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Zachary Harrison April 15, 2019

Characterization of Jagged1-Notch signaling in collective invasion and determination of ALCAM expression in non-small cell lung cancer models

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Abstract

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By Zachary Harrison

Non-small cell lung cancer (NSCLC) accounts for 85% of new lung cancer diagnoses and patients diagnosed with the disease typically have poor prognoses. Intratumoral heterogeneity complicates NSCLC treatment, which adds to the poor outcomes for patients. Designation of subpopulations of more-invasive leader cells and growth-promoting follower cells in these heterogenous populations is possible. This study sought to identify and characterize Jag1 as a biomarker for NSCLC leader cells and elucidate the function of Jag1 in collective invasion. In addition, ALCAM expression was determined in leaders, followers, and heterogenous patient-derived primary cells to assess biomarker potential. Cell cycle analysis by flow cytometry was performed on H1299 $Jag1^{+}$ and Jag1-knockdown cells to determine if Jag1 expression is correlated with cell cycle progression. No difference was observed in cell distribution in the cell cycle stages in relation to Jag1 expression status. Next, a simple proliferation assay was performed on H1299 $Jag1^{+}$ and Jag1-knockdown cells to investigate if Jag1 expression alters growth kinetics. Again, no difference was observed in the rate of proliferation between Jag1-expressing and Jag1-knockdown H1299 cells. ALCAM expression was determined in H1299 leaders and followers, in addition to heterogenous patient-derived primary cells, to determine if it is a biomarker for more invasive NSCLC subpopulations. ALCAM expression was high in H1299 leader cells, while it was low in followers. Interestingly, both patient-derived primary cell types analyzed, EUH 3123 and EUH 3174, exhibited high ALCAM expression, despite variable invasive potential *in vitro*. Despite no observed difference in cell cycle distribution or growth kinetics, it appears that Jag1 is a biomarker for H1299 leader cells on the basis of collective

invasion data not shown here. ALCAM, with high expression in two primary cell types that exhibit different invasion patterns, does not appear to be a biomarker for more invasive NSCLC subpopulations. Further studies are needed to fully characterize the role of ALCAM signaling in NSCLC collective invasion.

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CHAPTER 1: Introduction

Lung Cancer

Lung cancer is the second most common cancer in adults, accounting for over 230,000 new cases and over 150,000 deaths yearly (1). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of these new diagnoses (9). NSCLC is a heterogenous, constantly-evolving classification of tumors encompassing multiple subtypes including adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Patients diagnosed with NSCLC typically have advanced disease and poor prognoses (9). Recent advances in targeted therapies, including tyrosine kinase inhibitors and immune therapy, have improved outcomes for some patients (12). Despite this improvement, survival rates remain low for NSCLC and continued research is needed to identify additional druggable targets.

Intratumoral heterogeneity: leaders and followers

Intratumoral heterogeneity complicates the treatment of NSCLC, adding to poor patient outcomes (25). NSCLC tumors contain sub-populations of cells with distinct phenotypic characteristics and mutations. Driver mutations, in genes such as ALK, EFGR or, KRas, are ubiquitous in tumor cells and metastases, though other mutations may define different subpopulations (6). Subclones with mutations in tumor suppressors, such as LKB1, are selected for under treatment pressure and are associated with relapse (8). Further designation of subpopulations associated with tumor progression and collective invasion (leaders and followers) in these heterogenous populations is also possible (16). Leader cells are highly invasive and lead the cellular pack in collective invasion, while follower cells follow the leader cells and promote their growth via a decrease in mitotic defects. Identification of biomarkers associated with

leaders or other aggressive tumor subpopulations is crucial to better understanding NSCLC progression and developing novel treatment options for patients.

Jagged1-Notch signaling:

RNA sequencing of phenotypically-derived leader and follower subpopulations from the H1299 parental line identified Jagged1 (Jag1) as the most abundantly expressed receptor in leaders, 45-fold greater than in follower cells (data not published). Jag1 is one of five ligands for one of four Notch receptors (Notch1-4) supporting canonical Notch signaling in mammalian cells (17). Jag1 binding activates cleavage of the Notch intracellular domain, which subsequently enters the nucleus to activate transcription of Notch target genes (10). Notch signaling is essential for embryonic development, pluripotency, differentiation, and angiogenesis (24). Jag1 overexpression is associated with many cancer types, including NSCLC (17). Jag1-induced Notch signaling is implicated in maintaining cancer stem cell populations, preventing apoptosis, driving proliferation and metastasis, and promoting tumor angiogenesis (17). The multiple tumor-promoting effects of Jag1 make it an attractive target for novel therapies. The complete biological implications of Jag1 overexpression in NSCLC are yet to be fully characterized.

Sustained proliferative signaling:

One crucial hallmark of cancer is sustained proliferative signaling for chronic cell cycle proliferation (11). Normal cells regulate growth-promoting factor release to control entry and progression through the cell cycle and only divide when necessary. Cancer cells, on the other hand, regulate chronic progression through the cell cycle via dysregulation of cell signaling. Cancer cells utilize several methods to achieve chronic proliferative signaling. The cells may produce growth factors for self-stimulation in an autocrine fashion, may stimulate stromal cells to release factors, or overexpress receptor proteins for an amplified response to basal factor

concentrations (11). Previous work suggests that Jag1 can promote cell cycle progression in breast, colon, and prostate cancers via a variety of mechanisms (17). The role of Jag1 in chronic cell cycle proliferation in NSCLC is one of the foci of this study, given the overexpression of Jag1 is linked to driving proliferation and maintaining cancer stem cell populations.

