ANTI-UBIQUITIN ANTIBODIES AS A METHOD TO PREVENT METASTASIS

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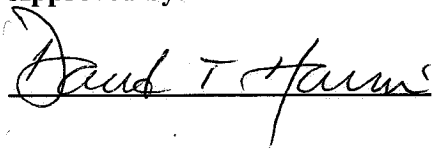

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Anti-ubiquitin Antibodies as a Method to Prevent Metastasis

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Abstract: Ubiquitin is a 76 amino acid protein that is known to be involved in a number of diverse cellular functions. Although ubiquitin is not found on the cell surface, it has been observed that antibodies against ubiquitin bind to a molecule on cell surfaces that expresses a cross-reactive peptide or structure, such as the Peripheral Lymph Node Homing Receptor molecule, which plays a role in in promoting and maintaining cell adhesion. Dysfunction of cell adhesion and cell migration occurs during metastasis when cancer cells travel into the circulation and then extravasates into a secondary site. Our hypothesis is that preventing the binding function of anti-ubiquitin mAb-reactive proteins in cancer cells will reduce metastasis. This inhibition can be achieved by antibodies that bind to ubiquitin or similar molecules. In vitro experimentation done by our laboratory has shown that blocking these ubiquitin-like molecules on cancer cells does, in fact, inhibit migration of cells prone to metastasis, although the mechanism by which this occurs is unknown. Cure Cancer Worldwide has engineered five different humanized anti-ubiquitin antibodies. This generation of antibodies should be capable of both inhibiting the binding of ubiquitin-like molecules involved in migration, and interacting fully with a functioning immune system, thus restricting the metastatic potential of a primary cancer. *In vivo* mouse experiments suggest the reduction in cancer growth of the highly invasive breast cancer cell line 4T1 is possible.

Introduction

Metastasis causes as much as 90% of cancer-associated mortality, thus the prevention of metastasis should be one of the most urgent missions of cancer treatment. Controlling metastasis may help control cancer; for example, in patients with colon cancer, the 5-year survival rate is approximately 80% to 90% for metastasis-free patients and only 10% to 20% for patients with metastases [1].

The process by with cancer cells metastasize is complex and involves several steps. In order to metastasize, a cancerous cell or cluster of cancerous cells, must locally invade the tissue it surrounds, and enter the microvasculature of the blood and lymph systems; also known as intraversion. Intraversion is an intricate process because the cells must not only escape from the primary tumor, but must change morphology such that instead of adhering to the tumor, they gain the ability to crawl away from the tumor and move long distances throughout the body. If the cells survives intraversion, then they must move through the bloodstream and finally exit it via the microvessels of distant tissues (known as extravasation). Normally cells that are not adhering to a surface or a matrix of some sort undergo apoptosis, so surviving in a state of suspension in the circulation is a feat in itself. If the cell survives extravasation, then it must adapt to the foreign microenvironment of the new tissue, establish itself, and then proliferate in order to form a secondary tumor, a process known as colonization [2, 3].

Blocking the ability of cancer cells to bind and exit the circulation may reduce their metastatic potential and increase the time the cancer cells are susceptible to removal by the immune system. This binding may be reduced by targeting cancerous cells with an anti-ubiquitin antibody. Ubiquitin is a highly conserved protein that is found in almost all eukaryotic tissues, and is best known for its role in apoptosis. However, ubiquitin has a myriad of other functions,

and it is now generally accepted that ubiquitin plays a role in most cellular signaling pathways [4]. Because ubiquitin-based regulation of cell signaling is so common, ubiquitin has been shown to be relevant to many human disorders and illnesses, including cancer, however there are no known cases of ubiquitin being found on the surface of cells, but antibodies against ubiquitin do bind on the cells surface because they act like the Mel-14 molecule, which adheres to the Peripheral Lymph Node Homing Receptor [5]. It is our hypothesis that our anti-ubiquitin mAb cross-reacts with Mel14 on the cells. Thus, ubiquitin and Mel-14 share a common sequence and structure, both of which bind to the Peripheral Lymph Node Homing Receptor.

