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Integrin related cell interaction in primary T lymphocytes and cancer cells

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Integrin related cell interaction in primary T lymphocytes and cancer cells

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Abstract

Integrins are a family of transmembrane adhesion proteins that mediate cell attachment to extracellular matrix or to another cell. The T cell LFA-1 integrin and its interaction with its predominant ligand, ICAM-1, is known to regulate antigen-driven differentiation of naive T cells into effector T cells, a process that is fundamental to adaptive immunity. The activation of LFA-1 on effector and naive T cells is still unclear and requires further investigation for a more detailed understanding. In this work we quantitatively study primary T cells (both naive and effector) and their interaction with ICAM-1 using atomic force microscopy (AFM). Measurements show that adhesion of the effector T cell is in general at least one order of magnitude higher than that of the naive T cells. OKT3 treatment has a profound impact on T cell–ICAM-1 interaction, resulting in at least a 2-fold increase in both detachment force and work. Our conclusions corroborate past research that shows that there is a higher expression of LFA-1 on effector T cells than naive T cells and that LFA-1 on effector T cells is predominantly in the high affinity state. Our results also possibly indicate that a mixed population of high and low affinity LFA-1 is present on naive cells. Furthermore, given the response to OKT3 treatment, it is likely that it induces populations of LFA-1 to change from low affinity to high affinity on both effector and naive T cells. Taken together, our studies reveal in more detail the regulation and conformational states of high affinity LFA-1 on T cells which is critical to T cell activation. Additionally, we use AFM to look at the interaction between neuropilin-2

(NRP-2), a type I transmembrane glycoprotein, which is expressed on cancer cells and show that it interacts with α5 integrin on endothelial cells. Most importantly, our studies reveal that the interaction mediates cancer cell vascular extravasation and promotes metastasis. This research further supports the highly promising route of preventing cancer metastasis by therapeutically blocking NRP-2 and thereby greatly improving the prognoses of cancer patients.

Part I

Integrin mediated cell adhesion in primary T lymphocytes

Chapter 1

Introduction

Integrins are obligate heterodimeric transmembrane adhesion molecules that contain both a distinct α- and β-subunit (of which 18 α-subunits and 8 β-subunits have been found) and are broadly expressed on a large variety of cells [1, 2, 3]. They function both as adhesion molecules and as receptors for outside-in signalling, a process that involves sending into the cell information about the mechanical and chemical properties of the extracellular milieu. Integrin is unique in that it usually remains in an inactive form on the surface of the cell until it is activated by the action of inside-out signaling in which stimulating signals from other receptors are received. The activation of integrin involves a dramatic conformation change from a closed, bent shape to an extended, open one [4, 5]. There are a few distinct conformations that integrin is capable of and each has its own ligand binding affinity based on conformational [6, 7, 8, 9, 10, 11] and structural [12, 13, 14, 15] studies.

The main integrin in T cells/lymphocytes is lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18, αLβ2) which is part of the β2 subclass of integrins [16, 17]. LFA-1 is known to be involved in two T cell activities: cell arrest and subsequent migration on surfaces expressing the main LFA-1 ligand, intercellular adhesion molecule-1 (ICAM-1; CD54), and formation of immunological synapses

by interacting with antigen-presenting cells (APCs). ICAM-1, mentioned earlier, is a cell surface glycoprotein that is the predominant ligand of LFA-1 and their interaction controls certain key lymphocyte roles such as cell migration, antigen presentation and lymphocyte extravasation [18, 19, 20, 21, 22, 23].

LFA-1 is expressed on the leukocyte surface in an inactive state. In order to bind to ICAM-1 with strong affinity, cellular activation is required; in particular certain avidity (receptor clustering) and affinity (molecule conformational change) enhancements are required [24, 25, 26]. In humans, modulation of LFA-1 activation performs a key role during inflammatory and immune responses [27, 28, 29, 30].

The ICAM-1 binding site of LFA-1 has been localized to the metal ion-dependent adhesion site (MIDAS) on the inserted-domain (I-domain) of the LFA-1 αL subunit [31]. The I-domain changes conformation upon activation from the low affinity (closed conformation) state to the high affinity (extended conformation) state, resulting in increased affinity for ligand binding [32, 33]. In T lymphocytes, a cascade of intracellular progressions that increase the affinity of LFA-1 for ICAM-1 occurs when the T cell receptors (TCRs) are engaged [34].

After T cell development, matured naive T cells, T lymphocytes that have not yet encountered the antigen they are programmed to respond to, leave the thymus and spread throughout the body. Fundamental to adaptive immunity is the direction of developmental cues from cells of the innate immune system for antigen-driven differentiation of naive T cells into effector T cells. It is known that this process is regulated by LFA-1 on the T cell and its interaction with ICAM on the APC through the immunological synapse [35, 36, 37]. Effector T cell LFA-1 is important in effector functions and migration and it has been shown that in effector T cells LFA-1 is highly expressed. Conversely, it has been also been shown that in naive cells there is a lower LFA-1 expression [38]. Recent research provides evidence that in primary effector T cells the I-domain of LFA-1 changes to the high affinity state

and that high affinity LFA-1 is crucial in T cell activation [39].

The activation of LFA-1 on the effector and naive T cells is still unclear and requires further investigation for a more detailed understanding. Herein, we study whole primary T lymphocytes (both naive and effector T cells) and their interaction with ICAM-1 in a more quantitative method utilizing atomic force microscopy (AFM) [40, 41].

Chapter 2

Materials and methods

2.1 T cells

Primary effector and naive T cells were kindly provided by the Ma Group at the MD Anderson Cancer Center. The cells were maintained at 37 °C in RPMI 1640 medium supplemented with 10 % serum prior to AFM measurements.

2.2 T cell–ICAM-1 adhesion measured by atomic force microscopy

The experimental apparatus used to measure cell adhesion is schematically illustrated in Figure 2.1. ICAM-1 was coated on standard 35 mm tissue culture dishes while a single T cell was coupled to the end of the cantilever via poly-L-lysine-mediated linkages. In order to prepare the poly-L-lysine-functionalized cantilevers, they were soaked in acetone for 5 min, irradiated by UV for 30 min, incubated in 100 mM $NaHCO_3$ (pH 8.6) for 20 min at room temperature, washed three times with phosphate buffered saline (PBS, 10 mM PO_4^{3-}, 150 mM NaCL, pH 7.3) and incubated in poly-L-lysine (PLL, 0.1 mg/mL in PBS; Sigma P4832) overnight at 4 °C in a humidified