ALCAM signaling:

Activated leukocyte cell adhesion molecule (ALCAM) was identified as the most differentially-expressed leader cell receptor during RNA sequencing of phenotypically-derived leader and follower cells (data not published). ALCAM was expressed nearly 400-fold higher in leaders than in followers, where it is likely epigenetically silenced. ALCAM (CD166) is an immunoglobulin cell adhesion molecule that engages in both homotypic interactions and heterotypic interactions with CD6 on T-lymphocytes, resulting in their activation (23). Furthermore, ALCAM is located at the junctions of endothelial cells, where it allows for adhesion of monocytes to endothelial cells and subsequent transendothelial migration (19). ALCAM is expressed in the pulmonary endothelium and its potential interaction with circulating tumor cells could result in transendothelial migration and formation of metastases (15).

ALCAM expression has been clearly associated with cancer progression in various tumor types (23). One primary confounding issue is that both high and low ALCAM expression have been linked to tumorigenesis in a cancer type-dependent manner. For example, low ALCAM expression in breast cancer tissues is correlated with skeletal metastases and poor prognosis (5), while high expression is linked to progression and lower survival in oral cancer (21). To further complicate ALCAM studies, ALCAM can be cleaved from the membrane and enter the cytoplasm (23). Membranous and cytoplasmic presence of ALCAM have both been linked to cancer progression, again in a cancer type-dependent manner. For example, the cytoplasmic

presence of ALCAM following cleavage by ADAM17/TACE is associated with ovarian cancer progression (20).

Investigation of ALCAM's role in NSCLC progression has yielded mixed results. ALCAM has been identified as a biomarker for cancer stem cells (14, 26), while others have shown ALCAM correlates with smaller tumors and fewer metastases (22). Therefore, it is necessary to characterize ALCAM's expression in leader NSCLC cells compared to followers to better understand its potential role in collective invasion.

Specific aims and hypothesis

Goals and hypothesis

The overall goal of the project is to 1) establish Jag1 as a biomarker for leader cells in NSCLC lines; 2) elucidate the function of Jag1 in NSCLC collective invasion; and 3) determine ALCAM expression in leader and follower cell lines, and heterogeneous patient-derived primary cells, to assess biomarker potential.

Since Jag1 is already implicated in promoting chronic proliferation in various other forms of cancer, we hypothesize that Jag1 overexpression will promote cell cycle progression in NSCLC. As such, we predict that cell cycle analysis of $Jag1^+$ cells will contain more cells in the S phase, actively dividing, compared to Jag1-knockdowns, with more cells in the G1 phase. In addition, we predict that $Jag1^+$ cells will proliferate at a more rapid rate than Jag1-knockdowns. Given ALCAM's enhanced RNA expression in phenotypically-derived leader cells compared to followers, we hypothesize that ALCAM is a biomarker for leader cells in NSCLC. As such, we predict that ALCAM expression will be high in leader cells and low in follower cells. To address our goals, we developed three specific aims:

Specific Aim 1: To investigate if Jag1 expression is correlated with changes in distribution of cells within the different stages of the cell cycle in a non-small cell lung cancer model.

Specific Aim 2: To investigate if Jag1 expression impacts growth kinetics in the NSCLC H1299 cell line.

Specific Aim 3: To investigate if ALCAM is a biomarker for leader cells in NSCLC lines and primary cell lines.

Strategy:

To this end, H1299 cells (ATCC), a line derived from a NSCLC patient harboring a p53 mutation, will be used to characterize the role of JAG1 in cell cycle progression and proliferation. H1299 cells, representative of a heterogenous NSCLC tumor, express Jag1 over a range of values (data not published). Jag1-expressing cells will be compared to Jag1-knockdown cells. Three lines of Jag1 knockdowns were generated using three individual short hairpin RNA (shRNA) plasmids, selected for with puromycin, and further selected via fluorescent activated cells sorting (FACS) (data not published). To contrast these Jag1-knockdowns, two biological replicates of JAG1 expressing cells will be used. One culture contains naïve H1299 cells, with no shRNA added and the second contains a control shRNA that does not target Jag1. These two lines were also sorted by FACS to verify Jag1 levels (data not published).

To elucidate if ALCAM is a biomarker for leader cells in NSCLC lines, H1299 leaders and followers will be stained with antibodies for ALCAM and IL13RA2, the cell surface marker identified during RNA sequencing as strongly correlated with follower cell phenotypes. H1299 leaders and followers that were both phenotypically-derived via spatio-temporal genomic and cellular analysis (SaGA) and flow-sorted for Jag1 (leader) and IL13RA2 (follower) will be used (16)

CHAPTER 2: Cell cycle analysis of Jag1-expressing and Jag1 knockdown cells

Specific Aim I:

To investigate if Jag1 expression is correlated with changes in distribution of cells within the different stages of the cell cycle in a non-small cell lung cancer model.

