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OVER-EXPRESSION OF ENDOTHELIN-3 INCREASES TUMOR VOLUME IN A TRANSGENIC MURINE MELANOMA MODEL

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To: Dr. Steven Oberbauer, Chairperson Department

of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Jesus Lopez entitled "Over-Expression of Endothelin-3 Increases Tumor Volume in a Transgenic Murine Melanoma Model", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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ABSTRACT

Melanoma is a cancer that is caused when pigment producing cells called melanocytes acquire cellular damage and begin to proliferate. Some major factors that affect melanoma progression include environmental carcinogens and genetic mutations. The tumor microenvironment is a rising field of interest in studying aggression in many cancers. A specific signaling pathway between endothelin 3 (Edn3) and endothelin receptor b (Ednrb) has been used as a marker for aggressive types of melanoma, but the role that Edn3 plays in tumor progression has not been addressed. This thesis studied the effects of Edn3 in mice with melanoma by using a model that overexpresses Edn3 through the Keratin 5 promoter and is thus referred to as a *K5-Edn3* mouse. Ten *K5-Edn3* and ten wild-type mice lacking the transgene were both injected with 10^6 B16F10-GFP. Primary tumors were measured biweekly for a full month. A second round of injection was done with 10^5 YUMM1.7-GFP cells, which carry three key driver mutations found in human melanomas. These tumors were grown and measured for 21 days. Flow cytometric analysis was done on the YUMM1.7-GFP tumors to check for Ednrb expression in the tumor microenvironment. In both cell lines, *K5-Edn3* mice grew statistically larger tumors at faster rates than the wild-type. Metastatic tissue was found only in the lungs of the *K5-Edn3* mice. Flow cytometric analysis showed that a subpopulation of tumorigenic cells and stromal cells express Ednrb in both mice groups. Together, the results indicate that overexpressing Edn3 promotes melanoma growth and increases the metastatic potential of primary tumors. Furthermore, Ednrb expression found in tumorigenic and stromal cells in the microenvironment may be contributing to the overall tumor progression in melanoma. Future directions should further characterize the stromal cell subpopulations expressing Endrb, which may give rise to novel therapies that inhibit their downstream effectors.

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INTRODUCTION

Cancer is estimated to affect approximately 1.8 million people in the United States alone (Siegel et al., 2019). This disease can take on different forms, from sarcomas to breast cancers. Melanoma, a particular type of skin cancer, is projected to affect 96,000 Americans in 2019 (Siegel et al., 2019). Although cancers vary widely in their phenotypes, all of them share common causes. Genetics play a predominant role in the generation of new cancer cells, as do many chemicals known as carcinogens (Siegel et al., 2019). Various factors may have a wide or narrow range on which cells they can affect. Melanoma specifically develops from pigment-producing cells known as melanocytes; these cells are predominantly found in the basal layer of the skin but can also be seen throughout different organs (Nelson et al, 2003). When melanocytes acquire damage to their cell cycle regulation, malignancies may begin to grow. The causes to such impairments have a wide range of sources, the most common being UV, or genetic predisposition (Mantovani et al., 2006) affecting mostly people of lighter pigmentation (Siegel et al., 2019).

The growth and progression of primary tumors are affected by factors in their environment, which can dictate whether a tumor will continue to grow in volume, become angiogenic, or metastasize (Sherr, 1996; Mantovani et al., 2006). These elements range from exogenous environmental factors to endogenous proteins. A hallmark trait of tumor succession is gene-expression manipulation (Sherr, 1996). Consequently, cell-signaling molecules and proteins are often over-or under-expressed in ways that promote growth (Sherr, 1996). Some new evidence has shown that the immune system might also aid in cancer progresison. Originally thought to only suppress cancers, the immune system may also be promoting tumor expansion (Schreiber et al., 2011). Understanding factors like these can lead to the development of novel therapies to decrease the mortality rate of aggressive cancers (Siegel et al., 2019; Barton & Yanagisawa, 2008). However, finding these specific factors can be challenging considering the complex and dynamic nature of cancer.

