serves as the negative control. Spent media refers to media that was exposed to cells. If the levels of aldehyde are higher in the spent media compared to the unspent media, then this indicates that the cell line is producing aldehyde. In the Aldehyde Assay, two questions were assessed: (1) are epidermal and oral keratinocytes similar in aldehyde production, and (2) are normal and malignant oral keratinocytes similar in aldehyde production? The prediction was that the level of aldehyde production would be similar across the cell lines.

The first finding was that there was no statistical evidence of aldehyde production in the epidermal (HaCat) and oral (TIGK, OKF4) keratinocytes relative to unspent media after 72 hours in culture (**Figure 2.3**). The second finding was that there was statistical evidence of aldehyde production in the malignant oral cell line ($FANCA^{+/+}$) (**Figure 2.4**). The $FANCA^{+/+}$ (oral SCC) cell line produced the most aldehyde of all the cell lines assessed after 72 hours. Interestingly, there was no evidence of aldehyde production in the $FANCA^{-/-}$ (oral SCC) cell line. This observation was unexpected because it is unclear how inactivation of FANCA would influence aldehyde production.

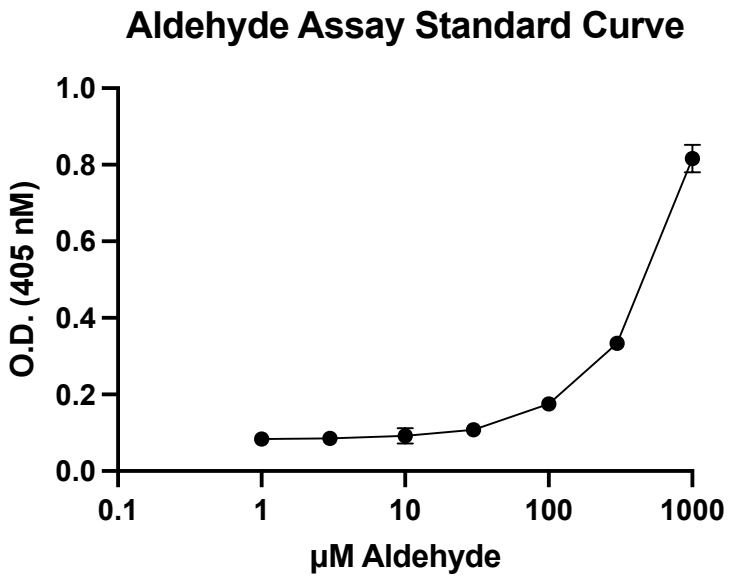

Figure 2.2: For the Aldehyde Assay, the aldehyde standards were prepared per the kit instructions, and the concentrations were as follows: 0, 1, 3, 10, 30, 100, 300, 1000 µM.

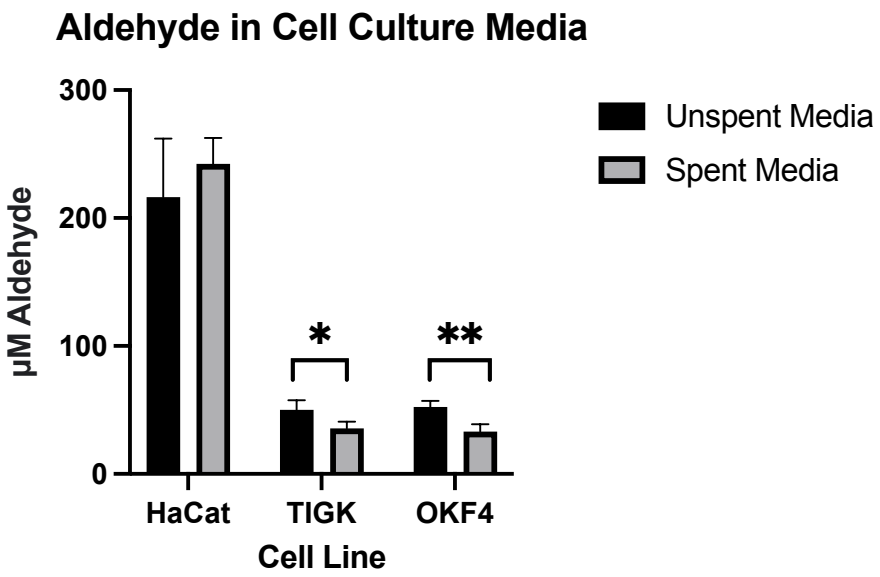

Figure 2.3: Aldehyde concentration was measured in cell culture media using the Aldehyde Assay. The results represent the means and standard deviations of a single experiment done in quadruplicate. Unspent media is control media that was not exposed to cells. Spent media is media procured from cells in culture for 72 hours. Spent media is from the epidermal keratinocyte (HaCat) cell line and the oral keratinocyte (TIGK, OKF4) cell lines. $P<0.05^{*}$, $P<0.01^{**}$, $P<0.001^{***}$

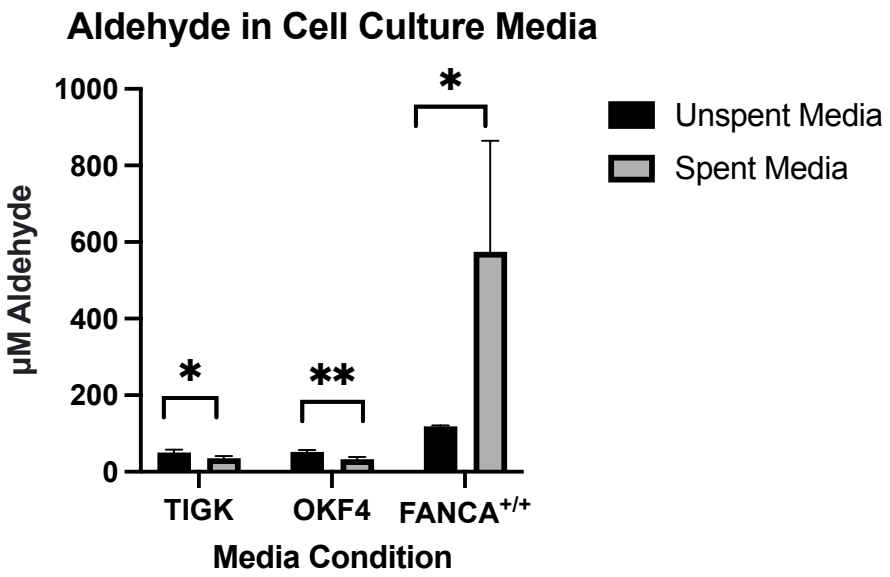

Figure 2.4: Aldehyde content was measured in cell culture media using the Aldehyde Assay. The results represent the means and standard deviations of a single experiment done in quadruplicate. Unspent media is control media that was not exposed to cells. Spent media is media procured from cells in culture for 72 hours. Spent media is from the oral keratinocyte (TIGK, OKF4) cell lines and the FANCA$^{+/+}$ (oral SCC) cell line. P<0.05*, P<0.01**, P<0.001*** (Note: FANCA$^{+/+}$ refers to the UM-SCC-01 FANCA$^{+/+}$ cell line.)

HS GC/MS:

While it was useful to gain information about the endogenous cellular production of aldehydes, the two aldehydes of chief interest are formaldehyde and acetaldehyde. These are the two reactive aldehydes that contribute to DNA crosslinking events that are relevant in FA. To learn whether epidermal and oral keratinocytes produced formaldehyde and acetaldehyde, HS GC/MS was performed. For the HS GC/MS experiments, derivatization was essential in preparing the samples (**Figure 2.5**). Since formaldehyde and acetaldehyde are highly volatile due to their extremely low boiling points, the derivatization process made it possible to create more stable forms of formaldehyde and acetaldehyde that could then be detected and measured via HS GC/MS. Calibration curves were created for each type of cell culture medium (DMEM and KSFM) (**Figure 2.7**). The intended purpose of the calibration curves was to convert the mass spectrometry signal (i.e., the relative abundance) of the samples to concentration. The question this assay addressed is: are epidermal and oral keratinocytes similar in formaldehyde and acetaldehyde production? The prediction was that the level of formaldehyde and acetaldehyde production would be similar across the cell lines.

There was no evidence of formaldehyde production in the epidermal and oral keratinocyte cell lines (**Figure 2.8A-C**). Interpreting the result for acetaldehyde production proved to be a bit more challenging due to assay sensitivity. **Figure 2.9** centers around understanding the acetaldehyde limit of detection. For example, in **Figure 2.9A**, the dotted line shown at a relative abundance of 0.47 represents the value measured on the corresponding KSFM calibration curve (**Figure 2.9B**) at 0 mM

acetaldehyde. In Figure **2.8D-F**, when assessing acetaldehyde production in the epidermal and oral keratinocytes, the signal for the relative abundance of acetaldehyde is greater in the spent media than the signal for the unspent media for all three cell lines, and the difference is statistically significant. At first sight, this data suggest that the cell lines *may* be producing acetaldehyde. However, the caveat here is that the values for the unspent and spent media for all three cell lines analyzed were below the limit of detection. This finding indicates that the assay is not sensitive enough for the levels of acetaldehyde present. Likewise, this assay indicates that the range in which the acetaldehyde levels fall, is outside of a useful range.

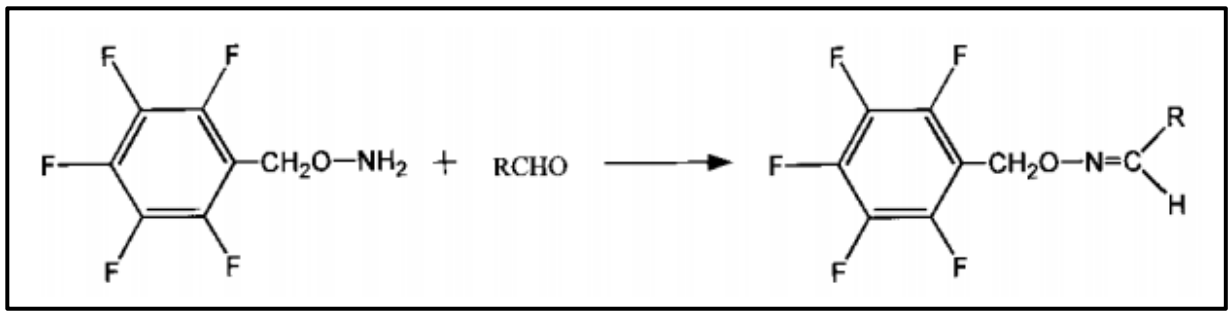

Figure 2.5: This figure has been previously published in (Sugaya N, et al. *Journal of Health Science*. 2001) [80] Derivatization of Aldehydes. Derivatization is essential in preparing the samples for HS GC/MS. In the reaction above, PFBOA and a generic aldehyde (RHCO) are the reactants. PFBOA reacts with the aldehyde to create a more stable version of the aldehyde (i.e., a newly-formed "derivatized aldehyde") that can then be detected and measured via HS GC/MS.