Extraversion has been compared in many aspects to the normal actions and movements of lymphocytes, and lymphocyte-endothelial cell interactions have been studied as a window for understanding the properties and mechanics of metastatic cancer cells [5]. Thus, it does not come as a complete surprise that cancer cells may express lymph node receptors. In lymphocytes, Peripheral Lymph Node Homing Receptors are shown to promote and maintain cell adhesion, which is key to the process of metastasis because as cancer metastasizes and cells migrate away from the primary tumor, the cells must adhere to other cells in the vasculature or lymphatics to re-establish a tumor at a secondary site [6]. Thus, we hope that blocking the function of migration-promoting molecules found on the surface of cells will reduce metastasis.

We can prevent the proper functioning of ubiquitin via anti-ubiquitin antibodies. Anti-cancer antibodies have increasingly become popular for the treatment of both solid and hematological cancers due to their specificity as well as their low toxicity [7]. Thus, antibodies against ubiquitin-like molecules should be able to target cell surface proteins that promote metastasis, as well as interact fully with a functioning immune system.

There are many mechanisms for tumor cell killing via antibodies. For example, cancerous cells can be killed through the direct actions of the antibody via receptor blockage, initiation of an apoptosis mechanisms, or if it is coupled with a cytotoxic compound or drug. Antibodies can also trigger immune induced defenses, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Additionally, antibodies have a variety of specific effects on tumor stroma and vasculature needed for tumor survival [7, 8].

Several antibody therapies have already been approved by the FDA and are being used in the clinical arena. For example, cetuximab (against Epidermal growth factor receptor) and trastuzumab (against the HER2/neu receptor) inhibit cell signaling, rituximab (against CD20) induces antibody-dependent cell mediated cytotoxicity (ADCC), and ipilimumab acts as an antagonist to CTLA-4, an antigen found on T cells which inhibits T cell attack, just to name a few [8]. The antibodies used clinically today all function by leading to cell death, our generation of antibodies is novel in the sense that they function to prevent the metastasis of cancerous cells, rather than kill them.

Materials and Methods

Cell lines and cell culture:

Five cancer cell lines (H640, AG49, MB231, CT and 4T1) were used in this study. The cancer cell lines were provided by the Experimental Mouse Shared Service (EMSS) at the University of Arizona. The cancer cells were maintained in MEM medium supplemented with 10% FBS, and 50 units/mL penicillin, streptomycin, essential amino acids, and L glutamine. Subconfluent cultures (10^6 cells/ml) were maintained to reduce down-regulation of surface proteins.

Monoclonal antibody:

All of the engineered human antibodies were obtained from Cure Cancer Worldwide (Tucson, AZ). 1OC2, used as a control antibody, was obtained from Becton-Dickinson (BD) or Biolegend. The monoclonal anti-human IgG antibodies used for flow cytometry labelled with florescent marker APC were also obtained from BD or Biolegend.

Flow cytometric analysis:

For the analysis of the mAb binding (to surface ubiquitin like molecules) on cells, cancer cell lines were incubated at 37C and 5% CO2, and then removed from flasks with trypsin. $0.5x10^6$ cells were then incubated for 30 minutes in buffer containing 20ul of 0.01mg/ml antibodies or the isotype control. The cells were then washed and incubated for 20 minutes with 10ul of 0.01 mg/ml APC conjugated anti-human IgG. The cells containing IOC2 control primary antibody were incubated with 10ul of 0.01 mg/ml anti-mouse IgG APC. The cells were washed and analyzed using a flow cytometer for florescence activity indicating whether the cells

expressed surface molecules that cross reacted with anti-ubiquitin antibodies and whether the engineered antibodies adhered those molecules.

In vivo work:

Primary tumors were established in 28 BALB/C Mice obtained from Jackson Labs by injection of with half a million 4T1 cancer cells that had been incubated for 20 minutes with either the engineered antibodies or the isotype control. The 500,000 4T1 cancer cells were injected directly into the mammary fat pad. There were no further administration of the antibody throughout the process.