A fundamental necessity of the immune system is to be able to distinguish self and non-self cells in the body. Tumors often bypass this process by overexpressing specific ligands or receptors on the surface of their cells that the immune system uses to recognize ‘self’ cells. This enables tumor cells from causing an immune response from being triggered and thus avoid attack. Current immune therapies usually comprise of drugs that target specific immune inhibitors (Larkin et al., 2015;Schreiber et al., 2011). An example of a drug therapy that works by using this principle is the use of PD-1 receptors and PD-L1, the ligands of PD-1 receptors (Topalian et al., 2012). When immune cells bind their PD-1 receptor to the ligand PD-L1 of cells, the immune system recognizes it as self; tumor cells thus have excess PD-L1 on their cells so they remain hidden from attacks of the immune system (Topalian et al., 2012). A similar mechanism of action is seen in the drug therapy used for melanoma with CTLA-4, which is another receptor-ligand relationship that is used to identify normal cells (Larkin et al., 2015).

Endothelin proteins also affect the aggression of melanoma tumors and have become recent candidates for cancer-drug therapy. These proteins consist of three small amino-acid peptides, called Endothelin-1, Endothelin-2 and Endothelin-3 (Edn-1,2,3 respectively). These peptides bind to four endothelin-specific receptors, but the most understood are Endothelin Receptor A and Endothelin Receptor B (Ednra,b respectively) (Nelson et al., 2003). Endothelins function in vasocontraction, although recent studies have also implicated their role in tumor progression (Mantovani et al., 2006; Barton & Yanagisawa, 2008). Of the three peptides, tumor growth and progression were primarily seen in Edn1 studies through its binding with Ednra.

Of the three peptides, Edn3 diverges the most from the group by differing in its amino acid sequence, causing it to bind primarily to Ednrb (Barton & Yanagisawa, 2008). Although the role of Edn1 has been well-studied and is associated with tumor progression, the function of Edn3 in melanoma development is not well known. This peptide has been associated with immune suppression and is used as a marker for aggressive types of melanoma (Schreiber et al., 2011). However, the role that Edn3 plays directly in cancer progression has not been addressed. I became interested in this signaling pathway and asked: what is the role of Edn3 in melanoma and metastasis? From previous work, I expected that an upregulation of Edn3 would increase both tumor volumes and the metastatic potential of primary tumors. To assess how Edn3 affects tumor growth, a transgenic mouse was used that over-expresses Edn3. With this model, the effect that Edn3 plays on melanoma tumors can be seen by comparing them to a wild-type strain.

MATERIALS AND METHODS

Cell Culture and Dilutions

Melanoma cells (Bl6F10 obtained from American Type Culture Collection, Manassas, VA and YUMM 1.7 obtained from Dr. Bosenberg, Yale University School of Medicine, New Haven, CT) were cultured in fetal bovine serum and stored in vapor phase nitrogen until confluent. The cells were labeled with GFP by using a recombinant lentivirus p-HIV-Ednrb-EGFP; the transduction was performed at Florida International University Tissue Cell Culture Facility. The cells were then counted and diluted to 100,000 cells per microliters in syringes using 25G needles.

Transgenic Mouse Model and Genotyping

Mice that overexpress endothelin 3 (Edn3) and endothelin receptor-B (Edrnb) were created by upregulating the Keratin 5 promoter on a C57BL/6J background in our laboratory (Garcia et al., 2018).. The mice were genotyped for the *K5-Edn3* gene to see if the transgene was present by clipping a small tail end (approximately 1 cm) from each mouse which served as the DNA source. The tails were digested by placing the tails in a 1 mL Eppendorf tube with 10 μL of proteinase-K (Fisher Scientific GSA, Waltham, MA) and 500 μL of tail lysis buffer, and the tubes were placed in a water bath at 55-60°C overnight. Once digested, DNA was extracted by centrifuging the Eppendorf tubes at 4°C for 10 minutes at 12000 xg. After centrifugation, 450 μL of the supernatant was pipetted and discarded without disturbing the crude DNA pellet. A total of 450 μL of 100% isopropanol was added to the tubes; the pellet was resuspended and centrifuged again as above. The complete supernatant was discarded after centrifugation and the centrifugate was dried for 10 mins. Finally, the DNA was resuspended in 1X TE Buffer (ThermoFisher Scientific, Waltham MA) and refrigerated at 4°C indefinitely. All samples were genotyped for both transgenes: *K5-tTA* and *TRE-Edn3-lacZ*, which make up the *K5-Edn3* transgenic mouse model. The *K5-tTA* gene was amplified using the primers 5’-CCAGGTGGAGTCACAGGATT-3’ and 5’-ACAGAGACTGTGGACCACCC-3’. The *TRE-Edn3-lacZ* was amplified using the primers 5’-AGGCCTGTGCACACTTCTGT-3’ and 5’-TCCTTGTGAAACTGGAGCCT-3’.

