GENETIC ANALYSIS OF SPECIALIZED TUMOR ASSOCIATED MACROPHAGES AND TUMOR ASSOCIATED FIBROBLAST

THESIS

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Tumor associated macrophages (TAMs) perform various task that are essential for tumor growth, angiogenesis, metastasis, and tumor invasion. Growth factors, chemokines, cytokines and the extra cellular matrix in the tumor microenvironments signal the TAMs to perform these tasks. The tumor microenvironment is not homogeneous and can be classified into several distinct regions such as sites of tumor invasion, perivascular, hypoxic and stromal regions. Each of these sites likely has different cytokines and signaling molecules in their microenvironment that might signal and direct the function of TAMs. The purpose of this project is to isolate and analyze TAMs from distinct tumor regions to gain a better understanding of the mechanism behind the specialized role of these TAMs. As a first step towards this goal, micro array analysis was conducted on flow sorted TAMs and developmental macrophages expressing yellow fluorescence protein (YFP). Micro array data from age matched developmental macrophages was compared to TAMS from early, premalignant stage of breast cancer, and late stage metastatic breast cancer to find the genes which were upregulated in TAMs versus developmental macrophages, and in late stage TAMs versus TAMs from the earlier premalignant stage of breast cancer. From this list of genes upregulated in TAMs, candidate cell surface macrophage markers that could be used to classify TAMs based on there association with one of the distinct tumor
microenvironments was selected. These candidate markers will be used to visualize macrophages associated with different regions within the tumor microenvironment using immuno-staining of tumor sections. In preliminary work, CD16 was selected as one potential candidate gene that was upregulated late in tumor development compared to earlier stages. Immunostaining indicated CD16 was expressed on a set of macrophages near blood vessels near the tumors, but not in macrophages located within tumors. Further evaluation of these markers would facilitate isolation of different subsets of macrophages within tumor and understanding the function of these macrophages in tumor microenvironment.

Another major cell group involved in all the changes in the tumor microenvironment discussed is the fibroblast. Originally the fibroblast inhibits the growth of the tumor. However through natural selection tumors evolve to change the microenvironment and like wise the function the fibroblast are performing to aid in tumor progression rather than suppress tumor progression. These fibroblast reprogrammed by the tumor cells are called tumor associated fibroblast TAFs. TAFs aid in tumor progression by angiogenesis, immune suppression, and matrix remodeling, providing growth and anti apoptotic factors and aid in metastasis by recruiting TAMs. A key gene involved with this is IKK. It is our hypothesis that ablating IKK in the fibroblast will lead to a reduction of tumor growth.
Dedicated to my friend Jing Liu and my family
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I would like to thank Dr Ostrowski for giving me the opportunity to work in a lab dedicated to the discovery and the better understanding of molecular biology.
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CHAPTER 1
INTRODUCTION

Breast cancer is the most commonly identified and one of the deadliest neoplasm in women in Western countries. Although cancer development proceeds through a multistep process in which epithelial cells undergo transformation as a result of multiple hits, it is becoming increasingly evident that discrete genetic alterations in epithelial cells alone cannot explain multistep carcinogenesis whereby tumor cells are able to express diverse phenotypes during the complex phases of tumor development and progression. The tumor microenvironment constitute the tumor cells and supportive platform that is composed of fibroblasts, endothelial cells, smooth muscle cells, adipocytes, hematopoietic cells like macrophages etcetera. Further, several distinct regions such as sites of tumor invasion, perivascular, hypoxic and stromal regions could be histologically determined in tumors.

In order to study the macrophage compartment in mammary tumors a myeloid specific yellow fluorescent protein (YFP) expressing transgenic mouse model was developed. Microarray experiments were conducted on macrophages either associated with the tumor (late or early) or developmental macrophages. We analyzed these data in order to develop specific markers for tumor associated macrophages
localized to distinct tumor microenvironment regions. We hypothesize that TAMs localized to different regions of tumor would be functionally different and analysis of these TAMs would throw light on tumor etiology and new therapeutic targets.

