

The Role of E2F3 in the Macrophage Assisted Metastasis of Breast Cancer

A Senior Honors Thesis

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by

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Abstract

Many studies have shown that macrophages play a critical role in metastasis within the tumor microenvironment. The aim of this study was to shed more light on the particular role of macrophages, and how the cell cycle transcription factor E2F3 affects tumor development and tumor metastasis. MMTV-PyMT was used to induce tumors in the mammary glands of female mice. It has been shown that Lys cre is very effective at specifically deleting genes in the macrophages. Lys cre was used to knock out the floxed E2F3 allele in the macrophages of these PyMT mice. E2F3 PCR was used to confirm that the Lys cre was working at an effective level in terms of deletion efficiency. Three groups of mice were generated from the breeding scheme. There was the control group (E2F3^{+loxP} - cre), the conditional group (E2F3^{+loxP} + cre), and the homozygous group (E2F3^{loxP/loxP} + cre). Tumor onset in the PyMT mice population was on average 80.2 days. After 110 days, the mice were harvested and their tumors and lungs removed for analysis. The analysis of the tumors showed that the three groups of mice had very similar tumor burden and dimensions. However, the analysis of the lungs showed more than a three fold difference in total area of metastasis between the *+loxP* -cre and *+loxP* +cre groups.

Aim of the Study:

The aim of this study is to find out more about the role that E2F3 plays in the macrophage during metastasis.

Introduction:

Breast Cancer:

The ACS estimates that in the year 2007 40,460 women in the United States will die from breast cancer. Only Lung cancer will claim more victims this year. This makes breast cancer one of the leading causes of death in women. What makes breast cancer so deadly is the tendency of tumors in the mammary gland to metastasize and spread to other locations within the body. Tumors tend to undergo many morphological changes before they metastasize.

Stages of Tumor Development:

Tumor cells undergo six major changes at the cellular level that allows them to metastasize and spread within the body. According to Hanahan and Weinberg (2000) these traits are self-sufficiency from external growth signals, non-responsiveness to negative growth signals, escape from apoptosis, unrestricted replication, stable angiogenesis, and the ability to migrate and invade other tissue. Tumors also undergo changes at the pathological level. With the model used for this experiment, there are certain tumor stages that can be clearly identified as the lesion progresses. The starting place is called the primary tumor, and is the site where the tumor first arose. The hyperplastic stage is characterized by clusters of densely grouped cells that aggregate around the milk-collecting duct (Lin et al., 2003). This is followed by Adenoma and early/late carcinoma stages. As the tumor progresses through these stages, the cell morphology becomes noticeably altered, and the cells increase in malignant potential until they metastasize.

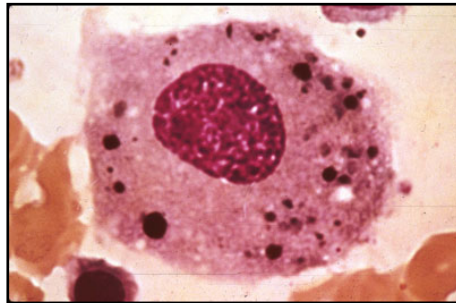
Tumor Microenvironment:

The tumor microenvironment is composed of many types of cells, not just cancerous ones. This complex and diverse organization of cells is comprised of

endothelial cells, fibroblasts, granulocytes, mast cells, T, B and NK cells, as well as dendritic cells, and macrophages (Albini & Sporn, 2007). All these cellular components within the microenvironment in some way affect the development and invasive properties of the tumor. As a result, recent studies have shown that the surrounding cells can modify primary oncogenic events in tumor cells (Pollard, 2004). Researchers have identified the products of genes such as epiregulin, and cyclooxygenase-2 that might be involved directly in metastasis in other cancer models (Christofori, 2007).

Macrophages:

Most of the research that has been done on breast cancer has focused primarily on the malignant epithelial cells. However, recently focus has shifted to other cell types and specially the macrophage. Macrophages originate from precursor cells called monocytes. Their primary role is in both innate and cell-mediated immunity.



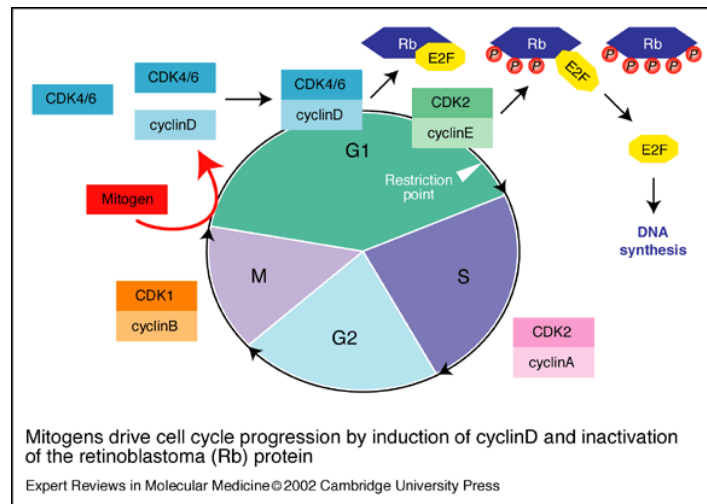
A macrophage

They phagocytize bacteria and other pathogens, and also serve as antigen presenting cells (APC). As an APC they are capable of signaling to other cells of the immune system such as lymphocytes, by secreting cytokines. However, within the tumor microenvironment the role of a macrophage expands and they are now called TAMs or tumor associated macrophages. Within the tumor, TAMs are able to secrete angiogenic factors, remodel and repair tissue, as well as release growth and migrations factors

(Condeelis & Pollard, 2006). As a result of this, macrophages are thought to play a critical role in tumor intravasation.

