

**REGULATION OF TUMOR VASCULAR PERFUSION AND
PERMEABILITY BY ENDOTHELIAL NCK**

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

	Page
ABSTRACT.....	1
ACKNOWLEDGMENTS	2
CHAPTER	
I. INTRODUCTION	3
Importance of tumor vasculature in tumor progression and metastasis.....	3
Current antiangiogenic therapies	3
Nck role in vascular biology.....	4
Objective.....	4
II. METHODS	6
Animals	6
Tamoxifen treatment.....	6
Western blot.....	6
Cell culture.....	7
Tumors	7
Tumor growth	7
Euthanasia & necropsy	7
Quantification of tumor vasculature	8
Statistics	8
III. RESULTS	9
Tumor growth in vivo	9
Tumor size ex vivo.....	10
Western blot analysis	12
Quantification of tumor vasculature	12
IV. CONCLUSION.....	14
Preliminary data	14
Future direction.....	14
REFERENCES	15
APPENDIX.....	18

ABSTRACT

Regulation of Tumor Vascular Perfusion and Permeability by Endothelial Nck

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Tumor progression and metastasis is increased by the unique vasculature found in tumors which can be characterized as having low perfusion and increased permeability. The role of Nck proteins is to control actin cytoskeletal remodeling in response to signaling by tyrosine phosphorylation, which is important in developmental angiogenesis. The role of Nck in tumor angiogenesis still remains unknown. Our goal is to determine role of endothelial Nck in tumor angiogenesis and cancer progression. In order to do this, we used a syngeneic melanoma tumor model in combination with inducible, endothelial-specific deletion of Nck gene in mice. Tumor growth rate, tumor size at sacrifice were compared in both mice with and without endothelial Nck. Tumor growth rates and excised tumor size were found to be smaller in the knockout mice, which suggests that endothelial Nck facilitates tumor growth. This study aims to have an important positive impact on the subsequent development of new strategies to improve functionality in the tumor vasculature, and therefore, the efficacy of anticancer therapy.

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CHAPTER I

INTRODUCTION

Importance of tumor vasculature in cancer progression and metastasis

The function of tissues in our body is dependent on a mature and organized vascular network. Therefore, angiogenesis, the creation of new blood vessels, is critical for healthy human development (Goel, 2012). In healthy tissue, angiogenesis is a highly regulated process (Dubrac, 2016). It is typically induced in hypoxic tissues by the production of proangiogenic factors, such as vascular endothelial growth factor (VEGF), which is important for endothelial cell migration and survival. Angiogenesis is also dependent on microenvironmental influences like extracellular matrix, cell-cell interactions, and other soluble factors (Goel, 2012). In addition, hypoxia which seems to be characteristic of the tumor microenvironment results in an increased production of proangiogenic factors (Semenza 2010). The abundance of these factors drives uncontrolled angiogenesis known as pathoangiogenesis as well as the production of a microvascular network with structural and functional abnormalities (Jain 2005; Goel, 2012). These abnormalities further modify the microenvironment. As a result both the abnormal vascular network and microenvironment of tumors lead to the progression of tumor growth and metastasis (Goel, 2012).

Current antiangiogenic therapies

Antiangiogenic therapies, such as anti-VEGF therapy have been used to treat cancer. However, the results have failed to show significant response rates or prolongation of survival in cancer patients (Jain et al. 2006; Giantonio et al. 2007). In addition, anti-VEGF therapy has been used in conjunction with other anti-cancer treatments such as chemotherapy and have shown

better outcomes than either treatment alone (Goel, 2012). While this combined approach has produced good initial outcomes, there is a lot of drawbacks. For one, these anti-angiogenic treatments have serious side effects which are hemorrhage and venous thromboembolism (Hapani, 2010; Nalluri, 2008) a meta-analysis; Risk of venous thromboembolism with the angiogenesis inhibitor bevacizumab in cancer patients: a meta-analysis.) Secondly, the incidence of tumor metastasis increases with this approach (Graeber, 1996). The benefits of anti-VEGF are short lived and are followed by tumor growth due to a response of adaptive resistance or preexisting non-responsiveness (Bergers, 2008). Therefore, there is the need for alternative targets to normalize tumor blood vessels in order to increase the effectiveness of anticancer therapies.

Nck role in vascular biology

The family of Nck adaptor proteins (Nck 1/ α and Nck 2/ β) are composed of three N-terminal Src homology 3 (SH3) domains and one C-terminal SH2 domain and have overlapping noncatalytic functions (Chaki et al., 2015). Nck proteins link tyrosine phosphorylation with downstream effectors that regulate the cytoskeleton. These modular adaptors are required for the development of mesoderm-derived embryonic structures (Bladt et al., 2003) as well as EndMT and cardiovascular development (Clouthier et al., 2015). Our lab, among others, have shown that Nck plays an important role in cytoskeletal remodeling (Rivera et al., 2004; Rivera et al., 2009), breast cancer progression (Morris et al., 2017), and sprouting angiogenesis (Chaki et al., 2013; Chaki et al., 2015).

