Using Image Segmentation to Computationally Interpret Loss of RASA3 Function and its Impact on Erythropoiesis and Epiphyseal Plate Development Through Dysregulation of Key Signaling Pathways

Research Paper

Abstract

In recent years significant progress has been made in understanding inherited bone marrow failure syndromes. Despite this, many pathological conditions relating to this group of disorders remain uncharacterized. Patients with these disorders often exhibit both bone and blood abnormalities, the underlying principles of which can be examined through the use of mouse models. The *scat* (severe combined anemia and thrombocytopenia) mouse model contains a missense mutation in the *RASA3* gene, which encodes the RASA3 protein and plays a critical role in hematopoiesis. The specific mutation in *scat* causes RASA3 to be mislocalized to the cytostol in reticulocytes, causing a loss of function resulting in impaired erythropoiesis. Homozygous mice enter periods of crisis and remission that characterize the phenotype, with erythroblasts failing to hemoglobinize properly and enter terminal differentiation. Furthermore, there is a consistent pattern of an increased proportion of cells in the S phase in late terminal erythroid differentiation, suggesting that the cell cycle itself is influenced by RASA3. Additionally, epiphyseal plate development appears severely compromised, as *scat* mice show chondrocyte dysplasia in the proliferating and hypertrophic zones, while heterozygotes occasionally display an abnormal hypothesized vascular invasion into the proliferating zone that disrupts the surrounding region, as confirmed by testing via boundary detection algorithms and color proportioning.

I. Introduction

Bone marrow failure syndromes (BMFS) are a group of inheritable disorders often characterized by compromised hematopoiesis and a reduction of mature erythrocytes. The complex interactions between genetic changes and alterations in the bone marrow itself that produce these disorders have yet to be identified, making it difficult to classify distinct etiologies in patients. Aplastic Anemia, a common BMFS, can be either acquired or inherited. Acquired Aplastic Anemia is the immune-mediated suppression of the bone marrow and responds well to immunosuppressive therapy.**[2]** The inherited variant of the disorder is less well understood, and thus cannot be treated to the same extent.

Models of bone marrow failure disorders- both spontaneous and engineered- allow for the

focused study of these uncharacterized molecular pathways.**[1]** Better treatment of patients is a priority, and models that mimic the phenotype of BMFS are essential in determining important effects of the disorders. One such spontaneous model is the autosomal recessive *scat* (severe combined anemia and thrombocytopenia) mouse model; *scat* carries a missense mutation in the protein-coding *Rasa3* gene.**[1]**

RASA3, a Ras-GTPase Activating Protein (GAP), functions as a negative regulator of the Ras signalling pathway, which is often studied in cancer research. RASA3 has previously been found to play a key role in normal blood formation, with the specific mutation in *scat* resulting in the mislocalization of RASA3 to the cytosol and thus loss of RASA3 function. Homozygous s*cat* mice cycle between hematologic crisis and remission, in a way similar to some BMFS patients.**[2]** The first crisis begins *in utero* and lasts until ~P9. Mice in crisis are smaller, with bone abnormalities limiting their growth. The exact mechanism mediating the crisis-remission transition is not well understood, but it offers an opportunity to study the resolution of bone-marrow failure.

Bone marrow failure disorders may manifest with physical abnormalities. Bone structure and development may be affected, causing microcephaly and hypertelorism, among other malformations. At the end of each long bone lies cartilage known as the growth, or epiphyseal plate. The epiphyseal plate is composed of several sections, those being the proliferating, hypertrophic, calcification, and ossification zones. The chondrocytes that the growth plate is composed of are stacked in columns, and should theses columns be interrupted, debilitating defects can occur. Some of the physical abnormalities present in BMFS, such as stunted growth, can be attributed to such dysphoria in the proliferating zone.**[3]** Analyzing the phenotypes present in heterozygous and homozygous *scat* mice can aid in the diagnosis and treatment of BMFS patients who exhibit physical irregularities.