Rationale:

Sustained proliferative signaling for chronic cell cycle proliferation is one of the hallmarks of cancer (11). Cancer cells regulate chronic progression through the cell cycle via dysregulated cell signaling. Jag1 has been observed to promote cell cycle progression in breast, colon, and prostate cancers (17). The role of Jag1 in cell cycle progression has not yet been studied in non-small cell lung cancer. Observing if Jag1 impacts cell cycle distribution is necessary to determine if it is a biomarker for leader cells in NSCLC lines.

Methods:

Cell line: NCI-H1299 cells were maintained in RPMI supplemented with 10% FBS and penicillin, streptomycin, and kanamycin. Cultures were maintained at 37°C in 5% CO_2.

Generation of Jag1 knockdowns: Three lines of JAG1 knockdowns were generated using three individual short hairpin RNA (shRNA) plasmids, selected for with puromycin, and further selected via fluorescent activated cells sorting (FACS). $Jag1^+$ controls included naïve H1299 cells (no shRNA added) and a control shRNA that does not target Jag1.

Cell cycle analysis by flow cytometry: Cell cycle analysis using FACS was performed on $Jag1^+$ and Jag-knockdown cell lines. Several methods were used to prepare cells for cell cycle analysis. In the first method, cultures were maintained in the log phase of growth in RPMI with 10% FBS with no further modifications. The second method involved serum-starvation, to sync cells in G1, and a time course of release into complete media. Cultures were first serum-starved for 24 hours.

Following serum starvation, cells were released using media with 10% FBS for either 24 hours, 48 hours, or 72 hours. In addition, samples were collected following serum starvation with no release, to verify that serum-free media was syncing cultures. Regardless of culture method, cells were collected by trypsinization while still in the log phase of growth and fixed in 95% ethanol for at least 30 minutes at 4°C. Cells were then stained with DAPI (4',6-diamidino-2-phenylindole) (4μg/mL DAPI in 0.25% Triton-X in PBS) for 15-30 minutes at room temperature. Samples were then read on the BD Biosystems flow cytometer, setting the G1 peak at 100K Pacific Blue intensity and G2 peak at 200K intensity. Results were analyzed using FlowJo. Curves were fit using the Dean-Jett-Fox model with no sync for S phase.

Proliferation: Proliferation was also measured in conjunction with the time course of serum starve release. 150,000 cells were plated at time 0h. Serum starvation occurred from 0h-24h. No counts were taken at 24h. Counts were taken at 48h (corresponding to 24h release from serum starvation), 72h (corresponding to 48h release from serum starvation), and 96h (corresponding to 72h release from serum starvation. Values were normalized to the density at 0h. Error bars represent standard deviation from the calculated means of the various $Jag1^+$ and Jag1 knockdown cell types tested. Cell numbers were obtained using the Bio-Rad automated cell counter.

Results

To determine whether Jag1 expression impacted cell cycle progression, we examined the distribution of cells in the stages of the cell cycle in $Jag1^+$ and Jag1 knockdown H1299 NSCLC lines. First, cells were maintained in the log phase of growth in complete media (supplemented with 10% FBS) and analyzed via FACS. No difference was observed in the distribution of cells in G1, S, or G2 between the $Jag1^+$ cells and Jag1 knockdowns (Figure 1A). Second, cells were maintained in serum-free media for 24 hours prior to a time course of release in serum-

containing media. Serum starvation for 24 hours with no release resulted in a partial G1 sync, with approximately 70% of cells found in G1, while 20-25% remained in S and fewer than 5% were found in G2 (Figure 1B). Next, cells were serum starved and released in serum-containing media for 24h, 48h, or 72h. Again, no difference was observed between the $Jag1^{+}$ and Jag1 knockdown cells at 24h, 48h, or 72h (Figure 1B). Interestingly, cells were differentially distributed in G1, S, and G2 depending on the length of release from serum starvation. Cells released for 24h exhibited a larger proportion of cells in S phase (~40%) compared to those released for 48h and 72h (~30%) (Figure 1B). For 48h and 72h release, a larger proportion of cells were present in G1 (50%) compared to 24h release (30-40%) (Figure 1B).

Proliferation was also measured during the time course of release from serum starvation. No difference in growth kinetics between $Jag1^{+}$ and Jag knockdown cells was observed (Figure 1C). Cells doubled within 48 hours, likely hindered by serum-free media conditions, then began a more rapid growth pattern. Error bars indicate a wide amount of variability in measured values for the different biological replicates for both Jag^{+} and Jag1 knockdown.

CHAPTER 3: Proliferation of Jag1-expressing and Jag1 knockdown cells

Specific Aim II: To investigate if Jag1 expression impacts growth kinetics in the NSCLC H1299 cell line.

Rationale:

Jag1 overexpression is associated with many cancer types, including NSCLC (17). Jag1-induced Notch signaling is associated with maintenance of cancer stem cell populations, prevention of apoptosis, driving proliferation and metastasis, and promoting angiogenesis. To establish Jag1 as a biomarker for leader cells, it is crucial to observe if the presence or absence (via stable knockdown) of Jag1 in NSCLC H1299 cells is correlated with differences in proliferative rate.

Methods:

Cell lines: NCI-H1299 cells were maintained in conditions as previously described. Two biological replicates for $Jag1^{+}$ cells were naïve cells, not exposed to shRNA, and a control shRNA that does not target Jag1. Three biological replicates for Jag1 knockdown were formed via targeting with three separate hairpins as previously described.