Another major cell group involved in all the changes in the tumor microenvironment discussed is the fibroblast. Originally the fibroblast inhibits the growth of the tumor. However through natural selection tumors evolve to change the microenvironment and like wise the function the fibroblast are performing to aid in tumor progression rather than suppress tumor progression. These fibroblast reprogrammed by the tumor cells are called tumor associated fibroblast TAFs. TAFs aid in tumor progression by angiogenesis, immune suppression, and matrix remodeling, providing growth and anti apoptotic factors and aid in metastasis by recruiting TAMs. A key gene involved with this is IKK. It is our hypothesis that ablating IKK in the fibroblast will lead to a reduction of tumor growth

1.1 The Tumor Microenvironment

To fully understand tumor progression it is important to understand not only the tumor cells and the changes they undergo to progress but also the tumor microenvironment and the changes they make in order to aid or allow the tumor cells to progress.

Originally the microenvironment is harsh towards the tumor. Immune cells signaled by inflammation, begin to attack the tumor cells. The microenvironment doesn’t have sufficient blood vessel to provide the tumor cells with the nutrients and oxygen the tumor cells need. However because the tumor cells are rapidly dividing and mutating in a harsh environment that is killing the weaker cells, a sort of biochemical intelligence is
developed by natural selection to favor tumor cells that secret factors which alter the microenvironment to aid in tumor progression rather than suppress tumor growth.

The tumor microenvironment can aid tumor progression by angiogenesis the process in which blood vessels is formed from a preexisting vascular network (1). These newly formed blood vessels can provide the tumor with the nutrients and oxygen in which the tumor needs to grow.

The tumor microenvironment can aid tumor progression by providing growth and anti apoptotic factors to the tumor. Much research has shown that fibroblast and macrophages, in the tumor microenvironment, secrete growth and anti apoptotic factor (2,3). These factors provide the signals needed for the tumors to grow and survive.

The tumor microenvironment can also aid in tumor progression by matrix remodeling. Cells in the microenvironment remodel the basement membrane to allow the tumor to escape the confines of the basement membrane.

The tumor microenvironment can allow for progression by immune-suppression. The tumor cells and microenvironment cells secrete factors that directly or indirectly suppress the immune cells.

The tumor microenvironment is not homogeneous and can be classified into several distinct regions such as sites of tumor invasion, perivascular, hypoxic and stromal regions (4). Each of these distinct regions have different cytokines and chemokines that signal the microenvironment cells to perform distinct task which aid in tumor progression. In the border of the tumor the basement membrane is being remodeled to allow for tumors to grow. In perivascular regions cells of the microenvironment secrete
chemokines that allow the tumors to chemotaxis into the blood vessels and then to metastasize. In hypoxic regions the microenvironment cells aid in tumor progression by initiating angiogenesis. In stromal regions microenvironment cells are secreting chemokines and growth factors that aid in tumor growth and metastasis.

1.2 Tumor associated Macrophages

A major cell group involved in all the changes in the tumor microenvironment discussed is the macrophages (5). Originally the macrophages attack the tumor. However through natural selection discussed earlier tumors evolve to change the microenvironment and likewise the function the macrophages are performing to aid in tumor progression rather than suppress tumor progression. These macrophages reprogrammed by the tumor cells are called tumor associated macrophages TAMs. TAMs aid in tumor progression by angiogenesis, immune suppression, and matrix remodeling, providing growth and anti-apoptotic factors and aid in metastasis through a process call intravasation. (6).

TAMs aide the angiogenesis process by secreting factor such as VEGF and MMPs. These factors signal endothelial cells to divide and migrate to form blood vessels. TAMs are involved in immune suppression by secreting factor that suppresses immune cells from attacking the tumor. TAMs aid in tumor invasion by matrix remodeling. They do this by secreting factors that degrade the basement membrane. TAMs provide the growth factors and anti-apoptotic factors the tumors need. TAMs aide in metastasis by secreting chemokines which allow the tumor cells to chemotaxis into the blood vessels.

In each of the distinct regions of the tumor microenvironment discussed earlier TAMs are reprogrammed by a unique set of cytokines associated with each of the distinct
regions to perform distinct task. In perivascular regions TAMs are aiding in metastasis. In tumor border regions TAMs are aiding in tumor invasion, In Stromal regions TAMs aide in tumor growth, In hypoxic regions TAMs aide in angiogenesis.