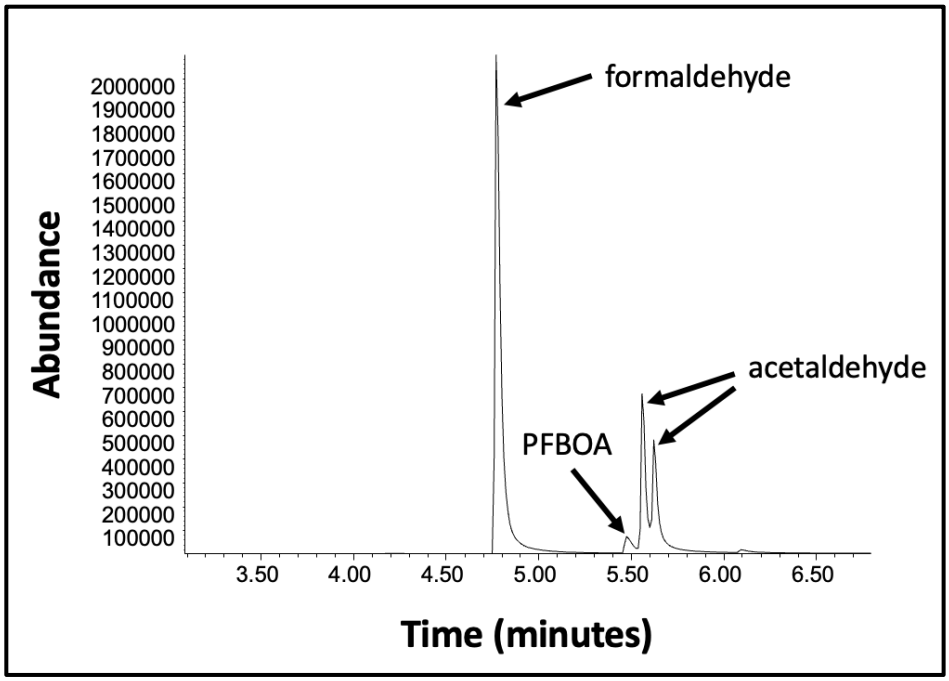

Figure 2.6: Representative chromatogram from HS GC/MS experiment. The peaks for formaldehyde, PFBOA, and acetaldehyde are shown.

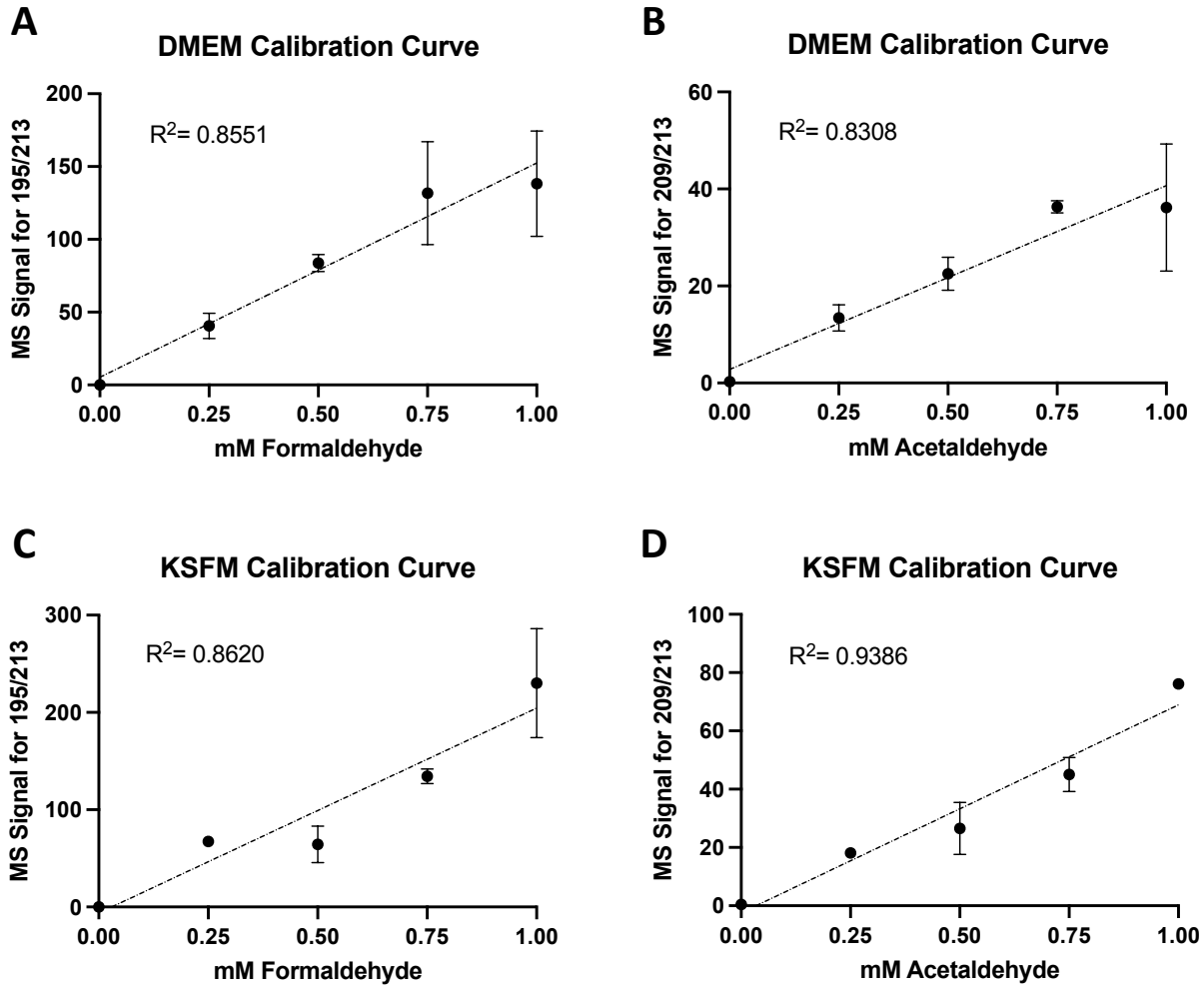

Figure 2.7: DMEM and KSFM Calibration Curves. For HS CG/MS, formaldehyde and acetaldehyde standards were prepared using the following concentrations: 0, 0.25, 0.5, 0.75, 1 mM. The standards shown in this figure were prepared in Dulbecco's Modified Eagle Medium (DMEM) media and Keratinocyte Serum-Free Media (KSFM). The results represent the means and standard deviations of a single experiment done in triplicate (DMEM) and in duplicate (KSFM).

DMEM Calibration Curve Values	
Formaldehyde Concentration (mM)	MS Signal for 195/213
0	0.00
0.25	40.53
0.5	83.71
0.75	131.78
1	138.30

Table 2.2: DMEM Formaldehyde Calibration Curve Values from HS GC/MS. Values correspond with **Figure 2.7A**. MS Signal for 195/213 corresponds to formaldehyde.

DMEM Calibration Curve Values	
Acetaldehyde Concentration (mM)	MS Signal for 209/213
0	0.27
0.25	13.42
0.5	22.53
0.75	36.31
1	36.18

Table 2.3: DMEM Acetaldehyde Calibration Curve Values from HS GC/MS. Values correspond with **Figure 2.7B**. MS Signal for 209/213 corresponds to acetaldehyde.

KSFM Calibration Curve Values	
Formaldehyde Concentration (mM)	MS Signal for 195/213
0	0.12
0.25	67.40
0.5	64.31
0.75	134.29
1	230.01

Table 2.4: KSFM Formaldehyde Calibration Curve Values from HS GC/MS. Values correspond with **Figure 2.7C**. MS Signal for 195/213 corresponds to formaldehyde.

KSFM Calibration Curve Values	
Acetaldehyde Concentration (mM)	MS Signal for 209/213
0	0.47
0.25	18.13
0.5	26.54
0.75	45.08
1	76.15

Table 2.5: KSFM Acetaldehyde Calibration Curve Values from HS GC/MS. Values correspond with **Figure 2.7D**. MS Signal for 209/213 corresponds to acetaldehyde.

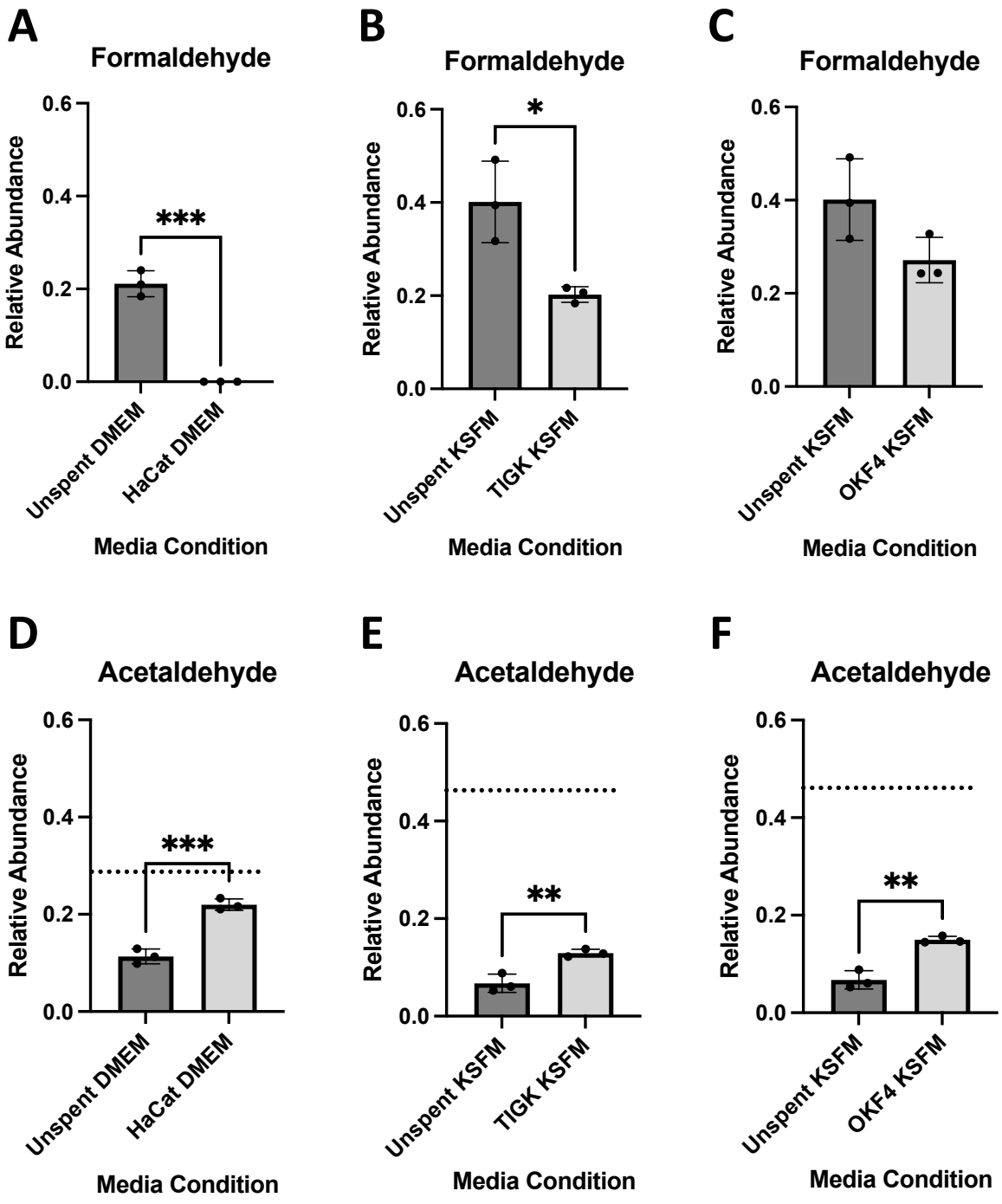

Figure 2.8: Formaldehyde and acetaldehyde production in unspent and spent media measured via HS GC/MS.