The analysis of protein expression in the growth plate is usually achieved through immunohistochemistry (IHC). IHC is the process of identifying antigens in a tissue section through the binding of antibodies, which can be viewed using a microscope. The images taken of each cross section are fluorescent and, when the brightness and area are standardized, able to depict the proportion of protein present. The fluorescent area can be separated using a color proportioning algorithm which uses the values for hue, saturation, and lightness (HSL) to create "color clusters", where pixels with similar properties are grouped.**[4]** HSL is an alternative to traditional red, green, blue, color models developed in the 1970's by computer graphics researchers to more closely align with the way humans perceive hues.**[4,5]** Creating a program able to both identify these color clusters and utilize boundary detection would determine both the proportion of protein present and the dysplasia of each epiphyseal plate zone based on size.

II. Materials & Methods

Cell Cycle Analysis

$CD34^+$ from umbilical cord blood were separated and placed into a culture to differentiate into red cells. The cells were incubated at 100,000/mL in 50μM EdU in normal culture media for 4 hours at 37°C, and the ClickIT manufacturer's protocol followed. RASA3 was knocked down to produce an shRASA3 batch of cells. The samples were resuspended in 250μL PI/RNAse staining buffer and analyzed using flow cytometry.

Western Blots

Cells were lysed with RIPA then loaded into wells along with a weight marker and separated using gel electrophoresis (10% gel). The resulting proteins on the gel were transferred for 90 minutes at 90V onto a membrane and incubated with a primary antibody overnight, washed with TBST, and incubated with a

secondary antibody. Following another set of washes chemiluminescence substrate was applied and the blot imaged.

Animals

All experimental mice were 5–35 days old, which corresponds to both the second crisis event of the *scat* mice, and a period of remission. The mice were sacrificed prior to the start of the research, and their tissues were used All protocols were performed according to NIH animal care guidelines, as approved and enforced by the Institutional Animal Care and Use Committees at Northwell Health and The Jackson Laboratory.**[6]**

Growth Plate Development

Femurs and tibias were taken from mice ranging from P5 to P35, fixed in 10% neutral buffered formalin overnight, decalcified with 10% ethylenediamine tetraacetic acid, and embedded in paraffin. Longitudinal sections were prepared on slides. After deparaffinization and rehydration, the sections were incubated with primary antibody overnight, then incubated with a secondary antibody and finally mounted using DAPI. The growth plates were then imaged at 20X.

Image Analysis

Image segmentation is done with the intent of changing the representation of an image into something more easily analysed. The techniques used for the IHC images were color clustering, edge detection, and a linear regression followed by distance calculation. The Canny Edge Detector operator was used to segment the growth plate image into lines. The five steps of the algorithm are as follows:

1. Apply Gaussian filter to smooth the image in order to remove the noise
2. Find the intensity gradients of the image
3. Apply non-maximum suppression to get rid of spurious response to edge detection
4. Apply double threshold to determine potential edges
5. Track edge by hysteresis: Finalize the detection of edges by suppressing all the other edges that are weak and not connected to strong edges.

Images were processed through the first two steps, and the intensity gradient in Y was used as the final segmentation. The gradient is able to visualise abrupt changes in morphology, allowing the borders of the growth plate to become more easily visible. The skew lines composing the borders were approximated into two parallel lines, and the distance between them calculated in pixels. Each image was 512x512 pixels for consistency.

The original images were analyzed for proportions of color clusters to determine the expression of the protein that was stained for during each IHC procedure. K-means clustering was used, with 4 color clusters being the optimal segmentation.

The Javascript implementation of the Canny Edge Detector algorithm was first developed by the Biomedical Imaging Group. The color clustering algorithm is based off of the work of bioinformatician Martin Krzywinski.

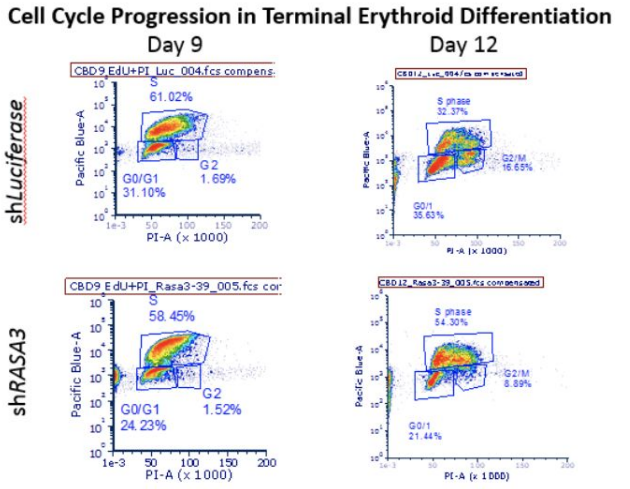
III. Results

Flow cytometry revealed that shRASA3 cells are found in the S phase more often than the control Luciferase cells, as demonstrated in Figure 1. The decreased percentage of cells reaching G2 indicates that RASA3 plays a role in cell cycle regulation.