Tumor size and body weights were obtained approximately 2 times per week. The mice were also evaluated for signs of sickness, such as ragged fur. If the mouse lost 10-20% of its original weight, it was assumed that they were sufficiently sick from the cancer and could then be sacrificed. Mouse lungs were then evaluated for metastasis. Measurements of the primary tumors were taken in addition to measurements for visible secondary tumors, and mean tumor burden calculated for each mouse. The tumor sizes were calculated from millimeter caliper measurements of tumor dimensions using the formula for an ellipsoid

$$\frac{(Lengh * width^2)}{2}$$

Results

As seen in Table 1, flow cytometric analysis showed that of the five cell lines chosen for this study, the highest percentage of mAb-binding cells were the 4T1 cells. 4T1 is a mammary carcinoma that is a highly tumorigenic cell line and invasive. It can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone. Thus, it is highly likely, that this cell line has surface molecules that promote metastasis, such as the Peripheral Lymph Node Homing Receptor.

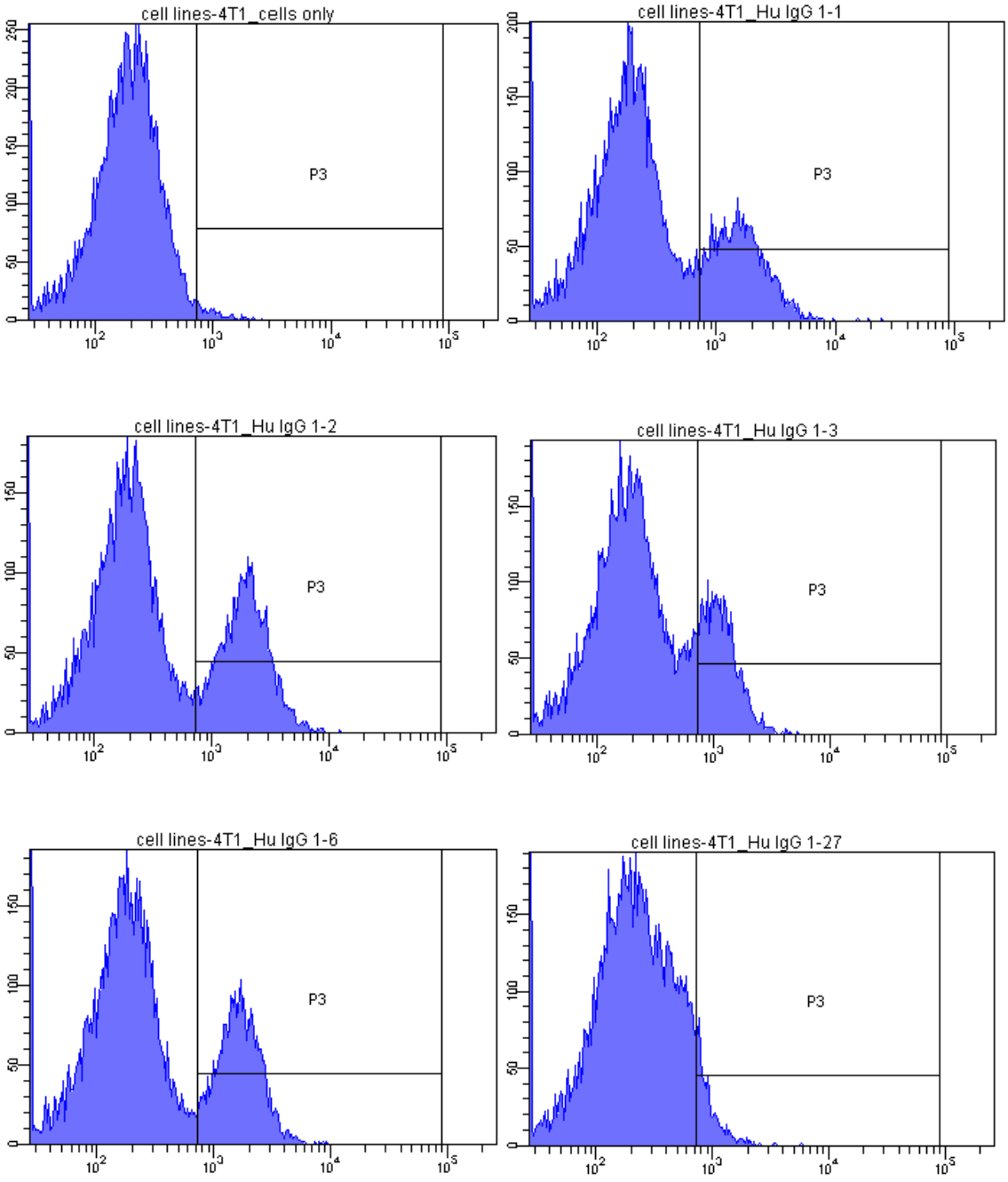
Flow cytometric analysis of the ubiquitin specific positive control monoclonal antibody show that a fraction of 4T1 cells express ubiquitin-like molecules on the cell surface, and that the antibody was able to bind to these molecules. In comparison, the negative control shows no fraction of cells with a positive florescence stain (Figure 1). Analysis of the generation of engineered antibodies show a much larger fraction of the antibodies adhere to the ubiquitin-like molecules on cell surface than the positive control antibody of IOC2. This is expected because this generation was made to have higher specific binding to ubiquitin. All the antibodies except Human IgG 1-3 show more binding than the positive control (> 12.1 percent of cells).

