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TITLE: Investigating the Role of Fatty Acid Metabolism and DecR1 Expression ErbB2-Driven Mammary Tumorigenesis and Metastasis Using a Transgenic Mouse Model Approach

PRINCIPAL INVESTIGATOR: Jason Turpin

CONTRACTING ORGANIZATION: McGill University
Montreal, QC, Canada H3A 1A3

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14. ABSTRACT Cancer cell metabolism has become an increasingly active area of research in recent years, elucidating the importance of Warburg metabolism and its role in tumorigenesis. Fatty acid oxidation is diminished in favor of synthesis during the tumorigenic process. DecR1 is a fatty acid oxidation protein that is frequently downregulated in models of breast cancer. Using a mouse model approach, expression of DecR1 in mammary epithelial cells results in a delay in ductal outgrowth, part of normal mammary gland development. Additionally, when expression of DecR1 is driven in an ErbB2 mouse model of breast cancer, it confers a slight delay in tumor onset. However, expression is lost in the end-stage tumors, suggesting induction of fatty acid oxidation is selected against during tumorigenesis. This is further supported by the finding that naïve, uninduced tumor cells retain the capacity to undergo induction. Finally, orthotopic injection of naïve tumor cells into nude mice shows induction of DecR1 expression has a growth-suppressive effect. Taken together, this suggests fatty acid oxidation is antagonistic to tumorigenesis, however the long term impact of DecR1 expression is still not known.					
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Introduction:

Cancer cell metabolism has become an increasingly active area of research in recent years, elucidating the importance of Warburg metabolism and its role in tumorigenesis. Increase in ATP production is an essential step in tumor initiation, and this shift towards a Warburg metabolism results in an increase in anabolic processes, providing necessary ATP and other macromolecule to fuel rapid cell growth and proliferation, such as amino, nucleic, and fatty acids. In order to meet this latter requirement, there is a shift towards the anabolic process of fatty acid synthesis and away from more catabolic fatty acid oxidation. Normal cells meet the majority of their energy demands through oxidative phosphorylation, whereas tumor cells truncate this process, and utilize primarily glycolysis even in the presence of normal oxygen [1]. This shift in metabolic processes, termed the Warburg Effect, was initially described by Otto Warburg [2] in the 1930s, but was largely ignored until recent years [3].

During the transition from a normal to malignant cell, a key change is the reliance upon a constant growth signal [4]. These growth signals are often provided by growth factor receptors, notable the EGFR-family of receptor tyrosine kinases (RTKs) [5]. ErbB2, a member of the EGFR family of RTKs, is amplified and overexpressed in around 30% of human breast tumors, correlating with poor prognosis [6]. Much of what has been learned about ErbB2 in breast cancer has been developed through the use of mouse models. Specifically, ErbB2 was expressed under the control of the Mouse Mammary Tumor Virus (MMTV) long terminal repeat, which drives expression of the transgene in the mammary epithelium [7]. Interestingly, ErbB2-expressing tumors have a concomitant increase in the overall glycolytic rate, as well as net fatty acid synthesis [8].

Another important aspect of cancer cell metabolism is the increase in *de novo* fatty acid synthesis, which ensures an adequate supply of lipids to the rapidly dividing cell for membrane biosynthesis [9]. In tumor cells, the excess pyruvate that exits glycolysis is rapidly converted to citrate, where the enzyme ATP Citrate Lyase (ACL) converts citrate to oxaloacetate, then to acetyl CoA, the principle substrate for *de novo* fatty acid synthesis [9]. Since knockdown of ACL reduces lipid synthesis, decreases cell proliferation and impairs tumor outgrowth *in vivo* [10], continual fatty acid synthesis can also be seen as an important requirement to support tumor cell proliferation.

More recently, gene expression profiling on of mammary tumors from transgenic mice expressing Neu (rat ErbB2) in the mammary gland reveal that the mitochondrial enzyme 2,4-dienoyl CoA Reductase (DecR1) is repressed during mammary tumorigenesis [11, 12]. This has since been confirmed internally using the polyoma virus mT (PyVMT), and activated Neu (NDL2-5) mouse models [13]. DecR1 catalyzes the rate-limiting step of the auxiliary pathway of fatty acid β oxidation [14, 15], which allows polyunsaturated fatty acids, with double bonds at even or odd positions, to be oxidized by reducing them to monounsaturated substrates [16]. Therefore, since tumor cells require a constant source of fatty acids for rapid cell division [10],

the expression of DecR1 in malignant cells would be counterproductive and result in impaired cell growth [13]. Tissue microarray data on a panel of human breast tumors has shown a similar decrease in DecR1 expression as seen in the spontaneous mouse models [13]. Not only does this help validate the relevance of these mouse models when studying the human disease, but provides support to the idea that this metabolic shift towards increased glycolysis is required for tumor initiation.