E2F Family:

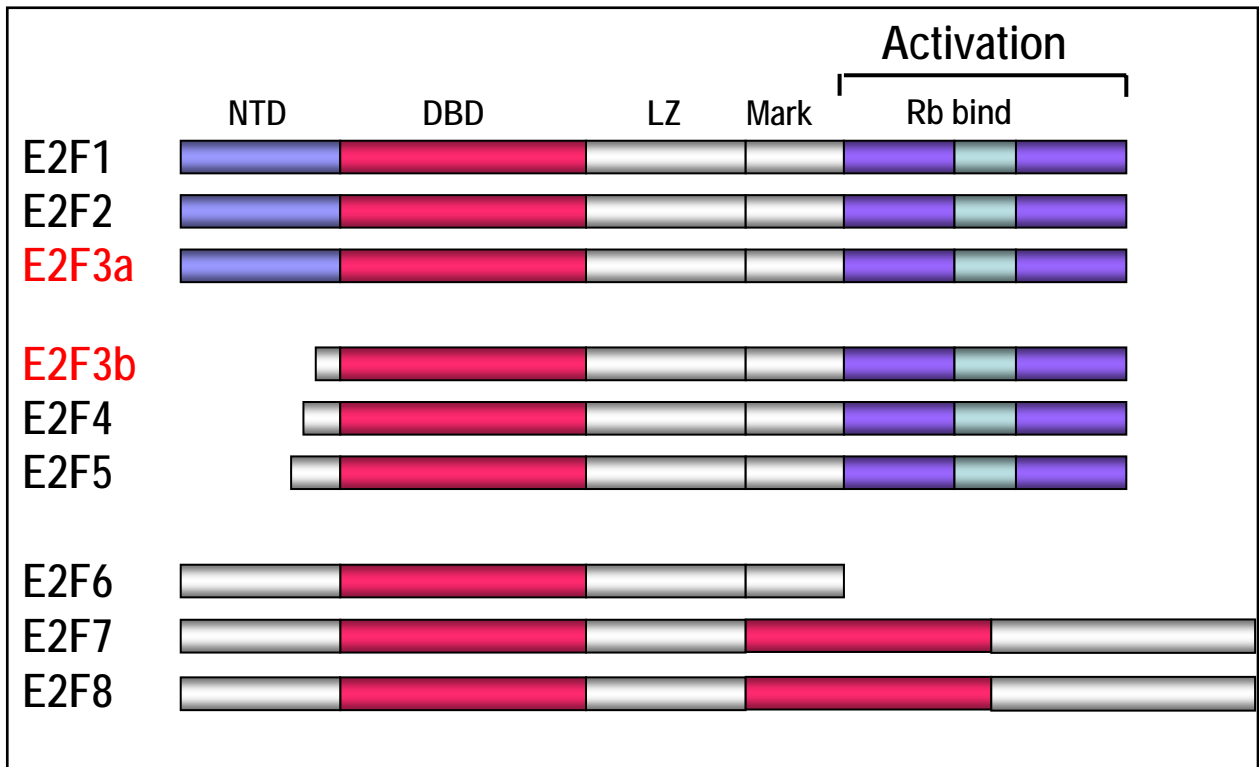
The genes that allow macrophages to infiltrate and aid in the intravasation of malignant epithelial cells are still unknown. However, research is currently being done on the E2F family of genes. The E2F gene family can be separated into three groups based on their different functions. E2F 1-3 are transcriptional activators of downstream genes such as *c-myc* involved in the progression of the cell towards S-phase. E2F 4 and 5 have a transcription repressing role (Trimarchi & Lees, 2004).



E2F6 also represses certain genes through a different transcriptional pathway (Trimarchi & Lees, 2004). E2F7 and 8 are recently discovered and their function is still under investigation. Evidence has shown that the E2F1–3 subgroup are transcription factors that are required for cells to enter the S-phase of the cell cycle (Wu et al., 2001). The tumor suppressor pRB inhibits the activity of the E2F genes. Only through cell-cycle dependent hyper-phosphorylation by CDK4/6 and cyclin D does it release the active E2F. This mechanism is being carefully studied as defects in pRB and over expression of the genes that the E2Fs activate are common mutations in many human cancers. This paper focuses primarily on the E2F3 gene, which shows two transcriptional variations in E2F3a and E2F3b. They are both similar however; E2F3b lacks the amino terminal domain

(Trimarchi & Lees, 2004). The E2F3 gene is believed to have a critical role in the proliferation and differentiation of the macrophage.

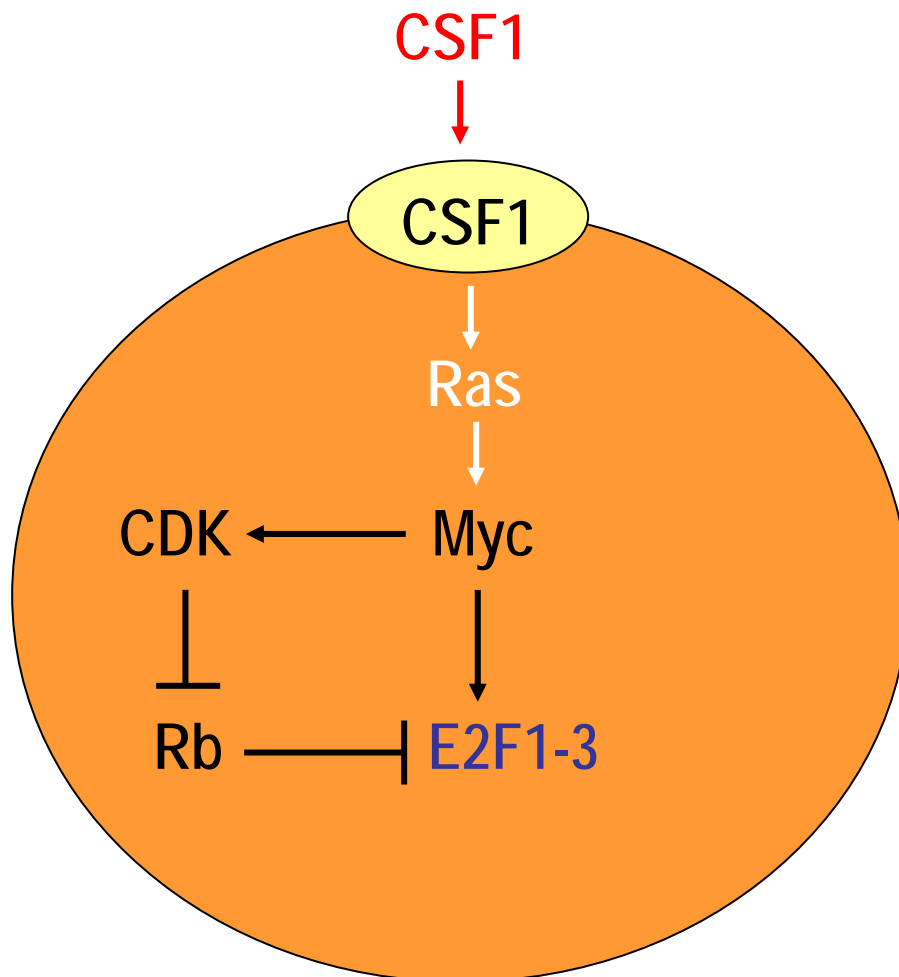
E2F Family of Transcription Factors



The domains of the E2F genes and their respective subgroups.

