Memorial Sloan Kettering
Cancer Center

October 19, 2020

Dear Andy, Directors, and Supporters of the Cure Breast Cancer Foundation:

As Scientific Director it gives me great pleasure to report on progress and plans. As you know the goal of the CBCF is the eradication of breast cancer by scientific research. Thefocus of our research is the phenomenon of cancer self-seeding. This powerful and novel concept has opened many exiting areas for discovery. Until recently the entire emphasis of cancer research has been on the division of cancer cells, ca lled mitosis. This approach has certainly led to many gains, but cancer remains a serious set of diseases, indeed a common cause of illness and death. Clearly, we need new targets for cancer therapy and prevention beyond attacking cancer cell division. We have only recently recognized that cancer is much more than a problem of abnormal mitosis. It also involves such characteristics as (1) metastasis, the spreading of cells from a primary tumorto other parts of the body, (2) angiogenesis, the development of new blood vessels to feed the cancer cell masses, and of the cancer cells with their micro-environment, which is the "normal" cells in the organ hosting the cancer cells.

CBCF research is based on the discovery that all three of these features—metastasis, angiogenesis, tumor microenvironment—are related by a single common pathway. That is, cancer cells can not only metastasize to distant organs (breast to lymph nodes, bone, lung, liver etc.) but also back to the site it came from or between different metastatic sites. In other words, cancer movement is not a one-way phenomenon. We call these travelling cancer cells seeds. When seeds travel, they bring with them bone marrow cells that can grow into blood vessels. Hence: angiogenesis. They also bring with them white blood cells—called leukocytes—that infiltrate the tumor and are hence called tumor-infiltrating leukocytes, or TILs. Leukocytes are one of the major components of the tumor microenvironment. When we think of leukocytes—the prime a ctors of the immune system—we often see them as killing cancer cells. But some can help the cancer grow or suppress other cancer-killing leukocytes.

CBCF research has made many discoveries regarding the biology of self-seeding. One of the most surprising and far-reaching is that TILs are often mutant, meaning that their DNA is not normal. Indeed, the mutations are often the same mutations that appear in a cancer of leukocytes called leukemia even though the patients have no evidence of leukemia. These mutations—DMNT3A, TET2, ASXL1 and others—modify the function of other genes. Using highly sensitive methods we have with our colleagues found such mutant leukocytes circulating in the blood stream of individual with and even without cancer, particularly this with certain genetic breast cancer predispositions. This is called *clonal hematopoiesis*, or CH. But they are more common in cancer patients and are found in particularly large amounts infiltrating the cancers themselves: mutant TILs. Moreover, one mutation—TET2—has been shown to make leukocytes(a type called macrophages) secrete molecules—interleukin 1-beta, interleukin 6—that have profound effects on other types of leukocytes. In that regard they very likely are important in the causation of at heart disease (heart attacks) and might play a role in severe illness from Covid-19.

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An important aspect of our current research is based on the hypothesis that mutant TILs promote tumor growth directly and indirectly by their effects on other cells in the tumor microenvironment. We have already found that mutant TILs inhibit the ability of chemotherapy drugs to kill cancer cells. Experiments are underway to examine the relationship between mutant TILs and response to immunotherapy.

In addition, we have discovered that when, rarely, a breast cancer patient later develops leukemia, those leukemic cells often have the same mutations that were found in their TILs, sometimes many years earlier. CH is known to be a risk factor for the development of hematological (blood) cancers. This observation might be crucial in assessing the likelihood of a breast cancer patient later developing a blood cancer, which could influence clinical decisions. The role of mutant TILs in increasing the likelihood of non-hematological cancers is also being explored.

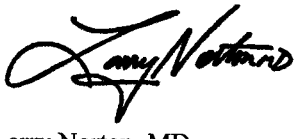
To do this work we have assembled a team of leaders at MSK in the fields of CH and hematologic (blood) malignancies (Dr. Ross Levine), single cell DNA analysis (Drs. Levine and Jorge Reis-Filho), breast cancer molecular pathology (Reis-Filho) and breast medical oncology (Dr. Elizabeth Comen and myself) and a team of researchers at the Dr. Larry Norton Institute in Israel (Dr. Nir Peled and colleagues) to define the impact of CH on breast cancer development and therapeutic response. We will employ an integrated approach combining cutting-edge genomic/single cell DNA sequencing methods for the study of human samples with laboratory systems aimed to identify novel mechanisms and therapeutic targets. Specific aims are included as Appendix 1.