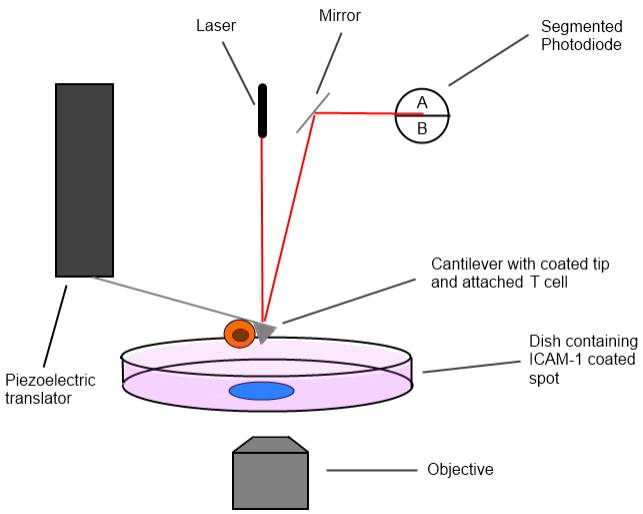
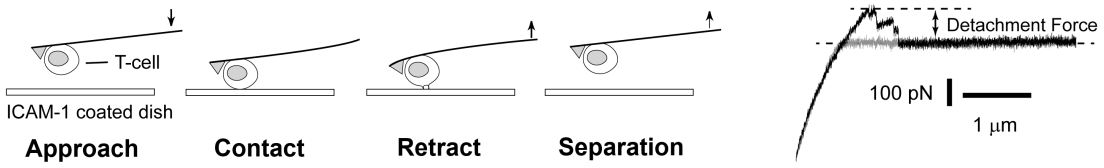


Figure 2.1: **AFM schematic for measuring contact forces between T cells and ICAM-1.** Shown in this schematic are the major components of a custom-built AFM coupled to an inverted optical microscope, which allows for viewing of the cells. A single T cell (designated in orange in the figure) is attached to a poly-L-lysine-functionalized cantilever and is lowered onto the portion of the dish coated with ICAM-1 (designated in blue) by the action of the piezoelectric translator. A laser (~635 nm) is reflected off the cantilever into the photodiode. The voltage difference between segments A and B of the photodiode gives the amount of cantilever deflection.



(a) (b)

Figure 2.2: **Cycle of AFM measurement and retraction trace.** (a) Schematic representation of a typical cycle of the AFM measurement: 1) approach of the T cell to the ICAM-1 coated spot, 2) contact between the T cell and the ICAM-1 coated spot, 3) retraction of the T cell, and 4) separation of the T cell from the ICAM-1 coated spot. Arrows indicate the direction of cantilever movement. (b) Typical force spectrum trace after a 5 s contact and with a compression force limit set at 500 pN. Grey and black curves are approach and retract traces, respectively. Two-headed arrow indicates the magnitude of detachment force on the retract (black) trace.

chamber.

Measurements of T cell–ICAM-1 interaction were conducted in RPMI 1640 medium at 25 °C. With the aid of an optical magnification system situated below the AFM, the tip of the poly-L-lysine-functionalized cantilever was positioned above a T cell in the medium and gently lowered to the surface of the dish for approximately 1 s to attach the cell to the cantilever. At the onset of the measurements the T cell, coupled to the AFM cantilever, was positioned directly above the portion of the dish coated with ICAM-1.

The relative position of the interacting T cell and ICAM-1 surface was controlled via expansion or contraction of the piezoelectric translator. The force acting between the surface and cell was derived from the deflection of the AFM cantilever, which was monitored by reflecting a focused laser beam off the back of the cantilever into a 2-segment photodiode. Cantilevers were individually calibrated by thermal fluctuation analysis [42] and had spring constants of approximately 0.015 N/m.

As illustrated in Figure 2.2a, AFM measurements of the interaction between an individual T cell and ICAM-1 involves a series of four steps. First, the cantilever with an attached T cell is lowered onto the ICAM-1 coated spot. At a certain point

contact is made, allowing for ICAM-1–receptor interaction to take place. A trigger signal is set so that the piezoelectric translator will stop its downward motion and prevent crushing of the cell or damaging the cantilever. The cantilever is subsequently retracted via the contraction of the piezoelectric translator, pulling the T cell and ICAM-1 apart until complete separation is achieved. During this process, the AFM continuously monitors the force of the cantilever in order to report on the interaction between the T cell and ICAM-1.

A typical AFM force-displacement record of AFM single cell adhesion assay is presented in Figure 2.2b. In this figure, the y-axis plots the cell–surface interaction force as a function of the relative distance between the base of the cantilever and surface (i. e. the ICAM-1). At the beginning of the force measurement, when the attached cell (T cell) is several microns above the surface, there is no strain on the cantilever. At this position, which is marked by the lower dashed line, the force is zero. Expansion of the piezoelectric translator lowers the attached cell onto the surface. Following cell–surface contact, further expansion of the translator presses the attached cell against the spot on the dish coated with ICAM-1. The compression force felt by the cells is determined from the upward deflection of the cantilever. For the measurements of T cell–ICAM-1 adhesion, the predefined limit of the compression force was set at 500 pN. Once this force value is reached the expansion of the translator ceases. This compression force is held for a predefined cell–surface contact time before the translator contracts to initiate cell–surface separation. We used a contact duration of 5 s. Upon retraction of the cantilever, molecular linkages established between the cell and surface pull the cantilever downward. The cell detachment process typically involves a series of rupture events. Each of these rupture events resulted in a rapid jump in force and may correspond to the unbinding of one or more adhesive ligand–receptor bonds.

To quantify the adhesion between the T cells and ICAM-1, we measure the

"detachment force" and "detachment work." As indicated by the double-headed arrow in Figure 2.2b, the detachment force is the maximal pulling force applied by the cantilever during the detachment process. The detachment work is derived from integrating the adhesive force over the distance traveled by the cantilever up to the point of the last bond rupture. Both parameters have been used to quantitate single cell adhesion in the literature.

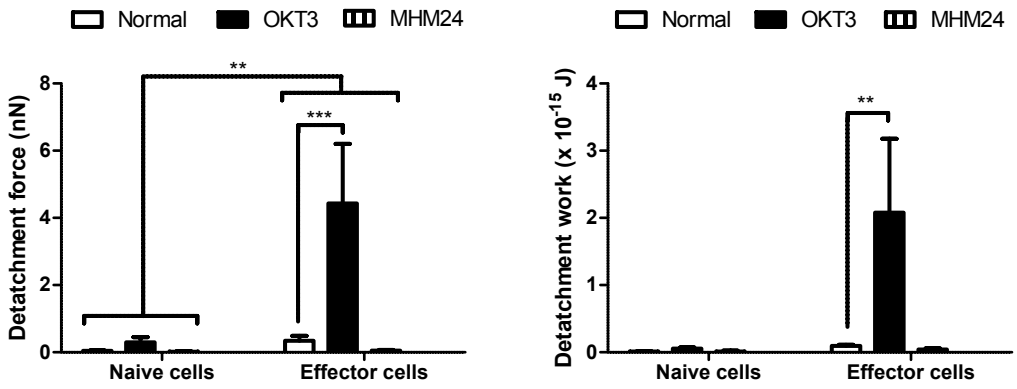
2.3 Statistical analysis

ANOVA or t-test was used for statistical analysis, with $p < 0.05$ considered statistically significant. Standard error mean is indicated in the data.

Chapter 3

Results

Figure 3.1 shows the detachment force and work of T cells bound to the ICAM-1 coated surface. In general, adhesion of the effector T cell is at least one order of magnitude higher than that of the naive T cell. OKT3 treatment, a mAb which leads to T cell action [43], has a profound impact on T cell–ICAM-1 interaction, resulting in at least a 2-fold increase in both detachment force and work. MHM24, a mAb for the I-domain of human LFA-1, was used for a negative control and its addition significantly reduced adhesion, suggesting that the measurements being made are primarily of LFA-1–ICAM-1 interactions.