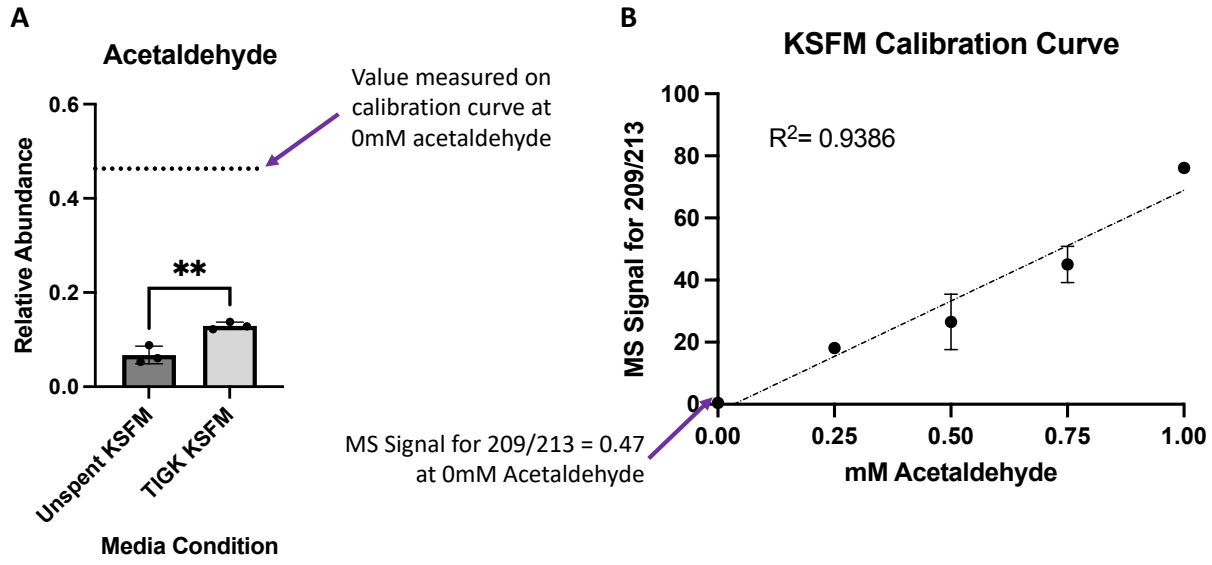

Figure 2.9: Understanding the acetaldehyde limit of detection in HS GC/MS. Values that fall below the dotted line shown are lower than the limit of detection of the assay.

2.4 Conclusions

I predicted that levels of endogenous aldehyde production would be similar across the epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines. I also predicted that levels of endogenous aldehyde production would be similar in the non-malignant (TIGK, OKF4) and malignant ($FANCA^{+/+}$) oral cell lines. The normal, non-malignant epidermal and oral keratinocyte cell lines did not produce detectable levels of aldehydes as measured via the Aldehyde Assay. This finding supports the favored hypothesis that oral and epidermal keratinocytes are similar in vitro. Additionally, the oral keratinocyte cell lines did not make more aldehyde compared to the epidermal keratinocyte cell line. This finding does not support the possibility that high endogenous aldehyde production is associated with the increased risk for HNSCC in FA patients. These findings suggest that exogenous sources of aldehyde are likely most important.

The Aldehyde Assay also demonstrated that the malignant oral cell line ($FANCA^{+/+}$) produced aldehyde at a level that was statistically significant and produced more aldehyde compared to the non-malignant oral cell lines (TIGK, OKF4). Aldehyde production in the malignant oral cell line ($FANCA^{+/+}$) was greater than aldehyde production of all the cell lines assessed. Observing aldehyde production in the malignant cell line ($FANCA^{+/+}$) was an interesting finding which indicates that it may be worthwhile to investigate aldehyde production in other malignant cell lines (e.g., epidermal SCC). Another surprising finding is that there was no statistically significant evidence of aldehyde production in the malignant oral cell line with deleted FANCA ($FANCA^{-/-}$). It was unexpected to learn that the two malignant oral cell lines ($FANCA^{+/+}$, $FANCA^{-/-}$) that differed genetically in FANCA activity also differed in endogenous aldehyde production

because it is unclear how the presence or absence of a functional FA DNA repair pathway influences aldehyde production. Aldehyde production in the $FANCA^{+/+}$ cell line could suggest that there is a threshold of endogenous aldehyde production that determines whether a normal cell type becomes malignant. The result for $FANCA^{-/-}$ could also suggests that there is some crosstalk between the FA pathway and catabolizing enzymes like ALDH2. For example, the $FANCA^{-/-}$ cell line lacks a functional FA DNA repair pathway so it would not be beneficial for the $FANCA^{-/-}$ cell line to produce interstrand crosslink-inducing aldehydes if there is no system in place to repair the DNA damage. Additional studies need to be done exploring aldehyde production and aldehyde catabolism in the context of malignant cells.

The HS GC/MS assay showed no evidence of formaldehyde production in the epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines. The readings for acetaldehyde production were below the limit of detection for all three cell lines assessed. The values measured for acetaldehyde were too low to be confident that the cell lines were producing acetaldehyde; the data indicate that there is a need for some troubleshooting to increase the sensitivity of the assay. Some options for troubleshooting include increasing the volume of media that is analyzed and increasing the volume of gas that is injected into the gas chromatograph. Nonetheless, the results from the HS GC/MS assay are consistent with the results from the Aldehyde Assay, and both methods of aldehyde detection imply that endogenous sources of aldehyde are likely not primary contributing factors to HNSCC carcinogenesis in FA. Thus, I turned to exploring exogenous sources of aldehydes and their impact on the cell lines used in this study.

CHAPTER 3:
ORAL AND NON-ORAL EPITHELIA HAVE DISTINCT RESPONSES TO EXOGENOUS REACTIVE ALDEHYDES

3.1 Overview

In individuals who do not have FA, it is thought the FA DNA repair pathway is found in all the tissues throughout the body. In patients with FA, unfortunately, the FA DNA repair pathway is non-functional due to a mutation in one of the 22 genes that code for the proteins that makeup the FA DNA repair pathway. Most FA patients born in the United States have a mutation in FANCA. The FANCA protein is part of the multiprotein FA core complex, and the purpose of the FA core complex is to stimulate the mono-ubiquitination of FANCD2. FANCD2 is one the final proteins in the FA DNA repair pathway cascade, and mono-ubiquitination of FANCD2 indicates that the proteins upstream of FANCD2 are properly functioning and that the pathway is operational. The FA DNA repair pathway is critical, and its purpose is to orchestrate the repair of DNA interstrand crosslinks. Individuals who lack a functional FA pathway are hypersensitive to DNA crosslinking agents such as mitomycin C and diepoxybutane as well as formaldehyde and acetaldehyde.

In understanding HNSCC development in FA patients, in addition to investigating endogenous aldehyde production, which was covered in Chapter 2, it was also imperative to assess three additional parameters: cellular viability, FA DNA repair pathway activity, and DNA interstrand crosslink formation in response to exogenous aldehydes. In this chapter, to test the impact of reactive aldehydes on the human cell lines, I completed a cellular viability assay, I monitored levels of mono-ubiquitinated FANCD2, and I investigated DNA alterations. Cellular viability was measured to gauge cellular health at

baseline and in response to the genotoxins formaldehyde and acetaldehyde. Measuring cellular viability was an indirect way to assess the FA DNA repair pathway because it was surmised that sensitivity to the crosslinking genotoxins was related to the activity of the FA DNA repair pathway. To directly monitor the activity of the FA DNA repair pathway, Western blots to detect mono-ubiquitinated FANCD2 were done. Mono-ubiquitinated FANCD2 protein activity was assessed at baseline and in response to treatment with formaldehyde and acetaldehyde. I initially intended to use DNA adduct analysis to gauge DNA damage at baseline and in response to acetaldehyde treatment by screening for acetaldehyde-induced DNA adducts. However, γH2AX was used to gauge DNA dynamics instead; γH2AX is an early cellular response that is prompted when DNA double-strand breaks are induced [81]. Western blotting to detect γH2AX was completed to observe whether formaldehyde and acetaldehyde could induce double-strand breaks in the DNA of the cells upon treatment with the aldehydes. Formaldehyde and acetaldehyde induce formaldehyde-specific and acetaldehyde-specific DNA adducts, and unrepaired DNA adducts ultimately lead to double-stranded breaks in the DNA and mutagenesis. Additionally, double-strand breaks are also purposely induced when DNA crosslink damage is resolved by the FA DNA repair pathway. The results from the assays were not in alignment with the favored hypothesis and instead suggested that there are some inherent differences between the epidermal and oral keratinocytes that could potentially explain the high incidence of HNSCC that is observed in FA patients.

3.2 Methods

3.2.1 Cellular viability

Cellular viability was measured to gauge the cells' sensitivity to the genotoxins (i.e., formaldehyde and acetaldehyde). Hoechst 33342 dye (Invitrogen) was used to measure cellular viability. This dye works by staining the nuclei of cells. An independent experiment was done in triplicate. Using a 12-well plate, 20,000 cells were seeded per well in 1 mL cold media. The treatment conditions were as follows: 0.5 mM formaldehyde, 1 mM formaldehyde, 0.5 mM acetaldehyde, and 1 mM acetaldehyde. The cells were treated for 48 hours. After 48 hours of exposure to treatment, 1 microliter of Hoechst 33342 dye was added to each well, and the plate was placed in the incubator for an additional hour. After the one-hour incubation, images were taken with 4x magnification using the ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices, San Jose, CA). Per well, four equivalent fields were imaged, and the cell count data from these images were used for quantitative analysis. We assessed cellular health and viability. This experiment was done in collaboration with Dr. Ahmed M. Diab.

3.2.2 FANCD2 protein activity

Western blot analysis to identify mono-ubiquitinated FANCD2 was used to assess the activity of the FA DNA repair pathway and to ascertain whether differential activity of FANCD2 expression exists across the cell lines under basal conditions (untreated) and when treated with formaldehyde and acetaldehyde [82]. Mono-ubiquitinated FANCD2 serves as a reporter for the activity of the FA DNA repair pathway. The molecular weight of the FANCD2 protein product is present at ~150 – 160 kDa.