In vitro studies, using a Neu-transformed mammary epithelial cell line, suggest re-expression of DecR1 decreases the tumorigenic potential of cells, as evidenced by the reduced tumor outgrowth upon mammary fat pad injection [13]. Furthermore, DecR1-expressing cells lose their glucose-dependency, and show decreased *de novo* fatty acid synthesis from glucose, indicating a return to a less transformed phenotype.

Body:

The first and most important accomplishment was the establishment and characterization of an inducible mouse model. This model is doxycycline-inducible, such that administration of doxycycline in the drinking water of mice induces expression in the mammary epithelium. In this two-construct system, MMTV-driven reverse tetracycline-dependent transactivator (MMVT-rtTA, henceforth referred to as MTB) maintains expression of the transactivator in the mammary epithelium. In the presence, but not the absence, of doxycycline, doxycycline binds to the transactivator, driving DecR1 expression in the mammary epithelium (Figure 1A). EGFP fluorescence is coupled to gene expression through an internal ribosomal entry site (IRES), allowing easy visualization of gene expression. As expected, induced mice show regulated gene expression by both EGFP in whole mounted mammary glands and immunoblot analysis for the V5 tag on the transgene (Figure 1B)

Specific Aim1 set out to characterize the effect of DecR1 expression during normal mammary gland development. The first step was to examine normal ductal outgrowth, which is the process in which the mammary gland develops from a rudimentary bud to invade the fat pad and form a functional mammary gland, a process that occurs from week 3 to week 8-10 post-natally. Outgrowth is characterized by high rates of cell proliferation and apoptosis and is assessed by measuring the distance of migration of the mammary epithelium, with respect to the position of the lymph node, in the inguinal mammary gland (Figure 2A). Expression of DecR1 modestly delays ductal outgrowth in mice of 6 and 8 weeks of age (Figure 2B), significant by student's t-test. The cause of this moderate delay in outgrowth could be through delay of proliferation, or stimulation of apoptosis. This would be determined by PCNA staining of histological sections to assess proliferation, or a TUNEL assay on histological sections to measure cell death. This has not yet been accomplished, but will be determined in the near future.

Further characterization of the role of fatty acid oxidation in normal mammary gland function involves a study of pregnancy/lactation and involution. Lactation is a process that relies heavily on fatty acid synthesis, so induction of DecR1 expression could have a dramatic impact. This will be measured by monitoring the health and weight of litters during development. Involution requires rounds of apoptosis to remodel the mammary gland back to a virgin state following weaning. These two assays have not yet been performed, since priority was placed on the tumorigenesis aspect of the project in terms of allocation of mouse resources.

Aim 2 examined the role of DecR1 and fatty acid oxidation on the tumorigenic process. For this aim, tri-genic mice were generated expressing the MTB transgene with inducible DecR1 and the oncogene NDL. The MMTV-NDL2-5 oncogene model, developed by Dr. Muller [17], develops multifocal mammary tumors with an average latency of 5-6 months. Initially, tri-genic mice, under induction as of 3 weeks of age, were compared to bi-genic mice without the MTB transgene. Subsequently, uninduced tri-genic mice were added to the tumor onset analysis. When comparing the two tri-genic groups, DecR1 expressing mice showed a very slight delay in tumor onset (Figure 3A). However, when end-stage tumors were analyzed, only one of 22 analyzed tumors retained expression of transgenic DecR1, while adjacent mammary glands were positive for the transgene (Figure 3B). Furthermore, EGFP fluorescence was clearly visible in adjacent mammary glands, indicating the problem was not due to complete lack of response to doxycycline (Figure 3C). Taken together, this data implies there is a strong negative selection against the expression of DecR1, and indirectly fatty acid oxidation, during the tumorigenic process. This type of result is not without precedence, as it was reported previously that MMTV-driven transgenes are not necessarily expressed in all mammary epithelial cells, and multiple MMTV-driven transgenes in the same mouse are not necessarily expressed in the same epithelial cells [18]. In that publication, it was suggested that manipulations that are deleterious to tumorigenesis can be selected against, a situation that appears to be analogous in this DecR1 study. Unfortunately, this result proved to be a set-back in the analysis, as the resulting tissues appeared to be wildtype for activation of several pathways by immunoblot, including Acetyl-CoA Carboxylase (ACC), Fatty Acid Synthase (FAS), and AMP-activated Protein Kinase (AMPK) (Figure 3B). Therefore, the approach to Aim 2 had to be modified.