(a) (b)

Figure 3.1: **Detachment force and work between T cells and ICAM-1.** (a) Average detachment forces between T cell and ICAM-1 coated surfaces. (b) Average detachment work between T cell and ICAM-1 coated surfaces. A 5 s contact time was used. Error bars are standard error mean from several sets of ~100 measurements. The error bar is standard error with $n \geq 3$ in each case. $^{**}p < 0.01$ and $^{***}p < 0.001$ between the indicated groups.

Chapter 4

Discussion

Our research quantitatively investigated LFA-1 expression on both primary effector and primary naive T cells. Adhesion to ICAM-1 is much greater in the effector T cells than naive T cells and it is likely that there exists a large population of high affinity LFA-1 on effector T cells. Based on the results it is possible that a large population of LFA-1 on naive T cells is in the low affinity state or a mixture of low and high affinity states. The OKT3 treatment induced significant enhancement of detachment force and work in naive cells which suggests that a population of low affinity LFA-1 changes to a high affinity state with the treatment. The increased adhesion in effector cells after OKT3 treatment also suggests that extra adhesion results from further activation of an inactive population of LFA-1 and/or the induced clustering of LFA-1 on the cell surface. Another question worth pondering and investigation is whether there is rebinding during the pulling of multiple bonds. Furthermore, to fully understand the molecular mechanism of LFA-1 mediated T cell adhesion more research utilizing molecular dynamic simulations and specific monoclonal antibodies and/or site-directed mutagenesis is necessary.

Overall, our results confirmed and elaborated in greater detail past studies of LFA-1 expression and confirmation on naive and effector T cells. Further investigation,

particularly in single molecule conditions will further reveal greater details of LFA-1 affinity state in various conditions and is currently in progress.

Part II

Neuropilin-2 promotes extravasation and metastasis by interacting with endothelial α5 integrin

Chapter 5

Introduction

Metastasis, the spread of tumor cells from the primary neoplasm to a non-adjacent organ, is the leading cause of death in cancer patients. The spread of the cancer cells occurs through multistep processes that are sequential and interrelated. In brief, it involves cancer cells from the primary tumor acquiring the ability pass through the walls of the blood and/or lymphatic vessels, intravasting into the blood stream, traveling through and extravasting the circulation, and subsequently colonizing a non-adjacent organ [44, 45]. Up to now, limited details on the molecular mechanisms that regulate the extravasation process and re-entry of the circulating cancer cells into distal tissues have been seen in the literature. As 90 % of mortality from solid tumors is a result of metastasis [45], a greater comprehension of how this mechanism functions which will aid in developing effective treatments to halt and treat cancer metastasis which will lead to a pronounced improvement in prognoses of cancer patients.

Recently, there has been mounting evidence that implicates neuropilins (NRPs) as playing a role in extravasation and metastasis of tumor cells. NRPs are type I transmembrane glycoproteins which include neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2). The two neuropilins were initially identified as multi-functional non-kinase

receptors for ligands of the class 3 semaphorin, vascular endothelial growth factor and other growth factors [46, 47, 48, 49, 50, 51, 52, 53, 54, 55]. Both aforementioned neuropilins have parallels and differences in their functions and share a 45 % homology in their protein sequences. NRP-2 is seen to be less developmentally crucial, as homozygous NRP-2 mutant mice are still viable with only slight defects [56, 57, 58]. In contrast, NRP-1 homozygous knockout mice suffer significant vascular and neural issues which lead to mortality [59]. Due to the more benign effects resulting from a lack of NRP-2, less is currently known about the biological attributes of NRP-2 than NRP-1.

NRP-2 is highly expressed on the surface of certain varieties of tumor cells, such as those of colorectal carcinoma [60], endocrine pancreatic tumors [61], and pancreatic adenocarcinoma [62]. The research suggests that NRP-2 expression is correlated in some form to cancer metastasis [63, 60, 64, 65]. In breast cancer [66] and osteosarcoma [67], a poor prognosis is associated with NRP-2 expression. Lymph node metastasis in papillary thyroid carcinoma [64] and breast cancer [66] has also been shown to be associated with NRP-2 expression. Additionally, endothelial cells of lymphatic vessels in tumor tissue show expression of NRP-2. It has been shown that administering blocking antibody to target NRP-2 on lymphatic endothelial cancer cells hinders tumor lymphogenous metastasis as it inhibits tumor lymphangiogenesis [63]. It is still unclear what molecular mechanisms with which NRP-2 influences tumor metastasis. However, regulation of CXCR4 [66] and survival signaling [62, 60, 68] has been shown to be correlated with NRP-2.

The following work was part of a collaborative effort with the Mukhopadhyay Group at the Mayo Clinic which resulted in a paper which was recently accepted for publication [69]. NRP-2 expression in renal cell carcinoma (RCC) and pancreatic cancer models was studied and together with our collaborator's data, cancer metastasis was shown to be promoted by NRP-2. This occurs uniquely through an interaction

between α5 integrin on the surface of endothelial cells and NRP-2 expressed on tumor cells, where NRP-2 functions as an adhesion molecule. The studies expose a potential mechanism for tumor cell vascular adhesion and extravasation which is mediated by NRP-2 and ultimately uncover the clinically important role of NRP-2 in promoting cancer metastasis.

Chapter 6

Materials and methods

6.1 Cell culture

Human RCC cell line 786-O (ATCC) was maintained in DMEM medium (10 % FBS, 1 % penicillin/streptomycin). Likewise, pancreatic cancer cell line AsPC-1 (ATCC) was maintained in RPMI-1640 (10 % FBS). Variants of these cell lines, in particular those with knocked down or overexpressed NRP-2 expression were prepared and provided by our collaborators [69]. All experiments used 5 mM EDTA in PBS to detach the cancer model cells from the culture dish as NRP-2 was found to be a trypsin sensitive membrane protein. Human umbilical vein endothelial cells (HUVECs; Lonza, San Diego, CA) were cultured in endothelial basal medium supplemented with EGM-MV Bullet kit (5 % fetal bovine serum (FBS), 12 µg/ml bovine brain extract, 1 µg/ml hydrocortisone, and 1 µg/ml GA-1000). In the experiments, HUVECs of passages 3–5 were used. Bovine collagen type I (BD Biosciences) was used to coat the dishes used in the HUVEC culture. α5 integrin antibody (Clone: 5H10-27(MFR5); Biolegend) was used for blocking.