Recent evidence suggests that CSF-1 and E2F3 may share a common pathway. CSF-1 is a protein that binds to the receptor CSF-1R. This receptor is primarily expressed in the monocyte lineage, the precursors to macrophages. CSF-1 is necessary during the G1 phase of the cell cycle because it regulates several important early and late response genes. Cells expressing the CSF-1R receptor on stimulation with CSF-1 lead to induction in the expression of E2F1-3.

CSF-1 Signaling



The CSF-1 signaling pathway showing a connection to E2F1, 2, and 3

Objectives:

The mice were allowed to develop tumors for 110 days and then their tumors and lungs were harvested and analyzed. The aim of this study is to find out more about tumor development and the role that E2F3 plays in the macrophage during metastasis. Analysis for tumors in the lungs allowed for a good measure as to the amount of metastasis that had occurred in each individual mouse. It is known that macrophages play a crucial role in metastasis perhaps by secreting certain proteases which allow malignant epithelial cells to escape or by other means.

Materials and Methods

Breeding:

Mice were used in this study which carried the polyoma virus middle T oncoprotein (PyMT). Expression of PyMT is controlled by the mammary tumor virus LTR (MMTV LTR) promoter. This promoter only allows PyMT to be active in the epithelial cells of the mammary gland. This model is used to induce tumors, and studies have shown that PyMT is a strong oncogene that has been linked to increase c-myc levels among other pathways (Lin et al., 2003). Other studies also suggest that PyMT associates with the Src family of tyrosine kinase signaling proteins (Webster et al., 1998)

Traditional E2F3 knockout mice are embryonically lethal and only survive to 11.5 days. To get around this problem, the Cre/*loxP* system was used (Kuhn and Torres 2002). Cre recombinase (Cre) is an enzyme present in the bacteriophage P1. This Cre binds to *loxP* sites on the DNA and modifies them. When there are two *loxP* sites flanking a segment of DNA, cre will excise that segment of DNA into a circular structure effectively knocking out the gene. In this study macrophage specific Lys-cre was used to knock out E2F3 in the macrophages of PyMT mice (Clausen et al., 1999).

Three experimental mice groups were used in this study: PyMT/ E2F3^{+/*loxP*} (control), PyMT/Lys^{+/-}/E2F3^{+/*loxP*} (conditional), and PyMT/Lys^{+/-}E2F3^{*loxP*} (homozygous). In order to generate the three groups of mice the following breeding scheme was used.

PyMT^{+/-}Lys^{+/-}E2F3^{+/*loxP*} X E2F3^{*loxP*}

↓

PyMT⁺Lys⁺E2F3^{+/*loxP*}

PyMT⁺Lys⁻E2F3^{+/*loxP*}

PyMT⁺Lys⁺E2F3^{*loxP*}

Genotyping and Sorting:

Mice were tailed in the 17-21 day range. DNA was isolated from these tails by the addition of tail buffer and proteinase K overnight in the incubator. The tail buffer allowed ProK to digest any protein present within the tail allowing for easy extraction of the DNA. After the digestion, the samples were centrifuged at 14,000rpm for 10 minutes. The supernatant was transferred to a fresh tube containing 500ul of isopropanol. The samples were shaken vigorously to allow the DNA to precipitate. The remnants of the tail buffer were removed by washing with 80% ethanol and then letting the DNA dry. The final step was the addition of low TE EDTA to the samples which allowed the DNA to dissolve in solution. After 30 minutes of incubation, the DNA was ready for PCR analysis.

Three PCR reactions were done (E2F3, PyMT, and Lys cre) to determine the genotype of the mice. A PCR (Polymerase Chain Reaction) is an enzymatic reaction that allows for the amplification of a specific segment of DNA. The reagents used in these PCR reactions were: 10x Buffer, MgCl₂, dNTPs, water, a set of PCR specific primers (2 or 3 depending on the PCR protocol), and a polymerase (usually *Therophilus aquaticus*). 6x loading dye was added to the reaction and it was run on a 2% agarose gel prepared with Ethidium Bromide. Afterwards it is analyzed on the Alpha imaging machine using UV light.

FACS

To test for E2F3 deletion in macrophages tumor and lung tissue samples were sorted using a flow cytometry technique. First, tumors were removed from the mice and digested with collagenase to make a cell suspension. Then they were sorted using PE conjugated F4/80 antibodies. After sorting, DNA was made from the macrophages and an E2F3 PCR was done to test for deletion. The lung was lavaged with cold PBS solution to isolate macrophages.

Tumor analysis:

Starting at 50 days of age the mice were monitored twice a week for tumor progression. They were palpitated to feel for tumors. When the tumors were ~ 5mm in size it, the size and position of the tumor was recorded as the primary tumor and it became the focus of the measurement. The tumor was measured using an electronic caliper, and once the primary tumor was labeled, it was the only tumor measured. Mice were sacrificed when they were 110 days of age; they were sacrificed earlier if they fit ERC (Early Removal Criteria). The ERC was based on how difficult it was for the mouse to move, and if the tumors had reached a point in which the mouse had become lethargic.

Necropsy:

After 110 days (or earlier depending on ERC), each mouse in the study was humanely sacrificed and dissected. The mammary tumor was carefully removed using scissors and forceps. Each tumor was individually weighed and measured using a caliper. The tumors were then stored in 50 ml conical tubes filled with formalin. The primary tumor was stored in a separate conical tube along with the liver. Finally, the lungs were inflated by inserting a syringe into the trachea of the mouse with formalin. The lung was then removed and placed in the same conical tube that contained the primary tumor. The mouse carcass was then properly disposed.