The results of numerous Western blots were inconclusive, but the knockdown of RASA3 appears to lead to slight and inconsistent increases in signalling down the RAF-MEK-ERK pathway, as conveys in Figure 2.

Figure 3. displays the results of the IHC stainings for each genotype of mouse tested. The heterozygous growth plates exhibit an unusual

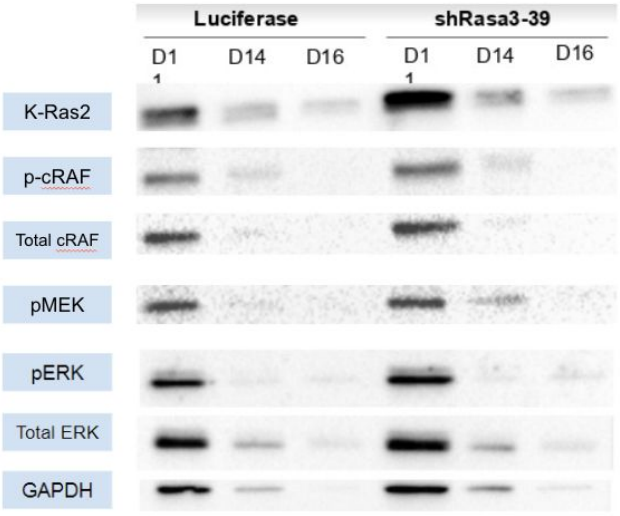
abnormality. Analysis of the color clustering of



the chondrocytes determined that Collagen II and

Figure 1. Flow Cytometry Analysis

PCNA expression were elevated in scat compared to wild type and heterozygous mice.



SOX9 was lowered in scat, while no solid trend

Figure 2. Western Blot Results

could be determined to MMP9 expression. All images were measured at 20X magnification. The trendlines for the proportion expressed in each image are shown in Figure 4.

The flow chart in Figure 5. demonstrates the image segmentation portion of analysis, where the original image's y-gradient is derived, and the contours converted into two parallel lines. Pixel distance was measured ImageJ. From these measurements it became evident that variation in the size of the growth plate correlates with specific genotypes. The length of

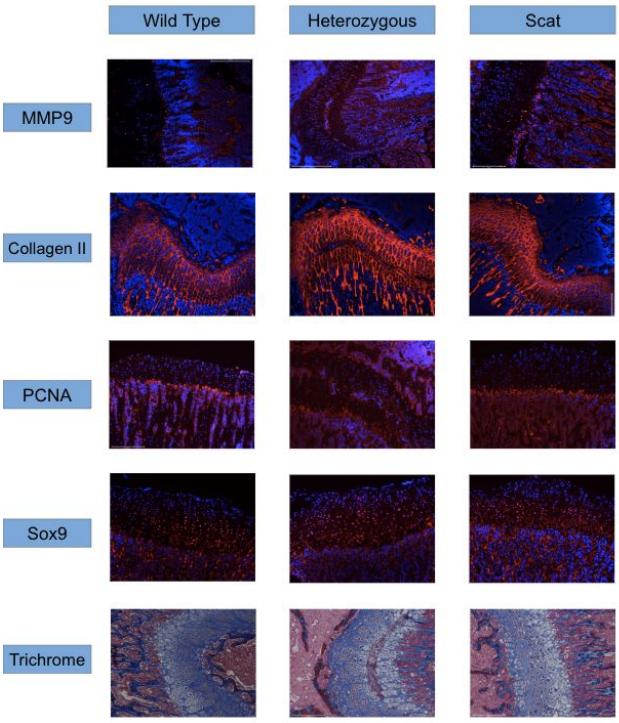


Figure 3. Growth Plate IHC Images at 20X

the epiphyseal plate increased from wild type to heterozygous, and increased again from heterozygous to scat. This demonstrates the increased dysplasia and irregularities in bone caused by a bone marrow disorder.