1.3 Tumor associated Fibroblast

Another major cell group involved in all the changes in the tumor microenvironment discussed is the fibroblast. Originally the fibroblast inhibits the growth of the tumor. However through natural selection discussed earlier tumors evolve to change the microenvironment and like wise the function the fibroblast are performing to aid in tumor progression rather than suppress tumor progression. These fibroblast reprogrammed by the tumor cells are called tumor associated fibroblast TAFs. TAFs aid in tumor progression by angiogenesis, immune suppression, and matrix remodeling, providing growth and anti apoptotic factors and aid in metastasis by recruiting TAMs.

TAFs aide the angiogenesis process by secreting factor such as VEGF and MMPs. These factors signal endothelial cells to divide and migrate to form blood vessels. TAFs are involved in immune suppression by secreting factor that suppresses immune cells from attacking the tumor. TAFs aid in tumor invasion by matrix remodeling. They do this by secreting factors that degrade the basement membrane. TAFs provide the growth factors and anti-apoptotic factors the tumors need in order to grow. TAFs can aide in metastasis by recruiting TAMs.

1.4 IKKB

It has been know for a long time that there is a link between inflammation and cancer. As early as the late 19\textsuperscript{th} century physicians noticed that cancer was accompanied by heavy
inflammation. At first this was considered a paradox because it was thought that the immune cells would be attacking the tumor cells. Later it became apparent that the tumor microenvironment was reprogramming the immune cells such as macrophages to aid in tumor progression rather than suppress tumor growth as discussed earlier. Tumors were considered wounds that wouldn’t heal since the body was now trying to fix the tumor rather than kill it. A major cell class involved in wound repair is the fibroblast. The fibroblast being manager cells manage the cell involvements of macrophages and endothelial cells. Originally they secrete factor that control the proliferation of epithelial cells however after being reprogrammed by the tumor they start to secrete growth and anti-apoptotic factors. As discussed earlier they also can feed the tumor by creating blood vessels for the tumor in a process called angiogenesis.

One of the key genes that link inflammation to cancer is the transcription factor NFKB. This gene was discovered as one of the transcription factors which was involved with the transcription of chemokines and cytokines up regulated in the biological process of inflammation. NFKB binds to the enhancer elements of inflammatory cytokines and chemokines and causes there transcription when inflammatory signals activate the cells. NFKB is also involved with the up regulation of MMP9 and VEGF. One of the major pathways that activate NFKB is the classical pathway. In this pathway extracellular signals from the inflammation process such as TNF bind to there respective receptor. This activates the receptor which in turn activates a slew of inter mediators. One of the major inter mediators is IKKB. Once IKKB is activated it activates NFKB which then relocates to the nucleus where it is involved with the transcription of thousand of genes
many of which are involved with the inflammation process. Since there are many NFkB scientist find it more practical to remove IKKB rather than all the NFkB in order to study its function. However one of the major drawbacks of this analysis of NFkB is that there exist other major pathways involved with the activation of NFkB. We will return to this issue in the future directions of this thesis.

Greten et al showed that when IKK was removed in the myeloid cells tumor growth was ablated because of lack of anti apoptotic and growth factors which would normally be up regulated by NFkB (10). Since Fibroblast provide anti apoptotic signals and growth factors to the tumor it is possible that by ablating IKK in the fibroblast one could slow tumor growth by providing less anti apoptotic and growth factor to the tumor. Another mechanism in which IKK could effect tumor growth is by activating mTOR and likewise VEGF(11)

1.5 Summary

Tumor associated macrophages (TAMs) perform various task that are essential for tumor growth, angiogenesis, metastasis, and tumor invasion. Growth factors, chemokines, cytokines and the extra cellular matrix in the tumor microenvironments signal the TAMs to perform these tasks. The tumor microenvironment is not homogeneous and can be classified into several distinct regions such as sites of tumor invasion, perivascular, hypoxic and stromal regions. Each of these sites likely has different cytokines and signaling molecules in their microenvironment that might signal and direct the function of TAMs

Tumor associated Fibroblasts (TAFs) perform various tasks that are essential for tumor
growth, angiogenesis, metastasis, and tumor invasion. Growth factors, chemokines, cytokines and the extra cellular matrix in the tumor microenvironment signal the TAFs to perform these tasks. A key gene involved with this signaling is IKK. It is our hypothesis that ablating IKK in the fibroblast will lead to a reduction of tumor growth.
CHAPTER 2
MATERIALS AND METHODS