Injections and Tumor Measurements

Ten *K5-Edn3* mice and ten wild-type mice 6 to 10 weeks old and weighing between 18 to 22 grams were selected. The mice were subjected to a 2:1 oxygen:isoflurane gas mixture to anesthetize them until movement has ceased and breathing slowed. The mice were then interperitoneally injected with 10 µL of the previously mentioned cell cocktail to inoculate a total of one million B16F10 cells. They were then allowed to re-acclimate and the tumor measurements were begun in the following week. Tumor volumes were measured by using a digital caliper, and the volumes were calculated by measuring their length (the longest dimension) and the width (the shortest dimension). Volumes were obtained by multiplying the length times the square of the width. Measurements were taken twice a week for 30 days after injections. Tumors were measured by restraining the mice and using digital calipers to obtain the dimensions.

Mice Sacrifice and Tissue Fixation

At 30 days after injection, the mice were euthanized with isoflurane and dissected. Tumors and lungs were taken and fixed overnight in a 4% paraformaldehyde solution in phosphate-saline buffer (PBS). The following day, the tissues were rinsed with PBS and placed in a 15% sucrose-PBS solution, which was increased to 30% the next day. Finally, the tissues were embedded in molds made of optimum cutting temperature (OCT) solution (Fisher Scientific, Hampton, NH). These molds were stored at -80°C and sectioned any time after 24 hours of freezing. Sectioning was performed in the Lecia CM3050 S Research Cryostat (Leica Biosystems, Wetzlar, Germany) at a constant temperature of -20°C. The sections were 10 μm thick and were obtained semi-continuously, where 4 sections were obtained and placed on glass slides. Excess OCT was melted away using a heat block set to 37°C for 4 hours post-sectioning and stored away in -80°C until needed.

Immunostaining: Signal Amplification

The tissues were taken to amplify the GFP signal to see if cells from the primary tumor had metastasized. Tissue slides were stained with GFP-1020 primary antibody (Aves Lab Inc., Tigard, OR) in a dilution buffer (1% normal serum, 0.3% Triton X-100, 1% bovine serum albumin powder) at 4°C overnight. The following day, the slides were washed excessively with 1X phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA) and stained with DAPI, 4’6-diaminidino-2-phenylindole (Abcam ab 104139, Cambridge, UK) before covering sections with a cover slip. The slides were visualized using the Leitz DMRB fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Flow Cytometric Analysis

After the mice were sacrificed, primary tumors were minced down into fine particles using a scalpel blade. The tumors were then dissociated using collagenase (Thermo Fisher Scientific, Waltham, MA) for 45 min, followed by the addition of trypsin 0.25% (Thermo Fisher Scientific, Waltham, MA) at 37ºC for 5 min. The cell suspension was stained for endothelin receptor b using an unconjugated rabbit Ednrb antibody (Proteintech Group Inc., Rosemont, IL) at 4ºC for 30 min. The secondary anti-rabbit PerCP/Cy5.5 antibody (Santa Cruz Biotechnology, Dallas, TX) was also stained at identical conditions as the primary antibody. The cell suspensions were analyzed using an Accuri C6 flow cytometer (BD Bioscience, Franklin Lakes, NJ).