To further support the idea that loss of transgene expression in end-stage tumors was a result of negative selection, the cohort of tri-genic animals not exposed to doxycycline (referred to as naïve tumors), and therefore not given the opportunity to select out transgene-expressing cells, were generated. Naïve tumors were harvested at clinical end-point, and either explanted into culture to generate primary cell lines, or transplanted orthotopically into athymic nude mice. In both cases, cell lines (Figure 4A) and transplanted tumors (Figure 4B) were determined by immunoblot analysis to retain the capacity for induction. With this result, it allows for the production of DecR1-expressing material, which can subsequently be analyzed as described in the Statement of Work. To assess the effect of sustained DecR1 expression on ErbB2-driven tumorigenesis, naïve transplanted tumor material was dissociated and immediately injected

orthotopically into athymic nude mice, with tumor outgrowth measured twice weekly by caliper measurement. Interestingly, there was a one-week delay in tumor formation in the doxycycline-induced cohort compared to the uninduced group (Figure 4C). Furthermore, the tumors grew at a slower rate, suggesting DecR1 expression suppresses tumor outgrowth in this model. The resulting tumors retained transgene expression at variable levels (Figure 4D).

Aim 3 set out to examine the effect of DecR1 expression metastatic burden. Unfortunately, much of these studies were rendered impossible with the initial result in Figure 3B, where the end-stage tumors did not express the transgene. This makes any analysis of metastatic burden irrelevant since transgene expression can never be assumed. Aim 3a and 3b will need to be carried out through orthotopic injections into athymic nude mice, as described previously. Aim 3c will also require the use of an orthotopic model. Aim 3d has partially been replaced by 3a due to the modification in procedure, however tail vein injections will be undertaken with the naïve inducible cell lines and transplanted material.

Aim 4 involves the metabolic characterization of DecR1 expression in a tumorigenic context. This aim suffers from the same limitations as described previously, the lack of a stably-expressing, inducible system. With the recent establishment of inducible primary cell lines and serially transplantable material, it will now be possible to undertake these types of experiments. Furthermore, the Metabolomics core facility at the Goodman Cancer Research Centre is still in the process of establishing protocols and assays, as well as purchasing new equipment. As this facility becomes more operational in the coming months, the capacity to undertake more specific metabolic studies will be dramatically increased.