One million cells (HaCat, TIGK, OKF4) were plated in 60mm petri dishes in cold (4°C) media and treated with 1 mM acetaldehyde or 1 mM acetaldehyde with Parafilm for 24 hours. For the 1 mM acetaldehyde treatment, the petri dishes were covered with Parafilm, or the petri dishes were left without Parafilm to allow for normal gas exchange. To detect the protein FANCD2 via western blot, 30-60 μg of protein were loaded to each lane of a 4-12% Bis-Tris Gel (Invitrogen) or 3-8% Tris-Acetate Gel (Invitrogen). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Merck Millipore Ltd.) and blocked in 1X Tris-Buffered Saline with 0.1% Tween 20 detergent (TBST) and 5% Bovine Serum Albumin (BSA). The blots were probed with a FANCD2 primary antibody (Novus) and a species-specific secondary antibody that was horseradish peroxidase (HRP)-labeled. Chemiluminescence (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific) was used to visualize the bands, and images were captured using the Bio-Rad ChemiDoc Imaging System (Hercules, CA). Western blots were quantitatively analyzed using the National Institutes of Health ImageJ program (Java 8) (Bethesda, MD).

3.2.3 DNA adduct analysis

DNA adductomic mass spectrometry profiling was utilized to examine the activity of the FA DNA repair pathway across the cell lines under basal conditions and in response to treatment with acetaldehyde. Acetaldehyde-induced DNA bifunctional adducts were quantitated to determine whether DNA adducts correlate with sensitivity to the genotoxin and whether there were specific adducts under basal (untreated) conditions. Three independent experiments were completed. One million cells (HaCat, TIGK, FANCA$^{-/-}$) were plated in 60mm petri dishes in cold media and treated with 0.5 mM acetaldehyde

for 48 hours. The cells were plated in cold (4°C) media to minimize vaporization of acetaldehyde which has a boiling point of 20.2°C. DNA was extracted from the harvested cells used the Gentra Puregene Core Kit B (Qiagen). DNA samples were shipped overnight on dry ice to Dr. Silvia Balbo, a collaborator at the University of Minnesota. The Balbo lab processed the samples as previously described in Guidolin, et al. (2021) [83]. **Table 3.1** shows a list of the candidate DNA adducts that were screen for in the analysis (courtesy of Silvia Balbo).

Precursor Ion (*m/z*)
278.0981
294.1083
304.1048
308.1234
310.1132
312.1132
322.1389
324.1297
332.1347
338.1456
340.1242
358.1506
366.1410
368.1546
382.1712
492.2031
505.2155
531.2089
587.1889

Table 3.1: List of candidate acetaldehyde-specific DNA adducts (interstrand crosslinks) that were screened for in the samples. List of screened adducts was provided courtesy of Silvia Balbo, collaborator at the University of Minnesota.

3.2.4 Gamma-H2AX (γH2AX) protein activity

One and a half million cells (HaCat, TIGK, OKF4, FANCA$^{+/+}$, FANCA$^{-/-}$) were plated in 60mm petri dishes in cold (4°C) media and treated with 50 μM formaldehyde and 1 mM acetaldehyde for 48 hours. Positive controls included cells treated with 60 ng/mL mitomycin C (FANCA$^{+/+}$, FANCA$^{-/-}$) for 48 hours and cells irradiated (FaDu) at Grade 4 (400 rads) using a Cesium-137 irradiator. To detect the protein γH2AX via Western blot, 60 μg of protein were loaded to each lane of a 4-12% Bis-Tris Gel (Invitrogen). The proteins were transferred to a PVDF membrane (Immobilon, Merck Millipore Ltd.) and blocked in 1X TBST and 5% BSA. The blots were probed with a γH2AX primary antibody (Millipore Sigma) and a species-specific secondary antibody that was horseradish peroxidase (HRP)-labeled. Chemiluminescence (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific) was used to visualize the bands, and images were captured using the Bio-Rad ChemiDoc Imaging System (Hercules, CA).

3.3 Results

Cellular Viability:

The purpose of the viability assay was to determine the impact that formaldehyde and acetaldehyde have on the epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines' survival. The question assessed was: what impact do formaldehyde and acetaldehyde have on cellular viability in the epidermal and oral keratinocyte cell lines? The prediction was that the cell lines would have similar sensitivity to formaldehyde and acetaldehyde.

The viability assay showed that the oral keratinocytes were not hypersensitive to formaldehyde and acetaldehyde relative to the epidermal keratinocytes. On the contrary, the epidermal keratinocyte cell line (HaCat) was more sensitive to treatment with formaldehyde and acetaldehyde compared to the oral keratinocyte cell lines (TIGK, OKF4), and the finding was statistically significant at all concentrations tested (i.e., 0.5 mM formaldehyde, 1 mM formaldehyde, 0.5 mM acetaldehyde, and 1 mM acetaldehyde) (**Figure 3.1**). In **Figure 3.1A**, when the cells were treated with 0.5 mM acetaldehyde, the percent viability was about 25% for the epidermal keratinocyte cell line (HaCat), 50% for one of the oral keratinocyte cell lines (TIGK), and 78% for the second oral keratinocyte cell line (OKF4). A similar trend was observed when the cells were treated with 0.5 mM formaldehyde, 1 mM formaldehyde, and 1 mM acetaldehyde. This result supported the alternative hypothesis which suggests that there are some inherent differences between the epidermal and oral keratinocytes. In this case, the purported inherent differences between the epidermal and oral keratinocyte cell lines would suggest resistance in the oral

keratinocytes relative to the epidermal keratinocytes following exposure to formaldehyde and acetaldehyde.

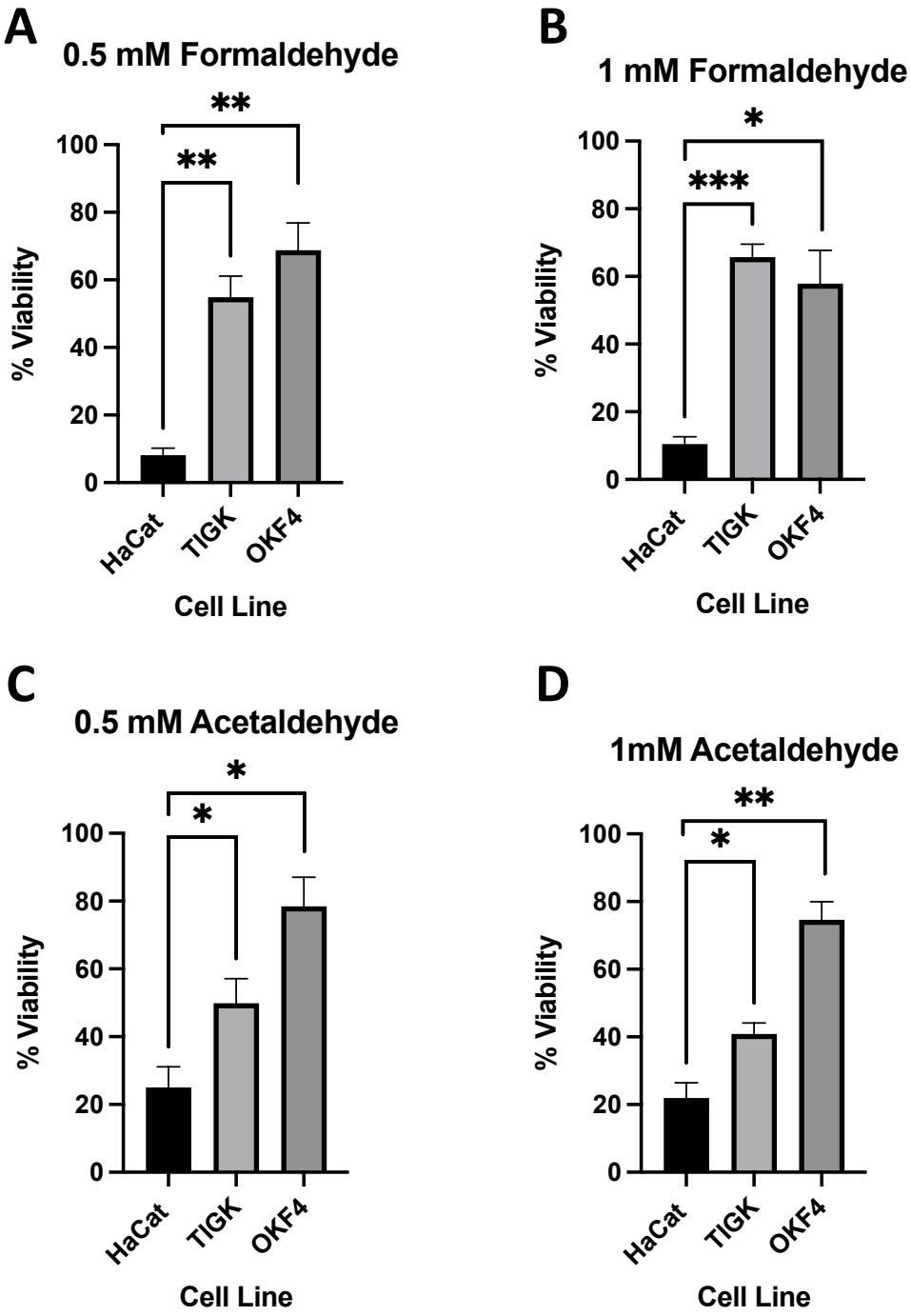

Figure 3.1: Epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines were treated with 0.5 mM formaldehyde, 1 mM formaldehyde, 0.5 mM acetaldehyde, and 1 mM acetaldehyde. Viability was measured by microscopic imaging following Hoechst 33342 dye staining. The results represent the means and standard deviations of a single experiment done in triplicate. Each bar was normalized to the untreated cell count. Percent viability below 100% indicates that cell death occurred. $P<0.05$*, $P<0.01$**, $P<0.001$***

Mono-ubiquitinated FANCD2:

FANCD2 is one of the key players in the FA DNA repair pathway, and when FANCD2 is mono-ubiquitinated, this event indicates that the proteins upstream of FANCD2 are functioning properly and that the FA DNA repair pathway is operational. FANCD2 activity (non-ubiquitinated and mono-ubiquitinated) was assessed via Western blot, and the question was: what is the status of the FA DNA repair pathway at baseline and in response to acetaldehyde in the epidermal and oral keratinocyte cell lines? I predicted that cell lines would have similar FA DNA repair pathway activity.