RESULTS

The melanoma cell line B16F10 was injected into a group of 10 *K5-Edn3* mice and 10 wild-type mice. Throughout the course of the experiment, tumors were measured twice weekly for a total of 30 days. Detectable tumors formed within 17 days after injection. The aggressiveness in tumor growth was about the same between *K5-Edn3* and wild-type groups until day 27. After this point, *K5-Edn3* tumors grew larger than wild-type tumors and were statistically different on day 30 according to an independent sample t-test with unequal variance ($p<0.05$). At the end of the experiment, the average *K5-Edn3* tumor volume was six times larger than the average wild-type tumor volume.

The results indicate that endothelin 3 does play a role in cancer progression. Figure 1 shows an increase in tumor size, which becomes strongly developed in the final two measurements. The large standard deviation exhibited in the *K5-Edn3* group compared with the wild-type group suggests that Edn3 might be phenotypically plastic, having a range of effects depending on the individual. For example, some *K5-Edn3* mice had tumors that grew larger and faster than others within the same group.

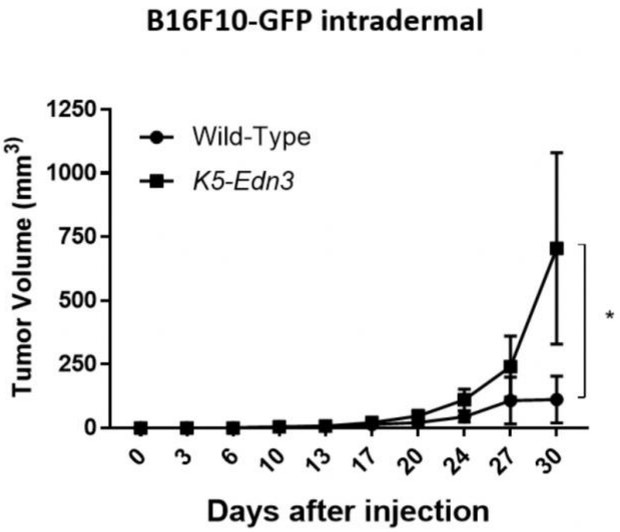

Figure 1. Endothelin-3 overexpression increases aggressiveness of intradermal B16F10-GFP melanoma tumors. Tumor volume of mice that received intradermal injections (n= 11) of B16F10-GFP melanoma cells throughout the course of 30 days. $*p<0.05$

The metastatic potential of *K5-Edn3* tumors was also increased. All lungs collected from the two groups were fixed in 4% PFA and stored in OCT. The tissues were then sectioned at a thickness of 10 µm and placed on glass slides. Because the GFP signal was weak, a signal amplification technique was used to stain the sections with an antibody that binds to GFP. The sections were then analyzed under a fluorescent microscope. A green fluorescence indicated a metastatic cell since the tissue came from the primary tumor injected with GFP-labeled B16F10 cells (Figure 2). No metastasis was seen during the 30-day experiment in wild-type lungs, while metastasis was seen in the *K5-Edn3* lungs. This implicates that Edn3 signaling with Ednrb plays an important role that increases the metastatic potential of melanoma tumors.