Lung analysis and Histology

Each lung was separated into its 5 individual lobes, and sent into histology for staining. The staining protocol is as follows; Dehydration (70% ethanol for 30 minutes, 95% ethanol 30 minutes, 100% ethanol 30 minutes, xylene 30 minutes), Rinse with water 10 minutes, Hydrate (100% ethanol for 30 minutes, 95% ethanol 30 minutes, 70% ethanol 30 minutes), Rinse with water 10 minutes, Dry the cassettes, Mayer Hemotoxylin

for 1 minute, Rinse with water for 20 minutes, 1% HCL for 20 minutes, Rinse with water 20 minutes, Dehydrate, then store in glass container with methyl salicylate.

Lung statistical analysis

Lung pictures are taken using a dissecting microscope, and they are analyzed for metastasis using the ImageJ program. This program allows for the measurement of the area of the total tumor mass present in the lung. First, each individual tumor on the lung picture is circled. The total number is recorded. After all the tumors have been circled, ImageJ allows for the area of all the tumors to be calculated. After this calculation, the entire lobe of the lung is circled and its area is measured. Dividing the tumor area by the lung area gives the area of metastasis.

Results

Lys cre efficiency:

The purpose of the E2F3 PCR was not only for genotyping purposes, but also to **check** the efficiency of Lys cre. Lung and tumor macrophages were isolated using the flow cytometry technique. Lungs were lavaged with cold PBS and tumors were digested with collagenase. The macrophages were sorted using PE conjugated F480 antibody. An E2F3 PCR was done to determine E2F3 deletion as seen in (Figure 1). Macrophages isolated from tumors and lungs of mice showed 50% deletion in E2f3^{+/*lox*p} and E2f3^{*lox*p/*lox*p} groups.

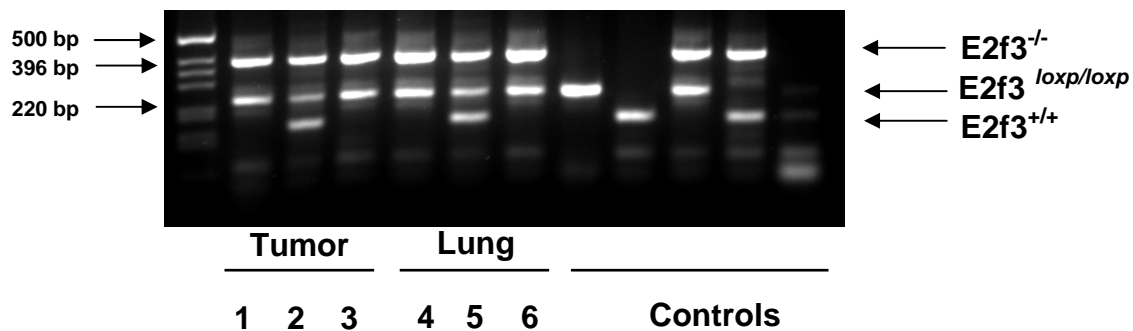


Figure 1. Macrophage samples isolated from tumors and lungs. E2f3^{*lox*p/*lox*p} (Lane 1,3, 4, and 6) and E2f3^{+/*lox*p} (Lane 2 and 5).

Rosa lox staining was done to study the expression in the mammary gland of Lys cre. Mice were created with the Rosa-LacZ gene expression in the macrophages by crossing Rosa-lox mice with Lys-cre mice. Lungs and mammary glands from these mice were isolated then stained with X-gal. The results show that only macrophages were staining positive for Lac-Z as indicated by Figure 2. Also, macrophages appear to be congregating around the exterior boundary of the tumors. Therefore, Lys cre was indeed targeting the macrophages.