2.1 c-fms-YFP

In order to study the macrophage compartment in mammary tumors a transgenic mouse which expressed a yellow fluorescent protein (YFP) under the control of The c-fms promoter a myeloid/macrophage specific promoter was developed. The plasmid construct and the generation of these transgenetic mice were previously described in (7). To examine the expression of the YFP, liver and spleen tissue were fixed in 4% paraformaldehyde in PBS in ice followed by over night incubation in 18% sucrose. The next day the tissue was fixed in OCT and frozen with dry ice. The OCT sections were sliced 5 um thin. Then the slides were examined under a flouresect microscope to characterize the expression of the YFP. Figure 1 shows that the yellow fluorescent protein under the control of the c-fms promoter is expressed throughout the mononuclear phagocyte cells of a mouse.

2.2 PyMT

A PyMT model was used to recreate in the mouse the tumor progression which occurs in most case of breast case. See (8) for a review of the PyMT model. The PyMT gene was under the control of the MMVT promoter, a promoter that activates gene expression in the mammary epithelial cells. The plasmid constructs and the generation of the MMVT;
PyMT transgenic mice were previously described in (8). The characterization of the MMVT; PyMT model is previously described (8).

2.3 FACS

Flow activated cell sorter FACS was used to isolate YFP expressing cells in mammary tumors harvested from PyMT, c-fms-YFP female mice which were 75 and 110 days and developmental mammary gland from c-fims-YFP female mice age 75 and 110 (figure 2). These tumors were excised, digested with collagenase and the resultant single cell population was flow sorted. FACS was done with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and the data were analyzed on CellQuest software (Becton Dickinson). Forward side scatter analysis was used. Figure 2 shows that two distinct subpopulations were isolated.

2.4 Microarray

Micro array analysis was performed on isolated RNA from the cells gathered with the FACS. RNA was isolated with TRIzol reagent and dissolved in RNase free H$_2$O. 25 micorgrams of total RNA was treated with DNAse using Qiagen RNase-free DNase kit and RNeasy spin columns. DNAse treated RNA was dissolved in RNase free H$_2$O to a concentration of .2ug ul$^{-1}$.

cRNA was labeled by in vitro transcription using T7 RNA polymerase with Cy3 or Cy5. Labeled cRNA was fragmented to on average 50-100 nucleotide by heating to 60 C in the presence of an hybridization buffer containing 10mM ZnCl$_2$, 1M NACL, .5% sodium sarcosine, 50mM MES, ph 6.5, and formamide to a final concentration of 30%, final volume 3 ml. Mouse430A microarray representing 22691 genes in the Mouse.
After hybridization slides were washed and scanned with a confocal laser scanner (Agilent Technologies). Fluorescent intensities were corrected for background noise and normalized using RMA normalization.

2.5 CSF-1R correlation coefficient

The correlation between CSF-1R and all other genes on an affymetrix array across 260 breast cancer samples. The CSF-1R correlation data was generated by Professor David A. Hume Director, ARC Special Research Centre for Functional and Applied Genomics Node head, CRC for Chronic Inflammatory Diseases, Institute for Molecular Bioscience University of Queensland

2.6 Genes with ets-2 tumor effect

To find genes with an ets-2 tumor effect in the macrophages, a Cre under the control of a Lys promoter was used. The Lys-Cre specifically removes flox genes from cells of the macrophages lineage. The micro array data from RNA collected from YFP expressing cells isolated by FACS from ets-2^{db/fl};Lys-Cre,c-fms-YFP,PyMT mice and the micro array data from RNA collected from YFP expressing cells isolated by FACS from ets-2^{db/fl};Lys-Cre,c-fms-YFP mice was used to find genes with an ets-2 tumor effect. The genes which were differential unregulated in the tumor microenvironment when ets-2 was removed in the macrophages (i.e. ets-2^{db/fl};Lys-Cre,c-fms-YFP,PyMT versus ets-2^{db/fl},c-fms-YFP,PyMT) but were not differential regulated by ets2 in development (i.e. ets-2^{db/fl};Lys-Cre,c-fms-YFP versus ets-2^{db/fl};c-fms-YFP) were considered to have an ets2 tumor effect in the macrophages

2.7 Hypoxia
Micro array collected from the GEO datasets of macrophages cultured under hypoxia versus macrophages cultured under normoxia. GEO dataset GSE4360.