Proliferation: Cells from each of the five lines were plated evenly (50,000 cells). Cultures were trypsinized, counted using the automated cell counter (Bio-Rad), and re-plated at 24 hour intervals for 72 hours. Values shown in figures are normalized to t = 0h, or time of initial plating. Results shown are based on two independent replicates of the entire procedure.

Results:

To determine whether Jag1 expression impacts proliferation kinetics in NSCLC H1299 cells, we conducted a proliferation assay on various Jag1-expressing controls and Jag1 knockdowns. Proliferation assays revealed no difference in growth kinetics between $Jag1^{+}$ and Jag1 knockdown cells (Figure 2). The doubling time for these cells is around 24 hours (Figure 2).

The error bars indicate a wide amount of variability in the biological replicates used for each group.

Similar results were observed when proliferation was measured in conjunction with cell cycle analysis in Chapter 2. The cells exhibited slow growth correlated with serum starvation, then a more rapid pace of growth when released into serum-free media (Figure 1C).

Chapter 4: Analysis of ALCAM expression in NSCLC lines and primary cell lines

Specific Aim III: To investigate if ALCAM is a biomarker for leader cells in NSCLC lines and primary cell lines.

Rationale: ALCAM, or activated leukocyte cell adhesion molecule, was the most differentially-expressed leader cell receptor following RNA sequencing of leaders and followers (data not published). ALCAM expression was 400-fold higher in leaders than followers, where it is likely silenced. ALCAM is associated with both cell adhesion and enabling migration (23). Investigations of ALCAM's role in NSCLC has yielded mixed results: some say it is a biomarker for cancer stem cells (14, 26), while others say it is correlated with smaller tumors and fewer metastases (22). It is necessary to observe if ALCAM expression is correlated with leader cell phenotypes, in addition to any potential correlation with tumor invasiveness.

Methods:

Cell lines and antibodies: Several variations of NCI-H1299 cells were cultured as previously described. These include parental cells (ATCC) and two cell populations previously sorted via FACS as leaders (high Jag1) and followers (high IL-13RA2). In addition, NCI-H1299 cell populations that stably express H2B-dendra2 protein were utilized following isolation using SaGA (15). Dendra2 is a photoconvertible fluorophore that allows for conversion from green fluorescence to red fluorescence when exposed to a 405nm laser (15). Following photoconversion of a user-defined cell population, the red fluorescent cells can be sorted via FACS into phenotypically-isolated populations (15). These populations included a parental, in addition to leader and follower populations, based on properties during collective invasion. Primary cells derived from a patient-derived organoid (PDO), EUH3123 and EUH3174, were maintained in modified M87 medium, consisting of DMEM/F12 with 15 mM HEPES and

bicarbonate supplemented with 2.5mM L-glutamine, 2% FBS, 1% Insulin-Transferrin-Selenium-X, 1% Penicillin-Streptomycin, 0.5% Fungizone, 0.2% Normocin, 0.05% hydrocortisone, 0.001% Cholera toxin, 0.05% β-estradiol, 0.005% ethanolamine, 0.005% O-phosphorylethanolamine, and 0.0025% triiodothyronine supplemented with 0.05% human EGF following every media change. Antibodies against ALCAM and IL13RA2 were used according to manufacturer's protocol per test (Sino Biologics; Miltenyi).

Staining and flow cytometry: Cells were trypsinized and washed prior to resuspension in flow buffer (1X PBS, 25mM HEPES, 1mM EDTA, 1% dialyzed FBS). For each cell type tested, 300,000 cells were stained with antibody and 100,000 cells were used as an unstained control. Antibody controls were formed for gating: one with beads only, a second with beads and one antibody, and a third with beads and the other antibody. Antibody mix was added to the stained samples according to manufacturer's protocol per test. Samples were incubated on ice while gently rocking for 1 hour. Samples were read on the flow cytometer (BD Biosystems).

Results

To probe if ALCAM expression is correlated with leader cells in NSCLC, we analyzed previously flow-sorted cells via flow cytometry. First, H1299 leaders (Jag^+) and followers ($IL13RA2^+$) were stained for both IL13RA2 (APC) and ALCAM (PE). The majority of leader cells were ALCAM-positive, while most followers were ALCAM-negative (Figure 3A). Leaders were mostly IL13RA2-negative and followers were mostly IL13RA2-positive, validating previous flow sorting. Interestingly, around 2-3% of leader cells and 4-5% of follower cells were observed to be both ALCAM- and IL13RA2-positive (Figure 3A).

Next, we stained primary cells from PDOs, EUH3123 and EUH3174, for both IL13RA2 (APC) and ALCAM (PE). EUH3174, previously shown to express Jag1 at high levels (data not

shown), were >90% ALCAM-positive (Figure 3A). EUH3123 cells were previously shown to be Jag1-negative (data not shown). Here we observe that 93% of EUH3123 cells sampled also express ALCAM (Figure 3A).

To further probe if ALCAM expression is associated with leader cells in NSCLC, we analyzed phenotypically-sorted H1299 populations. These cells were sorted using SaGA microscopy following transfection with H2B-dendra2 protein (FITC). Due to fluorochrome overlap of FITC with the PE channel, we stained only for ALCAM (APC). As previously observed, leader cells were majority ALCAM-positive, while followers were majority ALCAM-negative (Figure 3B). Parental H1299 cells were majority ALCAM-negative, with a small minority of cells ALCAM-positive (Figure 3B).