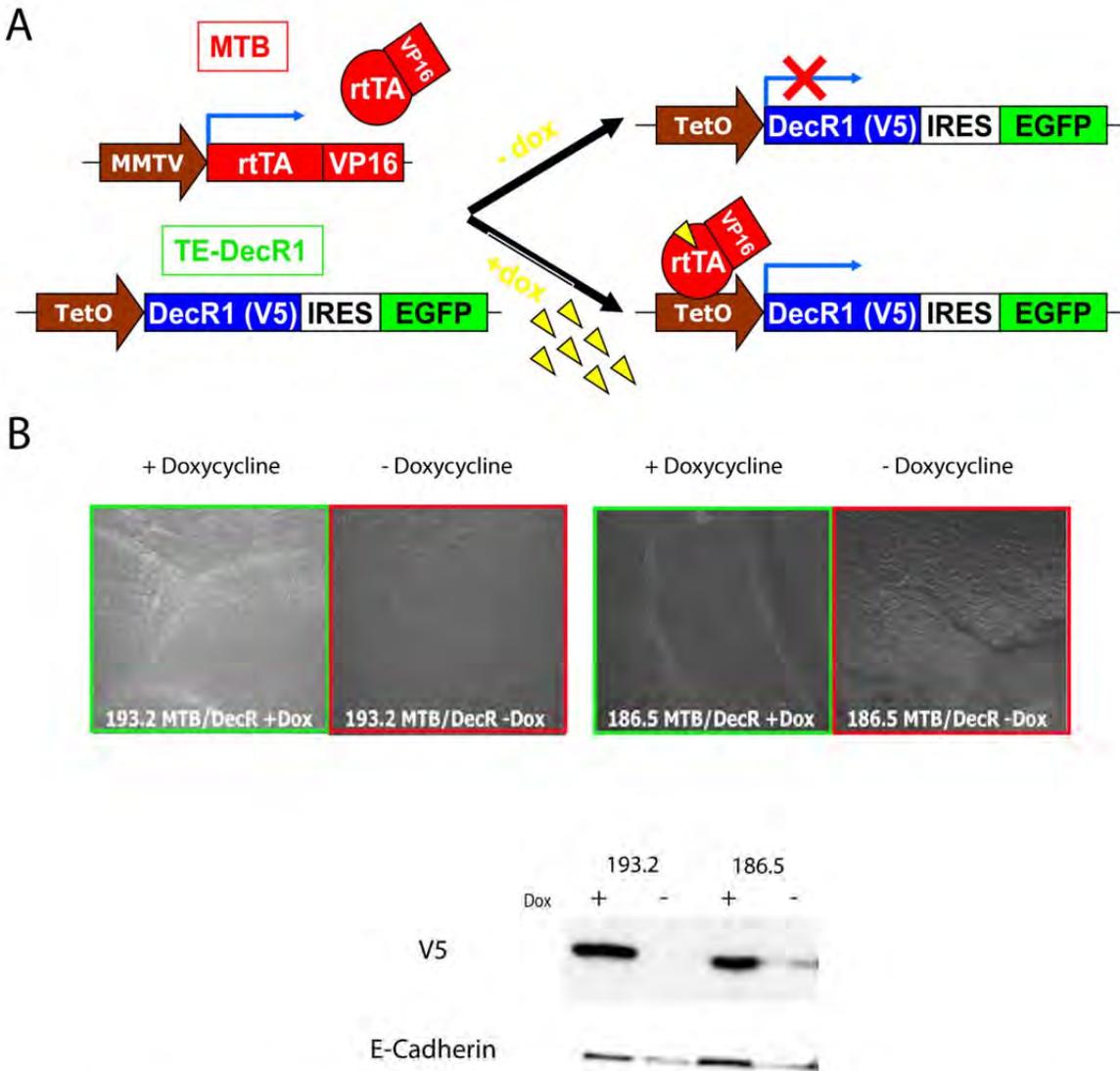


Figure 1: Generation of an inducible DecR-1 mouse model. A) A schematic diagram of a doxycycline inducible system. In this bigenic system, the first transgene (MTB) uses MMTV to drive expression of the reverse tetracycline-dependent transactivator (rtTA) in the mouse mammary epithelium . In the presence, but not the absence, of doxycycline, rtTA binds to the tetracycline response element (TetO) of the inducible transgene (TE-DecR1) to induce expression of DecR1 in the mouse mammary gland. B) Characterization of inducibility reveals administration of 2mg/ml doxycycline in drinking water is sufficient to induce robust transgene expression, as measured by wholemount mammary gland fluorescence and by immunoblot analysis for the V5 tag of DecR1.