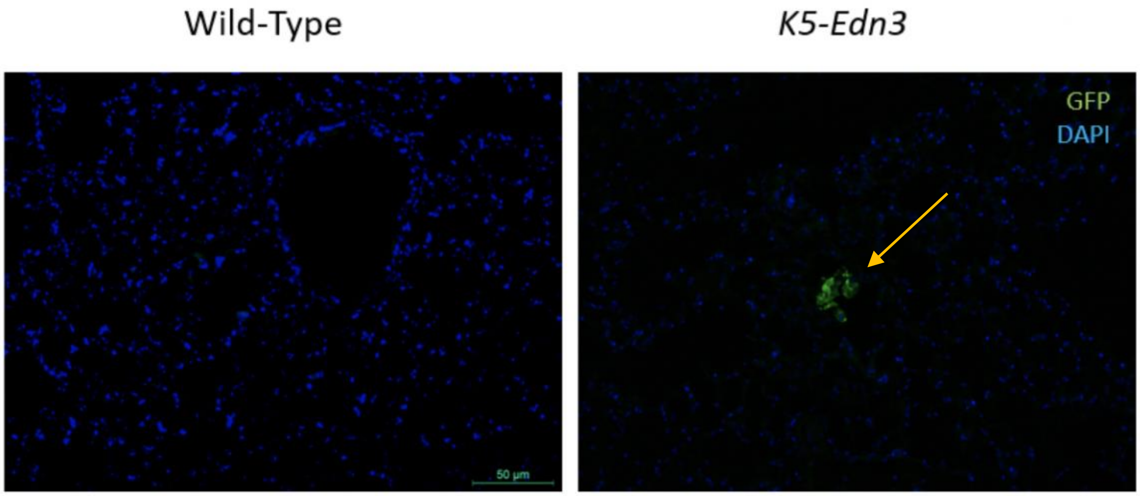

Figure 2. GFP$^+$ cells (green) in lungs of wild-type (left) and *K5-Edn3* (right) mice intradermally injected with B16F10-GFP cells (n=11).

To validate the results, a cell line that is more relevant to human melanoma cells was used. The YUMM 1.7 cell line, provided by Dr. Bosenberg at Yale University, was used to replicate the experiments because they carry three key drive mutations that are commonly found in human melanoma (Meek et al., 2016). As in B16F10 tumors, those from YUMM 1.7 grew at a much faster rate in *K5-Edn3* mice at the last two time periods and had developed tumors that were statistically larger than in the wild-type mice according to a t-test assuming unequal variance ($p<0.05$) (Figure 3). The experiment was cut short at the 21-day period because of the large tumor volume. This further corroborates earlier results and the case that Edn3 plays a role in increasing tumor size.

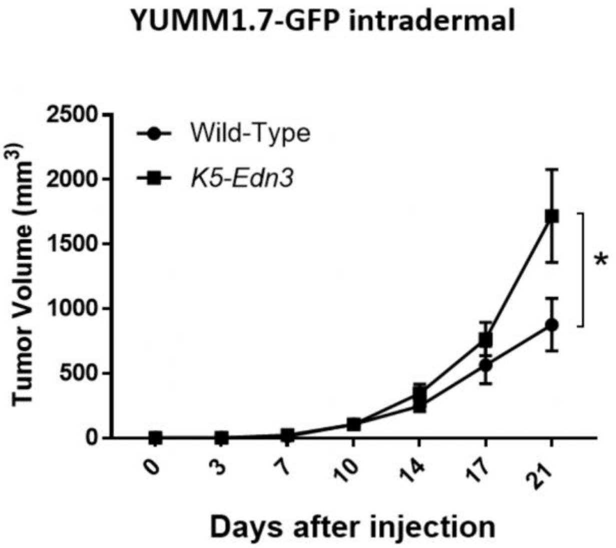

Figure 3. Endothelin-3 overexpression increases aggressiveness of intradermal YUMM1.7-GFP melanoma tumors. Tumor volume of mice that received intradermal injections (n= 20) of YUMM1.7-GFP melanoma cells throughout the course of 21 days increased dramatically after day 10, $*p<0.05$

Of the lung tissues that had been stained to amplify the GFP signal and analyzed under a fluorescent microscope only the lungs of the *K5-Edn3* mice had visible metastasis (Figure 4); none was found in the lungs of the wild-type mice. These results mimic those of the B16F10 tumors and additionally suggest that Edn3 signaling plays a significant role of metastasis in melanoma.