For further validation, we stained the same H1299 and primary cells analyzed in Figure 4A for both IL13RA2 (PE) and ALCAM (APC), flipping the fluorophore conjugated to each antibody. Parental H1299 cells revealed three distinct populations, with approximately 76% of cells ALCAM-negative and IL13-positive, 16% ALCAM-positive and IL-13-negative, and 7% double-positive (Figure 3C). A similar trend was observed for both H1299 leaders and followers as previously mentioned (Figure 3C). The primary cells, EUH3123 and EUH3174 also exhibited a similar trend as mentioned above (Figure 3C). Interestingly, EUH3174 displayed a small, yet distinct population of ALCAM- and IL13-positive cells not observed in the first experiment.

In summary, ALCAM is observed to be expressed in H1299 leader cells, regardless of whether phenotypically-sorted or flow-sorted for Jag1. Both primary cell types analyzed, EUH3123 and EUH3174, exhibit abundant ALCAM expression, despite different characteristics and Jag1 expression levels.

CHAPTER 5: Discussion and Conclusions

Specific Aim I

Cell cycle analysis was performed on H1299 lines to examine if Jag1 expression impacts the distribution of cells in each stage of the cell cycle. No difference in the distribution of cells in G1, S, or G2 was observed when Jag1-expressing cells were compared to Jag1 knockdowns (Figure 1). Similar observations were made when cells were analyzed during the log phase of growth and when cells were synced to G1 and analyzed on a time course of release into serum-containing media (Figure 1A,B).

Several interesting observations were made during the experiment run with serum starvation and a time course of release. First, only around 70% of serum-starved cells were actually synced in G1 during analysis by FACS (Figure 1B). The 24 hour serum starvation period was our balance between an insufficient G1 sync and keeping the cells in serum-free media for too long, to the point of massive cell death. This value was also adapted from a laboratory protocol that optimized serum-starve period length for H1299 to 20 hours. This protocol was used to observe the cell cycle distribution difference between H1299 followers and leaders phenotypically-sorted via SaGA (16).

Second, a differential distribution of cells in G1, S, and G2 was observed depending on the time of release from serum starvation. We attribute this difference to differential fixation periods for these cells in 95% ethanol. All samples were read following the 30 minute ethanol fixation and DAPI staining of the 72h release experimental group. As a result, samples released for 24h were fixed in ethanol for 48h, while those released for 48h were fixed for 24h. Ethanol fixation results in cell dehydration due to cell membrane permeabilization (13). Several protocols

state that cells can be fixed stably in ethanol for a week, though we suspect that the differential fixation period is likely behind the difference in results.

Ultimately, we observe and conclude that Jag1 expression does not alter the gross distribution of cells in G1, S, or G2 of the cell cycle in our H1299 NSCLC model. This observation, however, does not preclude Jag1 expression from having an impact on the cell cycle in NSCLC leader cells. In breast cancer, it has been observed that Jag1 can induce cell cycle progression by inducing cyclin D1 (3), while in colon cancer, Jag1 plays a similar role by inducing cyclin D1, cyclin E, and c-Myc expression (4). Contrary to this published data on the role of Jag1 in other cancers, preliminary data from our H1299 leader and follower RNA sequencing data set suggests that follower cells have upregulated expression of a variety of cell cycle-promoting genes (data not shown). Upregulated cell cycle signaling in followers is consistent with previous observations that follower cells proliferate more rapidly than leaders (16). Since H1299 Jag1-knockdown cells exhibit a similar distribution of cells in the cell cycle stages to $Jag1^+$ cells, it does not appear that Jag1 promotes cell cycle progression as part of its role in driving invasive leader cell phenotypes.

Specific Aim II

Proliferation analysis was performed to identify if Jag1 expression impacts growth kinetics of H1299 cells *in vitro*. No difference in growth kinetics was observed between $Jag1^+$ and Jag1-knockdown cells (Figure 2). Regardless of Jag1 expression level, we observed that these cells proliferate fairly slowly (Figure 2).

Even with multiple replicates, the variability in mean values for $Jag1^+$ and Jag1-knockdown proliferation was very high (Figure 2). We are unsure why the variability between

our biological replicates was so large for each experimental group. Reduction of this variability will require additional replicates and potentially different techniques for analyzing proliferation.

We conclude that Jag1 expression status does not influence growth kinetics in H1299 cells. It was previously observed that H1299 phenotypically-sorted follower cells proliferate at a faster rate than leader cells (16). Here, we observe no difference in $Jag1^{+}$ flow-sorted leader cells and Jag1-knockdown cells, which should assume a more follower-like phenotype. Despite this apparent contradiction, leader cells are not solely defined by Jag1 and other pathways will impact leader cell growth kinetics, incorporating our observations with those previously made by the lab.