Objective

In this application the objective is to determine the role of endothelial Nck in tumor angiogenesis and cancer progression. Our central hypothesis is that endothelial Nck facilitates

tumor growth and metastasis by prompting the development of a structurally disorganized, leaky, and poorly perfused tumor vasculature. This hypothesis was formulated based on existing literature demonstrating that Nck plays a role in actin remodeling (Rivera et al., 2004; Rivera et al., 2009) and that this remodeling plays a role in the abnormal endothelial response that leads to pathological angiogenesis and the destabilization of blood vessels (Weis and Cheresh, 2011; Chaki et al., 2015). The rationale for this research is that identification of endothelial Nck as a critical target to help normalize tumor vasculature will decrease tumor growth and metastasis and increase treatment efficacy.

CHAPTER II

METHODS

Animals

We established a colony of Nck^{ECKO} (*Cdh5Cre*^{ERT2}; *Nck1*^{-/-}; *Nck2*^{loxP/loxP}) mice in C57BL/6J background by backcrossing (5 generations) mixed breeding founders (kindly provided by Anne Eichmann)⁴⁷ with C57BL/6J mice obtained from the Jackson Laboratory. More information can be found in provided in Appendix I. This mouse line enables the inducible, Cre-mediated excision of Nck2 specifically in the endothelium. Nck appears to be dispensable for the quiescent/mature adult vasculature since mice with postnatal Nck deletion maintained normal weight and did not exhibit an overt phenotype.

Table 1: Animal groups. Description of genotype, sex, and number of mice in each group.

Group	Genotype	Female	Male	Total
iEC-NcK(KO)	<i>Nck1</i> ^{-/-} ; <i>Nck2</i> ^{f/f} ; Cre ⁺	n=9	n=7	n=16
Control	<i>Nck1</i> ^{-/-} ; <i>Nck2</i> ^{f/f} ; Cre ⁻	n=6	n=6	n=12

Tamoxifen treatment

Eight week-old NckECKO mice received 5 intraperitoneal injections of tamoxifen (75mg/kg) dissolved in corn oil, every other day, in a time period of 10 days to induce Cre activity. A step by step description of the procedure is provided in Appendix II.

Western blot

Levels of Nck in the tumor endothelium (CD31-expressing cells) were determined by western blotting performed on extracts of endothelial cells isolated from lungs (ML, 2009)

Cell culture

B16-F10 melanoma cells were removed from liquid nitrogen storage and plated in HG DMEM supplemented with 10% heat-inactivated FBS (Gemini Bio) and 1% pen/strep (Invitrogen) 6 days before tumor cell injection. Cells were passaged one to two days before injection (Tian et al., 2017). A step by step description of this procedure is provided in Appendix III.

Tumors

A subcutaneous syngeneic tumor model of B16-F10 melanoma cells, widely used in studies of tumor angiogenesis (Park et al., 2016; Steri et al., 2014; Tsai et al., 2011) was utilized. Three days after the last tamoxifen injection, mice were anesthetized with isoflurane for subcutaneous allograft injection of 2.5×10^5 cultured B16F10 cells in 100ul PBS on each flank (Park et al., 2016). A step by step description of the procedure is provided in Appendix IV.

Tumor growth

Tumors were measured using a digimatic caliper daily starting on day 8 post allograft transplantation. Tumor volume was calculated using the following formula: (Long side x (short side 2) x .5 (; Tsai et al., 2011).

Euthanasia & Necropsy

Euthanasia was carried out by CO₂ asphyxiation 18 days after tumor cell injection. Tumors were excised and measured using a digimatic caliper. In addition, the lungs, liver and spleen were excised in order to determine extent of metastasis (Tsai et al., 2011). A step by step description of the procedure is provided in Appendix V. Ex vivo tumors were divided in halves for cryopreservation and paraffin embedding for further pathological and immunohistochemical analyses.

Quantification of the tumor vasculature

Due to restrictions imposed by the Covid-19 pandemic, determinations of tumor vascular density were halted. These experiments will be resumed in the near future and quantitative analysis of tumor vascularization will be performed as follows. Briefly, the vascular density in tumor sections will be determined by immunohistochemistry using an anti-CD31 antibody (Abcam, AB28364) to visualize the endothelium (Maione and Giraudo, 2015; Stockmann et al., 2008) as well as H&E staining. A step by step description of the immunohistochemistry procedure that will be followed is provided in Appendix VI.

Imaging of immunohistochemistry will be done using Zeiss LSM 780 NLO Multiphoton Microscope with laser lines of 488 and 543 nm as well as an objective of Plan-Apo 20X/0.8. Tumor vasculature will then be quantified using Image J, a Java application used to analyze images. In addition, the surface area of blood vessels will be quantified using the surface area covered by antiCD31 and comparing it to that of the rest of the tumor cells (Maione and Giraudo, 2015).

Statistics

Minitab and Excel were used to conduct statistical analysis. Normal distribution was confirmed by the Kolmogorov-Smirnov test. The Student's t test was used to evaluate differences between means. $P < 0.05$ was considered statistically significant. A Grubbs outlier test on Minitab will also be performed.

CHAPTER III

RESULTS

Tumor growth in vivo

Measurements that were taken in vivo were used to calculate the volumes of individual tumors. The sums of the volumes (right and left) were found per each mouse, and a mean for all of these measurements was taken. These daily mean values were plotted to show a continuous growth curve (Fig. 1). The results suggest that overall mice lacking endothelial Nck experience slower tumor growth compared to our control group, which had both Nck genes.