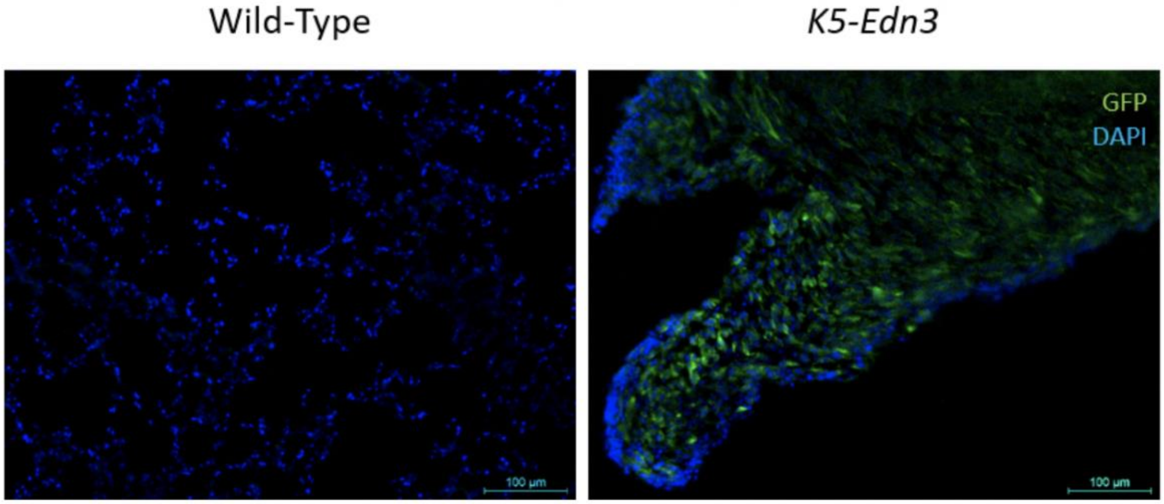

Figure 4. GFP$^+$ cells (green) in lungs of wild-type (left) and *K5-Edn3* mice intradermally injected with YUMM1.7-GFP cells at right (n=20). Metastatic tissue is clearly developed in K5 mice.

To investigate which subpopulation of cells in the tumor microenvironment is responding to Edn3, a flow cytometry procedure using GFP and Ednrb staining was used. Tumorigenic cells in YUMM1.7-GFP were distinguishable from stromal cells through the GFP signal while cells expressing Ednrb were labeled through antibody staining. Figure 5A shows the cells expressing GFP on the top row. The cells expressing Ednrb are found on the right column of each plot. Those that colocalize with GFP are in the upper right quadrant where tumorigenic cells that express Ednrb are found. This subpopulation of cells are tumorigenic cells that are also responsive to Edn3. These compose 6.78% of wild-type tumors and 7.03% of *K5-Edn3* tumors. However, an independent t-test assuming equal variance showed that there was no statistical difference between the expression of Endrb in tumorigenic cells between the wild-type and transgenic mice (Figure 5B).

Overall, a large subpopulation of cells in both wild-type and *K5-Edn3* tumors were in the bottom right quadrant which are GFP negative and Ednrb positive. This means that 24.2% of cells in the wild-type tumors and 17.2% of *K5-Edn3* tumors are stromal cells that can conduce Edn3 signaling. This suggests that melanoma aggression and metastatic potential is mediated through the interaction of both tumorigenic and stromal cells within the tumor microenvironment.