In Figure 2, we observed a reduction, a difference as large as 150 mm^3 in some cases, in the mean volume and weight of the primary tumors established using cells incubated with the engineered generation of antibodies as compared to the control. In fact, all groups of antibody treated mice show lower mean tumor burden after 24 days compared to control animals. We also observed that, in general, antibodies that have higher specific binding to ubiquitin or ubiquitin like molecules as revealed by the flow cytometric analysis are the most effective at reducing mean tumor burden in the mice.

Table 1: Percentage of various cancer cells that are mAb-positive for ubiquitin show that 4T1 cancer cells show the highest binding to the engineered antibodies, and that the Human IgG 1-2 experimental antibody against the 4T1 demonstrates highest affinity.

		AG49	4T1	H460	MB231	CT
Human IgG 1-1	Experimental	1.7	14.9	3.7	1.0	1.2
Human IgG 1-2	Experimental	1.8	20.7	4.5	2.5	1.9
Human IgG 1-3	Experimental	1.7	11.8	3.9	1.5	0.8
Human IgG 1-6	Experimental	1.2	18.0	7.2	2.8	1.0
Human IgG 1-27	Experimental	1.9	12.0	2.8	1.0	0.5
IOC2	Positive control	1.9	12.1	7.0	2.1	1.9
Rat IgG2a	Negative Control	0	0	0	0	0

Figure 1: Flow cytometric results for the adhesion of anti-ubiquitin specific antibodies to the cancer cell line 4T1 show a clear separation between negative and positively stained cells in all but Human IgG 1-1 antibody. Additionally, the highest fraction of positive cells occurs for the Human IgG 1-2 antibody. Negative controls show no fraction of positive cells.