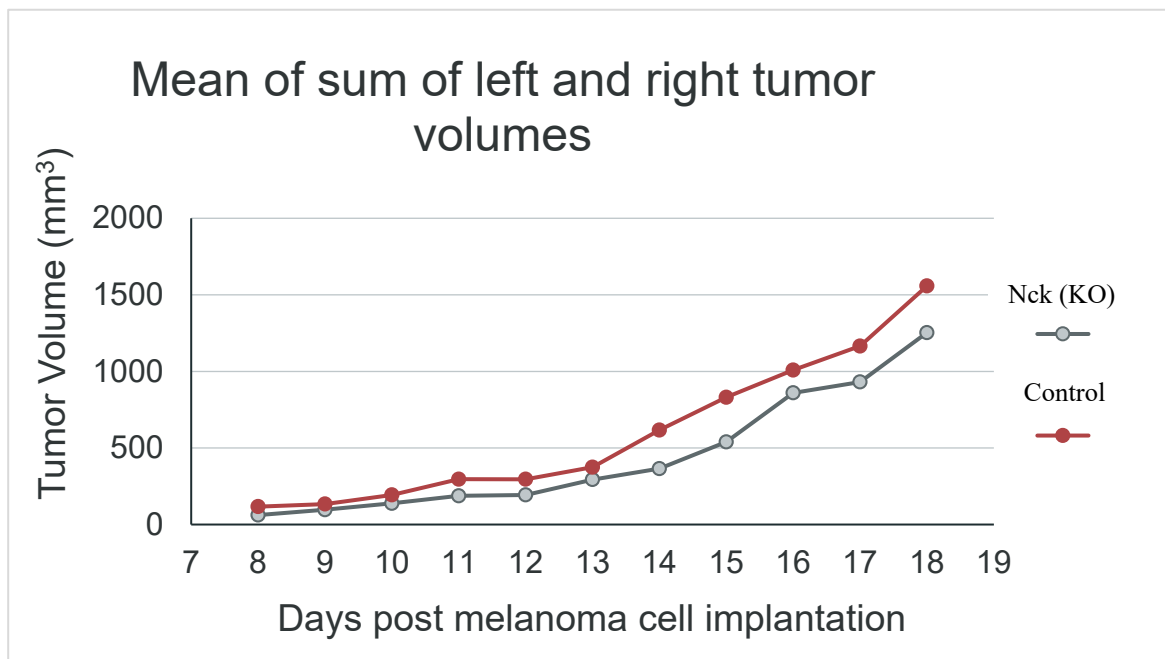


Figure 1: Growth curve. Average in vivo tumor volumes were plotted daily. The graph shows a decrease growth in Nck (KO) mice.

Tumor size ex vivo

Once these mice were sacrificed, the measurements of the excised tumors were taken. Volumes of these measurements were calculated and the sums (right and left) were found per each mouse. These sums were then used to create a box plot (Fig. 2), which once again suggests that mice lacking endothelial Nck have decreased ex vivo tumor volume. In addition, statistical analysis showed that the difference between these two groups was statistically significant with a p value less than .05.

Furthermore, these measurements were discriminated by sex. The boxplot (Fig. 3) showed that males and females seem to display differential tumor growth in response to endothelial Nck deletion.

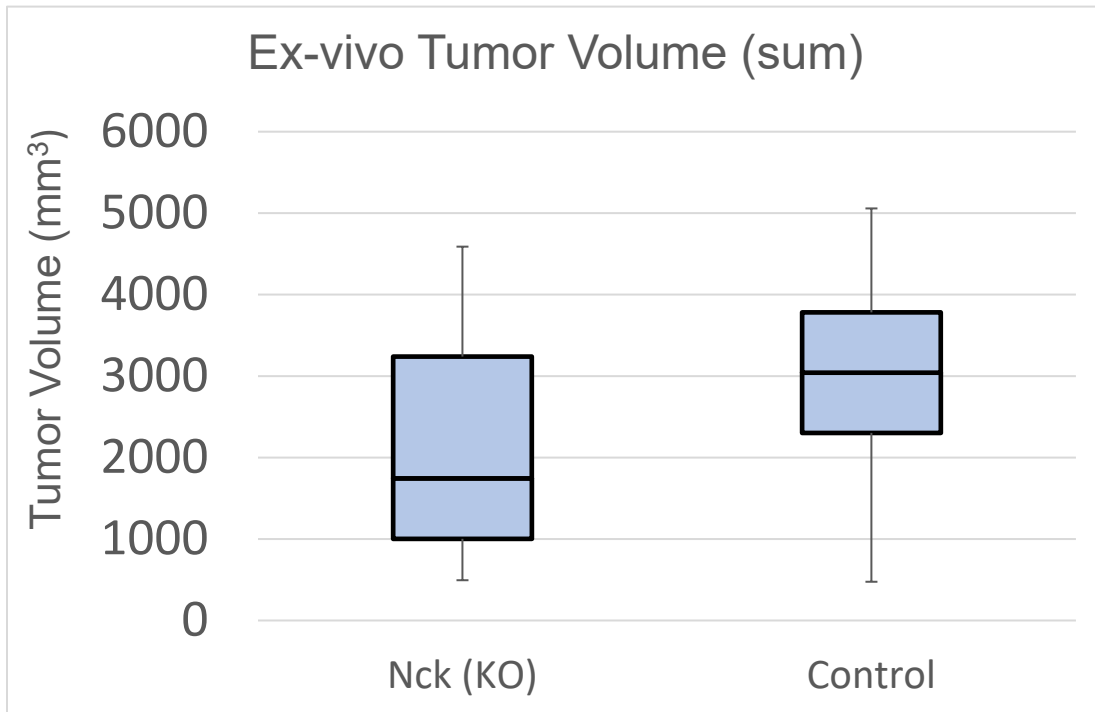


Figure 2: Ex-vivo tumor volume box plot. Average ex-vivo tumor volumes were plotted. This plot shows the median of the control group being higher than the Nck (KO) group