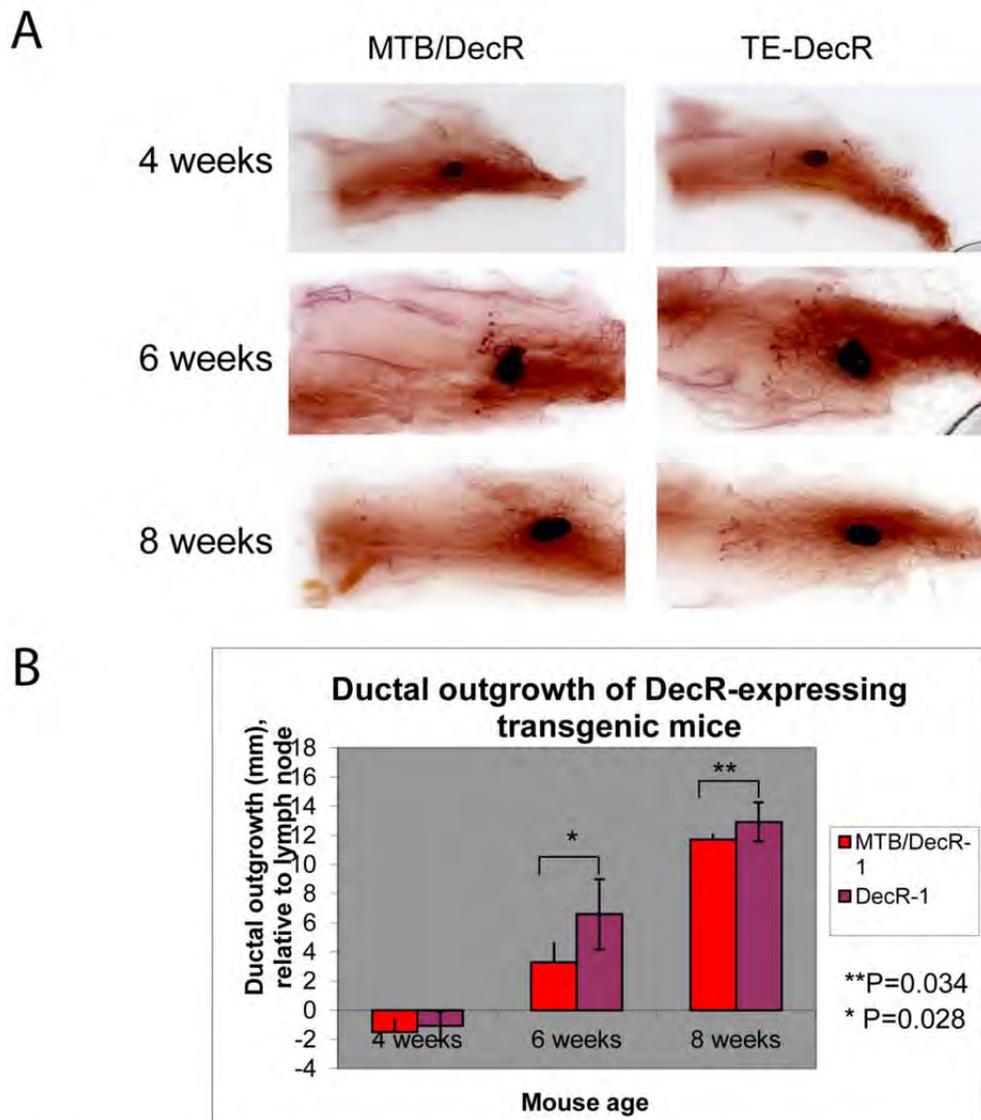


Figure 2: Ductal outgrowth of developing mice is impacted by DecR1 expression within the mammary epithelium. A) Transgenic mice expressing DecR (MTB/DecR) were sacrificed at the indicated time, and the inguinal mammary gland was wholemounted and stained with hematoxylin. B) Ductal outgrowth was quantified by measuring the distance from the center of the lymph node to the leading edge of the developing ducts.