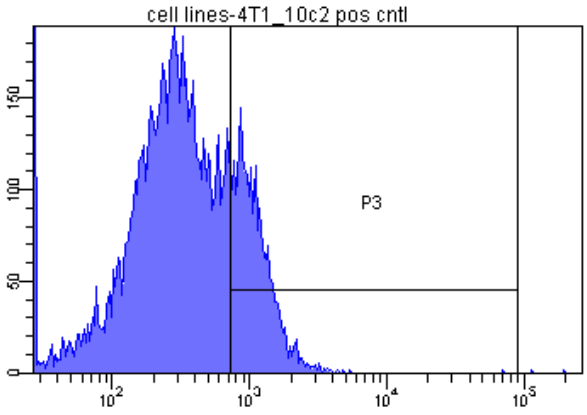

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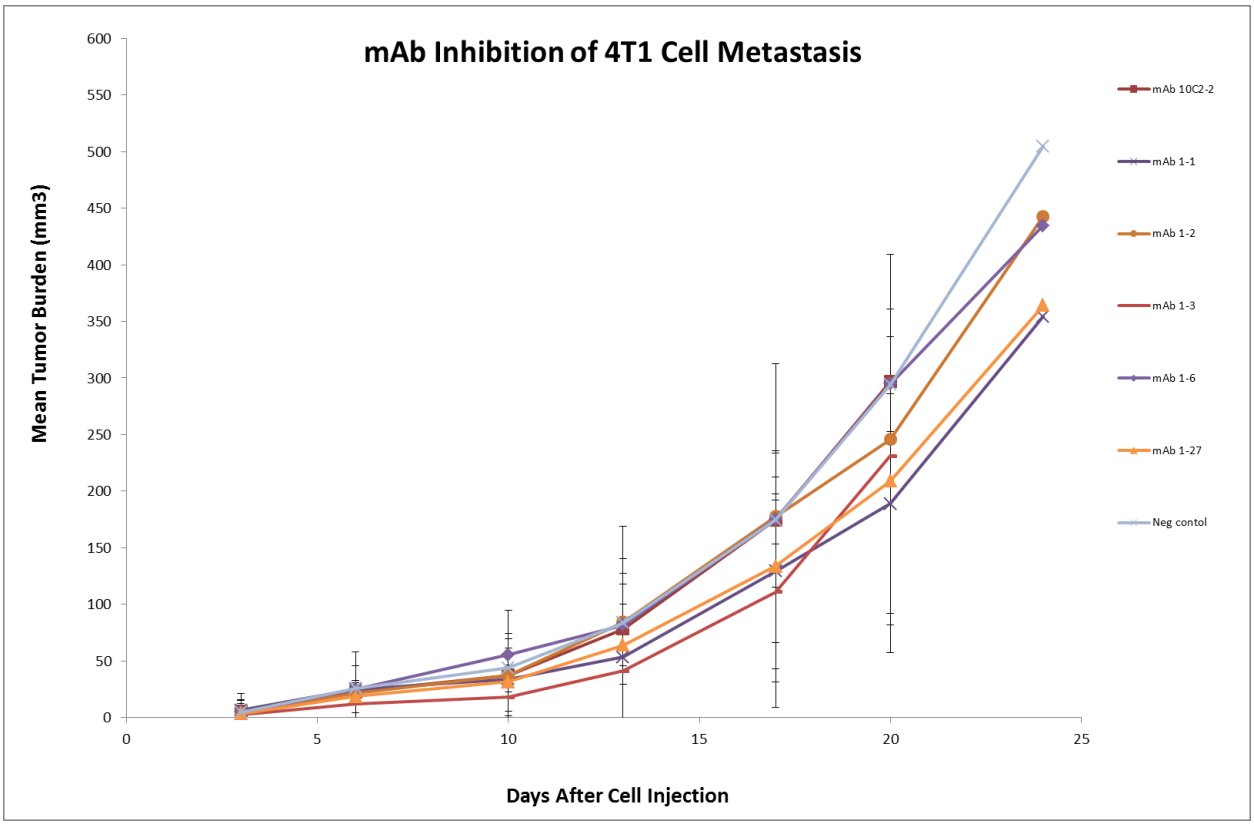
Figure 2: There is a reduction in the mean volume and weight of all the primary tumors established using cells incubated with the engineered generation of antibodies as compared to the control. The largest difference in mean tumor burden as compared to the negative control is 150 mm^3.



Discussion

Justification of Model:

In this experiment, the model used to study the metastatic spread of cancer in vivo was the mammary fat pad injection of the 4T1 cancer cells. This method provided an excellent in vivo model of metastasis because breast cancer cells were injected into mammary tissues and allowed to establish a palpable primary tumor. These cancer cells must be able to form tumors, invade, enter the circulation, and successfully metastasized, and therefore this model provides a more physiologically involved assay [9, 10]. A model that relied on the hematogenous spread of cancer was not used. In this model, as cells are injected directly into the tail vein of mice, their ability to form colonies in the lung are evaluated. This model does not measure the stage of cancer in which cells must detach from a primary tumor and crawl into the circulation, and therefor is an incomplete measure of metastatic capability [9].