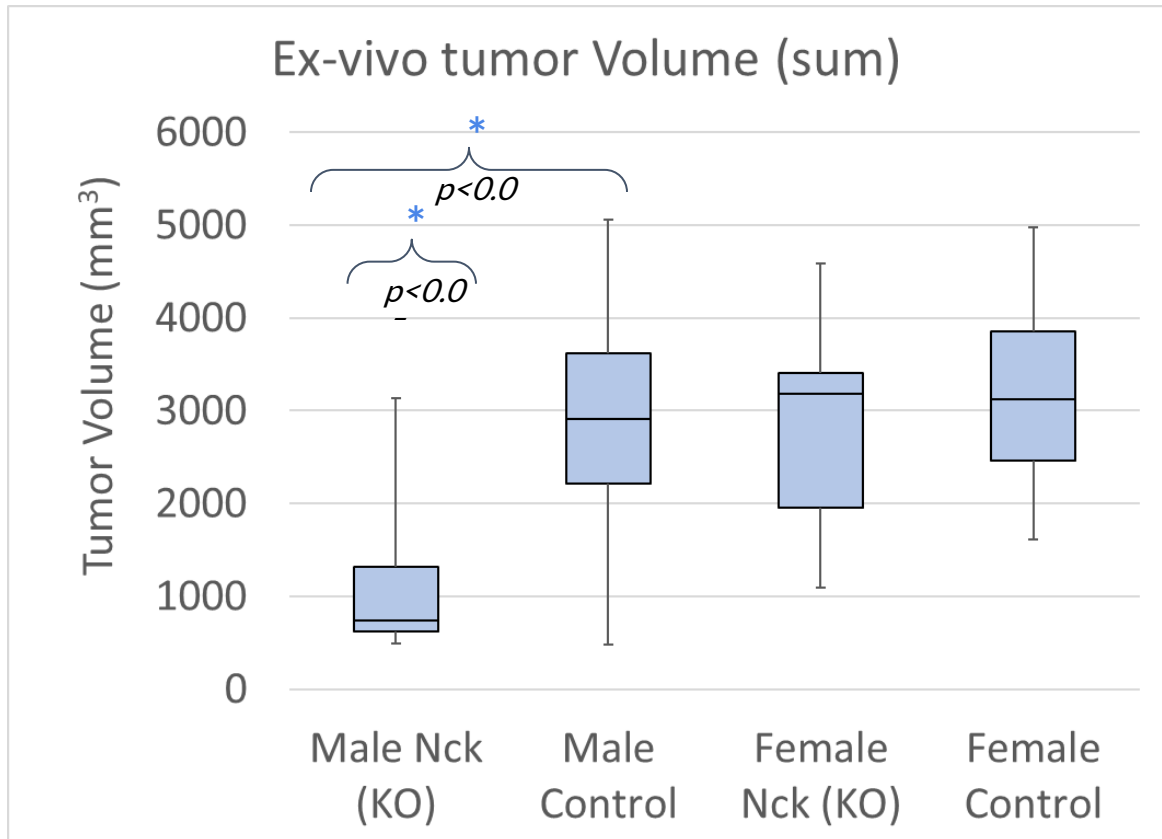


Figure 3: Ex-vivo tumor volume by gender box plot. Average ex-vivo tumor volumes were plotted by sex. This plot shows the median of the male control group being higher than the median of the male Nck(KO) group.

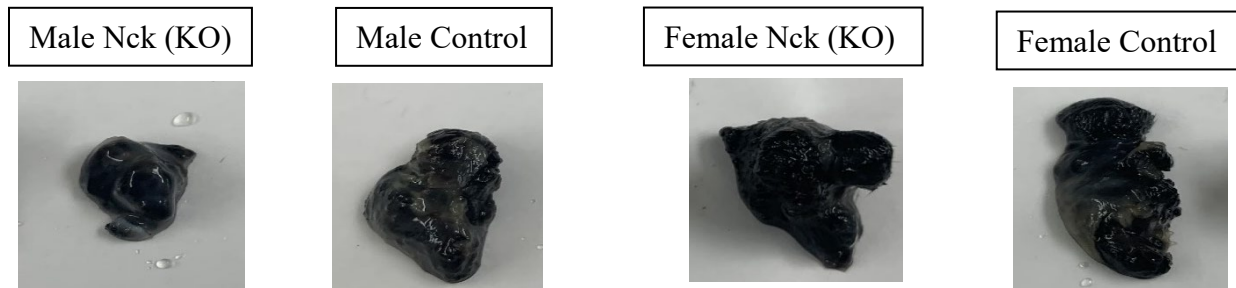


Figure 4: Pictures of extracted tumors. Each picture is a tumor that is representative of each group.

