Nicotinamide Phosphoribosyltransferase Expression in Developing Mice Embryo and Adult Tissues

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Introduction:

Metabolic rates and the cell cycle are highly regulated in living organisms. Measuring the regulatory, enzymatic contributors can lead to the prediction of pathologies such as cancer and diabetes\textsuperscript{4}. Nicotinamide phosphoribosyltransferase, known as Nampt is an enzyme which assists in the production of nicotinamide adenine dinucleotide, NAD, an essential molecule in living organisms. NAD is widely known to be responsible for the metabolic processes in an organism to allow for survival, such as facilitating electron transport. NAD is degraded when it performs cell signalling effects that lead to the determination of lifespan and survival of the cell\textsuperscript{6}. Nampt replenishes the store of NAD in the cell allowing for further signalling effects to continue. The expression of this enzyme has been reported in adult tissues in various mammals but the localized expression has not been shown in the developing embryo\textsuperscript{13}. To explore potential effects of cell signalling during development at various locations of the embryo, immunostaining is applied to detect the expression of Nampt.

To compare relative expression, an approach is needed to quantify the abundance of localized Nampt expression in the cells through immunohistochemical staining. The area of expression can be isolated from the background after digital processing of the red green blue, RGB, image of the stained tissue, allowing one to quantify the area fraction of expression. Image processing is an alternate method or precursor to molecular detection of protein expression.

Theory:

*Enzyme of interest: Nampt*

Nicotinamide adenine dinucleotide, NAD is essential for the survival of a cell, as it acts in several reactions that relate to metabolic processes and cell signalling. It performs reduction and oxidization reactions in glucose metabolism where it acts as an electron carrier where NAD is cycled to, and back from NADH. NAD also acts as a cofactor in enzymatic reactions that relate to the rate of gene transcription, DNA repair and lifespan of the cell through association with cell division cycle proteins\textsuperscript{6}.
Enzymatic reactions lead to the degradation of NAD. NAD is synthesized from various precursors, most of which is created from the essential nutrient tryptophan through the kynurenine pathway\(^8\). Nampt salvages the NAD that is consumed by enzymes during cell regulatory processes, acting as a rate limiting enzyme\(^6\).

Nampt catalyses a condensation reaction between nicotinamide (NAm) with 5-phosphoribosyl-1-pyrophosphate (PRPP), to produce nicotinamide mononucleotide (NMN), which is a NAD intermediate\(^8\), as shown below.

\[
\text{NAm} + \text{PRPP} \xrightarrow{\text{Nampt}} \text{NMN} \rightarrow \text{NAD} (1)
\]

The deletion or knockout of the Nampt or Pre \(\beta\) cell colony Enhancing Factor (PBEF) gene would lead to early embryonic death, at the stage of 10.5 days in embryonic mice. Nampt is expressed in various tissue types at different degrees. For example, it is expressed in the smooth muscle where NAD acts as a cofactor to an enzyme that relates to calcium mobilization\(^14\).

The expression of Nampt has been shown in adult tissues where there is a variation in different organ tissue types\(^4\). It is expected that there will also be a variation in the abundance of expression in each developmental stage as there is modulations to the levels of NAD in various tissue types.

**Immunohistochemistry**

To visualize the location of Nampt expression, immunohistochemical (IHC) staining is applied to embryo sections. The staining of the tissues is based on the use of primary antibodies and polymeric oxidation products for visualization\(^9\). For Nampt, the primary antibody used is polyclonal anti-Pre \(\beta\) cell colony enhancing factor (PBEF or Nampt) which binds to Nampt. The antigen retrieval process involves high pressure and high temperature to allow binding of Nampt to the antibody. The primary antibody is visualized with diaminobenzidine (DAB), which creates a localized, brown hue oxidation product. This allows for the visualization of the primary antibody and the location of Nampt expression in turn\(^12\).
Red Green Blue to Hue Saturation and Brightness

The red green blue, RGB colour model defines colour in an image using red, green, and blue light. The value of the light will range from the minimum intensity of zero to the maximum intensity of 255. A coloured image defined with the three light values will consist of 3 bytes of information. The RGB model is additive where it uses the transmitted light to display colour. For example, the human eye or digital cameras attached to computer screens use RGB to determine colour. Each pixel will include a series of values which will determine the level of each light colour.

The hue saturation brightness, HSB colour model is based on three properties of colour, hue saturation and brightness. It is based on the property that every colour results from a pure colour. The pure colour is added to white or black to result in different shades of the value of pure colour. Hue is a representation of the pure colour value measured in degrees, from zero to 360 where zero is red, 60 is yellow, 120 is green, 180 is cyan, 240 is blue and 300 is magenta. Saturation is the degree to which the pure colour has been mixed with white, where desaturated is the colour mixed with white and, which is measured in a percentage where a higher percentage indicates a more pure hue. Brightness is also measured as a percentage where it determines the intensity of the colour which is measured with black, the higher the percentage the brighter the colour.

Quantification

Quantifying the expression of Nampt involves measuring the area fraction of immunostaining to the background using the equation below.

\[
\text{Area fraction of expression} = \frac{\text{Area of IHC staining}}{\text{Total area of interest}}
\]

The area of IHC staining is indicated by the area of tissue stained a brown hue due to DAB oxidization and the total area of interest includes the stained and surrounding tissue of the section. The ratio of expression can be localized and quantified.
The RGB image of the immunostained tissue is taken by a digital imaging system and processed by conversion to HSB stacks in order to isolate the brown hue of the DAB secondary antibody. The expression of Nampt is localized and the abundance measured based on the area fraction of the immunostain. Quantifying the area of immunostaining is a precursor or alternative to Western blotting, which evaluates the level of protein expression using a molecular approach, also utilizing antibody for visualization but is does not localized the expression in embryonic tissues. IHC staining allows for discrete, localized values of expression.