IV. Conclusions

shRASA3 cells fail to make it to G2 to the same extent Luciferase cells do. The exact mechanism that causes the increased proportion of cells in the S phase is unknown, further flow experiments will be necessary. What is clear is that RASA3 negatively regulates the RAS signalling pathway, and has a profound impact on the cell cycle, which differs from previous thoughts about its role.

More proteins will need to be imaged and

quantified for a solid conclusion to be drawn about the effect of the knockdown of RASA3,

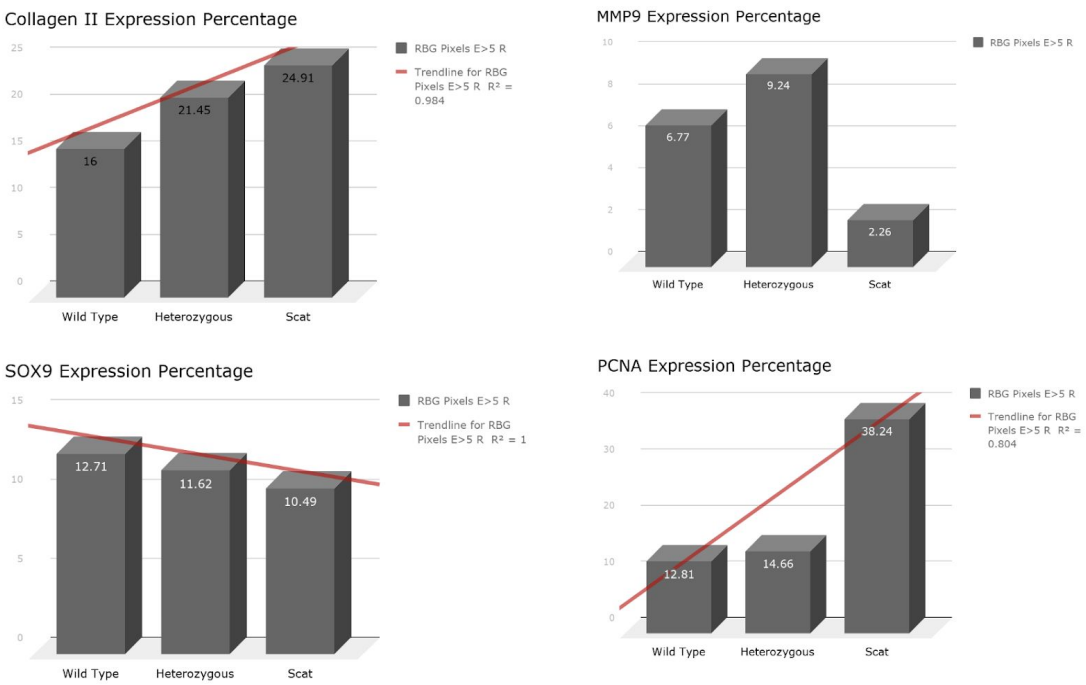


Figure 4. Color Cluster Proportions

but the upregulation of proteins downstream is consistent with the results of the flow cytometry experiments.

The abnormality in the heterozygous growth plates is hypercellular and expresses MMP9, suggesting it may be the result of aberrant osteoclast activity. Further staining with osteopontin will reveal more.

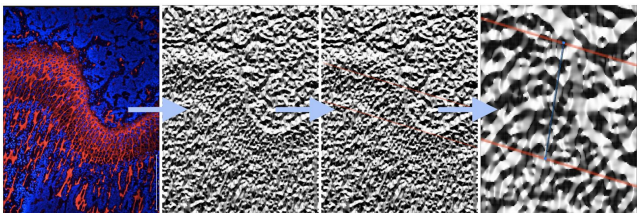


Figure 5. Distance Processing Representation

Enlarged growth plates in scat and heterozygous mice are evidence that the chondrocyte differentiation is affected in mutated mice. As columns of cartilage cells extend toward metaphysis, their lengthening by cell division occurring at the base would be extended if there was a change in differentiation time.

V. References

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