Western blot analysis

In order to ensure that deletion of endothelial Nck took place, a western blot analysis was used (Fig. 4). Expression levels of Ve-cadherin, an endothelial specific marker and Nck were tested in two conditions. The first condition applied was tamoxifen and the second was corn oil, a control condition. The decreased levels of Nck shown when tamoxifen was present in comparison to the normal levels shown in the control group, showed the effective deletion of Nck. In addition, the expression of Ve-cadherin in both conditions, showed that this gene is not being expressed specifically in the endothelium.

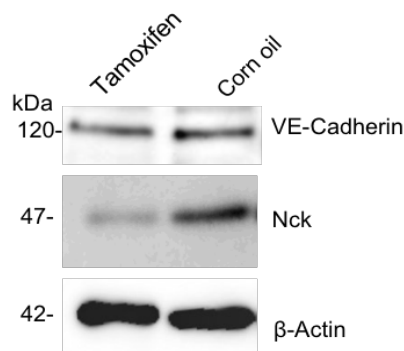


Figure 5: Western blot analysis. Nck and VE-Cadherin markers were subjected to western blot analysis with tamoxifen being present in one group and corn oil in the control group. B-Actin served as a loading control.

Quantification of the tumor vasculature

Due to restrictions imposed by the Covid-19 pandemic, determinations of tumor vascular density were halted. These experiments will be resumed in the near future and quantitative analysis of tumor vascularization will be conducted using anti-CD31 antibody (Abcam, AB28364) to visualize the endothelium. Anti-CD31 was optimized Figure 6 shows the results of immunohistochemistry optimization of antiCD31 on tumor tissue. This antibody fluoresces red and allows for the visualization of the endothelium, which will allow the quantification of tumor

vascular density with Image J. In addition, H&E staining will also be conducted to measure the vascular density as shown in **Figure 7**.

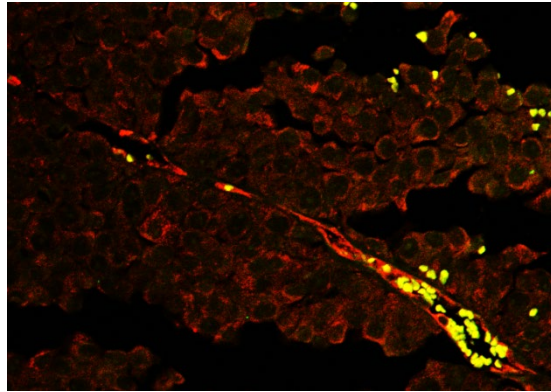


Figure 6: Immunohistochemistry. Optimization of anti-CD31, which allows for the visualization of the endothelium that lines blood vessels, as shown in red.

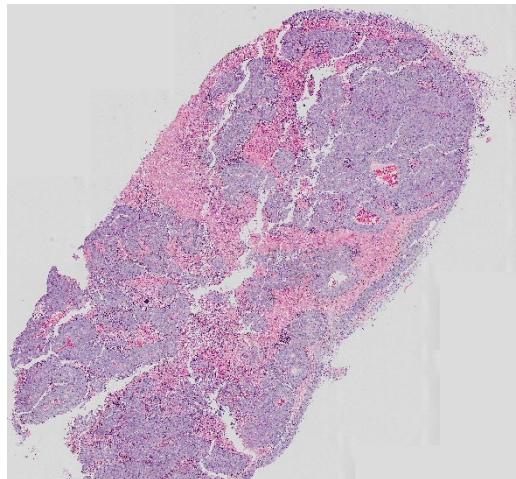


Figure 7: H&E staining. Allows for the distinction of certain cell types like blood cells shown in dark pink, and therefore quantification of tumor vascular density.

CHAPTER IV

CONCLUSION

Preliminary data

The growth curve of the in vivo tumors showed that overall mice lacking endothelial Nck experience slower tumor growth compared to the control group. Additionally, the box plots of the excised tumor volumes showed that mice lacking endothelial Nck have decreased ex vivo tumor volume. In conclusion, the preliminary data shows that deletion of endothelial Nck results in decreased tumor growth.

Future direction

Analysis of density of vasculature in tumors will be done using immunohistochemistry and H&E staining will be done. To ascertain functionality of tumor vasculature, vessel perfusion will be quantified by retroorbital injection of FITC-lectin (Maione and Giraudo, 2015). Tumor vascular permeability will be quantified using retroorbital injection of FITC-conjugated 40kDa dextran (Maione and Giraudo, 2015). Additional tumor models will also be implemented.