Two groups of macrophages were examined. One group was exposed to normoxia (21%) oxygen and the other groups were exposed to hypoxia (0%) oxygen for 24 hours. There were 4 donors in each group. 2 ug of RNA pooled from 4 donors belonging to the group exposed to normoxia and 2 ug of RNA pooled from 4 donors belonging to the group exposed to hypoxia were used as target preparations for DNA microarrays.

2.8 IHC on consecutive sections

Immunohistochemistry was performed with paraffin imbedded tumors. Tumor sections were fixed in Zn Formalin over night and then washed with distilled water for 30 minutes. Afterwards they were imbedded in paraffin. 5 um thick consecutive sections from paraffin imbedded 75 day old PyMT tumor were probed with F-480 and CD16 antibodies. Sections were stained with primary anti–rat F-480 (Caltag) 1:50 o/n 4C followed by secondary donkey anti- rat (BDPharmagin) 1 hour room temperature. Consecutive sections were stained with primary anti-rat CD16 (Caltag) 1:100 o/n 4C followed by secondary donkey anti-rat (BDPharmagin) 1 hour room temperature. Antigen retrieval was done with Daco reagent in a steamer followed by blocking with MOM blocking reagent and stained with DAB reagent

2.9 Double staining Immuno florescents.

The double staining was performed on paraffin imbedded tumors. Tumor sections were fixed in Zn Formalin over night and then washed with distilled water for 30 minutes. Afterwards they were imbedded in paraffin. 5 um thick consecutive sections from
paraffin imbedded 65 day old PyMT tumor were double stained with F-480 and CD16 antibodies. Antigen retrieval was done with Daco reagent in a steamer followed by blocking with MOM blocking reagent 1 hour room temperature. Sections were stained with primary anti–rat F-480 (Caltag) 1:50 o/n 4C followed by secondary donkey anti- rat (BDPharmagin) 1:500 1 hour room temperature, followed by streptavidin-FITC 1:500 15 min room temperature. Sections were blocked again with MOM blocking reagent 1 hour room temperature. Sections were then stained with primary anti-rat CD16 (Caltag) 1:100 o/n 4C followed by secondary donkey anti-rat (BDPharmagin) 1:500 1 hour room temperature. followed by streptavidin-Texas RED 1:300 15 min room temperature. Then Sections were stained with DAPI 1 min room temperature.

2.10 IHC of tumor from injection study

Immunohistochemistry was performed with paraffin imbedded tumors. Tumor sections were fixed in Zn Formalin over night and then washed with distilled water for 30 minutes. Afterwards they were imbedded in paraffin. 5 um thick consecutive sections from tumor harvested from the injection study were stained with primary anti–rat F-480 (Caltag) 1:50 o/n 4C followed by secondary donkey anti- rat (BDPharmagin) 1:500 1 hour room temperatures. Sections were also stained with primary anti-rat Caspase-3 (Caltag) 1:100 o/n 4C primary followed by secondary donkey anti-rat (BDPharmagin) 1:500 1 hour room temperature. Antigen retrieval was done with Daco reagent in a steamer followed by blocking with MOM blocking reagent and stained with DAB reagent

2.11 Western

Mammary Glands 5 and 9 were dissected from 6 weeks old female FVBN mice with
geneo-types FspCre; IKKf/- and IKKf/f. The tissue was minced and digested in collagenase o/n 32 C. Following day digested tissue was suspended in DMEM/F12 media pipetted up and down until the digested tissue was homogenously distributed through out the media. The resulting mixture was centrifuged 1000 rpm 3 min. the supernatant was discarded. The pellet was resuspened in 12 mL of DMEM/F12 media. Fibroblasts were separated from the mixture by means of the gravity separation method. The 12 mL mixture sat for 12 min to let the heavy cells sink to the bottom. The fibroblast due to there shape would sit on the top. Then 8 mL from the top of the mixture were placed in a new tube and sat for 12 min. Then 6 mL from the top of the mixture were placed in a different tube and sat for another 12 min. Then 4 mL from the top of the mixture were placed in a different tube and sat for 12 min. Then 3 mL from the top of the mixture were plated onto a cell plate. The cells then incubated 24 hours in 32C.