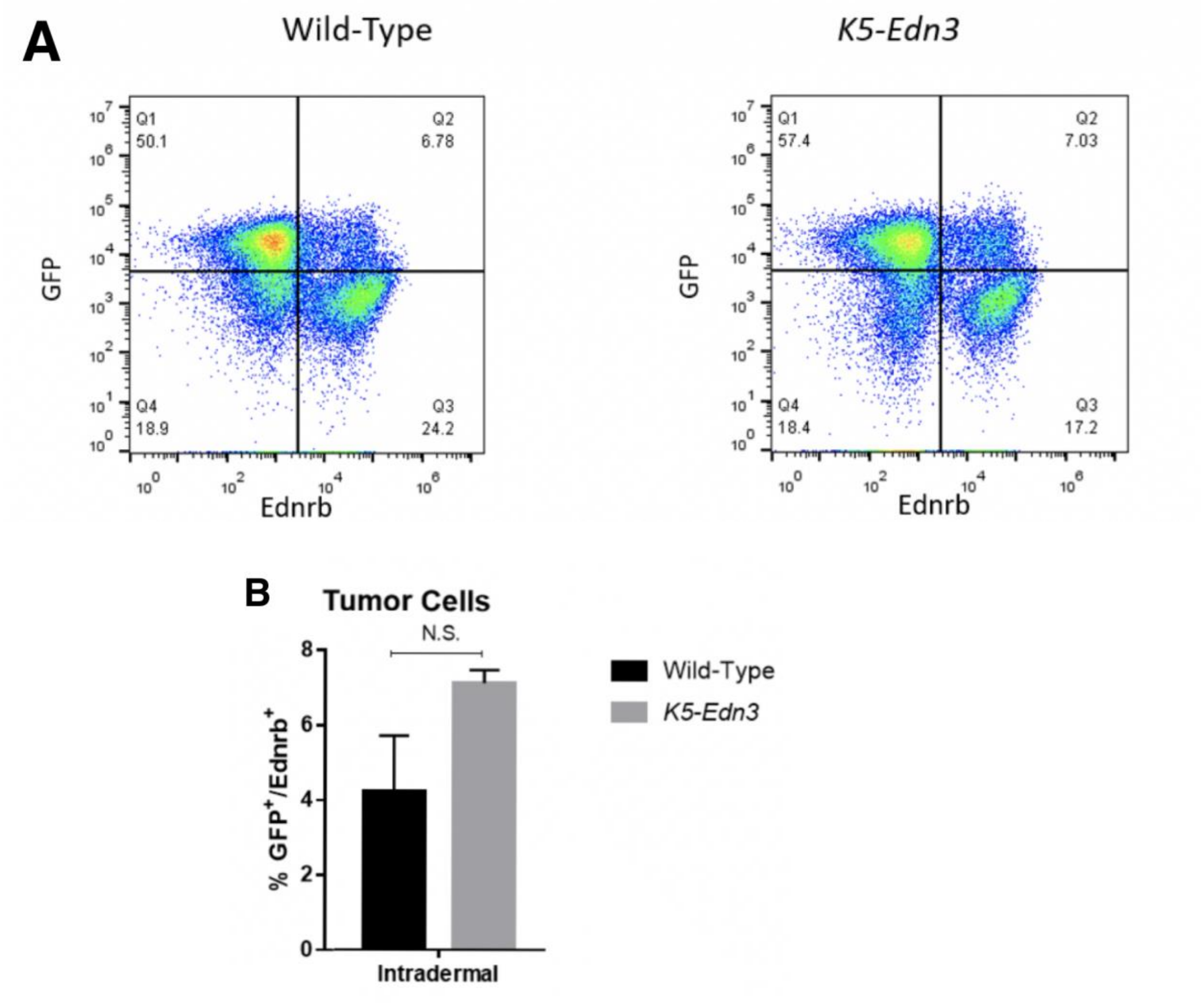

Figure 5. Ednrb is expressed in tumorigenic and stromal cells. **A.** Flow cytometry plots of GFP$^+$/Ednrb$^+$ melanoma cells of intradermal YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice.
B. Comparative percentages of GFP-positive cells detected using flow cytometric analysis (n=3/group).

DISCUSSION

The role of Edn3/Ednrb signaling has been used previously as a marker for aggressive melanomas (Demunter et al., 2001), though with some opposition (Kikuchi et al., 1996). However, this study provides support to the body of work investigating the role of endothelin signaling in increasing tumor growth and metastatic potential in melanomas. In fact, the results show that when Edn3 is over-expressed, melanoma tumors grow at a faster rate and have larger volumes, and more rapid rates of metastasis. These results have also been corroborated in both cell lines used in this study (Meeth et al., 2016; Melnikova et al., 2004). The YUMM1.7 cell line carries an important mutation in the *Braf* gene (Meek et al., 2016), which is commonly found in about 50% of melanoma cases (Akbani et al., 2015) while the B16F10 cell line is considered to be wild-type for the *Braf* gene (Melnikova et al., 2004). This experimentation thus suggests that Edn3 plays a role independent of the *Braf* mutation and affects a gamut of melanomas. Recent work has shown that inhibition of mutated *Braf* melanomas upregulate Ednrb and Edn1 (Asundi et al., 2014; Smith et al., 2017), and suggests that future combinatory therapies could be more effective by targeting both endothelin signaling and *Braf* mutations.