Consistent with our interest in how white blood cells support tumor growth the CBCF supported the groundbreaking work of Dr. Clare Yu of the University of California, Irvine. She is a world-class mathematician who studied the geometric arrangement of TILs in human breast cancers that lack estrogen, progesterone, and HER2 receptors (called *triple negative*). TILs can be B cells (that make antibodies) and T cells (that kill directly). She found that both B and T cells are clustered together in cancers that metastasize. It seems that the clusters might form because they are gathered around self-seeds, which is exactly what we saw in the original observations that proved self-seeding in the laboratory. In cancers with a good prognosis there are more TILs but they are dispersed evenly throughout the tumor, which might be because these tumors are not active seeders. As we have shown before, seeding and metastases are linked. Moreover, the special arrangement of B cells in the clusters is important and is consistent with the hypothesis that these cells are "educating" (called *antigen presentation*) the killer T cells. This work is important not only in predicting which cancer have a good prognosis (so they might not need adjuvant chemotherapy) but in the evaluation of drugs specifically designed to attack mutant TILs, as mentioned above (see also Appendix 1).

Lastly, over this past year Dr. Rachel Hazan has made significant advances in studying the cellular molecule p21. This protein plays an unusual role in breast cancer metastasis by promoting cell migration while inhibiting cell division. p21 levels go up during chemotherapy, thereby encouraging tumor cells to become seeds and metastasize to distant organs while stopping them from dividing and thereby escaping chemotherapy (which kills only dividing cells). Using the CBCF funds, she discovered that p21 promotes seeding by a specific mechanism: increasing the production of a molecule called TCF1 that in turns activates a molecule called Wnt and thence cyclin D1. She proposes that a combination of drugs that inhibit cyclin D1 and TCF1 might be effective in stopping the seeding process. Indeed, inhibition of cyclin D1 activation by palbociclib, a drug that inhibits CDK4/6 activation by cyclin D1, seems to have this effect. Her current research is focused on finding drugs that augment the effect by modulating TCF1. Her specific aims are included as Appendix 2.

With CBCF funding we have made significant progress in exploring the basis for and implications of cancer self-seeding and are rapidly approaching the point where these advances will translate into major strides toward the control, eradication, and even prevention of breast cancer. I speak for the teams of scientists that you have supported with your generosity, enthusiasm, encouragement, and confidence when I express our deep gratitude and appreciation. Together we are not only discovering fundamentals of biology of far-reaching importance but doing so with a dedication toward ridding the world of breast cancer and many other cancer types as well.

Sincerely,



Larry Norton, MD

Appendix 1.
The CH and Mutant TIL Project

Specific Aim 1: To perform a detailed genomic, immunophenotypic and functional characterization of human breast cancer and its microenvironment in the context of CH at single-cell resolution.

Specific Aim 2: To interrogate the *in vitro/vivo* consequences of CH on solid tumor biology, progression and metastasis utilizing murine models engrafted with *Dmnt3a*, *Tet2* and *Asxl1* mutant hematopoietic cells.

Specific Aim 3: To define the impact of CH, including infiltrating clonal leukocytes (mutant TILs), on the response of triple-negative breast cancers (TNBCs) to chemotherapy ± immunotherapy.

Specific Aim 4: To perform *in vitro* screening and *in vivo* testing of epigenetic and immunomodulatory drugs with known activity in hematologic malignancies in order to identify therapeutic targets which attenuate CH and which enhance the response of the solid tumor/CH microenvironment to tumor-directed therapies.

Specific Aim 5: To identify the clinical and CH molecular features associated with the development of secondary leukemia in breast cancer patients in order to develop a clinically tractable model to predict secondary hematopoietic malignancies.