The fibroblasts were collected by means of a cell scraper and suspended in 200 ul of Lysate buffer. Cells sat on ice for 20 min. Spin at 14000 rpm 10 min at 4C transfered supernatant to new tube discarded pellet. Protein concentration was determined by Bradford assay from Bio-Rad. 50 ug of protein with 2x sample buffer was loaded into a 8% polyacylyermide gel ran for 2 hours on 100V. Then the sample was transferred unto a nitrocellulose membrane for immunoblotting. The membrane was blocked with 10 % Milk in TBS-t. The membrane was blotted with primary rabbit anti-IkkB (Abcam) 1:500 o/n 4C the followed by donkey secondary anti-rabbit conjugated horse radish peroxidase (Abcam) 1:1000 2 hours room temperature. Then incubate with 2mL substrate (Biorad). Membrane was exposed to x ray film and developed (figure 7).
CHAPTER 3

FINDING CANDIDATE MARKERS FOR SPECIALIZED TUMOR ASSOCIATED MACROPHAGES

3.1 Isolating TAMs

Transgenic mice with a PyMT gene under the control of the MMVT promoter were used to create mice with mammary gland tumors which resemble the breast cancer tumor of humans. These mice were crossed with transgenic mice with an YFP gene under the control of the c-fms promoter. These mice have Yellow florescent protein expression in the mononuclear phagocyte lineage. Mammary tumors were isolated from 75 and 100 day female FVBN cfms-YFP; PyMT mice. Normal mammary glands were isolated from 75 and 100 day female FVBN cfms-YFP mice. Next FACS was used to isolate YFP expressing cells from the 75 and 100 day Normal mammary glands and the 75 and 100 day PyMT mammary gland tumors. RNA was isolated from the cells. Micro array experiments were down with the isolated RNA. This analysis gave us a set of genes which were up regulated in the tumor associated macrophages versus developmental macrophages.

3.2 Finding candidate markers

To find candidate markers to label macrophages localized to specific regions of the
microenvironment discussed in the introduction, a bioinformatics methodology discussed in this section was developed. Since the purpose of finding the candidate markers was to use FACS to isolate TAMs localized to distinct regions of the microenvironment, only genes localized to the membrane were considered as possible candidate markers. We first began with the genes up regulated in the tumor associated macrophages versus the developmental macrophages discussed in section 3.1. We then filtered out the genes which were not localized to the membrane. To find genes which were more specific to macrophages we only considered genes with a high CSF-1R correlation coefficient.

Next to categorize the genes we considered three different categories. Category one were genes which had an ets2 tumor effect these genes could be expressed near blood vessels since ets2 has shown to be involved with metastasis. Category two was genes up regulated under hypoxia. These Genes would be most likely expressed in hypoxia regions of the tumor. Category three were genes with biological process which represent the biological process going on in the microenvironment. For example genes which are involved in angiogenesis would be most likely expressed near tumor vessels or in hypoxia regions. Figure 3 displays a diagram representing the above methodology. Table one show us interesting genes. Three genes were chosen for further analysis. These genes were CD16, CCR2, Cx3cr1.

CD16 was chosen for further analysis because it was up regulated in hypoxia. Being up regulated in hypoxia we would expect CD16 expressing macrophages to be localized to hypoxic regions. CD16 also known as the Fc gamma receptor 3a is involved in the biological process of antibody mediated phagocytosis. CD16 attaches to the Fc portion of
an antibody. Once attached a signaling cascade occurs initializing the CD16 expressing phagocyte to engulf the antibody coated body. Since macrophage phagocyte debris and dead cells which occur in abundance in hypoxia regions and CD16 is essential for antibody mediated phagocytosis, it makes sense that CD16 expression would be upregulated in hypoxic regions. CD16 has been shown to be unregulated in TAMs (9). CCR2 and Cx3CR1 are important chemokine receptors, these receptors were chosen because they had an ets2 tumor effect.

### 3.3 Testing Candidate Markers

IHC was performed to test whether CCR2, Cx3cr1, and CD16 label a subpopulation of Macrophages localized to a distinct region of the tumor microenvironment. F480 was used to label all the macrophage. Consecutive sections 5 um apart were used to compare F480 stained tumor sections with CCR2, Cx3cr1 and CD16 stained tumor sections. CCR2 and Cx3cr1 antibodies label the tumor cells as well as the macrophages therefore could not be used as a candidate marker.

Immunostaining indicated CD16 was expressed on a set of macrophages near blood vessels near the tumors, but not in macrophages located within 75 day tumors (figure 4). This was confirmed with double staining (figure 5).