The Western blot data showed that acetaldehyde stimulated FA DNA repair activity in the oral keratinocytes to a greater degree compared to the epidermal keratinocytes (**Figure 3.2**). At baseline (control), bands for non-ubiquitinated and mono-ubiquitinated FANCD2 were present for all three cell lines. When the cell lines were treated with acetaldehyde without Parafilm (ACET) or acetaldehyde with Parafilm (ACET + P), in the oral keratinocytes a shift of non-ubiquitinated to mono-ubiquitinated FANCD2 was observed. This shift was especially evident in **Figure 3.2A** for the first oral keratinocyte cell line (TIGK). For this oral cell line (TIGK), in the first lane (control), there were relatively equal levels of non-ubiquitinated and mono-ubiquitinated FANCD2. This finding was confirmed by density quantification of the bands in **Figure 3.2B** which shows the ratio of the upper band (mono-ubiquitinated FANCD2) to the lower band (non-ubiquitinated FANCD2). A ratio of one indicates that the lower and upper bands are equal. When the oral cell line (TIGK) is treated with acetaldehyde, there is a noticeable shift in which the lower band becomes fainter, and the upper band becomes darker. This indicates very clearly that the FA DNA repair pathway was stimulated by the acetaldehyde

treatment in this cell line. A similar trend was observed in the second oral keratinocyte cell (OKF4), albeit the shift was more subtle. This result supported the alternative hypothesis which suggests that there are inherent differences between the epidermal and oral keratinocyte cell lines. This experiment was done once and would need to be repeated to establish statistical significance.

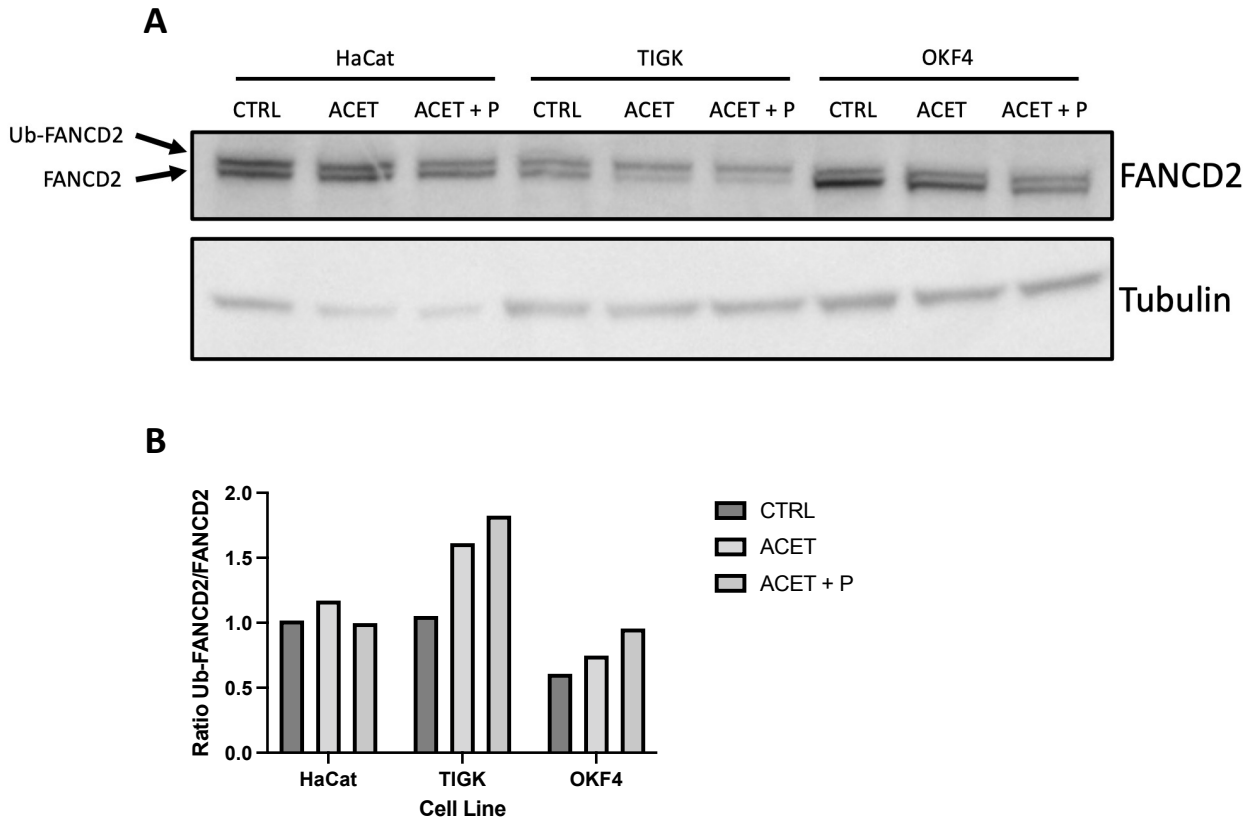

Figure 3.2: (A) Western blot visualizing the two versions of FANCD2 across the epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines. The lower band (FANCD2) is the inactive form, and the upper band (Ub-FANCD2) is the active, mono-ubiquitinated form which indicates that the FA DNA repair pathway is active. ACET indicates treatment with 1 mM acetaldehyde. ACET + P indicates treatment with 1 mM acetaldehyde and subsequently sealing the petri dish with Parafilm (P) to prevent the acetaldehyde from escaping the petri dish due to its volatile nature. Cells were treated for 24 hours. **(B)** Western blot quantification of Ub-FANCD2/FANCD2 ratio. Loading control (Tubulin) was also integrated into western blot quantification. n=1.

DNA Adductomic Profiling:

DNA Adductomic Profiling is a mass spectrometry technique used to assess DNA damage (e.g., aldehyde-induced DNA adducts). The questions the assay was intended to address was: are DNA adducts present at baseline, and can acetaldehyde-specific DNA adducts be induced, detected, and measured in the epidermal and oral keratinocyte cell lines? The prediction was that equal quantities of DNA adducts would be induced by acetaldehyde in the cell lines. The oral SCC FANCA$^{-/-}$ cell line was used a positive control, and the prediction was that higher quantities of DNA adducts would be induced in the FANCA$^{-/-}$ cell line due to absence of the FA DNA repair pathway.

Acetaldehyde-induced DNA adducts were not observed as the assay did not work for unknown reasons. To confirm that the assay did not work, a positive control sample, a cell line deficient in the FA DNA repair pathway (FANCA$^{-/-}$), was examined. Due to lack of a functional FA DNA repair pathway, the FANCA$^{-/-}$ cell line should have developed DNA adducts following acetaldehyde exposure. Detecting bi-functional acetaldehyde-induced DNA adducts (i.e., acetaldehyde-induced DNA interstrand crosslinks), is a relatively novel method for the collaborating lab (Silvia Balbo Lab at the University of Minnesota) that completed the adduct analysis, and more troubleshooting needs to take place. Possible reasons for the assay not working include preparation of the samples (e.g., DNA extraction procedure), not having enough DNA, and potentially the need to modify the mass spectrometry protocol. The result for an adductomic assay includes chromatograms for each DNA adduct detected. From each adduct-specific chromatogram, the area under the curve can be measured, and this is quantitative data that is used for analysis. In this experiment, no DNA adducts were detected and no

chromatograms were generated, hence there is no true data for this assay to show here. Again, this assay needs to be repeated, and detection methods need to be optimized using the positive control sample as a reference.

The DNA Adductomic Profiling assay was uninformative as no data was captured. However, there was still an interest in understanding the impact of formaldehyde and acetaldehyde on the genome, so a marker for DNA double-strand breaks was evaluated: γH2AX.

γH2AX:

When a DNA double-strand break occurs, the histone H2AX is phosphorylated on the Ser-139 residue, creating γH2AX. This signature phosphorylation event is an early cellular response specific for DNA double-strand breaks. An increase in the cellular concentration of γH2AX is indicative of an increase in DNA double-strand breaks and can be measured using Western blot following treatment with aldehydes. The question addressed via Western blot detecting γH2AX was: are double-strand breaks induced in the epidermal and oral keratinocyte cell lines when treated with formaldehyde and acetaldehyde? The prediction was that there would be similar levels of activation of γH2AX in the cell lines. γH2AX levels were also examined in the oral SCC FANCA$^{+/+}$, FANCA$^{-/-}$, and FaDu cell lines as positive controls. One oral keratinocyte cell line (OKF4) had higher levels of double-strand breaks compared to the epidermal cell line (HaCat) (**Figure 3.3**). The other oral keratinocyte cell line (TIGK), like the epidermal cell line (HaCat), did not show evidence of double-strand breaks. The double-strand breaks observed in the oral keratinocyte cell line are likely due to double-strand breaks intentionally induced by the FA DNA repair pathway to resolve DNA interstrand crosslinks.

γH2AX levels were also observed in the malignant cell lines. For the FANCA$^{+/+}$ (oral SCC) and FANCA$^{-/-}$ (oral SCC) cell lines, γH2AX expression was present at baseline and in response to treatment with formaldehyde, acetaldehyde, and mitomycin C. γH2AX levels were greatest in the FANCA$^{-/-}$ compared to the FANCA$^{+/+}$ cell line for the control as well as the formaldehyde and acetaldehyde treatment. This result was expected as the FANCA$^{-/-}$ lacks the ability to repair DNA damage via the FA DNA repair pathway so more

double-strand breaks would be expected. γH2AX levels were greater in the FANCA$^{+/+}$ compared to the FANCA$^{-/-}$ cell line for the mitomycin C treatment. In general, γH2AX expression was greatest when the FANCA$^{+/+}$ and FANCA$^{-/-}$ cell lines were treated with mitomycin C, a known and well-studied DNA crosslinker. Irradiated FaDu cells, a positive control, also showed γH2AX activation. While the double-strand breaks induced in the oral keratinocyte cell line likely resulted from intentional action of the FA DNA repair pathway to facilitate repair, double-strand breaks observed in the malignant cell lines, particularly for the FANCA$^{-/-}$ cell line, likely resulted from blatant chromosomal breakage due to lack of DNA repair capabilities. This experiment was only performed once, and thus statistical analysis of quantitated band densities could not be performed. Repeat experiments are needed to confirm results and alignment with the preferred hypothesis.