Although the role of Edn3/Ednrb signaling has been debated in melanoma progression, studies have yet to expand on the identity of cells in the microenvironment that are responsive to endothelin. Studies have shown that melanoma cells express Edn3 (Tang et al., 2008) and Edn1 both *in vivo* and *in vitro* (Chiriboga et al., 2016). The expression of Edn1 has been associated with increased angiogenesis in melanomas (Spinella et al., 2014) and is expressed in intratumoral macrophages as well (Chiriboga et al., 2016). Although Ednrb and Edn3 are a marker for aggressive melanomas (Demunter et al., 2001), the subpopulation of cells mediating the signaling pathway has not been characterized. This study helped elucidate this problem by addressing which cells in the tumor microenvironment expressed Ednrb. The tumorigenic cells from the YUMM1.7 injections were labeled with GFP, and the flow cytometry data showed that indeed, a small percentage of tumorigenic cells were Ednrb$^+$/GFP$^+$ in both *K5-Edn3* and wild-type tumors. The transgenic mice and wild-type mice did not show a statistical difference in tumorigenic cells expressing Ednrb. However, both groups had non-tumorigenic cells that were expressing Ednrb, which indicates that these cells take part in endothelin signaling. This is suggestive that stromal cells might be participating in the role of Edn3/Ednrb signaling in aggressive melanoma tumors. Similarly, Binder et al.,(2009) showed that stromal cells lacking Endrb in rats developed breast cancer tumors with impaired growth, largely due to an immune driven response.

The tumor microenvironment is composed of a rich diversity of cells. Tirosh et al.,(2016) investigated the identity of different cell populations in human metastatic melanomas; the majority of the stromal cells present consisted of T cells – including CD8, CD4 and Treg – and B lymphocytes. Although my current study did not investigate the exact composition of stromal cells, future experimentation should look at the possible role of Edn3 signaling in immunogenic cells from the tumor microenvironment. The role of the microenvironment has been studied previously in different cancers, but none has been done on melanoma. For example, the blockade of Ednra and Ednrb in colorectal cancer cells has been implicated in a reduction of tumor aggression (Haque et al., 2013). Perhaps Edn3 signaling plays a role in immunosuppression in the tumor microenvironment that might allow tumors to grow at faster rates and metastasize as seen in the results of this study.

A different mechanism of action that Edn3 might play in the promotion of tumor aggression and increased metastatic potential is through the angiogenic properties that the family of endothelins have. Previous studies have shown that upregulation of Edn1 was associated with higher vasculature in ovarian cancer (Salani, et al., 2000). It is also known that endothelin signaling increases the recruitment of endothelial cells to create new blood vessels (Huang et al., 2013). However, while the role of Edn3 in angiogenesis has been studied in ovarian cancers (Buckanovich et al., 2008), its role in melanoma remains unexamined. Future work should elucidate the role that endothelial cells play with Edn3 in melanoma angiogenesis. Perhaps by increasing the recruitment of endothelial cells to the tumor microenvironment by Edn3/Endrb signaling might be a key player in providing oxygen and nutrients to the primary tumor through vascular growth. This could in large explain why the tumors of the *K5-Edn3* mice were able to grow to without restraint unlike the tumors of the wild-type mice.

The present study supports the body of work implicating the diverse role of endothelin signaling in melanoma progression. Previous research has shown that Ednrb and Edn3 can be used as markers for aggressive melanomas in humans, but have not investigated the role of Edn3/Ednrb signaling in its progressions. I show that a tumor microenvironment rich in Edn3 increases tumor volume, aggression, and the propensity for primary tumor cells to disseminate and metastasize to distant organs. The results affirm that not only are tumor cells responsive to Edn3, but the stromal cells present in the microenvironment are responsive as well. Together, stromal and tumor cells may both be contributing to the growth and expansion of primary melanoma tumors.

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