### 3.4 Phenotype

500,000 F305 cells were injected into the mammary gland 5 and 9 of FVBN mice and FspCre; Ikkf/- mice to see if there was a difference in tumor growth between mice in which IKK was ablated in the fibroblast and wild type mice. After 3 weeks the mice were sacrificed and the tumors were harvested and measured to see if there was a difference in
tumor growth between the two groups (see table 2). The average volume of the tumors harvested from the experimental group was 65 with a standard deviation of 40.2 and the average volume of the tumors harvested from the control was 76 and standard deviation of 49.2. This was not significant. Next to see if there was a difference in the collagansse density around the tumor, the tumors were formalin fixed and embedded in paraffin. The 5 um sections of the paraffin blocks were trichromed stained. These slides were than scanned and avarege collanagansse density of the two group was accessed. The experimental group had .1565 collananges density were as the control group had a .136544 collanganes density. Again no significant difference was reported between the two groups.

Since IKK has been shown to be involved with recruitment of macrophages, our hypothesis was that the tumors from the FspCre; IKKf/- mice would have less TAMs then tumor harvested from the control mice. To test this hypothesis 5um sections for paraffin emembed tumors harvested from the control and experimental mice were stained with F480 a macrophage specific marker figure 6. As you can from figure 6 both tumor groups exhibit a macrophage infiltration concluding that IKK in the fibroblast did not effect macrophage recruitment to the tumor.

Since IKK has been shown to be involved with transcription of growth factors, our hypothesis was that TAFs in the FspCre; IKKf/- would be secreting less anti apoptotic factors than the TAFs in the control mice. To test this hypothesis 5um sections for paraffin emended tumors harvested from the control and experimental mice were stained
with caspase 3 anti apoptotic marker figure 7. As you can from figure 7 both tumor
groups exhibited the same degree of apoptosis, concluding that IKK in the fibroblast did
not effect anti apoptic factors.
4.1 Discussion on TAMs

The tumor micro environment is essential for tumor progression. Macrophages are an important player in the tumor micro environment. From this list of genes upregulated in TAMs, candidate cell surface macrophage markers that could be used to classify TAMs based on their association with one of the distinct tumor microenvironments was selected. These candidate markers will be used to visualize macrophages associated with different regions within the tumor microenvironment using immuno-staining of tumor sections. In preliminary work, CD16 was selected as one potential candidate gene that was upregulated late in tumor development compared to earlier stages. Immunostaining indicated CD16 was expressed on a set of macrophages near blood vessels near the tumors, but not in macrophages located within tumors. Further evaluation of these markers would facilitate isolation of different subsets of macrophages within tumor and understanding the function of these macrophages in tumor microenvironment.

4.2 Discussion on TAFs

As you can see from the experiments that was no phenotypic difference in regards to tumor growth when IKKbeta was removed in the fibroblast. One reason for this could be that there are other major pathways involved in NFKbeta activation for example the non
classically pathway in which IKKalpha dimmers are responsible for activated NFKbeta are a redundancy pathway. In which case, it would require both the ablation of IKKbeta and IKK alpha in order to see the effect of NFK beta on tumor progression. So a future direction would be to create a FspCre IKKalpah f/-, IKKbeta f/- mice and repeat the experiments discussed earlier.
1. Pia Nyberg et al. Tumor microenvironment and angiogenesis; Frontiers in bioscience 13, 6537-6553, May 1 2008

2. Claire E. Lewis and Jeff Pollard. Distinct Role of Macrophages in Different Tumor Microenvironment Cancer Res. 2006; 66 (2) pg 605-612

3. Pollard, Tumor educated macrophages promote tumor progression and metastasis; nature review; cancer, volume 4 January 2004 pg 71-77


5. Lin et al; Colony-stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy, J Exp Med Volume 193, 6, March 2001, 727-739


7. Sasmono et al. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse; Blood 2003 (101) (3) pg 1155-1163

8. Lin et al; Progression to Malignancy in the Polyoma Middle T Oncoprotein Mouse Breast Cancer Model Provides a Reliable Model for Human Diseases; American Journal of Pathology, 2003 Vol 163, No 5 pg 2113-2125