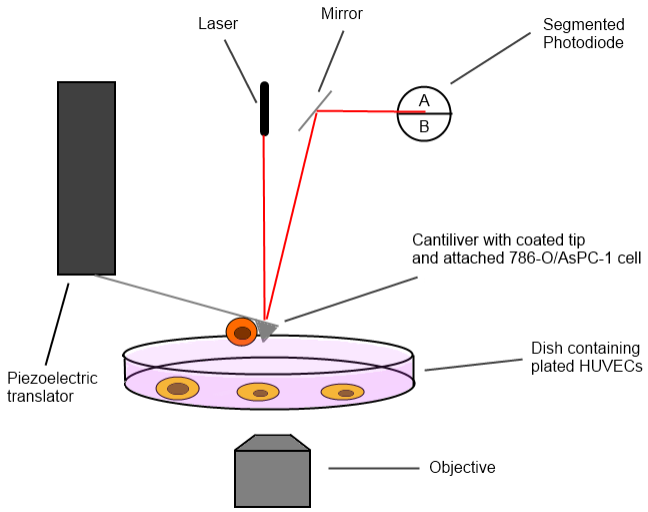


Figure 6.1: **AFM schematic for measuring contact forces between 787-O/AsPC-1 cells and HUVECs.** Shown in this schematic are the major components of a custom-built AFM coupled to an inverted optical microscope, which allows for viewing of the cells. A single 786-O cell (designated in orange in the figure) is attached to a Con A-functionalized cantilever. This in turn is lowered onto the plated HUVECs (designated in yellow) by the action of the piezoelectric translator. A laser (~635 nm) is reflected off the cantilever into the photodiode. The voltage difference between segments A and B of the photodiode gives the amount of cantilever deflection.

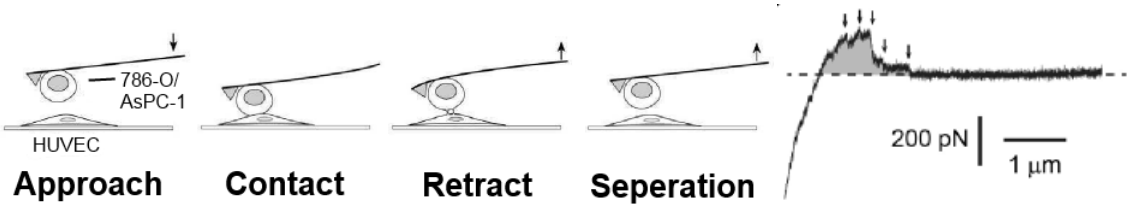
6.2 786-O/AsPC-1 cell–HUVEC adhesion measured by Atomic force microscopy

Atomic force microscopy (AFM) is a technique used to measure the mechanical contact force between cells [40, 41] and is utilized in this experiment to study the role NRP-2 plays in mediating interaction between cancer cells and endothelial cells. The experimental apparatus used to measure cell adhesion is schematically illustrated in Figure 6.1. In methods previously described [70, 71], HUVECs were plated on standard 35 mm tissue culture dishes while a single 786-O/AsCP-1 cell was coupled to the end of the AFM cantilever (MLCT, Bruker Nano, Camarillo, CA). Atomic force microscopy was used to control the relative position of the interacting cells via expansion or contraction of the piezoelectric translator. The force acting between

the cells was derived from the deflection of the AFM cantilever, which was monitored by reflecting a focused laser beam off the back of the cantilever into a 2-segment photodiode. Cantilevers were individually calibrated using the equipartition theorem [42] and had spring constants ranging from 0.015 N/m to 0.025 N/m.

The AFM force measurements were carried out by a custom-built AFM with the individual cancer cells attached to the tip of an AFM cantilever via Concanavalin A (Con A)-mediated linkages. In order to prepare the Con A-functionalized cantilevers, they were soaked in acetone for 5 min, irradiated by UV for 30 min, incubated in biotinamidocaproyl-labeled bovine serum albumin (biotin-BSA, 0.5 mg/mL in 100 mM $NaHCO_3$, pH 8.6; Sigma, St. Louis, MO) overnight at 37 °C, rinsed three times with phosphate-buffered saline (PBS, 10 mM PO_4^{3-}, 150 mM NaCl, pH 7.3) and incubated in streptavidin (0.5 mg/mL in PBS; Pierce, Rockford, IL) for 30 min at room temperature. Unbound streptavidin was removed by rinsing the cantilevers with PBS and the cantilevers were incubated in biotinylated Con A (0.5 mg/mL in PBS; Sigma) for 15 min at room temperature and rinsed with PBS. With the aid of an optical magnification system situated below the AFM, the tip of the Con A-functionalized cantilever was positioned above the center of a cell and gently lowered onto the cell for approximately 1 s to attach the cell to the cantilever. Measurements of cancer cell–endothelial cell interaction were conducted in EBM medium at 25 °C. At the onset of the measurements the cancer cell, coupled to the AFM cantilever, was positioned directly above either the center of an isolated HUVEC, or a HUVEC–HUVEC cell junction in a Petri dish seeded with HUVECs to approximately 50 % confluency.

As illustrated in Figure 6.2a, AFM measurement of the interaction between an individual 786-O cell and a HUVEC involves a series of four steps. The cantilever with an attached 786-O/AsPC-1 cell is first lowered onto a HUVEC. A trigger signal is set so that the piezoelectric translator will stop its downward motion and



(a) (b)

Figure 6.2: **Cycle of AFM measurement and retraction trace.** (a) Schematic representation of a typical cycle of the AFM measurement: 1) approach of the cancer cell to the HUVEC, 2) contact between the cancer cell and the HUVEC, 3) retraction of the cancer cell, and 4) separation of the cancer cell from the HUVEC. Arrows indicate the direction of cantilever movement. (b) Typical force spectrum retraction trace for a cancer cell bound to a HUVEC cell body. Measurements were acquired with a compression force of 500 pN, 1 s contact, and a cantilever retraction speed of 3.5 µm/s. Shaded area in the trace is the "detachment work." Arrows point to rupture events, i. e. breakage of adhesive bond(s). Dashed line indicates zero forces.

prevent crushing of the cell or damaging the cantilever. Following a 1 s contact at a compression force of 500 pN, the cantilever is subsequently retracted with a speed of 3.5 µm/s, pulling the 786-O/AsPC-1 cell–HUVEC pair apart until complete separation of the two cells is achieved. During this process, the AFM continuously monitors the force of the cantilever in order to report on the interaction between the 786-O/AsPC-1 cell and the HUVEC. A typical AFM retraction trace is presented in Figure 6.2b. Upon retraction of the cantilever, molecular linkages established between the cells pull the cantilever downward. The cell detachment process typically involves a series of rupture events, as indicated by arrows, and may correspond to the detachment of one or more adhesive ligand–receptor bonds. To quantify the adhesion between 786-O/AsPC-1 cells and HUVECs, we measured the "detachment work," which is derived from integrating the adhesive force over the distance traveled by the cantilever up to the point of the last bond rupture (shaded area in Figure 6.2b) [72, 73]. In the experiments the detachment work was determined for 786-O/AsPC-1 cells that had normal NRP-2 expression (control), NRP-2 knocked down, and NRP-2 overexpressed. These measurements were also performed with anti-α5 integrin

antibody added at a concentration of 10 μg/mL and preincubated for 30 min before commencing measurements.

6.3 Statistical analysis

ANOVA or t-test was used for statistical analysis, with $p < 0.05$ considered statistically significant. Standard error mean is indicated in the data.