Antibody Safety:

The safety and effectiveness of antibodies used as therapy vary, but typically, the nature of the targeted antigen is a good indicator of the safety of the antibody. An ideal antigen for antibody targeting should be abundant and easily accessible, meaning that it should be expressed in large amounts and consistently for each cancer cell. Furthermore, the safest antibodies for oncological therapy are specific to antigens exclusively on cancer cells, thus preventing the antibodies from binding to normal cells and inducing death in healthy cells [7]. Ubiquitin as an antigen is not readily found on cancer cells, however, molecules that cross react with ubiquitin, such as Peripheral Lymph Node Homing Receptor, are readily found on the cell surface, as shown by the flow cytometric analysis. It is important to note however, that because only a

fraction of the cells presented as positive for ubiquitin-like molecules, there may be an issue of accessibility and or the abundance of ubiquitin on the surface of cells.

Additionally, because only a small number of cells that form a tumor can metastasize, namely cancer stem cells, it is possible that the antibody is specific for an antigen only found on these cancer stem cells. This would explain why only a fraction of the cells were positive for the antibody, as well as why this antibody prevents the spread of metastasis. Also, Epithelial to Mesenchymal transition (EMT) in cancer, a process permitting cancer cells to become mobile and metastatic, has been shown to be regulated by many ubiquitin dependent processes [14]. If the antibodies were taken up by the cancer stem cells, they may have inhibited signal transduction processes involved in EMT.

Most of the antigens that are targeted for antibody based therapies are specific to cancerous cells, however, ubiquitin is found in all cells. These antibodies are not expected to harm or interfere with the function of normal cells because most of ubiquitin's functions are preformed inter-cellularly. Further bioassays need to be performed in order to see if the cells took up any of the ubiquitin specific antibody, leading to the interference of intracellular ubiquitin. Additionally, to address the abundance and availability problem addressed in the previous paragraph, assays to increase the expression of ubiquitin on the surface of cells could be used.

Novelty of Antibody:

Many antibody clinical therapies facilitate the direct killing of tumor cells by eliciting receptor agonist activity that leads to programed cell death [11]. These antibodies generally perform this function by blocking downstream signaling. An antibody can also bind to an enzyme, effectively neutralizing it, and reducing proliferation by means of hindering the function

of that enzyme. Antibodies conjugated with a secondary molecule can deliver a drug, toxin, or radioisotope to a specific antigen found on a tumor cell. Immune mediated tumor killing also fall under the mechanisms in which antibodies facilitate cell death. Antibodies can induce phagocytosis, activate complement, and engage in antibody-dependent cellular cytotoxicity (ADCC), in which antibodies activate NK cells [11]. All these antibodies seek to kill tumor cells, and may reduce the likelihood of metastasis as a result of cell killing, however, treatment methods that focus only on the primary cancer usually fall short in aiding the patient after the cancer has metastasized. No antibodies have yet been engineered specifically to reduce metastasis. Prevention of metastasis in clinical settings today revolve around hormonal therapies, such as Myoset (a drug used to treat metastatic breast cancer), and metastasis are usually treated in much the same way as primary cancers both through systemic and local therapies [12].

The antibodies used in this experiment provide a way for preventing metastasis alone because they work to inhibit molecules on the surface of cells that specifically allow cancer cells to move throughout the body and establish in different areas. By creating an antibody specific to a protein that promotes cell adhesion, they can not only prevent a cell from forming malignancies in various regions of the body, but can also keep cancer cells localized in a specific region, thereby increasing the time that white blood cells and other immune related defenses can work to eliminate them. In this way, these antibodies are truly novel, and could provide patients with already established cancers a treatment to prevent the onset of metastasis or further malignancy.

Crossreactivity with Mel 14:

The antibodies engineered by CCW crossreact with the Mel 14 antigen, acting much like the anti-Mel 14 antibody. Mel 14 is known to bind to the lectin domain of the peripheral lymph node homing receptor. This receptor is a surface glycoprotein that allows cells to home into

peripheral lymph nodes by virtue of an adhesive interaction with the postcapillary venule endothelium. It has been demonstrated that anti-Mel 14 blocks the binding of murine lymphocytes to peripheral lymph node endothelium both in vitro and invivo, and does play a function in regulating cell adhesion [5]. If these anti-ubiquitin antibodies cross-react with Mel 14, then they may also be adhering to peripheral lymph node homing receptors, and thereby preventing metastatic cells from adhering to new tissues. In fact, it has proposed and there is evidence that that ubiquitin is actually a part of the lymph node homing receptor [13].