10. Florian et al; IKKB links Inflammation and Tumorigenesis in a Mouse Model of Colitis-Associated Cancer; Cell, 118, pg 285-296
11. Lee et al; IKKB Suppressio of TSC1 links Inflammation and Tumor Angiogenesis via the mTOR Pathway; Cell; 130;p 440-455

12. Kalluri et al; Fibroblast in Cancer; Nature; May 2006 vol 6; p392
APPENDIX FIGURES AND TABLES
### Category 1 genes with an ets2 tumor effect (expression in Log2 scale)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>control</th>
<th>late tumor</th>
<th>ets2KO</th>
<th>ets2K0 late tumor</th>
<th>ets2 tumor effect</th>
<th>csf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itga4</td>
<td>8.135917546</td>
<td>9.098818076</td>
<td>8.31678179</td>
<td>7.720251622</td>
<td>-1.55943</td>
<td>0.456233</td>
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<td>CD16</td>
<td>8.678015568</td>
<td>11.5924933</td>
<td>8.9848652</td>
<td>10.41150068</td>
<td>-1.48784</td>
<td>0.560032</td>
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<tr>
<td>Tnfrsf17</td>
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<td>6.027805286</td>
<td>9.561518723</td>
<td>5.040754608</td>
<td>-1.43613</td>
<td>0.358142</td>
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<tr>
<td>Cd47</td>
<td>7.234698245</td>
<td>7.318835704</td>
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<td>Cx3cr1</td>
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<td>8.06122591</td>
<td>5.21955672</td>
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### Category 2 genes upregulated in hypoxia (expression in Log2 scale)

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<tr>
<th>Gene Symbol</th>
<th>control</th>
<th>late tumor</th>
<th>Hypoxia/Normoxia</th>
<th>csf</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2</td>
<td>6.237198641</td>
<td>7.437392804</td>
<td>2.49134945</td>
<td>0.488636731</td>
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<tr>
<td>CD16</td>
<td>8.678015568</td>
<td>11.5924933</td>
<td>1.430160774</td>
<td>0.560031967</td>
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<tr>
<td>CD66</td>
<td>11.03611061</td>
<td>12.08679327</td>
<td>1.511263622</td>
<td>0.637667401</td>
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### Category 3 genes with interesting biological process (expression in Log2 scale)

<table>
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<tr>
<th>Gene Symbol</th>
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<th>late tumor</th>
<th>csf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vcam1</td>
<td>6.537699587</td>
<td>9.832518255</td>
<td>0.462346573</td>
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Table 1. List of candidate markers
<table>
<thead>
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<th>Group</th>
<th>Volume</th>
<th>Collage</th>
<th>Volume</th>
<th>Collage</th>
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</thead>
<tbody>
<tr>
<td>Exp.16mg9</td>
<td>32.9</td>
<td>61</td>
<td>40.2</td>
<td>65.7</td>
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<tr>
<td>Exp.13mg9</td>
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<td>0.1673</td>
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<td>Exp.8mg9</td>
<td>32.9</td>
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<td>Exp.12mg4</td>
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<td>0.1835</td>
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<td>Exp.7mg4</td>
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<tr>
<td>Control</td>
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<td>Control.21mg9</td>
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<td>Control.18mg9</td>
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<tr>
<td>Control.22mg9</td>
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<td></td>
<td>116.1</td>
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<tr>
<td>Control.12mg9</td>
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<td>Control.10mg4</td>
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<td>0.0824</td>
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Table 2. Tumor volumes for injection study
Figure 1: YFP expression in c-fms YFP transgenic mice. Data collected from Tahera Zabuawala
Figure 2: Mammary tumors from 65 day old mice were excised, digested with collagen and the resultant single cell population was flow sorted. Data collected from Tahera Zabuawala.
Figure 3: Diagram modeling the Bioinformatics approach to finding candidate genes.
Figure 4: Immunostaining indicated CD16 was expressed on a set of macrophages near blood vessels near the tumors, but not in macrophages located within tumors.
Figure 5: Immunostaining indicated CD16 was expressed on a set of macrophages near the tumor border, but not in macrophages located within tumors.
Figure 6: Testing the efficiency of the FspCre. Western blot with protein extracted from isolated fibroblast from mammary glands of FspCre; IKK f/- mice and IKK f/- mice and antibodies IKK and Tublin.
Figure 7: F-480 staining of tumors from the injection study.
Figure 8: Caspase 3 staining of tumors from the injection study