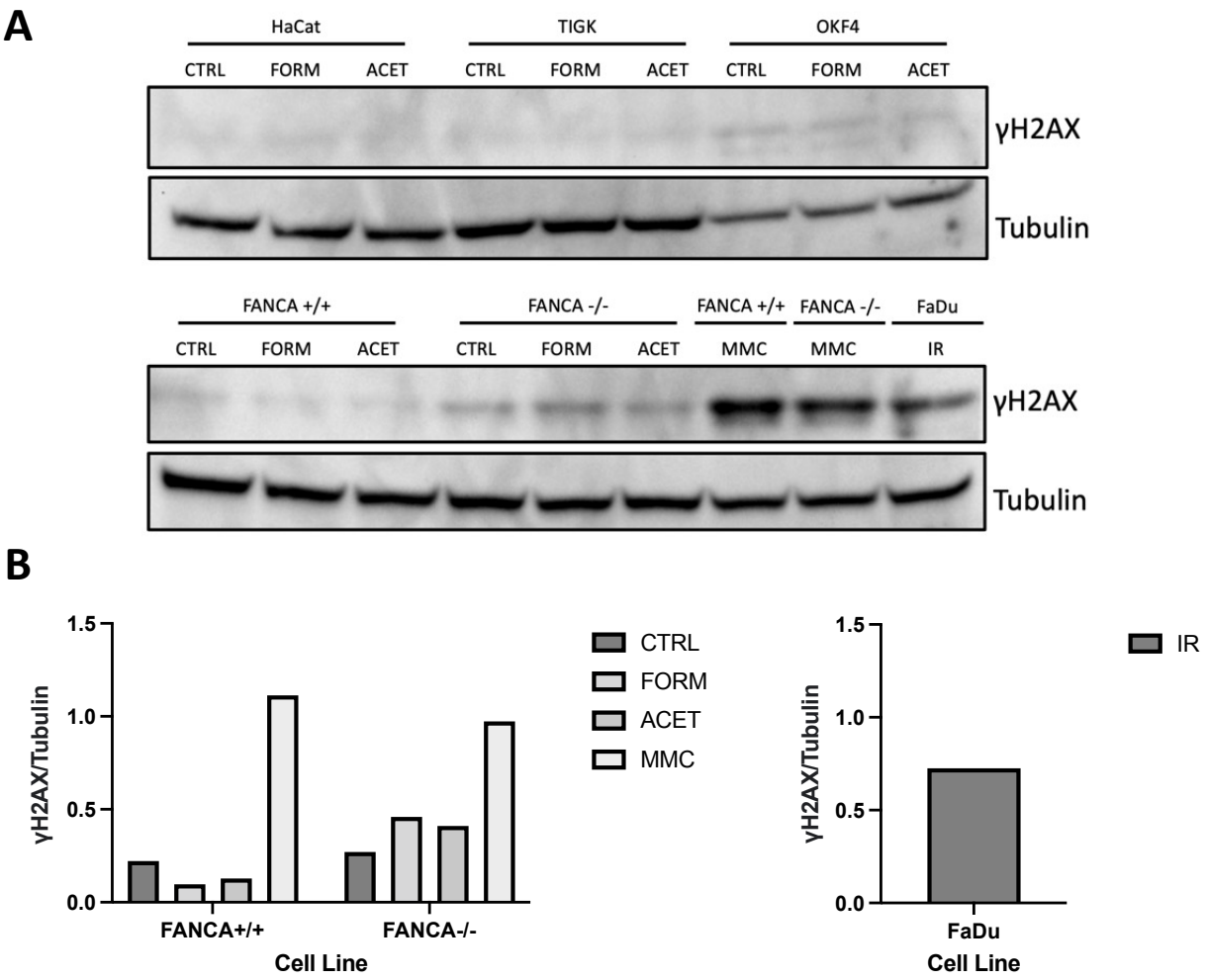

Figure 3.3: (A) Western blot visualizing γH2AX across the epidermal keratinocyte (HaCat), oral keratinocyte (TIGK, OKF4), and oral SCC (FANCA$^{+/+}$, FANCA$^{-/-}$, and FaDu) cell lines. FORM indicates treatment with 50 μM formaldehyde. ACET indicates treatment with 1 mM acetaldehyde. MMC denotes treatment with 60 ng/mL mitomycin C. IR refers to cells irradiated at Grade 4 (400 rads). Cells were treated for 48 hours. **(B)** Western blot quantification of γH2AX/Tubulin for the oral SCC cell lines (FANCA$^{+/+}$, FANCA$^{-/-}$, and FaDu). (Note: FANCA$^{+/+}$ and FANCA$^{-/-}$ refer to the UM-SCC-01 FANCA$^{+/+}$ and UM-SCC-01 FANCA$^{-/-}$ cell lines, respectively.)

3.4 Conclusions

The viability assay showed that the oral keratinocyte cell lines were more resilient compared to the epidermal keratinocytes when treated with formaldehyde and acetaldehyde at 0.5 mM and 1 mM concentrations. The finding was statistically significant at each concentration tested. The viability data suggests that in normal, non-FA cell types, the oral tissues are more resistant to formaldehyde and acetaldehyde genotoxic assault, possibly due to the activity of the FA pathway. It is also possible that surviving oral keratinocytes have may have aldehyde-induced DNA damage (i.e., genomic instability and mutations) which could then contribute to HNSCC carcinogenesis.

To monitor the activity of the FA DNA repair pathway, expression of non-ubiquitinated FANCD2 and mono-ubiquitinated FANCD2 (indicator that the FA DNA repair pathway is functional) was observed. Basal FA DNA repair activity was present in the epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines. Acetaldehyde exposure activated the FA DNA repair pathway to a greater degree in the oral keratinocyte cell lines (TIGK, OKF4) compared to the epidermal (HaCat) cell line. The ability of oral keratinocyte (TIGK, OKF4) cell lines to activate the FA DNA repair pathway more readily in response to genotoxic stress (i.e., formaldehyde and acetaldehyde) also hints at a greater reliance on the FA pathway for protection compared the epidermal (HaCat) keratinocyte cell line which may rely more on other forms of protection. It is possible that epidermal keratinocytes rely more on ALDH2 as a mechanism of protection compared to reliance on the FA DNA repair pathway. ALDH2 is the enzyme responsible for catabolizing acetaldehyde into acetic acid, and there is data that suggest that the expression of ALDH2 is greater in the epidermal tissues compared to expression in the

oral mucosa [84, 85]. The FANCD2 Western blot results did not support the favored hypothesis and instead supports the notion that there are some inherent differences between the oral and epidermal keratinocytes which contribute to aldehyde response.

Aldehyde-specific DNA adducts were not detected in any of the samples, including the positive control sample, making this experiment uninformative. This was the first time this assay was attempted. This experiment needs to be repeated, and much troubleshooting will be necessary to optimize the protocol to detect acetaldehyde-specific DNA interstrand crosslinks. Some troubleshooting aims can include increasing the number of cells that are treated which would increase the amount of DNA harvested; additionally, modifying the DNA extraction protocol could be useful. The goal of the suggested troubleshooting options would be to increase the amount of DNA that is used for the adduct analysis and to increase the quality of the DNA harvested. Importantly, troubleshooting attempts should be completed with the positive control sample to validate the assay prior to testing additional experimental conditions.

Since the DNA Adductomic Profiling assay was uninformative and did not yield useful data, γH2AX levels were measured by Western blot to assess DNA double-strand breaks downstream of aldehyde treatment which gave some insight into the impact of exogenous formaldehyde and acetaldehyde on the genome. γH2AX expression was observed in one of the oral cell lines (OKF4), and the indicated double-strand breaks likely resulted from FA pathway-induced double-strand breaks that occur to resolve DNA damage (i.e., interstrand crosslinks). It was interesting that double-strand breaks were observed in one oral keratinocyte cell line (OKF4) and not the other (TIGK). This finding

suggests that within the oral cavity (and presumably other cells of the head and neck), there are inherent differences that are unique to the specific cell type. γH2AX expression was also observed in the malignant oral cell lines (FANCA$^{+/+}$, FANCA$^{-/-}$, and FaDu) which served as positive controls for the experiment.

CHAPTER 4:
DISCUSSION AND FUTURE DIRECTIONS

The question that inspired my dissertation is as follows: why are FA patients predisposed to HNSCC at a higher rate compared to other non-hematopoietic malignancies? The intention of my dissertation research was to understand whether formaldehyde and acetaldehyde contributed to DNA interstrand crosslinking events which could consequently lead to genomic instability and HNSCC. Since aldehyde exposure varies for different cell types and tissues, and since differences in cellular response to aldehyde exposure could be impacted by an FA diagnosis, both cell type and FA status were examined as variables in this study. Endogenous aldehyde production was measured across various human cell lines including oral keratinocytes, which are exposed to exogenous aldehydes and can be involved in HNSCC, especially in FA patients, and epidermal keratinocytes, which are not generally exposed to high levels of exogenous aldehydes and are not considered to be at increased risk for malignancy in FA patients. Cell lines which are considered FA-functional and FA-defective were used as controls for the experiments. Two sources of aldehydes were examined: endogenous and exogenous. For endogenous aldehydes, cell lines were assessed for typical aldehyde production during normal cellular metabolism in standard culture conditions. For exogenous aldehydes, the cell lines were treated with formaldehyde and acetaldehyde to gauge cellular viability, activity of the FA DNA repair pathway, and DNA double-strand break induction.

My data suggest that endogenous aldehyde production is unlikely to be a contributing factor to genomic instability in the cell lines examined. There was no

evidence of endogenous aldehyde production in the non-malignant oral keratinocyte cell lines (TIGK, OKF4) nor the epidermal keratinocyte cell line (HaCat). Since the oral keratinocytes did not produce more aldehydes compared to the epidermal keratinocytes, this supports the premise that endogenous aldehyde production is not responsible for the high incidence of HNSCC that is seen in FA patients. Unlike the results of the Aldehyde Assay and the HS GC/MS assay which were in alignment with the favored hypothesis, the viability assay demonstrated that inherent differences exist between the oral and epidermal keratinocytes. Likewise, the viability findings also hinted that there is a system of protection in place that makes the oral keratinocyte cell lines (TIGK, OKF4) more resistant to formaldehyde and acetaldehyde compared to the epidermal cell line (HaCat).

The systems of protection come in two forms: the FA DNA repair pathway and ALDH2. Acetaldehyde exposure activated the FA DNA repair pathway to a greater degree in the oral keratinocyte cell lines, and this finding suggests that the oral keratinocytes may rely more on the FA DNA repair pathway for protection against DNA crosslinking agents. There was no evidence of formaldehyde- and acetaldehyde-induced double-strand breaks for the epidermal cell line (HaCat) and one of the oral keratinocyte cell lines (TIGK). There were faint γH2AX bands for the OKF4 oral cell line, which aligns with the observation of increased activation of the FA DNA repair pathway. When the FA pathway works to resolve DNA interstrand crosslinks, double-strand breaks are intentionally induced to aid in the removal of the interstrand crosslinks. Additionally, for the viability assay, the cell line that had the greatest viability for three out of the four treatment conditions (i.e., 0.5 mM formaldehyde, 1 mM formaldehyde, 0.5 mM acetaldehyde, and 1 mM acetaldehyde), was the OKF4 oral keratinocyte cell line. γH2AX is an early cellular response to the

induction of DNA double-strand breaks so it is plausible that the reason for the increased survivability of the OKF4 cell line is that the FA DNA repair pathway is activated more robustly in this cell line (OKF4) compared to the other two cell lines assessed (HaCat, TIGK). γH2AX levels in the OKF4 cell line support this notion.

If the cell line data can be reasonably extrapolated in vivo, this would suggest that endogenous aldehyde production is unlikely to be a contributing factor to HNSCC development in FA patients. However, the best way to extrapolate these findings would be to measure endogenous aldehyde levels across multiple tissues including oral and epidermal sources from individuals diagnosed with FA and from healthy controls.

Exposure to exogenous formaldehyde and acetaldehyde is a possible contributor to HNSCC if the cell line data can be reasonably extrapolated to FA patients; however, exogenous aldehyde exposure is not the full narrative because some inherent differences were observed between the cell lines. The oral keratinocyte cell lines activated the FA DNA repair pathway to a greater extent compared to the epidermal keratinocyte cell line. The increased activation of the FA DNA repair pathway in the oral keratinocyte cell lines suggests greater dependence on the repair pathway for protection. It is important to note that in an FA patient, the FANC mutation is present in every cell within the body, which results in a systemically non-functional FA DNA repair pathway; this is what makes FA patients who they are on a genomic level. The presence of the FANC mutation in all the cells of an FA patient is in alignment with the systemic nature of the genetic disorder. Therefore, loss of the FA pathway in the oral cavity and presumably in surrounding tissues of the head and neck, could be more detrimental since these tissues rely more on the FA

DNA repair pathway for protection from exogenous aldehyde exposure compared to other tissues such as the epidermal tissues. The epidermal keratinocytes may be relying more on ALDH2 for protection. Some data suggest that there is greater expression of ALDH2 in epidermal tissues compared to ALDH2 expression in the oral mucosa, and FA patients have functional ALDH2 [84, 85]. If the epidermis depends more on ALDH2 for protection, then this tissue would be less affected in FA patients.