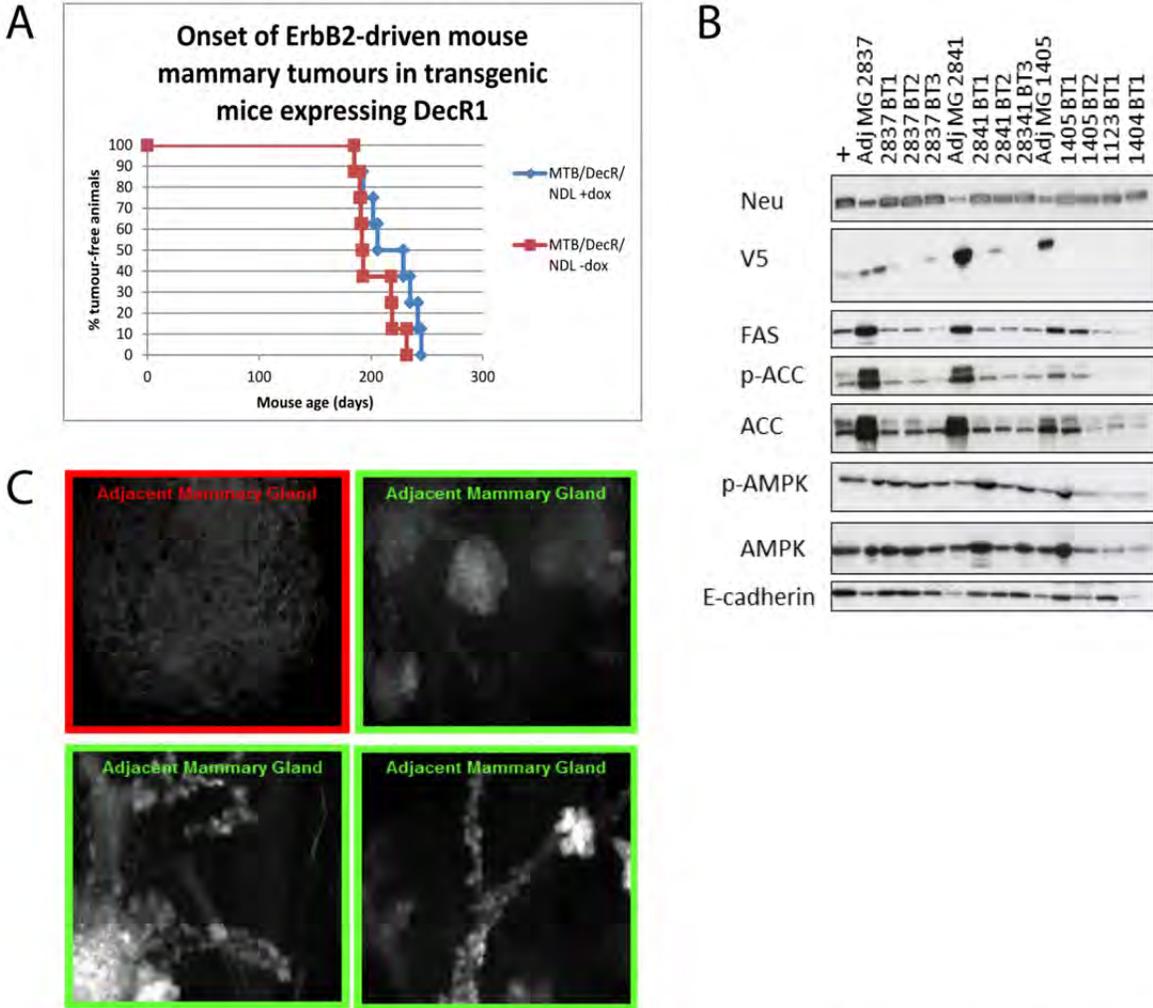


Figure 3: Sustained DecR1 expression in an ErbB2-driven model of tumorigenesis has minimal impact on tumor onset, however results in loss of transgene expression. A) Ectopic expression of DecR1 results in a minimal delay in tumor onset, with a t-50 of 206 days, compared to 185 for the uninduced cohort. B) Immunoblot analysis reveals that end-stage tumors have lost expression of the inducible transgene, which is retained in the adjacent mammary glands. There is also no change in metabolic protein expression and activation, such as ACC, FAS, and AMPK. C) Fluorescence wholemount microscopy confirms inducibility in adjacent mammary epithelium at time of sacrifice, supporting the concept that lack of inducibility in tumors is a result of selective exclusion of the transgene, and not a global failure to induce DecR1 expression in the mouse.