Specific Aim 6: To explore the reasons why mutations on leukocytes and in other non-malignant tissues—including ductal carcinoma in situ of the breast—do not always progress to frank malignancy.

Single cell sequencing platforms, including the MissionBio Tapestri and the 10x single cell sequencing platforms are available in the Levine and Reis-Filho laboratories and the protocols for both liquid samples and solid tumor DNA and RNA sequencing have been fully established. The methods to be employed for Specific Aims 2 and 3, including C57BL/6J CH mouse models with SCL:CreERT2 deletion of *Dmnt3a*, *Tet2* and *Asxl1* with a TdTomato reporter, are fully established in the Levine laboratory. Samples for the studies proposed in Specific Aims 1 and 4 are being collected under MSKCC IRB approved protocols and available in the Precision Pathology Biobanking Center (Department of Pathology) and Reis-Filho laboratory. Additional samples may

be obtained in the Dr Larry Norton Institute in Soroka Hospital in Beer Sheva, Israel. The MSK Clonal Hematopoiesis Program (Levine, Norton, Comen, Reis-Filho) provides a unique opportunity to follow solid tumor patients with CH, to bank serial samples, and to support discovery science and cutting-edge trials aimed to prevent secondary malignancies. Moreover, particularly for Aim 6, we will be utilizing the extensive mathematical analysis capabilities of Dr. Norton's mathematical Oncology Initiative (collaborations with Dr. Allen Tannenbaum of Stony Brook University and the Department of Mathematics, Ben Gurion University).

Appendix 2
The p21 Project

Specific Aim 1: *To determine the effect of p21 and TCF1 on EMT, self-seeding and metastasis* we propose to test the effect of p21 and TCF1 on self-seeding using a human xenograft model of breast cancer. We will (1) overexpress p21 or TCF1 in human breast cancer cells that are non-metastatic and non-seeding cells (MCF7); and conversely (2) knock out p21 or TCF1 in highly metastatic cells that are self-seeding (LM2). We will evaluate effects of p21 and TCF1 gain or loss of function on EMT leading to de-differentiation and cell migration, cancer stem cell activity, and importantly self-seeding and metastatic abilities. If the data point to an important effect of p21 or TCF1 on self-seeding, we will use RNA sequencing to identify gene candidates that can be used to design therapeutic approaches that can inhibit self-seeding and/or metastasis.

Specific Aim 2: *To identify breast tumor cell types and genes driving self-seeding and metastasis.* Breast cancer is a heterogenous disease comprised of diverse carcinoma cell clones that collaborate or compete in controlling tumor behavior, involving dominant clones that drive a worse tumor phenotype. Interestingly, tumor cell heterogeneity is also present in distant metastases. We hypothesize that the self-seeding population heterogeneity might select for clones that fuel metastasis. We will use single cell RNA sequencing to determine whether the self-seeding population is heterogeneous and identify tumor cell clusters and genes that drive self-seeding and hence metastasis. Technically, we will generate primary tumors by injection of unlabeled lung metastatic cells (MDA-MB-231/LM2) cells in the left mammary gland (recipient) and with GFP/luciferase expressing LM2 on the right contralateral mammary fat pad (donor). Following self-seeding (60 days later), we will isolate GFP labelled cells from the recipient tumor and compare them by single cell analysis to the unlabeled (non-seeding) resident population (in the recipient site) or to the remainder GFP labelled population in the donor tumor. Alternatively, we will inject by tail vein GFP labelled LM2 cells into mice bearing tumors generated by orthotopic injection of RFP labelled LM2 cells. We will determine the kinetics of self-seeding of circulating GFP+ tumor cells into the RFP+ tumor. We will then use the optimal time to isolate self-seeding GFP+ LM2 cells and compare them to resident RFP+ primary tumor cells using single cell analysis. This experiment will determine whether the self-seeding population is homogenous (monoclonal) or made of different tumor cell clones (heterogenous) that are driving self-seeding. Elucidation of the genes and signaling pathways driving one or multiple self-seeding clones might shed light into drivers of self-seeding that can be exploited for therapeutic intervention in metastatic breast disease.