There were several limitations that were specific to this study. All the experiments were completed using cell lines derived from non-FA patients. It would be useful and relevant to complete these experiments using primary cells from FA patients representing a variety of FANC mutations to capture a better understanding about the cellular and molecular dynamics that influence HNSCC development in this population. Likewise, this work was completed using established cell lines, and my analysis of non-malignant oral keratinocytes was limited to two cell lines. For many analyses, the oral keratinocytes (TIGK, OKF4) behaved similarly, but not always. Therefore, it would be necessary to study additional cell types from the head and neck tissues. Additionally, formaldehyde and acetaldehyde are highly volatile due to their extremely low boiling points. Due to the volatile nature of the two compounds, it can be challenging to detect and quantify formaldehyde and acetaldehyde. Additional troubleshooting needs to be done to increase the sensitivity of the HS GC/MS assay. One idea is to double the concentration of media that is added to the glass vials used for derivatization. This would ideally increase the concentration of aldehyde derivatives in the headspace of the glass vials, the region from which the sample is collected and subsequently injected to the gas chromatograph.

Additional limitations exist that are related to the field of FA research. Since FA is a rare disorder, it may be challenging to find FA patients and harvest biological samples (e.g., primary cells). Acquiring biological samples from FA patients would help move the field forward because the investigative findings would be more relevant to the patients that this research is centered around. This is a challenge faced by many FA researchers. Furthermore, animal models would be helpful in studying mechanisms of HNSCC initiation and progression in FA, and to my knowledge, at this time, there are no reported animal models which develop HNSCC in a FA or FA-like background. Perhaps generation of such a model would benefit the field by providing the complex biological system necessary to best understand the interactions between various cells and tissues with endogenous and exogenous genotoxic exposures.

ALDH2 expression in tissues has been inadequately studied, and much of the data regarding ALDH2 expression has been generated in mice [86]. There is a need to understand ALDH2 expression in human tissues. It would have been beneficial to assess ALDH2 expression in the epidermal (HaCat) and oral (TIGK, OKF4) keratinocyte cell lines as well as the malignant oral ($FANCA^{+/+}$, $FANCA^{-/-}$, FaDu) cell lines that were used for this study (Chapters 2 and 3). Evaluating ALDH2 levels in the cell lines could have provided another layer of information that could have informed data interpretation. Matsumoto et al. (2019) showed that when mice deficient in ALDH2 consumed ethanol, ethanol-induced skin hyperpigmentation occurred [87]. This finding was interesting because it confirms that there is a link between ALDH2, ethanol/acetaldehyde, and the skin. Acetaldehyde (and formaldehyde) vapors can also permeate through the skin [88].

Skin pigmentation issues are commonly reported in FA patients [89]. More investigation needs to be done exploring ALDH2 in FA patients.

Exploring ALDH2 levels in the context of FA can lead to the development of novel pharmaceutical tools such as ALDH2 activators [90, 91]. There is a need to thoroughly explore ALDH2 levels across different tissues in FA patients, and it would be beneficial to complete this assessment in non-FA patients as well. Increasing the activation of ALDH2 in the head and neck tissues of FA patients could help with the metabolism of acetaldehyde. FA patients may not have a functional FA DNA repair pathway, but they do have functional ALDH2 which can be used to their advantage. Though it is recommended that FA patients avoid products with alcohol such as alcoholic beverages and mouthwashes that contain alcohol, it is important to note that acetaldehyde (and formaldehyde) have been found in foods and beverages [75-77]. It would be wise to consider protecting FA patients against potential aldehyde exposure from daily eating and drinking. Understanding more about ALDH2 levels in non-FA patients could also help individuals who are at risk for developing HNSCC such as individuals who consume (or abuse) alcohol. The ethanol that is found in alcoholic beverages (and mouthwashes) is metabolized to acetaldehyde, and the acetaldehyde is catabolized to acetic acid by ALDH2. Individuals who drink alcohol in excess are at risk for developing HNSCC and could benefit from stimulating the activity of cellular levels of ALDH2.

In addition to investigating ALDH2 activity, it would be relevant to collect saliva samples from FA patients to screen for acetaldehyde and additional analytes. Studying the saliva of FA patients can serve as an entryway into investigating the oral microbiome

of FA patients. From the literature, it is known that oral microbes can metabolize ethanol to produce acetaldehyde, and oral microbes can also produce acetaldehyde from sugar alcohols in the diet [74]. Sugar alcohols are artificial sweeteners that are used in sugar-free products [92].

Furthermore, I am interested in oral health, specifically the relationship between head and neck cancer and the oral microbiome. Studies have elucidated the importance of the oral microbiome in the maintenance of health and the progression of disease [93-95]. Investigating the oral microbiome in FA patients, a population that is cancer-prone, could be a valuable contribution to the field. There is a need for further identification and characterization of the oral microbiome. The link between HNSCC and the oral microbiome in FA patients is an area that has not been extensively explored. After alcohol consumption, it has been demonstrated that acetaldehyde levels are higher in the saliva compared to the liver, the organ where ethanol is primarily metabolized [96]. As mentioned, the oral microbiota can metabolize ethanol, and the first metabolite of ethanol is acetaldehyde, a Class I carcinogen [97, 98]. Studies also show that oral microbes can produce acetaldehyde from sugar alcohols which can be found in the diet [74]. Alcohol consumption is a known risk factor for head and neck cancer, and consequently, it is recommended that FA patients avoid consuming alcoholic beverages. Since FA is typically diagnosed in childhood and in early adolescence (prior to the legal drinking age in many countries), FA patients are typically not drinking alcohol due to their age. However, a potential source of exposure to alcohol that must be avoided is mouthwash [63, 64].

The composition of oral microbes in the mouth is influenced by factors such as diet and oral hygiene, and poor oral hygiene is associated with an increased risk for developing oral cancer [99, 100]. Some literature alludes to the notion that FA patients tend to have poor oral hygiene [19]. In a study that reported oral findings in 26 Turkish children with FA, 62% of the children had never received care from a dentist [19]. This is concerning because dentists are an essential part of the care team for FA patients. It is imperative that FA patients are continuously engaged in dental healthcare and are seen by dentists regularly to ensure the maintenance of oral health. Additionally, since FA patients are susceptible to developing HNSCC, dentists can help monitor oral tissues for abnormal changes (e.g., precancerous lesions). The sample size of the study was small (i.e., 26 children) [19]. However, this study demonstrates the need for more investigation to assess the oral health state across a plethora of FA patients to avoid making broad generalizations and so that an accurate picture can be captured. It is critical to note that FA patients may face social and economic barriers (and other challenges related to the social determinants of health) that hinder them from consistent engagement in quality dental care.

Furthermore, there is a need for oral healthcare professionals who are knowledgeable in disorders like FA and who are invested in addressing oral healthcare needs that are unique to FA patients. Tailored education about the importance of oral health and oral conditions that are commonly found in FA patients may be beneficial to this population. Some of the oral conditions observed in FA patients include gingivitis, periodontitis, caries (cavities), and inflammation of the oral mucosa [17, 18, 101]. FA patients also experience dry mouth (xerostomia) [19, 102]. Dry mouth can result from

radiotherapy and immunological complications [103-106]. Additionally, dry mouth can influence the composition of the oral microbiome. It is important to maintain normal levels of saliva production because saliva lubricates the oral cavity and protects the oral mucosa [107, 108].

Lyko et al. (2013) collected saliva samples from FA patients to screen for four bacteria that contribute to periodontal disease [109]. However, minimal work has been done to investigate the link between the oral microbiome of FA patients in relation to HNSCC, and again, oral healthcare professionals can help to bridge this knowledge gap. It is plausible that the oral microbiome of FA patients may predispose them to developing HNSCC or at least contribute to the progression of HNSCC. The aim of this proposed study would be to investigate and characterize the oral microbiome in FA patients and measure microbial acetaldehyde production. A non-invasive way to investigate and characterize the oral microbiome of FA patients would be to collect saliva samples from FA patients and analyze the microbes present. In addition to investigating and characterizing the oral microbiome in FA patients, I would also measure acetaldehyde production in the saliva samples using HS GC/MS [110].

One of the biggest breakthroughs of my research project was the HS GC/MS work. A lot of time was dedicated to optimizing the protocol. After much effort, each of the expected peaks on the chromatogram were distinctly visible (i.e., the peaks for PFBOA (the derivatization agent), formaldehyde, acetaldehyde, and the internal standard). I think the HS GC/MS work has potential clinical applications. The HS GC/MS work can extend into the clinical realm by substituting the media samples that I used in my

experiments with biological samples from FA patients, specifically saliva. Collecting saliva samples would be non-invasive and relatively easy to acquire from the patients assuming that a pool of patients is available. The purpose of collecting the saliva samples would be to investigate whether there are detectable levels of formaldehyde and acetaldehyde, and if so, quantitate the levels and monitor for other oral complications which may be correlated to aldehyde levels. Such a study could take place in a dental office during biannual visits; it is recommended that individuals seek dental care every six months for routine checkups. Compared to cell culture media, saliva is quite viscous which could impact the ability of the derivatized analytes to enter the headspace of the glass vial upon heating. However, the viscosity of saliva can be easily overcome by adding a reagent to make the saliva a bit thinner. The viscosity of the saliva could also be overcome by heating the samples for a longer period (i.e., longer than the 60-minute heating at 60°C for derivatization). Using the protocol that I employed in my HS GC/MS experiments, it is likely that formaldehyde and acetaldehyde could be observed.

The HS GC/MS protocol that I used to run my samples is known as Selected Ion Monitoring (SIM). With the SIM protocol, I tell the instrument which ions are of interest, and these are the only ions that are screened in the samples. There is another method to run the samples known as Full Scan (FS) monitoring. With this method, the instrument can collect data regarding all the compounds that are present in the samples. In the case of this proposed experiment, it would be compounds that are able to undergo derivatization with PFBOA and enter the headspace of the vial. I think it would be valuable to run the SIM and FS monitoring on the saliva samples. FS monitoring can detect derivatized compounds beyond the compounds observed in SIM and make it possible to

find derivatized compounds beyond formaldehyde and acetaldehyde. FS monitoring could open doors to compounds that were not specifically screened for in my analyses and may detect the presence of other aldehydes that are larger than formaldehyde and acetaldehyde. In previous experiments, when running the FS protocol on samples, acetic acid was detected, and acetic acid is a metabolite of acetaldehyde. Acetic acid was observed when sampling directly from the media. With FS detection, the mass spectrometry software has thousands of compounds in its database that make it possible to match the unknown analytes in the samples with known compounds in the database.