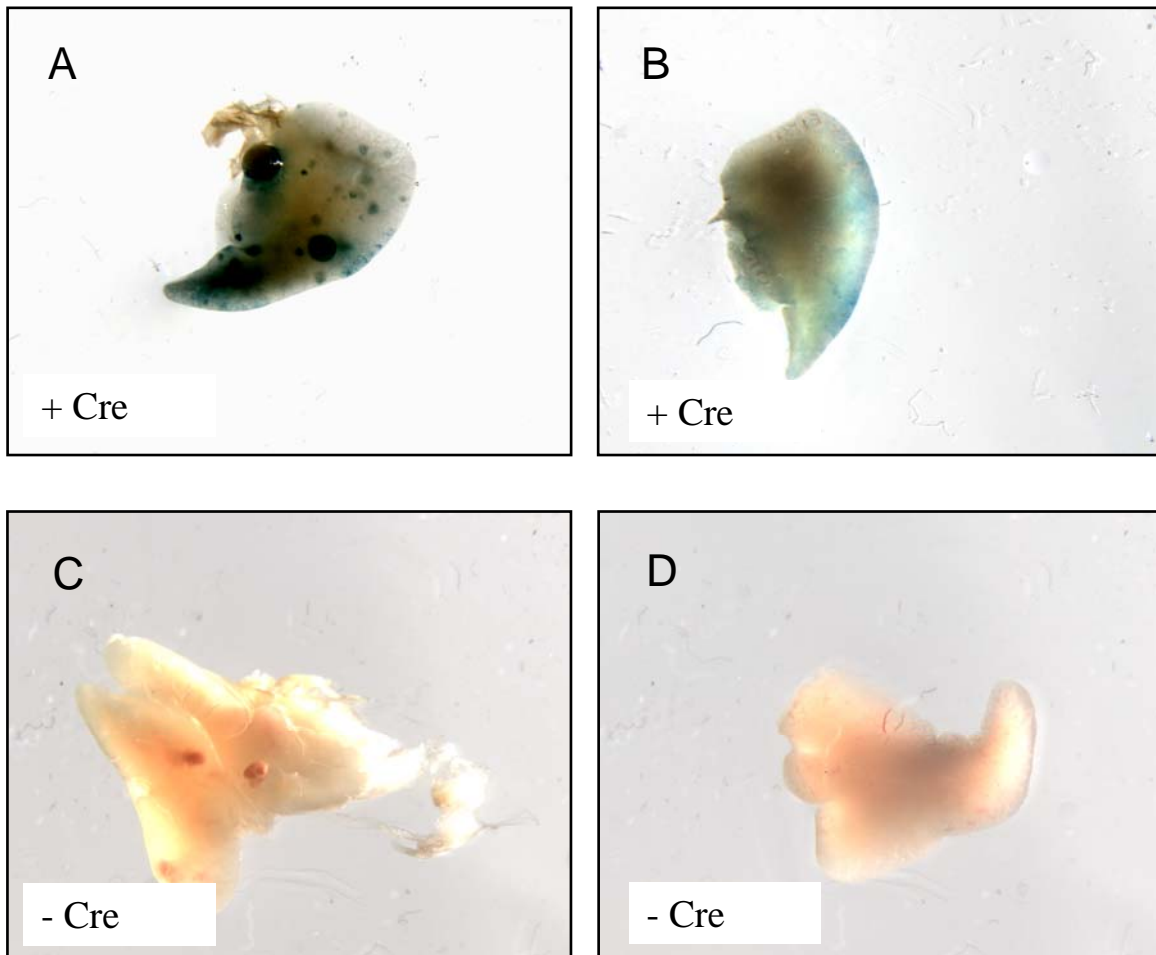


Figure 2. Rosa-lox mice with LacZ staining of lungs and tumors. **A** shows a Lys-cre+ lung with tumors. The circular dots are presumed to be macrophages gathered around a tumor. **B** Lys-cre+ but normal lung has no tumors. Macrophages are still present. **C** and **D** show lungs with and without tumors however without Lys-cre.

Tumor data collection:

The groups of mice were generated for this study: PyMT/E2F3^{+loxP} (control), PyMT/Lys^{+/-}/E2F3^{+loxP} (conditional), and PyMT/Lys^{+/-}E2F3^{loxP} (homozygous). The control group contained 37 mice, the conditional group had 20, and the homozygous had 15. As described in Materials and Methods, mice were palpitated for tumors twice a week in the mouse facility. Mice have 10 mammary glands (5 on each ventral lateral, Figure 3).

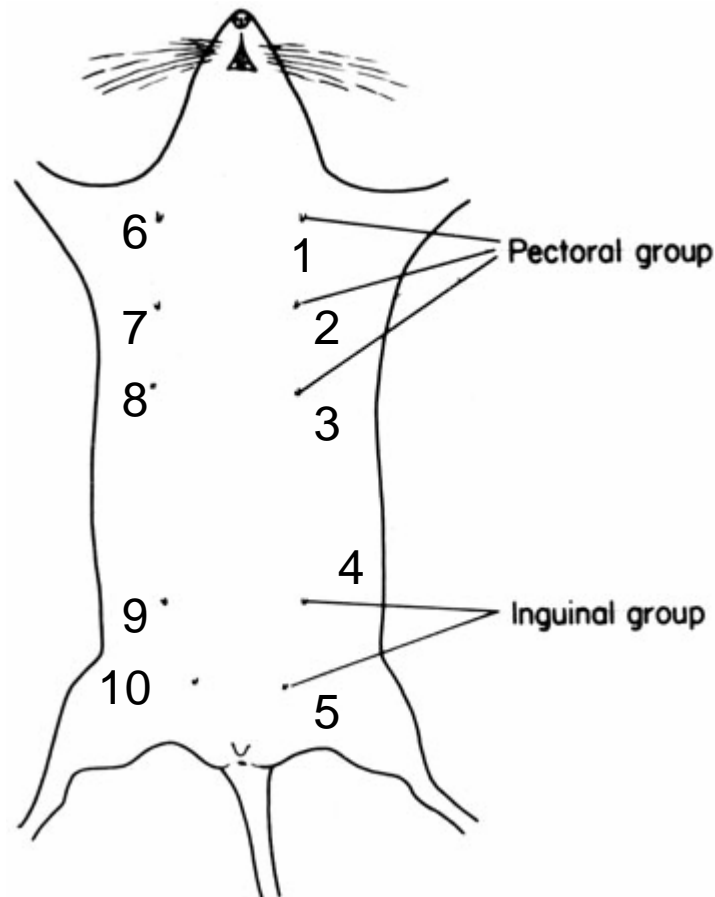


Figure 3. Ventral side of mouse showing the location of the mammary glands

Once tumors met reached a size of ~5 mm they were then measured weekly with an electronic caliper. After mice reached 110 days of age or if they meet the ERC

criterion they were harvested for their tumors and lungs. Tumors were collected, weighed, and a final measurement was taken with a caliper. The tumor data for all three groups is presented in Figure/Table X. The control ($E2F3^{+/loxP} - cre$) group had an average age of onset of 78.71 (± 10.91) days. The conditional ($E2F3^{+/loxP} + cre$) and ($E2F3^{loxP/loxP}$) *homozygous* groups had 79.24 (± 11.37) and 81.81 (± 8.08) days respectively. Figure X better demonstrates the similarities in age of onset and data points. As it shows, each group of mice are similar in terms of age of onset. Tumor burden (%) was calculated by weighing all the tumors and dividing it by the weight of the mouse during necropsy. It represents the % body weight that the tumors encompass. In the control ($E2F3^{+/loxP} - cre$) group, tumor burden was 43.78 (± 8.03) percent. The conditional ($E2F3^{lox+/p}$) group and *homozygous* ($E2F3^{loxp/loxp}$) had 43.34 (± 8.44) and 46.31 (± 8.44) %, respectively (Figure 4). Data tables for all the groups are shown in figure 5a-5c.