Chapter 7

Results

7.1 Quantification of cancer cell–endothelial cell interaction mediated by NRP-2

Figure 7.1a shows the detachment work of HUVEC bound to 786-O control, NRP-2 knockdown or overexpressing 786-O cells. In general, HUVEC cell–cell junctions are about 40 % to 60 % more adhesive to the 786-O cell. Compared to NRP-2 knockdown 786-O cells, an approximately 200 % increase ($p < 0.05$) of detachment work was detected on NRP-2 overexpressed 786-O cells. Anti-α5 integrin antibody has a profound impact on 786-O cell–HUVEC adhesion. In both HUVEC bodies and cell junctions, incubation of the 786-O cells with the antibody completely diminished the enhanced adhesion induced by NRP-2 overexpression. In addition, α5 integrin blockages also inhibited adhesion between 786-O control cells and HUVEC cell junctions. Figure 7.1b shows that in AsPC-1 pancreatic cancer cells, knockdown of NRP-2 significantly decreased detachment work of HUVEC junctions. Furthermore, AsPC-1 cell–HUVEC adhesion in bodies and junctions was abrogated by anti-α5 integrin antibody. These findings suggest that NRP-2 is an adhesion molecule which promotes the adhesion between cancer cells and endothelial cells and that α5 integrin

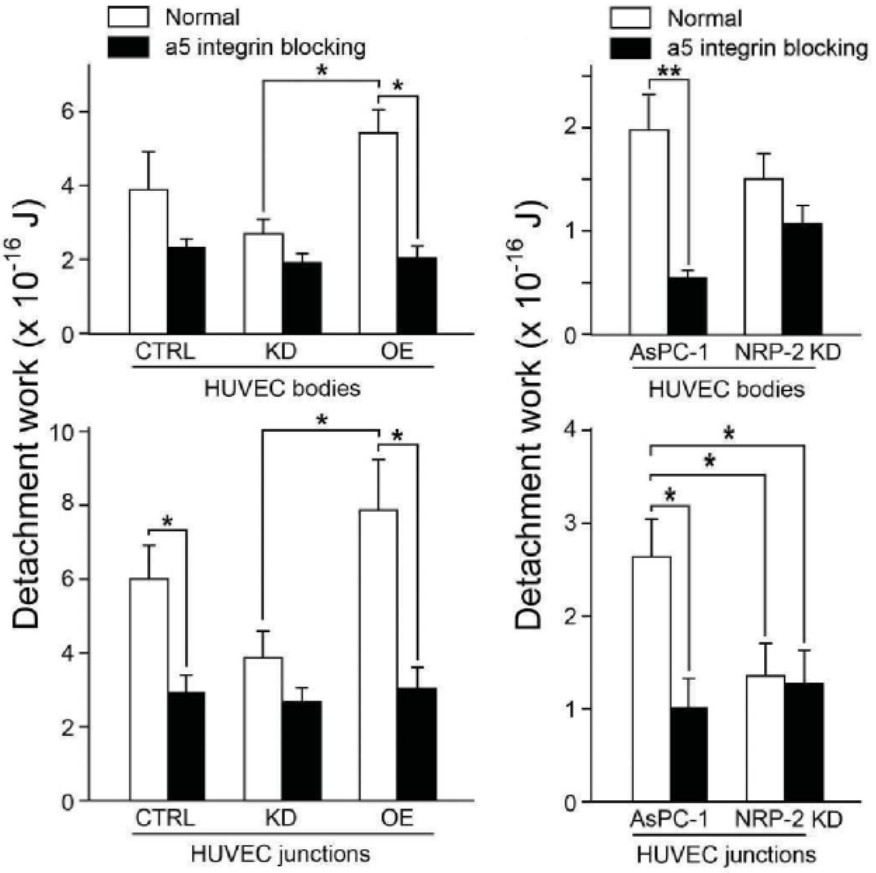


Figure 7.1: **Detachment work between cancer cells and HUVEC.** (a) Adhesion strength between a single pair of 786-O cell and HUVEC (upper panel: HUVEC cell body; lower panel: HUVEC cell junctions) measured by the detachment work. (b) Adhesion strength between a single pair of AsPC-1 cell and HUVEC (upper panel: HUVEC cell body; lower panel: HUVEC cell junctions) measured by detachment work. The error bar is standard error with $n > 5$ in each case. $^{*}p < 0.05$ and $^{**}p < 0.01$ between the indicated groups. CTRL, control 786-O; KD, NRP-2 knockdown; OE, NRP-2 overexpression.

is a trans-binding partner of NRP-2.

7.2 Results of collaborators

Our collaborators performed a variety of other approaches to studying NRP-2 which were further complemented and supported by the results of our experiments. They used an *in vivo* RCC xenograft tumor model (nude mice with subcutaneously injected tumor cells, 786-O among them) to show that NRP-2 depletion decreased metastasis but did not influence primary tumor growth. To further extend these results the collaborators examined clear cell RCC (ccRCC) patient samples utilizing a tissue microarray (TMA) and found that NRP-2 expression was higher in patients with more a more advanced tumor stage. Using another TMA they also found that NRP-2 was notably higher in metastatic carcinoma than primary tumors. This suggests that NRP-2 is involved particularly in cancer metastasis.

An important step in metastasis is extravasation of the cancer cells from the circulation to colonize a distant site from the original organ. To study NRP-2's role in this step the collaborators used an *in vivo* cancer metastasis extravasation model. Zebrafish embryos were injected with 786-O cells and they later found that cells with NRP-2 overexpressed were actively extravasating while the control cells exhibited none of this behavior. These results single out that a role in cancer metastasis that NRP-2 plays a part in is extravasation of cancer cells. Following this another study was done using an *in vitro* cell adhesion assay which further expounded upon the gathered data by showing that the interaction between cancer cells and endothelial cells is mediated by surface expression of NRP-2.

To bridge the above experiments to other types of tumor cells, the extremely metastatic pancreatic cancer cell line, AsPC-1 was similarly studied. *in vitro* NRP-2 depletion was found to reduce the ability of AsPC-1 to adhere to an endothelial

monolayer. In the same zebrafish extravastion model, knockdown of NRP-2 in AsPC-1 showed similar results as before, with an observation of reduced extravasation of cancer cells. In a similar mouse xenograft model, NRP-2 knockdown noticeably reduced cancer metastasis. In addition, a prospectively acquired cohort of patients with pancreatic cancer showed that high NRP-2 expression correlated with poor patient survival.

Finally, our collaborators hypothesized that during cancer metastasis, an integrin expressed on the surface of endothelial cells interacts with NRP-2. They were able to identify $\alpha 5$ integrin as one that interacts with NRP-2 and that HUVECs have a much higher expression of it then 786-O cells.

Chapter 8

Discussion

The results that we obtained confirmed and quantified the data that our collaborators collected. Taken together, it reveals a mechanism in which NRP-2 on a variety of tumor cells promotes cancer metastasis by mediating the interaction with α5 integrin on endothelial cells, particularly during the stage of metastasis in which the tumor cell adheres to the endothelium and extravasates from the circulation to colonize a distant organ. Other recent research [64, 66, 74] in addition to ours point to high expression of NRP-2 being correlated with cancer metastasis. Therapeutically targeting NRP-2 has already been shown to reduce metastasis in some cases in an *in vivo* model [63]. Our research uncovers the previously unknown mechanism of NRP-2 action and the players involved which aids in developing therapeutics to block NRP-2. This is a highly promising method of preventing cancer metastasis and greatly improving the prognoses of cancer patients.