However, it is has been shown that the antigenic determinant of Mel 14 is located in the carboxyl terminal 13-amino-acid proteolytic peptide of ubiquitin, but is undetected in intact undenatured ubiquitin and other cellular ubiquitinated proteins [13]. Thus, it is possible that this antibody is specific to an unusual or rare conformation of ubiquitin, or that in certain cancerous cells there is an altered form of the homing receptor in which the antigenic determinant that binds to Mel 14 is exposed.

In conclusion, these findings hold promise as a novel therapeutic strategy for treating metastasis because monoclonal antibody against ubiquitin has been shown to reduce cancer growth in mice with cancer caused by 4T1 cells, as well as reduce the number of metastasis. Because ubiquitin in not customarily found on the surface of cells, either normal or cancerous, it is hypothesized that the anti-ubiquitin antibodies binds to cell surface proteins that contain ubiquitin or ubiquitin like sequences such as the peripheral lymph node homing receptor. Anti-ubiquitin antibodies binding to this receptor provide a particularly promising explanation because of the receptors role in the migration of white blood cells, which move using a process very similar to the method that cancer cells undergo in order to metastasize.

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References:

1. Nelson, R. "Survival Rates Increasing in Metastatic Colorectal Cancer." Journal of Clinical Oncology 27.20 (2009): n. pag. Web.

2. J. Fidler, “The “seed and soil” hypothesis revisited”. Nat. Rev. Cancer 3, 453 (2003)

3. Valastyan, Scott, and Robert A. Weinberg. "Tumor Metastasis: Molecular Insights and Evolving Paradigms." Cell 147.2 (2011): 275-92. Print

4. A. Schaffer, M. Nethe, P. Hordijk, Ubiquitin links to cytoskeletal dynamics, cell adhesion and migration. Biochem. J. 442, 13–25 (2012).

5. Bowen, B. R. "The Mel 14 Antibody Binds to the Lectin Domain of the Murine Peripheral Lymph Node Homing Receptor." The Journal of Cell Biology 110.1 (1990): 147-53. Print.

6. L. K. Ferrarelli, Promoting Metastasis with Ubiquitin. Sci. Signal. 6, ec225 (2013).

7. Scott, Andrew M., Jedd D. Wolchok, and Lloyd J. Old. "Antibody Therapy of Cancer." Nature Reviews Cancer 12.4 (2012): 278-87. Print.

8. Desoize, Bernard. "“Antibodies in Cancer Treatment”." Critical Reviews in Oncology/ Hematology 62.1 (2007): 23-25. Print.

9. Mcclatchey, Andrea I. "Modeling Metastasis in the Mouse." Oncogene 18.38 (1999): 5334-339. Print.

10. Marsden, Carolyn G., Mary Wright, Latonya Carrier, Krzysztof Moroz, Radhika Pochampally, and Brian G. Rowan. "A Novel in Vivo Model for the Study of Human Breast Cancer Metastasis Using Primary Breast Tumor-initiating Cells from Patient Biopsies" BMC Cancer 12.1 (2012): 10. Print.

11. Weiner, George J. "Monoclonal Antibody Mechanisms of Action in Cancer." Immunologic Research 39.1-3 (2007): 271-78. Print.

12. Pinto, Álvaro. "Beyond Abiraterone: New Hormonal Therapies for Metastatic Castration-resistant Prostate Cancer." Cancer Biology & Therapy 15.2 (2014): n. pag. Print.

13. John, T. St, W. Gallatin, M. Siegelman, H. Smith, V. Fried, and I. Weissman. "Expression Cloning of a Lymphocyte Homing Receptor CDNA: Ubiquitin Is the Reactive Species." Science 231.4740 (1986): 845-50. Print.

14. Voutsadakis, Ioannis A. "The Ubiquitin–proteasome System and Signal Transduction Pathways Regulating Epithelial Mesenchymal Transition of Cancer." Journal of Biomedical Science 19.1 (2012): 67. Print.