Specific Aim III

ALCAM expression was analyzed in H1299 lines and NSCLC primary cells to potentially identify an additional biomarker for leader cells. ALCAM expression was high in H1299 leaders, while it was low in H1299 followers (Figure 3). This expression pattern was observed in both Jag1 flow-sorted and phenotypically-sorted cells (Figure 3A,B). Based on the H1299 results, ALCAM appears to be a marker for leader cells. Leader cells are characteristically more invasive and lead collective invasion (16). This observation is consistent with those previously made by Zhang et al. (26) and Ishiguro et al. (14) in NSCLC primary tumors and cell lines, that ALCAM promotes tumor invasiveness. Interestingly, neither of these papers provides a data for a specific mechanism by which ALCAM promotes invasion. A different study may offer that mechanism: they observe that intact ALCAM-mediated cell adhesion activates metastasis-promoting matrix metalloprotease (MMP) cascades to initiate invasion in melanoma (18). MMPs break down the extracellular matrix via proteolysis, which can promote invasion.

Investigation of two NSCLC primary cell types, EUH3123 and EUH3174, complicates the assessment of ALCAM as a leader cell biomarker. ALCAM expression was high in both EUH3123 and EUH3174 (Figure 3A,C). Prior to this investigation, we observed that EUH3123 invades poorly in collagen only, while EUH3174 invades aggressively in chain-formation regardless of medium (data not published). Furthermore, only EUH3174 was Jag1-positive, our marker for more invasive leader cells (data not shown). Given that the more follower-like EUH3123 cells are also ALCAM-positive, it does not appear that ALCAM is a biomarker for leader cells.

Clinical data accompanying the two primary tumor samples adds further complexity to the question. Both EUH3123 and EUH3174 were described as 'invasive' primary tumors, based on the criterion that the tumor grew through the basement membrane; however, only EUH3174 cells collectively invade via chain-growth *in vitro*. Chain-like invasion is defined as a single leader cell leading a chain of follower cells outward from an embedded cell spheroid (15). These cells are both described clinically as invasive, yet have different invasion properties *in vitro*. A recent paper suggests a mechanism that could explain this phenomenon (7). Invasive endometrial tumors were ALCAM-positive in their study, but ALCAM was shed by matrix metalloprotease-9 (MMP-9) at the surface of the tumor prior to invasion. They suggest that serum levels of ALCAM, as opposed to tumor expression, could be diagnostic for invasive carcinoma.

Ultimately, our results are not extensive enough to claim that ALCAM is a leader cell biomarker, given that this only applies to a single model of leaders and followers (H1299). While both EUH3123 and EUH3174 are described as clinically invasive, their invasive ability differs extensively *in vitro*. The observation that both of these cell types are ALCAM-positive does not support the claim that ALCAM is a biomarker for leader cells. Further studies are needed to

elucidate the function of ALCAM in these NSCLC primary cells and in H1299 leaders and followers.

Conclusions

Jag1 appears to be a biomarker for leader cells in NSCLC. This conclusion is not apparent from the data shown here; however, ongoing work in the lab has shown that Jag1-knockdown results in less collective behavior in invasion in both 2D and 3D (data not shown). In addition, similar results were observed when leaders and followers were targeted with antibodies for Jag1 or Notch (data not shown). The role of Jag1 in altered collective invasion is yet to be determined, though here we observe no difference in cell cycle distribution or proliferative rate between cells expressing Jag1 and Jag1-knockdowns.

ALCAM does not appear to be a biomarker for NSCLC leader cells. ALCAM expression was high in primary cells derived from NSCLC patients regardless of their invasive potential *in vitro*. How this high ALCAM expression impacts these cells or their invasive potential remains to be elucidated.