Chapter 9

Concluding remarks

In this work we quantified, using a highly sensitive method, the adhesive strength of T cells and cancer cells on a substrate and interacting cells, respectively. This technique is not limited to the chosen systems and can potentially be used to study a wide range of cell–cell and cell–substrate interactions in various disease-oriented settings. Our characterization of the T cell–ICAM-1 interaction is far from complete. A more thorough investigation under single molecule conditions will be needed to identify the affinity state of LFA-1 under various conditions. We hypothesize that on naive T cells, most LFA-1 molecules are in an inactive state. Upon OKT3 activation, some populations of LFA-1 will be induced to high affinity. In effector cells, the enhanced adhesion compared to naive cells suggests a significant population of LFA-1 is maintained in the high affinity state. Since OKT3 treatment further increased adhesion in effector cells, the extra adhesion may stem from further activation of the inactive population of LFA-1 and/or the induced clustering of LFA-1 on the cell surface. Another open question is the possibility of rebinding during the pulling of multiple bonds. Although AFM can reveal the dissociation kinetics of cell adhesion bonds, the association kinetics might be relevant in some cases such as after OKT3 treatment. Moreover, while AFM measurements reveal the force required to pull T

cells from the substrate, complementary studies using molecular dynamic simulations and specific monoclonal antibodies and/or site-directed mutagenesis will be necessary to fully address the molecular mechanism of LFA-1 mediated T cell adhesion.

Bibliography

[1] R O Hynes. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, 69(1):11–25, April 1992.

[2] Richard O. Hynes. IntegrinsBidirectional, Allosteric Signaling Machines. *Cell*, 110(6):673–687, September 2002.

[3] Bing-Hao Luo, Christopher V Carman, and Timothy A Springer. Structural basis of integrin regulation and signaling. *Annual review of immunology*, 25:619–47, January 2007.

[4] Ronen Alon and Michael L Dustin. Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen-presenting cells. *Immunity*, 26(1):17–27, January 2007.

[5] Martin A Schwartz and Douglas W DeSimone. Cell adhesion receptors in mechanotransduction. *Current opinion in cell biology*, 20(5):551–6, October 2008.

[6] Azucena Salas, Motomu Shimaoka, Avi N Kogan, Charlotte Harwood, Ulrich H von Andrian, and Timothy A Springer. Rolling Adhesion through an Extended Conformation of Integrin αLβ2 and Relation to α I and β I-like Domain Interaction. *Immunity*, 20(4):393–406, April 2004.

[7] Revital Shamri, Valentin Grabovsky, Jean-Marc Gauguet, Sara Feigelson, Eugenia Manevich, Waldemar Kolanus, Martyn K Robinson, Donald E Staunton, Ulrich H von Andrian, and Ronen Alon. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nature immunology*, 6(5):497–506, May 2005.

[8] Azucena Salas, Motomu Shimaoka, Uyen Phan, Minsoo Kim, and Timothy A Springer. Transition from rolling to firm adhesion can be mimicked by extension of integrin alphaLbeta2 in an intermediate affinity state. *The Journal of biological chemistry*, 281(16):10876–82, April 2006.

[9] Eilon Woolf, Irina Grigorova, Adi Sagiv, Valentin Grabovsky, Sara W Feigelson, Ziv Shulman, Tanja Hartmann, Michael Sixt, Jason G Cyster, and Ronen Alon. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nature immunology*, 8(10):1076–85, October 2007.

[10] Paula Stanley, Andrew Smith, Alison McDowall, Alastair Nicol, Daniel Zicha, and Nancy Hogg. Intermediate-affinity LFA-1 binds alpha-actinin-1 to control migration at the leading edge of the T cell. *The EMBO journal*, 27(1):62–75, January 2008.

[11] Ziv Shulman, Vera Shinder, Eugenia Klein, Valentin Grabovsky, Orna Yeger, Erez Geron, Alessio Montresor, Matteo Bolomini-Vittori, Sara W Feigelson, Tomas Kirchhausen, Carlo Laudanna, Guy Shakhar, and Ronen Alon. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity*, 30(3):384–96, March 2009.

[12] Junichi Takagi, Benjamin M. Petre, Thomas Walz, and Timothy A. Springer. Global Conformational Rearrangements in Integrin Extracellular Domains in Outside-In and Inside-Out Signaling. *Cell*, 110(5):599–611, September 2002.

[13] Motomu Shimaoka, Tsan Xiao, Jin-Huan Liu, Yuting Yang, Yicheng Dong, Chang-Duk Jun, Alison McCormack, Rongguang Zhang, Andrzej Joachimiak, Junichi Takagi, Jia-Huai Wang, and Timothy A. Springer. Structures of the αL I Domain and Its Complex with ICAM-1 Reveal a Shape-Shifting Pathway for Integrin Regulation. *Cell*, 112(1):99–111, January 2003.

[14] Tsan Xiao, Junichi Takagi, Barry S Coller, Jia-Huai Wang, and Timothy A Springer. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature*, 432(7013):59–67, November 2004.

[15] Noritaka Nishida, Can Xie, Motomu Shimaoka, Yifan Cheng, Thomas Walz, and Timothy A Springer. Activation of leukocyte beta2 integrins by conversion from bent to extended conformations. *Immunity*, 25(4):583–94, October 2006.

[16] D. Davignon, E. Martz, T. Reynolds, K. Kurzinger, and T. A. Springer. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2,3 that participates in T lymphocyte-mediated killing. *Proceedings of the National Academy of Sciences*, 78(7):4535–4539, July 1981.

[17] T A Springer, M L Dustin, T K Kishimoto, and Steven D Marlin. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annual review of immunology*, 5:223–52, January 1987.

[18] M L Dustin, R Rothlein, A K Bhan, C A Dinarello, and T A Springer. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function

of a natural adherence molecule (ICAM-1). *Journal of immunology (Baltimore, Md. : 1950)*, 137(1):245–54, July 1986.

[19] R Rothlein, M L Dustin, S D Marlin, and T A Springer. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *Journal of immunology (Baltimore, Md. : 1950)*, 137(4):1270–4, August 1986.

[20] Steven D. Marlin and Timothy A. Springer. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell*, 51(5):813–819, December 1987.

[21] G Siu, S M Hedrick, and A A Brian. Isolation of the murine intercellular adhesion molecule 1 (ICAM-1) gene. ICAM-1 enhances antigen-specific T cell activation. *Journal of immunology (Baltimore, Md. : 1950)*, 143(11):3813–20, December 1989.

[22] Donald E. Staunton, Michael L. Dustin, Harold P. Erickson, and Timothy A. Springer. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell*, 61(2):243–254, April 1990.

[23] Michael L. Dustin and Timothy A. Springer. Role of Lymphocyte Adhesion Receptors in Transient Interactions and Cell Locomotion. *Annual review of immunology*, 9:27–66, November 1991.

[24] Christopher V Carman and Timothy A Springer. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Current Opinion in Cell Biology*, 15(5):547–556, October 2003.

[25] Richard O Hynes. Structural biology. Changing partners. *Science (New York, N.Y.)*, 300(5620):755–6, May 2003.