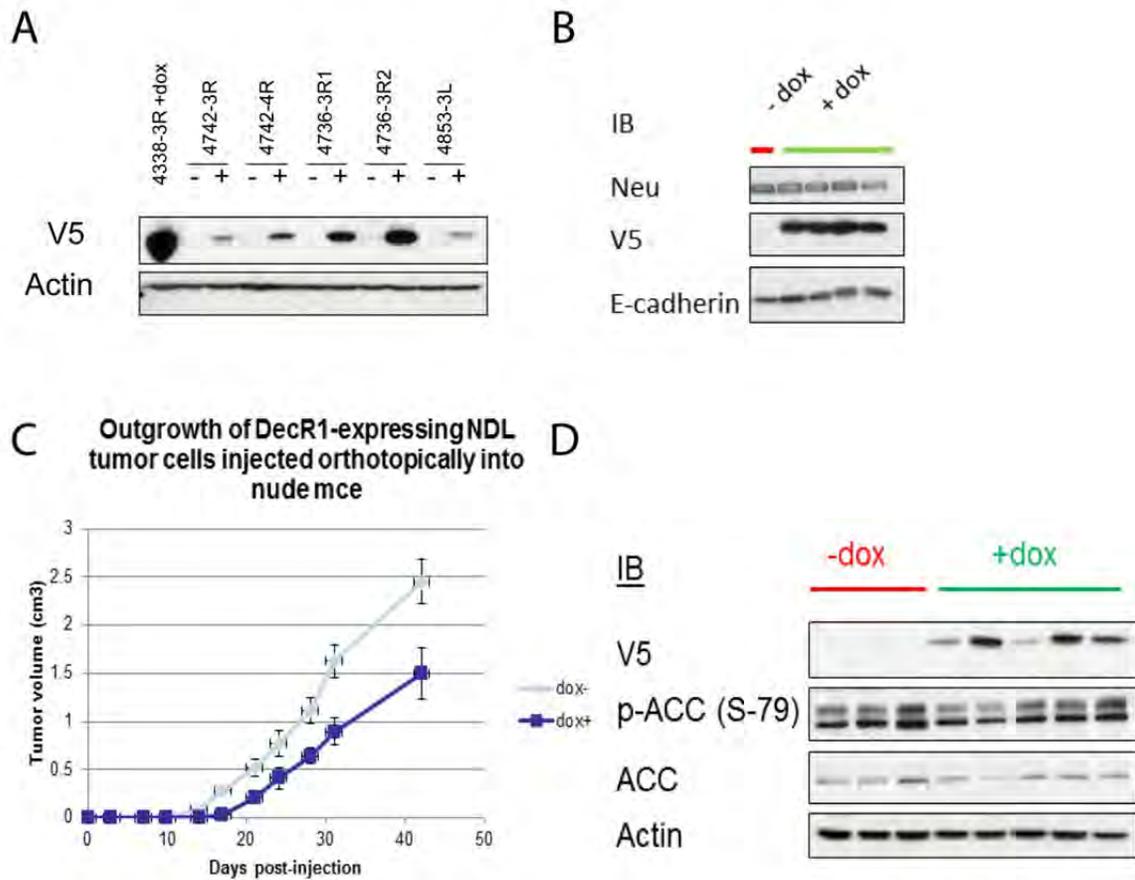


Figure 4: Naive NDL-driven transgenic tumors retain the ability to express DecR1 in an inducible manner. A) Naive transgenic tumors explanted into culture, when treated with 10ug/ml doxycycline in culture medium, retain the ability to express DecR1, as seen by immunoblot analysis for the V5 tag. B) A transgenic tumor that has been transplanted orthotopically into athymic nude mice also retains the ability to express DecR1. C) A transplanted transgenic tumor was dissociated and immediately injected orthotopically into athymic nude mice. Outgrowth was measured by twice weekly palpations and caliper measurements. DecR1-expressing mice (+dox) arise with a 1-week delay and subsequently grow at a slower rate. D) Explanted tumors retain a variable degree of DecR1 transgene expression, but do not seem to affect activity of ACC by immunoblot.

Key research accomplishments:

- Developed and characterized an inducible system for studying fatty acid oxidation in normal tissue development and ErbB2-driven tumorigenesis
- Determined that loss of fatty acid oxidation is important for tumorigenesis, and that ectopic expression of DecR1 can be selectively removed during the transformation process
- Expression of DecR1 in an established primary tumor suppresses the rate of tumor outgrowth.
- Explanted primary cells from naïve tumor material retain the capacity for induction, and have become critical tools in this study for elucidating the effects of fatty acid oxidation on Warburg metabolism.

Reportable outcomes:

- Developed a doxycycline-inducible mouse model of DecR1 expression.
- Submitted an abstract to and attended the 9th Conference on Signalling in Normal and Cancer Cells, held March 6th-10th, 2010 in Banff, Alberta, Canada.

Conclusion:

The main conclusion that can be drawn from the research to this point in the project is that fatty acid oxidation is not permissive to mammary tumorigenesis. Since end-stage NDL2-5-driven tumors did not express DecR1, and as such were a victim of the escapee phenomenon, this strongly suggests that fatty acid oxidation is not supportive of the transformation and tumorigenic process. This has an impact on much of the proposed work, as the approach will need to be changed. Metabolic analyses and doxycycline withdrawal will now be done using transplanted tumors or orthotopically injected cells in the presence or absence of doxycycline. Transgenic mice will be used to generate naïve, uninduced tumor material with which induction studies can be subsequently carried out. While this does not change the potential impact of the research, the approach to generating data will be slightly appended.

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