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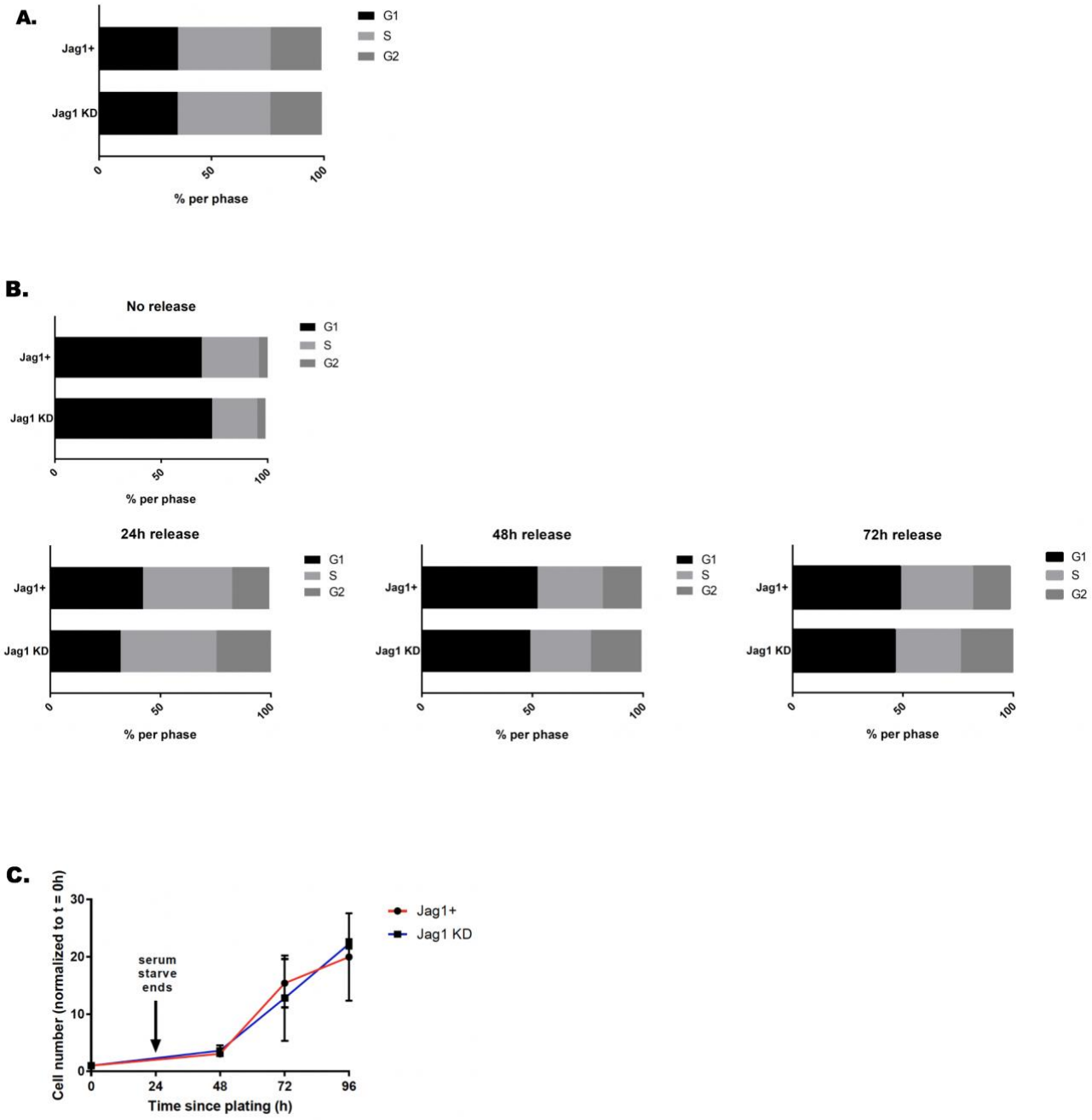


Figure 1. Cell cycle analysis of H1299 Jag1^{+} and Jag1-knockdown cells (A) Cell cycle analysis by FACS performed on cells maintained in the log phase of growth. (B) Cell cycle analysis by FACS performed on cells serum starved for 24h with no release or release into serum-containing media for 24, 48, or 72 hours. (C) Proliferation data to accompany the time course mentioned in (B). Error bars represent no standard deviation from the mean cell number.

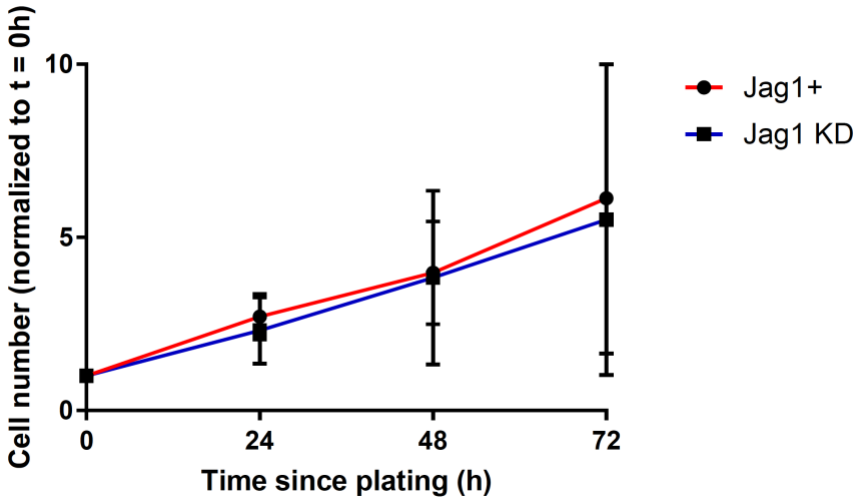
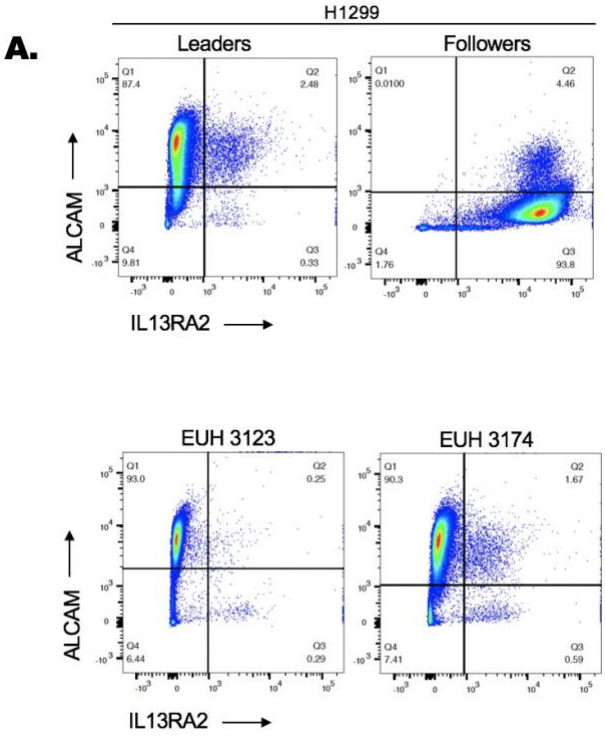
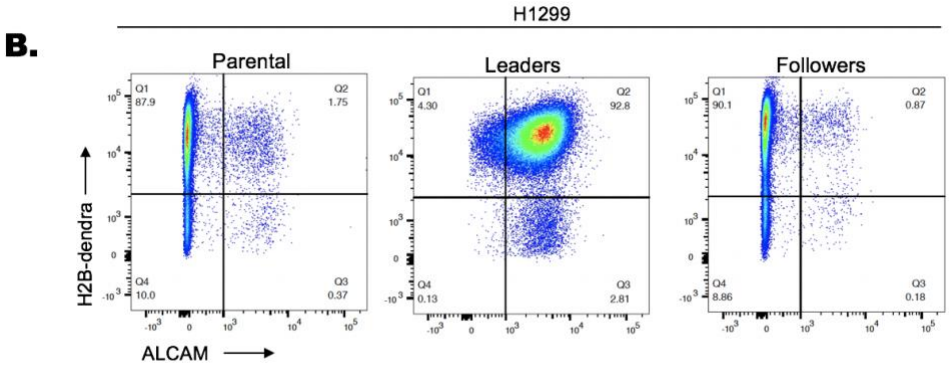