[26] Nancy Hogg, Andrew Smith, Alison McDowall, Katherine Giles, Paula Stanley, Melanie Laschinger, and Robert Henderson. How T cells use LFA-1 to attach and migrate. *Immunology letters*, 92(1-2):51–4, March 2004.

[27] BR Blazar, PA Taylor, A Panoskaltsis-Mortari, GS Gray, and DA Vallera. Coblockade of the LFA1:ICAM and CD28/CTLA4:B7 pathways is a highly effective means of preventing acute lethal graft-versus-host disease induced by fully major histocompatibility complex-disparate donor grafts. *Blood*, 85(9):2607–2618, May 1995.

[28] R. Schmits. LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. *Journal of Experimental Medicine*, 183(4):1415–1426, April 1996.

[29] C. Berlin-Rufenach, F. Otto, M. Mathies, J. Westermann, M. J. Owen, A. Hamann, and N. Hogg. Lymphocyte Migration in Lymphocyte Function-associated Antigen (LFA)-1-deficient Mice. *Journal of Experimental Medicine*, 189(9):1467–1478, May 1999.

[30] Mark Lebwohl, Stephen K Tyring, Tiffani K Hamilton, Darryl Toth, Scott Glazer, Naji H Tawfik, Patricia Walicke, Wolfgang Dummer, Xiaolin Wang, Marvin R Garovoy, and David Pariser. A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *The New England journal of medicine*, 349(21):2004–13, November 2003.

[31] J LEE. Crystal structure of the A domain from the a subunit of integrin CR3 (CD11 b/CD18). *Cell*, 80(4):631–638, February 1995.

[32] Michael S. Diamond and Timothy A. Springer. The dynamic regulation of integrin adhesiveness. *Current Biology*, 4(6):506–517, June 1994.

[33] Qing Ma, Motomu Shimaoka, Chafen Lu, Hua Jing, Christopher V Carman, and Timothy A Springer. Activation-induced conformational changes in the I domain region of lymphocyte function-associated antigen 1. *The Journal of biological chemistry*, 277(12):10638–41, March 2002.

[34] M P Stewart, C Cabanas, and N Hogg. T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. *Journal of immunology (Baltimore, Md. : 1950)*, 156(5):1810–7, March 1996.

[35] K Bottomly. A functional dichotomy in CD4+ T lymphocytes. *Immunology today*, 9(9):268–74, September 1988.

[36] T R Mosmann and R L Coffman. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology*, 7:145–73, January 1989.

[37] Dan Li, Jeffrey J Molldrem, and Qing Ma. LFA-1 regulates CD8+ T cell activation via T cell receptor-mediated and LFA-1-mediated Erk1/2 signal pathways. *The Journal of biological chemistry*, 284(31):21001–10, July 2009.

[38] Hwee San Lek, Vicky L Morrison, Michael Conneely, Paul a Campbell, David McGloin, Stefanie Kliche, Colin Watts, Alan Prescott, and Susanna C Fagerholm. The spontaneously adhesive leukocyte function-associated antigen-1 (LFA-1) integrin in effector T cells mediates rapid actin- and calmodulin-dependent adhesion strengthening to ligand under shear flow. *The Journal of biological chemistry*, 1:1–18, April 2013.

[39] Yang Wang, Dan Li, Roza Nurieva, Justin Yang, Mehmet Sen, Roberto Carreño, Sijie Lu, Bradley W McIntyre, Jeffrey J Molldrem, Glen B Legge, and Qing

Ma. LFA-1 affinity regulation is necessary for the activation and proliferation of naive T cells. *The Journal of biological chemistry*, 284(19):12645–53, May 2009.

[40] G. Binnig and C. F. Quate. Atomic Force Microscope. *Physical Review Letters*, 56(9):930–933, March 1986.

[41] William F Heinz and Jan H Hoh. Spatially resolved force spectroscopy of biological surfaces using the atomic force microscope. *Trends in Biotechnology*, 17(4):143–150, April 1999.

[42] Jeffrey L. Hutter and John Bechhoefer. Calibration of atomic-force microscope tips. *Review of Scientific Instruments*, 64(7):1868, July 1993.

[43] G A Van Seventer, Y Shimizu, K J Horgan, Stephen Shaw, and V A N Seventer. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *Journal of immunology (Baltimore, Md. : 1950)*, 144(12):4579–86, July 1990.

[44] Isaiah J Fidler. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nature reviews. Cancer*, 3(6):453–8, June 2003.

[45] Gaorav P Gupta and Joan Massagué. Cancer metastasis: building a framework. *Cell*, 127(4):679–95, November 2006.

[46] Shin Takagi, Tatsumi Hirata, Kiyokazu Agata, Makoto Mochii, Goro Eguchi, and Hajime Fujisawa. The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. *Neuron*, 7(2):295–307, August 1991.

[47] S Takagi, Y Kasuya, M Shimizu, T Matsuura, M Tsuboi, A Kawakami, and H Fujisawa. Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. *Developmental biology*, 170(1):207–22, July 1995.

[48] H Chen, a Chédotal, Z He, C S Goodman, and M Tessier-Lavigne. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron*, 19(3):547–59, September 1997.

[49] Z He and M Tessier-Lavigne. Neuropilin Is a Receptor for the Axonal Chemorepellent Semaphorin III. *Cell*, 1997.

[50] AL Kolodkin, DV Levengood, and EG Rowe. Neuropilin Is a Semaphorin III Receptor. *Cell*, 1997.

[51] Roman J Giger, Erica Rowe Urquhart, Susan K.H Gillespie, Dorothy V Levengood, David D Ginty, and Alex L Kolodkin. Neuropilin-2 Is a Receptor for Semaphorin IV Insight into the Structural Basis of Receptor Function and Specificity. *Neuron*, 21(5):1079–1092, November 1998.

[52] Shay Soker, Seiji Takashima, Hua Quan Miao, Gera Neufeld, and Michael Klagsbrun. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell*, 92(6):735–45, March 1998.

[53] Z Gluzman-Poltorak, T Cohen, Y Herzog, and G Neufeld. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. *The Journal of biological chemistry*, 275(24):18040–5, June 2000.

[54] Benoit Favier, Antoine Alam, Pauline Barron, Jacques Bonnin, Patricia Laboudie, Pierre Fons, Marie Mandron, Jean-Pascal Herault, Gera Neufeld, Pierre Savi, Jean-Marc Herbert, and Françoise Bono. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood*, 108(4):1243–50, August 2006.

[55] Laura S Gammill, Constanza Gonzalez, Chenghua Gu, and Marianne Bronner-Fraser. Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. *Development (Cambridge, England)*, 133(1):99–106, January 2006.

[56] Hang Chen, Anil Bagri, Joel A Zupicich, Yimin Zou, Esther Stoeckli, Samuel J Pleasure, Daniel H Lowenstein, William C Skarnes, Alain Chédotal, and Marc Tessier-Lavigne. Neuropilin-2 Regulates the Development of Select Cranial and Sensory Nerves and Hippocampal Mossy Fiber Projections. *Neuron*, 25(1):43–56, January 2000.