REFERENCES

- Bladt, F., E. Aippersbach, S. Gelkop, G.A. Strasser, P. Nash, A. Tafuri, F.B. Gertler, and T. Pawson. 2003. The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network. *Molecular and Cellular Biology*. 23:4586-4597.
- Bergers, Gabriele, and Douglas Hanahan. "Modes of resistance to anti-angiogenic therapy." *Nature reviews. Cancer* vol. 8,8 (2008): 592-603. doi:10.1038/nrc2442
- Clouthier, D.L., C.N. Harris, R.A. Harris, C.E. Martin, M.C. Puri, and N. Jones. 2015. Requisite role for Nck adaptors in cardiovascular development, endothelial-to-mesenchymal transition, and directed cell migration. *Mol Cell Biol*. 35:1573-1587.
- Chaki, S.P., R. Barhoumi, M.E. Berginski, H. Sreenivasappa, A. Trache, S.M. Gomez, and G.M. Rivera. 2013. Nck enables directional cell migration through the coordination of polarized membrane protrusion with adhesion dynamics. *J Cell Sci*. 126:1637-1649.
- Chaki, S.P., R. Barhoumi, and G.M. Rivera. 2015. Actin remodeling by Nck regulates endothelial lumen formation. *Mol Biol Cell*. 26:3047-3060.
- Dubrac, A., G. Genet, R. Ola, F. Zhang, L. Pibouin-Fragner, J. Han, J. Zhang, J.L. Thomas, A. Chedotal, M.A. Schwartz, and A. Eichmann. 2016. Targeting NCK-Mediated Endothelial Cell Front-Rear Polarity Inhibits Neovascularization. *Circulation*. 133:409-421.
- Fehrenbach, M. L., Cao, G., Williams, J. T., Finklestein, J. M., & Delisser, H. M. (2009). Isolation of murine lung endothelial cells. *American journal of physiology. Lung cellular and molecular physiology*, 296(6), L1096–L1103. <https://doi.org/10.1152/ajplung.90613.2008>
- GiantonioBJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson AB 3rd. 2007. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: Results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol*
- Goel, S., A.H. Wong, and R.K. Jain. 2012. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harbor perspectives in medicine*. 2:a006486.

- Graeber, T., Osmanian, C., Jacks, T. et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. *Nature* 379, 88–91 (1996). <https://doi-org.srv-proxy1.library.tamu.edu/10.1038/379088a0>
- Hapani S, Sher A, Chu D, Wu S (2010) Increased risk of serious hemorrhage with bevacizumab in cancer patients: a meta-analysis. *Oncology* 79:27–38. doi:10.1159/000314980
- Jain RK. 2005a. Antiangiogenic therapy for cancer: Current and emerging concepts. *Oncology* (Williston Park) (T., 1996)
- JainRK, DudaDG, Clark JW, Loeffler JS.2006. Lessonsfrom phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol*
- Maes, H., A. Kuchnio, A. Peric, S. Moens, K. Nys, K. De Bock, A. Quaegebeur, S. Schoors, M. Georgiadou, J. Wouters, S. Vinckier, H. Vankelecom, M. Garmyn, A.C. Vion, F. Radtke, C. Boulanger, H. Gerhardt, E. Dejana, M. Dewerchin, B. Ghesquiere, W. Annaert, P. Agostinis, and P. Carmeliet. 2014. Tumor vessel normalization by chloroquine independent of autophagy. *Cancer Cell*. 26:190-206.
- Maione, F., and E. Giraud. 2015. Tumor angiogenesis: methods to analyze tumor vasculature and vessel normalization in mouse models of cancer. *Methods Mol Biol*. 1267:349-365.
- Morris, D.C., J.L. Popp, L.K. Tang, H.C. Gibbs, E. Schmitt, S.P. Chaki, B.C. Bywaters, A.T. Yeh, W.W. Porter, R.C. Burghardt, R. Barhoumi, and G.M. Rivera. 2017. Nck deficiency is associated with delayed breast carcinoma progression and reduced metastasis. *Mol Biol Cell*. 28:3500-3516.
- Nalluri SR, Chu D, Keresztes R et al (2008) Risk of venous thromboembolism with the angiogenesis inhibitor bevacizumab in cancer patients: a meta-analysis. *JAMA* 300:2277–2285. doi:10.1001/jama.2008.656
- Park, J.S., I.K. Kim, S. Han, I. Park, C. Kim, J. Bae, S.J. Oh, S. Lee, J.H. Kim, D.C. Woo, Y. He, H.G. Augustin, I. Kim, D. Lee, and G.Y. Koh. 2016. Normalization of Tumor Vessels by Tie2 Activation and Ang2 Inhibition Enhances Drug Delivery and Produces a Favorable Tumor Microenvironment. *Cancer Cell*. 30:953-967.
- Rivera, G.M., C.A. Briceno, F. Takeshima, S.B. Snapper, and B.J. Mayer. 2004. Inducible clustering of membrane-targeted SH3 domains of the adaptor protein Nck triggers localized actin polymerization. *Curr Biol*. 14:11-22.

- Rivera, G.M., D. Vasilescu, V. Papayannopoulos, W.A. Lim, and B.J. Mayer. 2009. A reciprocal interdependence between Nck and PI(4,5)P(2) promotes localized N-WASp-mediated actin polymerization in living cells. *Mol Cell*. 36:525-535.
- Semenza GL. 2010. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene*
- Steri, V., T.S. Ellison, A.M. Gontarczyk, K. Weilbaeher, J.G. Schneider, D. Edwards, M. Fruttiger, K.M. Hodivala-Dilke, and S.D. Robinson. 2014. Acute depletion of endothelial beta3-integrin transiently inhibits tumor growth and angiogenesis in mice. *Circulation research*. 114:79-91.
- Stockmann, C., A. Doedens, A. Weidemann, N. Zhang, N. Takeda, J.I. Greenberg, D.A. Cheresh, and R.S. Johnson. 2008. Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature*. 456:814-818.
- Tian, L., A. Goldstein, H. Wang, H. Ching Lo, I. Sun Kim, T. Welte, K. Sheng, L.E. Dobrolecki, X. Zhang, N. Putluri, T.L. Phung, S.A. Mani, F. Stossi, A. Sreekumar, M.A. Mancini, W.K. Decker, C. Zong, M.T. Lewis, and X.H. Zhang. 2017. Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming. *Nature*. 544:250-254.
- Tsai, M.S., C.C. Chang, M.L. Kuo, and Y.T. Wu. 2011. Vascular endothelial growth factor-A and changes in a tumor-bearing mouse model with Lewis lung cancer. *Oncol Lett*. 2:1143-1147.
- Weis, S.M., and D.A. Cheresh. 2011. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nature medicine*. 17:1359-1370.