This study could be impactful and best conducted as a longitudinal study with FA patients of all ages and those without HNSCC and those with HNSCC. In addition to the data collected via HS GC/MS to capture information about the chemical species present in the saliva, it would be valuable to observe the bacterial community members that are also present in the saliva samples. Microbial communities shift based on health and disease. Many FA patients have adverse oral health situations and some lean more toward the disease end of the spectrum. The intent of the longitudinal study would be to collect relevant data from the saliva samples and follow the patients to see who develops HNSCC (if the patient does not already have HNSCC). A long-term goal would be to create a database with all the analytical chemistry and microbial data collected from the saliva samples. Additionally, based on the wealth of data gathered (including demographic information about the subjects), a second long-term goal would be to discern whether a pattern can be detected among the FA patients who at the start of the study do not have HNSCC and go on to develop HNSCC and among those who have HNSCC at the start of the study. It would be interesting to ascertain if a pattern exists among these two groups

based on compounds present in the saliva and based on the oral microbes detected in the saliva samples. Such a pattern may make it possible to predict the 1 in 7 FA patients who will then go on to develop HNSCC [7].

The HS GC/MS work that I presented in my dissertation has laid groundwork that has clinical relevancy. Coupling HS GC/MS with investigating the oral microbiome of FA patients could contribute to the FA field of research. It is possible that there will be a day when a physician can collect the saliva of an FA patient and use it to predict whether the patient will develop HNSCC and have preventative regimens available to prevent the development of HNSCC. Investigating the analytes and oral microbial community members present in saliva samples of FA patients holds promise. While investigating oral health in FA patients using HS GC/MS techniques could be beneficial to the field, there is an alternative approach that could yield relevant data. For example, before diving into aldehyde analysis, there could be value in examining broad factors that contribute to the adverse oral outcomes in FA patients. Factors such as saliva production, diet, and the immune system can all influence oral manifestations in FA patients and gathering data on these metrics could be helpful.

As mentioned previously, more research needs to be done investigating ALDH2 activity in FA patients. Acetaldehyde is metabolized by ALDH2. Novel pharmaceutical tools are available that can induce the activity of ALDH2 which can increase the rate of acetaldehyde metabolism. Increasing the expression/activation of ALDH2 could be a promising therapeutic for FA patients by ridding the body of unnecessary and damaging acetaldehyde.

FA provides a unique lens and opportunity to investigate carcinogenesis of the head and neck squamous epithelia. Investigating molecular mechanisms that contribute to head and neck epithelial carcinogenesis in FA patients is vital. Understanding the mechanisms that contribute to and drive HNSCC in FA patients can lead to the development and implementation of preventative measures that can mitigate carcinogenesis. Such prophylactic measures may not only help FA patients but may extend to the broader population of individuals who are at risk for developing HNSCC but do not have FA.

APPENDICES

APPENDIX A:
LIST OF FIGURES

APPENDIX B:
LIST OF TABLES

APPENDIX C:
LIST OF KEY TERMS

Fanconi anemia (FA): a rare genetic disorder characterized by bone marrow failure, developmental anomalies, organ defects, extreme sensitivity to DNA crosslinking agents, and increased susceptibility to certain cancers (e.g., acute myeloid leukemia (AML) and head and neck squamous cell carcinoma (HNSCC))

Acute myeloid leukemia (AML): a cancer that begins in the bone marrow and spreads to other blood-forming cells of the bone marrow; results in the buildup of immature bone marrow cells

Head and neck squamous cell carcinoma (HNSCC): a cancer that develops in squamous cells of the upper aerodigestive tract; sites that can be affected include the lips, the oral cavity, the tongue, the salivary glands, the nasal cavity, the pharynx, the paranasal sinuses, and the larynx

Reactive aldehydes: a group of organic compounds with the generic formula (RCHO) that are highly reactive; examples include formaldehyde and acetaldehyde

Formaldehyde: a natural organic compound and the simplest aldehyde (CH_2O); a well-known carcinogen

Acetaldehyde: a natural organic chemical compound and the second-most simplest aldehyde (CH_3CHO); a well-known carcinogen

Hematopoietic stem cell transplantation (HSCT): a type of cancer treatment intended to restore normal hematopoietic function; can also be used to treat progressive bone marrow failure

Mitomycin C: a chemotherapeutic drug that can be used to treat upper gastro-intestinal cancers and a potent DNA crosslinking agent ($C_{15}H_{18}N_4O_5$)

Disulfiram: a drug that inhibits aldehyde dehydrogenase 2, the enzyme that converts acetaldehyde into acetic acid (vinegar)

FA DNA repair pathway: DNA repair system that resolves DNA interstrand crosslinks; FA patients do not have a functional FA DNA repair pathway

DNA crosslinking: occurs when exogenous or endogenous agents react with two nucleotides of DNA strands resulting in the formation of a covalent bond; the covalent bond can form within the same strand (intrastrand crosslink) or between opposing strands (interstrand) of double-stranded DNA

DNA adducts: a segment of DNA that is tethered to a carcinogen; a type of DNA damage

HaCat: cell line derived from adult skin

TIGK: cell line derived from adult gingival tissue

OKF4: cell line derived from the floor of the mouth

UM-SCC-01 FANCA$^{+/+}$: cell line derived from the floor of the mouth in of a non-FA patient with HNSCC (mutations in TP53, CASP8, and EP300); cell line has a functional FA DNA repair pathway

UM-SCC-01 FANCA$^{-/-}$: cell line derived from the floor of the mouth in of a non-FA patient with HNSCC (mutations in TP53, CASP8, and EP300); cell line does not have a functional FA DNA repair pathway

JHU-SCC-FaDu FANCA$^{+/+}$: cell line derived from the pharynx of a non-FA patient with HNSCC (mutations in TP53, FAT1, and CDKN2A); cell line has a functional FA DNA repair pathway

FANCD2: a protein that is mono-ubiquitinated in response to DNA damage; FANCD2 is critical for orchestrating the repair of DNA interstrand crosslinks upon mono-ubiquitination

Headspace gas chromatography/mass spectrometry: a method used in analytical chemistry to separate and identify compounds within a mixture; this technique can be used to detect volatile compounds such as formaldehyde and acetaldehyde

APPENDIX D:
HEADSPACE GAS CHROMATOGRAPHY/MASS SPECTROMETRY METHOD

```
   INSTRUMENT CONTROL PARAMETERS:    Instrument 1
                                     ---------------------------------
-----------

   C:\MSDCHEM\1\METHODS\LAQUITA_SIM_UPDATED COLUMN_STEEPRUN.M
      Tue Mar 15 21:02:03 2022

Control Information
------- -----------

Sample Inlet      :  GC
Injection Source  :  Manual
Mass Spectrometer :  Enabled


 No Sample Prep method has been assigned to this method.

================================================================================
===
                                   6890 GC METHOD
================================================================================
===

OVEN
   Initial temp:  50 C (On)                Maximum temp:  320 C
   Initial time:  1.00 min                 Equilibration time:  0.50 min
   Ramps:
     #   Rate  Final temp  Final time
     1  20.00      300        1.00
     2    0 (Off)
   Post temp:  50 C
   Post time:  0.00 min
   Run time:  14.50 min

FRONT INLET (SPLIT/SPLITLESS)          BACK INLET (UNKNOWN)
   Mode:  Split
   Initial temp:  200 C (On)
   Pressure:  10.1 psi (On)
   Split ratio:  2:1
   Split flow:  1.6 mL/min
   Total flow:  4.8 mL/min
   Gas saver:  On
   Saver flow:  20.0 mL/min
   Saver time:  1.00 min
   Gas type:  Helium

COLUMN 1                               COLUMN 2
   Capillary Column                       (not installed)
```

```
   Model Number:  19091S-433
   Description: HP-5MS  5% Phenyl Methyl
   Max temperature:  325 C
   Nominal length:  30.0 m
   Nominal diameter:  250.00 um
   Nominal film thickness:  0.25 um
   Mode:  constant flow
   Initial flow:  0.8 mL/min
   Nominal init pressure:  10.1 psi
   Average velocity:  22 cm/sec
   Inlet:  Front Inlet
   Outlet:  (other)
   Outlet pressure:  ambient

FRONT DETECTOR (NO DET)                 BACK DETECTOR (NO DET)

SIGNAL 1                                SIGNAL 2
   Save Data:  Off                         Save Data:  Off

THERMAL AUX 2
   Use:  MSD Transfer Line Heater
   Initial temp:  220 C (On)

                                        POST RUN
                                           Post Time: 0.00 min

INJECTOR 1
   Solvent Wash Mode:  A, B
   Sample pumps:  1
   Sample Volume (uL):  5.000
   Syringe size (uL):     10.0
   Pre washes from bottle A:  0
   Pre washes from bottle B:  0
   Post washes from bottle A:  0
   Post washes from bottle B:  0
   Viscosity delay (seconds):  0
   Pre injection dwell (min):  0.00
   Post injection dwell (min):  0.00
   Sample skim depth (mm):   16.0  ( On)
   Plunger Speed:  Fast
   Solvent saver:  Off

TIME TABLE
   Time(min)      Parameter & Setpoint



 Column 1 Inventory Number :
 Column 2 Inventory Number :

                              MS ACQUISITION PARAMETERS


General Information
```

```
------- -----------

Tune File                : atune.u
Acquistion Mode          : SIM


MS Information
-- -----------

Solvent Delay            : 3.00 min

EMV Mode                 : Gain Factor
Gain Factor              : 1.00
Resulting EM Voltage     : 2341

[Sim Parameters]

GROUP 1
Group ID                 : 1
Resolution               : Low
Plot 1 Ion               : 75.00
Ions/Dwell In Group      (  Mass,  Dwell) (  Mass,  Dwell) (  Mass,
Dwell)
                         ( 75.00,     90) (181.00,     90) (195.00,
90)
                         (209.00,     90) (212.00,     90) (213.00,
90)

[MSZones]

MS Source                : 230 C   maximum 250 C
MS Quad                  : 150 C   maximum 200 C


                            END OF MS ACQUISITION PARAMETERS


                             TUNE PARAMETERS for SN: US82321663
                         ----------------------------

 Trace Ion Detection is OFF.

 EMISSION    :      34.610
 ENERGY      :      69.922
 REPELLER    :      30.123
 IONFOCUS    :      60.737
 ENTRANCE_LE :       7.000
 EMVOLTS     :    2352.941
                                Actual EMV  :    2341.18
                                GAIN FACTOR :       1.01
 AMUGAIN     :    1274.000
 AMUOFFSET   :     130.000
 FILAMENT    :       1.000
 DCPOLARITY  :       0.000
```

```
 ENTLENSOFFS :      13.553
 MASSGAIN    :    -622.000
 MASSOFFSET  :      -8.000

                        END OF TUNE PARAMETERS
                      ----------------------------------


                                                 END OF INSTRUMENT CONTROL
PARAMETERS
                                                 ------------------------------
------
```

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