[57] Roman J Giger, Jean-François Cloutier, Amar Sahay, Rabinder K Prinjha, Dorothy V Levengood, Stephen E Moore, Susan Pickering, David Simmons, Sohaila Rastan, Frank S Walsh, Alex L Kolodkin, David D Ginty, and Martin Geppert. Neuropilin-2 Is Required In Vivo for Selective Axon Guidance Responses to Secreted Semaphorins. *Neuron*, 25(1):29–41, January 2000.

[58] Li Yuan, Delphine Moyon, Luc Pardanaud, Christiane Breant, Marika J. Karkkainen, Kari Alitalo, and Anne Eichmann. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development*, 129(20):4797–4806, October 2002.

[59] T Kawasaki, T Kitsukawa, Y Bekku, Y Matsuda, M Sanbo, T Yagi, and H Fujisawa. A requirement for neuropilin-1 in embryonic vessel formation. *Development*, 126(21):4895–4902, November 1999.

[60] Michael J Gray, George Van Buren, Nikolaos A Dallas, Ling Xia, Xuemei Wang, Anthony D Yang, Ray J Somcio, Yvonne G Lin, Sherry Lim, Fan Fan, Lingegowda S Mangala, Thiruvengadam Arumugam, Craig D Logsdon, Gabriel Lopez-Berestein, Anil K Sood, and Lee M Ellis. Therapeutic targeting of

neuropilin-2 on colorectal carcinoma cells implanted in the murine liver. *Journal of the National Cancer Institute*, 100(2):109–20, January 2008.

[61] Tzafra Cohen, Yael Herzog, Asia Brodzky, Joel K Greenson, Samuel Eldar, Zoya Gluzman-Poltorak, Gera Neufeld, and Murray B Resnick. Neuropilin-2 is a novel marker expressed in pancreatic islet cells and endocrine pancreatic tumours. *The Journal of pathology*, 198(1):77–82, September 2002.

[62] Nikolaos A Dallas, Michael J Gray, Ling Xia, Fan Fan, George van Buren, Puja Gaur, Shaija Samuel, Sherry J Lim, Thiruvengadam Arumugam, Vijaya Ramachandran, Huamin Wang, and Lee M Ellis. Neuropilin-2-mediated tumor growth and angiogenesis in pancreatic adenocarcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 14(24):8052–60, December 2008.

[63] Maresa Caunt, Judy Mak, Wei-Ching Liang, Scott Stawicki, Qi Pan, Raymond K Tong, Joe Kowalski, Calvin Ho, Hani Bou Reslan, Jed Ross, Leanne Berry, Ian Kasman, Constance Zlot, Zhiyong Cheng, Jennifer Le Couter, Ellen H Filvaroff, Greg Plowman, Franklin Peale, Dorothy French, Richard Carano, Alexander W Koch, Yan Wu, Ryan J Watts, Marc Tessier-Lavigne, and Anil Bagri. Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer cell*, 13(4):331–42, April 2008.

[64] Hironao Yasuoka, Rieko Kodama, Mitsuyoshi Hirokawa, Yuuki Takamura, Akira Miyauchi, Michiya Inagaki, Tokio Sanke, and Yasushi Nakamura. Neuropilin-2 expression in papillary thyroid carcinoma: correlation with VEGF-D expression, lymph node metastasis, and VEGF-D-induced aggressive cancer cell phenotype. *The Journal of clinical endocrinology and metabolism*, 96(11):E1857–61, November 2011.

[65] Erica C Rushing, Megan J Stine, Sarah J Hahn, Sofia Shea, Mark S Eller, Alaa Naif, Sarika Khanna, William H Westra, Achim A Jungbluth, Klaus J Busam, Meera Mahalingam, and Rhoda M Alani. Neuropilin-2: a novel biomarker for malignant melanoma? *Human pathology*, 43(3):381–9, March 2012.

[66] Hironao Yasuoka, Rieko Kodama, Masahiko Tsujimoto, Katsuhide Yoshidome, Hiroki Akamatsu, Masaaki Nakahara, Michiya Inagaki, Tokio Sanke, and Yasushi Nakamura. Neuropilin-2 expression in breast cancer: correlation with lymph node metastasis, poor prognosis, and regulation of CXCR4 expression. *BMC cancer*, 9(1):220, January 2009.

[67] A Handa, T Tokunaga, T Tsuchida, Y H Lee, H Kijima, H Yamazaki, Y Ueyama, H Fukuda, and M Nakamura. Neuropilin-2 expression affects the increased vascularization and is a prognostic factor in osteosarcoma. *International Journal of Oncology*, 17(2):291–296, August 2000.

[68] Shaija Samuel, Puja Gaur, Fan Fan, Ling Xia, Michael J Gray, Nikolaos A Dallas, Debashish Bose, Cristian Rodriguez-Aguayo, Gabriel Lopez-Berestein, Greg Plowman, Anil Bagri, Anil K Sood, and Lee M Ellis. Neuropilin-2 mediated β-catenin signaling and survival in human gastro-intestinal cancer cell lines. *PloS one*, 6(10):e23208, January 2011.

[69] Ying Cao, Luke Hoeppner, Steven Bach, Guangqi E, Yan Guo, Enfeng Wang, Jianmin Wu, Mark J Cowley, David K Chang, Nicola Waddell, Sean M Grimmond, Andrew V Biankin, Roger J Daly, Xiaohui Zheng, and Debabrata Mukhopadhyay. Neuropilin-2 promotes extravasation and metastasis by interacting with endothelial α5 integrin. *Cancer research*, pages 0008–5472.CAN–13–0529–, May 2013.

[70] Xiaohui Zhang, Ewa Wojcikiewicz, and Vincent T Moy. Force spectroscopy of the leukocyte function-associated antigen-1/intercellular adhesion molecule-1 interaction. *Biophysical journal*, 83(4):2270–9, October 2002.

[71] Xiaohui Zhang, Aileen Chen, Dina De Leon, Hong Li, Eisei Noiri, Vincent T Moy, and Michael S Goligorsky. Atomic force microscopy measurement of leukocyte-endothelial interaction. *American journal of physiology. Heart and circulatory physiology*, 286(1):H359–67, January 2004.

[72] Pierre-Henri Puech, Anna Taubenberger, Florian Ulrich, Michael Krieg, Daniel J Muller, and Carl-Philipp Heisenberg. Measuring cell adhesion forces of primary gastrulating cells from zebrafish using atomic force microscopy. *Journal of cell science*, 118(Pt 18):4199–206, September 2005.

[73] Jens Friedrichs, Jonne Helenius, and Daniel J Muller. Quantifying cellular adhesion to extracellular matrix components by single-cell force spectroscopy. *Nature protocols*, 5(7):1353–61, July 2010.

[74] Adrian M Jubb, Susan M Sa, Navneet Ratti, Laura A Strickland, Maike Schmidt, Christopher A Callahan, and Hartmut Koeppen. Neuropilin-2 expression in cancer. *Histopathology*, 61(3):340–9, September 2012.

Biography

Steven Bach, originally from San Jose, CA, earned his B.S. degree in bioengineering at the University of California, Riverside in 2011. He then went on to Lehigh University to do graduate work with Dr. Xiaohui (Frank) Zhang where he studied integrin-mediated T cell and cancer cell adhesion using single-molecule force spectroscopic methods.