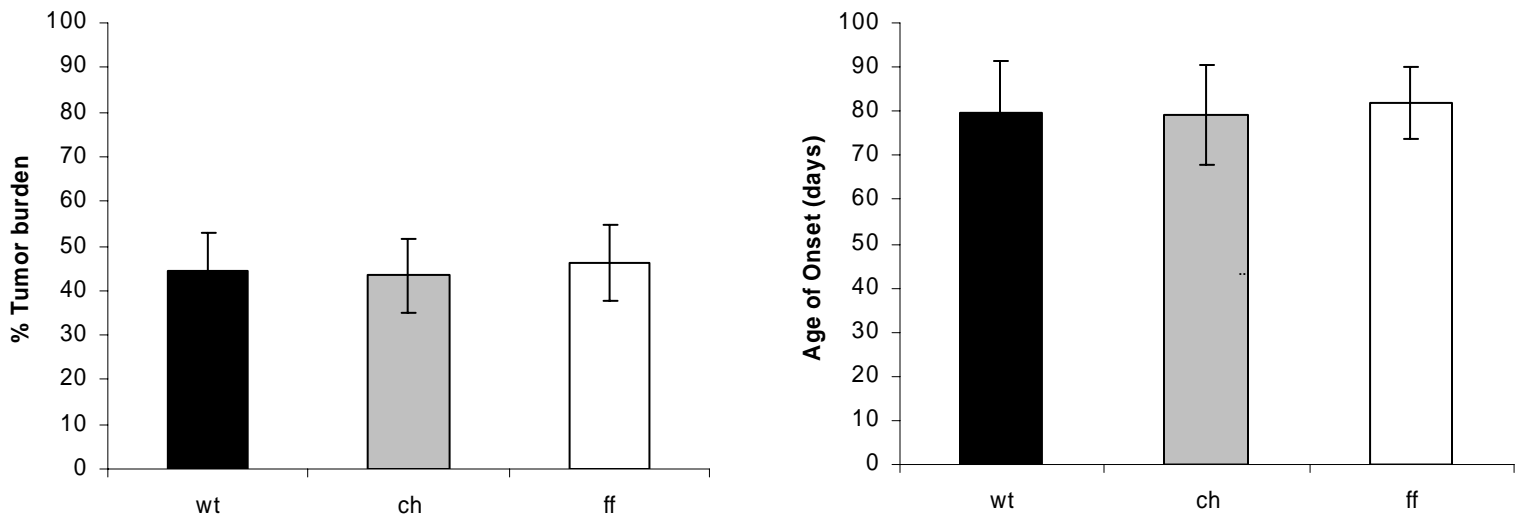


Figure 4. Two graphs showing % tumor burden and age of onset in the three experimental groups of mice. Wt represents the control group, ch is the conditional group, and ff is the homozygous group.

ID	Age of Onset	Age of Death	Animal	Tumor	Tumor Burden	Metastasis
	Days		mass (g)	mass (g)	(%)	
Pye3Fa3	61	110	37.8	19	50.26	14.98
Pye3Fb3	83	105	44	21.7	49.32	27.89
Pye3Fb4	83	105	43.2	23.1	53.47	22.06
Pye3Fc1	75	107	38.4	20	52.08	56.15
Pye3Ff13	97	112	38.7	11.9	30.75	4.07
Pye3Fe4	88	110	41.2	16.7	40.53	3.96
Pye3Fl3	74	101	38.6	12.7	32.90	11.52
Pye3Fm5	84	110	34.7	9.8	28.24	2.03
Pye3Fs1	78	110	44.6	19.1	42.83	17.73
Pye3Fu7	73	105	41.7	21.2	50.84	0.32
Pye3Fv5	91	110	33.8	14.8	43.79	5.3
Pye3Fu9	86	110	24.5	9.7	39.59	67
Pye3Fy6	61	110	33	17.8	53.94	7.32
Pye3F(II)b3	60	99	50	25.3	50.60	1.63
Pye3(II)c7	76	110	44.5	23.15	52.02	27.47
Pye3(II)c8	80	110	44	21.06	47.86	23.3
Pye3F(II)e6	68	110	46.9	22.4	47.76	5.70
Pye3F(II)e7	90	110	36	11.8	32.78	1.10
Pye3F(II)e9	90	110	31.5	11.8	37.46	8.40
Pye3F(II)g3	66	108	26.7	11.9	44.57	51.60
Pye3F(II)n13	89	110	37.8	14.3	37.83	4.9

AVG 78.71 43.78 17.35
SD 10.91 8.03

Figure 5a. Shows all the data for the control group (E2F3^{+loxP} – cre).

ID	Age of Onset	Age of Death	Animal	Tumor	Tumor Burden	Metastasis
	Days		mass (g)	mass (g)	(%)	
Pye3FI2	78	110	46.3	21	45.36	1.9
Pye3Fm7	70	110	41.6	17.9	43.03	5.29
Pye3Fn4	60	110	49.7	22.7	45.67	2.49
Pye3Fp2	56	110	47.6	21.1	44.33	17.64
Pye3Fs3	84	110	40.6	16.9	41.63	8.28
Pye3Fu11	104	110	40.5	18.7	46.17	11.4
Pye3Fx1	67	109	46.5	27.1	58.28	7.2
Pye3F(II)e12	68	110	33.5	9.9	29.55	1.5
Pye3F(II)g5	82	111	36.5	15.9	43.56	4.20
Pye3F(II)i2	97	110	32.2	9.5	29.50	0.6
Pye3F(II)j6	84	110	39.6	15.5	39.14	2.57
Pye3F(II)m2	85	110	42.7	17.7	41.45	1.02
Pye3F(II)m3	84	110	40.1	20.8	51.87	13.90
Pye3F(II)n15	89	110	43.3	22.1	51.04	4.30
Pye3F(II)p6	73	110	37.1	14.8	39.89	0.80
Pye3F(II)p10	87	110	34.4	11.7	34.01	2.78
Pye3F(II)q5	83	110	40.5	17.6	43.46	4.00
Pye3F(II)q7	73	110	44.5	25.3	56.85	2.10
Pye3F(II)s7	84	110	37.85	14.6	38.57	2.9
Pye3F(III)b6	78	110	43.9	25.5	58.09	8.7
Pye3F(III)b8	78	110	41.12	23	55.93	1.84

AVG 79.92 43.34 5.02
SD 11.37 8.44

Figure 5b. Shows all the data for the conditional group (E2F3^{+loxP} + cre).