Figure 2. Proliferation analysis of H1299 Jag1^{+} and Jag1 knockdown cells. Cell number values are arbitrary as listed following normalization to t = 0h. Error bars represent standard deviation from the mean of biological replicates for each experimental group. Results shown are average of two independent experiments.


A.
H1299
Leaders
Followers
Q1 87.4
Q2 2.48
Q4 9.81
Q3 0.33
Q1 0.0100
Q2 4.46
Q4 1.76
Q3 93.8
ALCAM
IL13RA2
EUH 3123
EUH 3174
Q1 93.0
Q2 0.25
Q4 6.44
Q3 0.29
Q1 90.3
Q2 1.67
Q4 7.41
Q3 0.59
ALCAM
IL13RA2


B.
H1299
Parental
Leaders
Followers
Q1 87.9
Q2 1.75
Q4 10.0
Q3 0.37
Q1 4.30
Q2 92.8
Q4 0.13
Q3 2.81
Q1 90.1
Q2 0.87
Q4 8.86
Q3 0.18
H2B-dendra
ALCAM

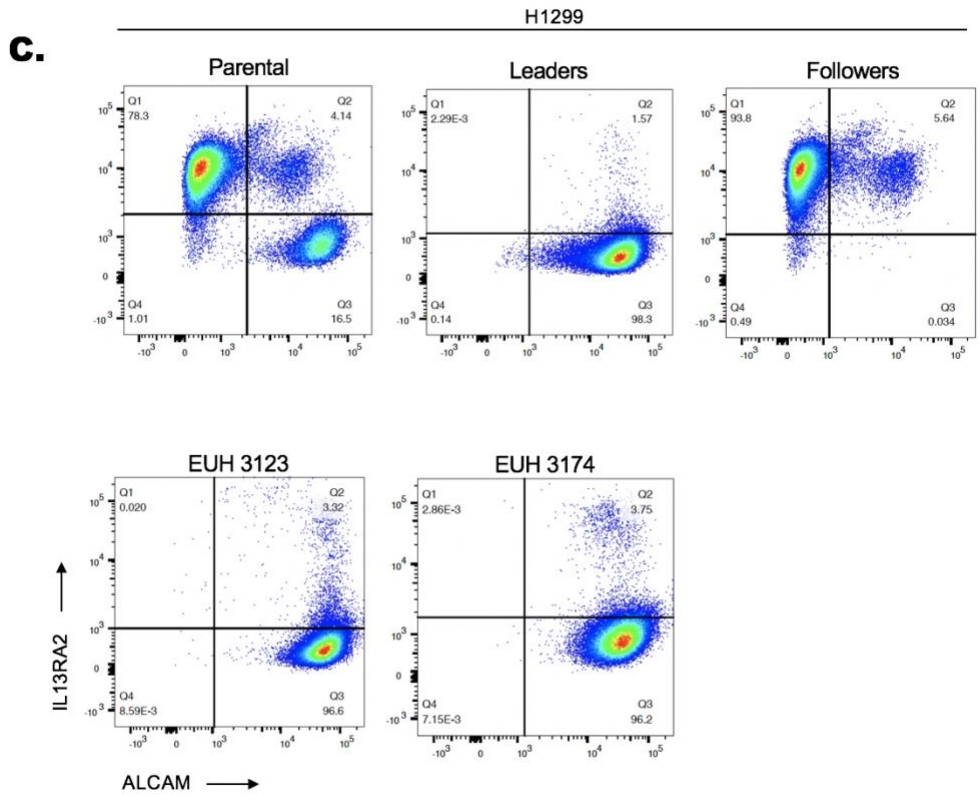


Figure 3. ALCAM expression in H1299 subpopulations, EUH 3123, and EUH 3174. A. ALCAM (PE) and IL13RA2 (APC) expression in previously flow-sorted H1299 populations and primary cell types derived from PDOs. B. ALCAM and H2B-dendra2 expression in phenotypically-sorted H1299 populations. C. ALCAM (APC) and IL13RA2 (PE) expression in previously flow-sorted H1299 populations and primary cell types derived from PDOs.