APPENDIX I

TAMOXIFEN PREPARATION

Tamoxifen Preparation Day 1:

Materials:

- Microcentrifuge tubes (VWR, cat# 89000-044)
- Tamoxifen (Cayman Chemicals, cat#13258)
- Corn oil

Tamoxifen is a potential carcinogen- be sure to wear appropriate PPE while handling: lab coat & mask

Procedure:

1. Aliquot 100 uL filter-sterilized corn oil per mouse to be injected into a 1.5 mL microcentrifuge tube. Note: Prepare an extra dose for every 3 mice.
2. Weigh 2 mg of tamoxifen per mouse to be injected and add to corn oil
3. Briefly vortex until tamoxifen is distributed equally throughout solution
4. Wrap microcentrifuge tube in aluminum foil (tamoxifen is light sensitive)
5. Incubate tamoxifen solution with shaking at 37°C overnight

Tamoxifen Preparation Day 2:

Materials:

Insulin syringes (True plus 29 gauge .3cc ½” insulin needles, cat#56151-1711-01)

Procedure:

1. Remove tamoxifen from the incubator shaker, and examine it in order to verify that it is fully dissolved. If not fully dissolved, place in a hot plate at 55°C and vortex every 5-10 minutes, until fully dissolved
2. Once tamoxifen is fully dissolved, pipette 130 uL of solution into an insulin syringe. Note: 100uL doses are required; extra is used to account for pipetting error

APPENDIX II

CELL CULTURING

Day One: Thawing and plating frozen cells

Materials:

- 10cm tissue culture dishes
- Complete media (High glucose DMEM with 10% FBS and 1% v/v penicillin streptomycin)
- DPBS
- DMEM
- Trypsin/EDTA (.05mg/mL)
- 50 mL tube
- Hemocytometer

Procedure:

1. One week before implantation, remove approximately one-third the necessary amount of cells from liquid nitrogen (ex: 2 mice require 1×10^6 cells, so remove 30×10^4 cells)
2. Thaw cells in 37°C water bath and resuspend in complete media
3. Aliquot 30×10^4 cells and plate in 10 mL media on 10 cm culture dishes
4. After plating, incubate cells at 37°C in 5% CO₂
5. Change media the next day and every other day from then until culture is 40-50% confluent
6. Passage cells once 50% confluent. Do this 1-2 days before injection to make sure that cells are in their logarithmic growth phase

Day when cells 50% confluent: Passaging of cells

1. Warm complete media, DPBS and DMEM at room temperature
2. Aspirate all media
3. Wash culture dishes twice using 3-5 mL of DPBS
4. Add 0.75mL of Trypsin/EDTA dropwise to each dish
5. Incubate dishes at 37°C for four minutes, and tap on the plates every two minutes to facilitate cell detachment
6. Checked dishes under the microscope for cell adhesion
7. Add 3 mL complete media to neutralize Trypsin activity
8. Transfer cells to 15 mL tube and wash dishes with 3-5 mL unsupplemented DMEM to collect residual cells
9. Centrifuge cells at 800 RPM for 5 minutes
10. Aspirate supernatant
11. Add and mix 10 mL fresh complete media

12. Count cells using a hemocytometer. Plate 30×10^4 cells in 10 mL complete media on new 10 cm culture dishes
13. Rock dishes gently to ensure even cell distribution and incubate at 37°C

APPENDIX III

TUMOR CELL INJECTIONS

Preparing Cells for Injection

Materials:

- Complete media (high glucose DMEM with 10% FBS and 1% P/S)
- DPBS
- Trypsin EDTA
- 1.5 mL microcentrifuge tubes

Procedure:

1. Aspirate media 1-2 hours before injection, and wash culture dishes twice using 3-5mL of DPBS at room temperature
2. Add 0.75 mL Trypsin/EDTA dropwise to dishes and incubate at 37°C for about 4 minutes
3. Check dishes under the microscope for cell adhesion
4. Use 1mL of complete media to neutralize trypsin activity
5. Collect cells in 15 mL centrifuge tubes and wash dishes with 3-5 mL DPBS to collect residual cells
6. Centrifuge cells for 5 minutes at 800 RPM
7. Aspirate supernatant
8. Resuspend cells in 1 mL DPBS per plate
9. Count cells using a hemocytometer and calculate viability using Trypan blue. Do not use cultures with less than 90% viability
10. Calculate the volume of DPBS needed to achieve 2.5×10^5 cells per 100 uL and adjust for it
11. Place cells on ice
12. Aliquot 200 uL for each mouse in a 1.5mL microcentrifuge. Keep and transport on ice packs

Injection

Materials:

- Isoflurane anesthesia cart
- Depilatory
- Cotton swabs
- Kimwipes
- Beaker of water
- Cell suspension
- Mice
- Insulin syringes

Procedure:

1. Weigh mouse
2. Anesthetize animals using 2L/min isoflurane
3. Apply depilatory cream to both flanks above hind limb
4. Continuously rub cotton swabs on the application area until depilation begins
5. Remove excess cream using wet cotton swabs and kimwipes to prevent chemical burns
6. Take out cell suspension from cooler and resuspended
7. Draw 125 uL into insulin syringe
8. Tent skin above hind limb for insertion of needle, bevel down, and slow injection of cell suspension
9. Remove needle 5 seconds after successful bleb formation to minimize leaking
10. Repeat the same process on the opposite side flank

APPENDIX IV

EUTHANASIA & NECROPSY

Materials:

- 1mL syringes
- 26G ¾ in needles
- Dissecting scissors and forceps
- 10% NBF
- Caliper
- 10mM EDTA in DPBS

Procedure:

1. Euthanize mice by CO₂ asphyxiation
2. Spray body with isopropyl
3. Perform exsanguination via cardiac puncture
4. Lift and cut skin transversely using dissecting scissors
5. Use small iris scissors to cut through abdominal wall
6. Cut femoral artery
7. Cut diaphragm through and pull back rib cage to expose the heart
8. Perfuse 3-5 mL PBS/EDTA at the apex of the heart at a rate of 1mL/min. Judge perfusion on paleness of liver
9. Perfuse 2mL 10% NBF through the apex of the heart
10. Carefully excise and weigh tumor w
11. Measure excised tumors (LxWxH) with digital caliper and store in 10% NBF at 4°C overnight
12. Excise lungs, liver and spleen and store in 10% NBF at 4°C

APPENDIX V

IMMUNOHISTOCHEMISTRY

Day 1:

Fixation (for frozen tissue samples only)

Materials:

Precooled acetone to about -20C

Procedure:

- Immerse slides in precooled acetone for 10 minutes
- Let slides dry at room temperature for 10 minutes

Rehydration (Steps 1-3 for paraffin embedded tissue samples only)

Materials:

- EtOH:100%, 95%, 80%, & 70%
- H₂O
- Xylene

Procedure:

1. Wash slides twice in Xylene for 3 minutes
2. Wash slides twice in 100% EtOH for 3 minutes
3. Wash slides for 3 minutes each in 95%, 80%, & 70% EtOH.
4. Wash slides twice in H₂O for 5 minutes

Antigen Retrieval (For paraffin embedded tissue samples only)

Materials:

- 10 mM sodium citrate
- Tween 20
- dH₂O
- Microwave

Procedure:

1. Note: Prepare antigen retrieval buffer before beginning IHC:
2. Mix 2.94g of sodium citrate with approximately 1000mL of dH₂O and bring to pH of 6 using HCl
3. Add 500uL of Tween 20 to the solution and it vortex until dissolved
4. Immerse slides with more than enough antigen retrieval buffer

5. Place immersed slides in the microwave for 4 minutes and take out to cool for 2 minutes.
Repeat this 5 times
6. Leave slides to cool for 20 minutes
7. Wash slides 3 times in dH₂O for 5 minutes

Blocking

Materials:

- Bovine Serum Albumin (BSA)
- PBS
- 10 mL syringe
- Syringe filter

Procedure:

1. Note: Make 5% BSA before IHC was started:
2. Add .5g of BSA to 10mL of PBS
3. Vortex solution for a couple of seconds, then leave sitting for 10 minutes
4. Filter solution
5. Add 200 uL of blocking solution (5% BSA) to each tissue and incubate at room temperature for 30 minutes
6. Dip slides in PBS briefly

Primary Antibody

Materials:

- Bovine Serum Albumin (BSA)
- PBS
- 10 mL syringe
- Syringe filter
- Primary antibodies:
 - Anti-CD31 (Ab28364)
 - Normal Rabbit Immunoglobulin G
 - Isolectin B4
- 15 cm plates
- Filter paper
- Parafilm

Procedure:

1. Note: Prepare 1% BSA before starting IHC :
 - a. Add 1g of BSA to 10mL of PBS
 - b. Vortex solution for a couple of seconds then leave sitting for 10 minutes
 - c. Filter solution
2. Dilute antibodies in 1% BSA and make enough for 250 uL per tissue sample
 - a. Anti-CD31 (Ab28364): 1:50
 - b. Normal Rabbit Immunoglobulin G & Isolectin B4: 1:100

3. Create humidifier chambers:
 - c. Place a rectangular piece of filter paper in a 15 cm plate and moisten using ddH₂O
 - d. Place a piece of squared parafilm (the same size as the filter paper) on top to protect the slides from the water.
4. Place slides in the humidifier chambers
5. Apply 200 uL of diluted primary antibody to each tissue sample on each slide.
6. Incubate slides in humidifier chambers at 4°C overnight

Day 2: Secondary Antibody

Materials:

- PBS
- BSA
- EtOH: 70%, 80%, 95%, and 100%
- Xylene
- Prolong diamond mounting media
- Secondary antibody: AF546

Procedure:

- a. Wash slides 3 times with PBS, while covered on a shaker, for 5 minutes
- b. Dilute secondary antibody AF546 (1:500) with 1% BSA and enough was made for 250 uL per sample
- c. Apply 200 uL of secondary antibody to each tissue sample
- d. Cover slides and incubate them at room temperature for 1 hour
- e. Wash slides 3 times with PBS, while covered on a shaker, for 5 minutes
- f. Dip slides 10 times into each solution of: 70%, 80%, and 95% EtOH
- g. Dip slides 20 times into solution of 100% EtOH and then into solution of Xylene
- h. Apply mounting media and coverslips to each tissue sample.