ID	Age of Onset	Age of Death	Animal	Tumor	Tumor Burden	Metastasis
	Days		mass (g)	mass (g)	(%)	
Pye3F(II)q8	83	110	36	17.1	47.50	0.3
Pye3F(II)s3	91	110	38.9	17.55	45.12	27.97
Pye3F(II)t4	89	110	39.7	15.41	38.82	2.79
Pye3F(II)y4	70	110	46.4	28.8	62.07	11.73
Pye3F(III)e6	79	111	39.6	17.6	44.44	23.51
Pye3F(III)f8	78	110	37.6	18.1	48.14	0.82
Pye3F(III)f11	85	110	37.7	17.1	45.36	4.64
Pye3F(III)i1	75	107	40	16	40	3.31
Pye3F(III)i5	72	107	41.6	18.3	43.99	1.43
Pye3F(III)k14	84	110	42	21.9	52.14	5.22
Pye3F(III)m4	78	111	37.8	15.7	41.53	0.31
Pye3F(III)r3	79	109	50.78	27.4	53.96	10.9
Pye3F(III)s3	72	109	44.3	22.7	51.24	2.9
Pye3F(III)s6	82	110	48	12.4	25.83	4.15
Pye3F(III)t6	97	111	34.7	11.7	33.72	0.16
Pye3F(III)u3	95	110	36.8	14.9	40.49	1.26

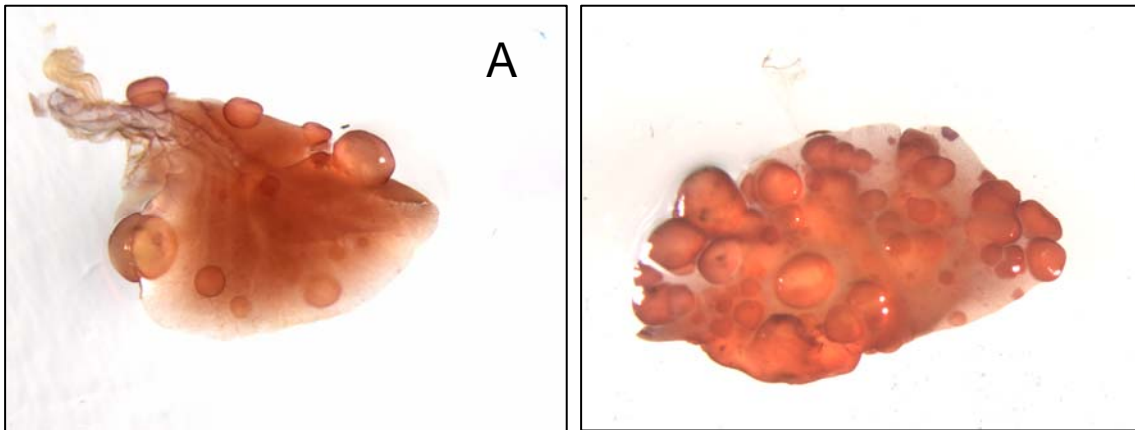
AVG 81.81 46.31 6.34
SD 8.08 8.44

Figure 5c. Shows all the data for the homozygous group (E2F3^{loxp/loxp} + cre).

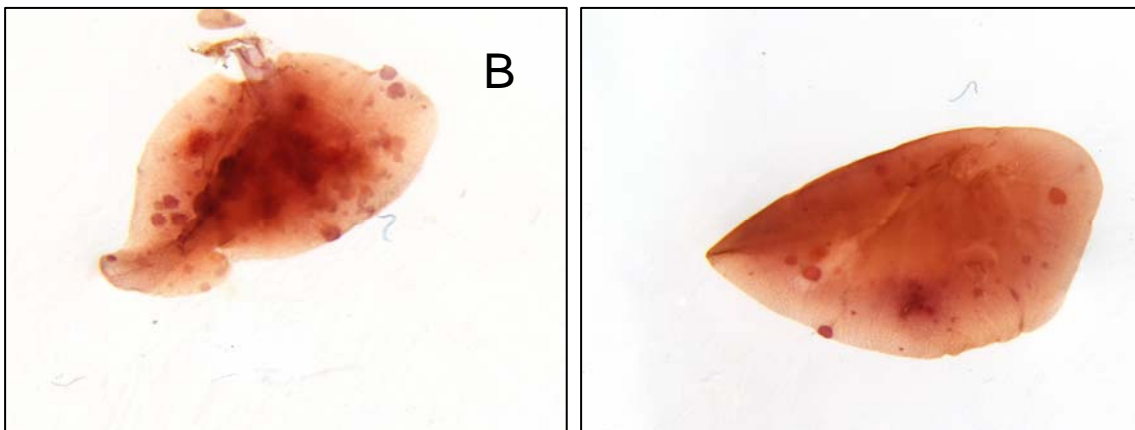
Lung Data collection:

The Lungs were kept in formalin and sent to the histology lab for staining with Mayer Hemotoxylin. The staining allowed the tumors to be easily distinguished from normal lung tissue (Figure 6).

PyMT E2F3^{+/*loxP*} - Cre



PyMT E2F3^{+/*loxP*} + Cre



PyMT E2F3^{loxP/loxP} + Cre

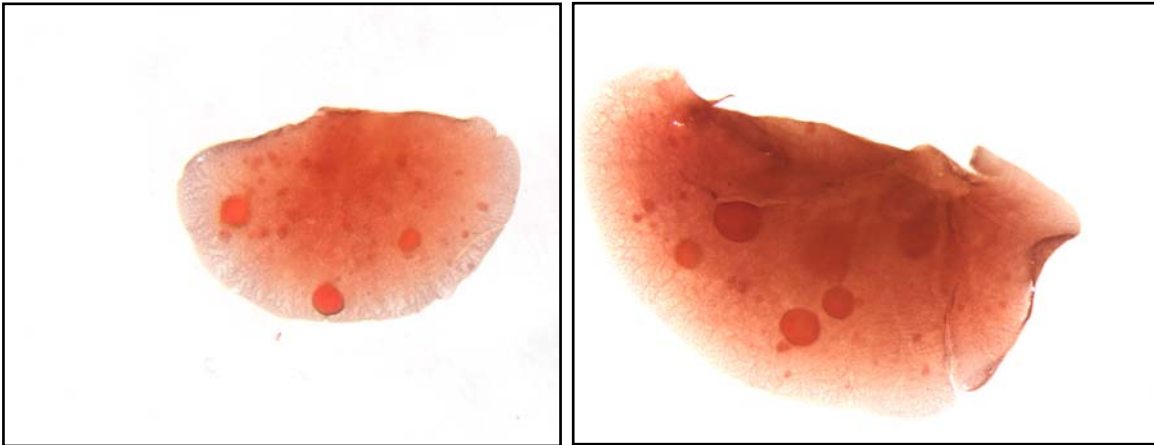


Figure 6. The 3 groups of experimental mice with Mayer hemotoxylin staining (further described in materials and methods).

The protocol for the lung staining is described in the materials and methods section of the paper. Once stained, pictures were taken of each of the 5 lobes of the lung using a dissecting microscope and the appropriate microscope software. These pictures were then analyzed using software provided by the NIH called ImageJ. With ImageJ, the lung picture (1 picture for each lobe, a total of 5 per lung) was opened and then analyzed. ImageJ allows for the measurement of area within a picture. First the total area of the tumors within the lobe was measured. Each tumor was completely encircled in order to account for its area. This was repeated for every tumor within the lobe until all were accounted. Once this was done, the area was summed up and measured. The number of tumors within each lobe was also recorded. After the total tumor area was calculated by ImageJ, the total lobe area was calculated by encircling the entire lung lobe. Thus the total tumor area was divided by the total lobe area and result was the % metastasis of the lobe. This process was repeated for all five lobes and the % metastasis and number of tumors was averaged.

The results of the Metastasis and number of tumors are summarized in Figure 7.

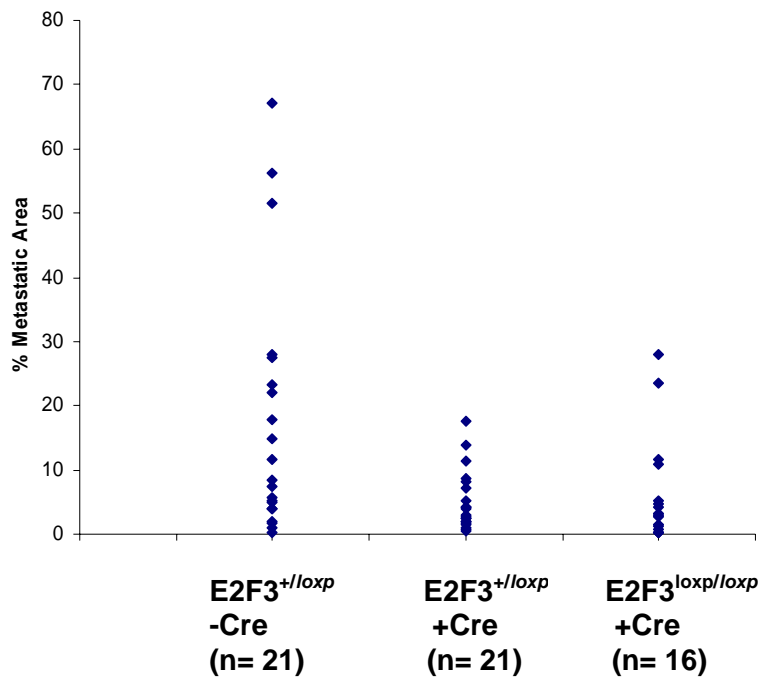


Figure 7. The graph shows metastatic area for all three groups of mice. For E2F3^{+/loxp} - cre The average metastatic area was 17.35; For E2F3^{+/loxp} + cre it was 5.02; for E2F3^{loxp/loxp} + cre it was 6.34

The average percent metastatic area for wild type is 17.35% while it is 5.02% for the conditional group is. The homozygous group shows results similar to the conditional group and its percent metastatic area is 6.34%. Lung metastasis was measured both qualitatively and quantitatively. The t-test between the conditional and control group shows the p-value to be **0.004** meaning that it is statistically significant. The t-test between the control and homozygous group shows a p-value of 0.013 which is also statistically significant.

Discussion

Tumor analysis

The results showed that age of onset and tumor burden was similar among the three groups of mice. Therefore, macrophages don't play a critical role in tumor growth. This data was expected since tumors are composed almost entirely of epithelial cells. Also, macrophages are post-mitotic cells, so their cell-cycle would not be affected. Deleting E2F3 in macrophages did not have an effect on the epithelial cells and their proliferation.

Lung analysis

The interesting results arose from the analysis of the lung. In terms of metastasis, the control group showed more than a three-fold difference when compared to the *+loxP* group. These results support the hypothesis that macrophages play a role in metastasis. Macrophages with 50% deletion of E2F3 led to a dramatic change in the size and number of metastasis in the lung. However, the homozygous group which theoretically should've had 100% deletion of E2F3 instead had similar results to the conditional group. The reason appears to be that Lys-cre dramatically loses efficiency when trying to delete two alleles as opposed to only needing to delete one allele. In other words, there is a limit to the Lys-cre efficiency.

Role of E2Fs in cell cycle and metastasis

E2Fs are important regulatory genes within the cell cycle. Based on tumor F4/80 staining, E2F3 doesn't seem to affect macrophage proliferation to any discernable degree. However, the results of the lung analysis study show that E2F3 does affect macrophages based on the decrease in metastasis. The question is how does E2F3 interfere with normal macrophage function? Previous studies on other cell types have shown that deletion of an E2F leads to a decrease in proliferation and a lower number of cells. The E2F3 gene is acting in another way within the macrophage and it certainly seems that the CSF-1 pathway might be involved. The current hypothetical mechanism for TAM

metastasis seems to be the ability of macrophages to release proteases. These proteases are thought to degrade the outer epithelial lining of the tumor and allow malignant epithelial cells to escape within the bloodstream.

Further hypothetical thinking suggests a link between E2F3 and CSF-1 an important macrophage pathway that controls the expression of early and late response genes. Since macrophage proliferation is being affected by E2F3 deletion, maybe gene expression of these proteases is being affected along with the CSF-1 pathway.

Clinical application

In the clinical sense avoiding metastasis is the physician's number one concern while treating a cancer patient. Metastasis is what makes cancer so deadly. Based on the results of the experiment and other publications, macrophages play a key role in metastasis. Thus, being able to control the macrophage population within a tumor might decrease the chances of the tumor spreading and therefore increase a patient's outlook on life. A drug that could somehow reduce macrophage activity within the tumor or repress the gene expressing the proteases in TAMs is a more viable alternative.

Areas of further research

Future experiments should seek to understand what genes are being affected within the macrophage by the deletion of E2F3. A micro-array or other gene expression tool could be used to answer this question. Most importantly, are proteases being down-regulated and are these proteases responsible for allowing metastasis occur.

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