Methods:

**Tissue preparation**

The wild type, normal, mice embryos are harvested from timed pregnancies at the development stages of 9.5, 10.5, 11.5, and 18.5 days. Adult tissues are extracted from major organs of the wild type mouse. The tissues are dehydrated and fixed in xylene and embedded in wax. Sagittal and transverse sections are cut of the embryos and adult tissues. The sections are attached to frosted glass slides in preparation for light microscopy.

The sections are stained with haematoxylin and eosin (H &E) to localize the sections that incorporate various tissue types and organs. The section adjacent to the H&E stained slide with the appropriate tissue types includes the same features, and is used for staining to localize Nampt.

**IHC stains**

The sections of interest are exposed to the antibody, polyclonal anti-Pre β cell colony Enhancing Factor (PBEF or Nampt). The antibody from Bethyl Labs involves antigen retrieval in citrate buffer. The primary antibody is then visualized with a diaminobenzidine (DAB) peroxidase substrate kit from Vector Laboratories. A brown hue appears where there is expression of Nampt.

Images are taken of the immunostained and control tissues through a light microscope at 400 times magnification at each area of interest for each developmental stage.
**Digital processing**

The RBG image taken through the light microscope is converted into hue, saturation and brightness stacks in the program ImageJ. The hue stack is then isolated and converted into an 8-bit greyscale image. The brown hue is then isolated by manual thresholding to include the range between zero and 90 degrees. The image is now black and white, with black indicating the brown hue and white representing the unstained tissue.

The area fraction of expression (AFE) is then calculated by selecting a circular area of interest and finding the area of expression by analysing particles in ImageJ. The AFE of the control is subtracted from the AFE of the immunostained tissues. Eight areas of interest are taken of the same tissue type and the average AFE taken to be the resultant value.

**Statistical analysis**

The one way ANOVA is obtained between major organ tissues and within vascular tissues for the various developmental stages. This allows for the detection of a variation of expression in the different tissue types or the variation of expression at different developmental stages.

**Results:**

The variation in the tissue types results in different distributions of Nampt expression in each area of interest. The section of tissue treated with immunostain and DAB oxidization in citrate buffer results in a brown hue, indicating localized Nampt expression as shown in Figure 1b and 1d. Tissue sections not treated with anti-PBEF results in no detection of Nampt where all other factors remain constant, as shown in Figure 1a and 1c. The abundance of brown hue is thresholded and quantified based on area fraction of expression, AFE.

The AFE of vascular tissue is summarized in Figure 2, where it indicates the abundance of Nampt expression in the aortic endothelium and media at each developmental stage. The ANOVA p-value for the sample is $4.46 \times 10^{-8}$, indicating that there is a variation in the sample. Individual p-values between
the expression of the endothelium and the aortic media indicate that there is significantly greater area of expression by an average of 218% in the endothelium compared to the aortic media at each developmental stage. There is an average decrease of 21.6% in the AFE of vascular tissue as the developmental stage increases.

The AFE of various differentiated tissues at the developmental stages of 11.5 days, 18.5 days and adult are shown in Table 1. There is an average decrease of 26.9% in expression, with the exception of the stomach lamina propria. Based on the p-values of between each developmental stage of the same tissue, there is a significant change in the expression of Nampt with the exception of the stomach epithelium. The ANOVA p-value for the sample is $3.58 \times 10^{-12}$, indicating a variant in the sample.

Figure 1: Control and immunostains of the adult aorta tissue and the adult heart tissue
Effect of the immunostaining in mice tissue sections result in a brown hue at the locations where there is positive expression of Nampt. Imaged under a light microscope at 400 times magnification, scale bar indicating 50 microns. (a) Adult aortic control tissue. (b) Adult aortic IHC stained tissue. (c) Adult heart control tissue. (d) Adult heart IHC stained tissue.
Figure 2: Area fraction of Nampt expression in vascular tissue vs. the developmental stage

Effect of immunostaining in the aorta with localized areas of interest on the aortic endothelium and aortic media. ANOVA p-value for the sample is $4.46 \times 10^{-8}$ with a sample size of 8 for each variant. The p-values between the aortic endothelium and media at each developmental stage are indicated below the horizontal axis.

Table 1: The average area fraction of Nampt Expression in major organ tissues ($\mu$m²/µm²)

<table>
<thead>
<tr>
<th>Tissue Type \ Tissue Age</th>
<th>11.5 days</th>
<th>18.5 days</th>
<th>Adult</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.562731</td>
<td>0.347584</td>
<td>-</td>
<td>0.00021</td>
</tr>
<tr>
<td>Stomach epithelium</td>
<td>0.525881</td>
<td>0.49283</td>
<td>-</td>
<td>0.16045</td>
</tr>
<tr>
<td>Stomach lamina propria</td>
<td>0.213988</td>
<td>0.69204</td>
<td>-</td>
<td>$2.16 \times 10^{-6}$</td>
</tr>
<tr>
<td>Heart</td>
<td>0.588467</td>
<td>0.369784</td>
<td>0.472135</td>
<td>0.00019</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>0.254005</td>
<td>0.173187</td>
<td>0.01913</td>
</tr>
<tr>
<td>Lung vessel</td>
<td>-</td>
<td>0.379304</td>
<td>0.299319</td>
<td>0.01985</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
<td>0.845749</td>
<td>-</td>
</tr>
</tbody>
</table>

*ANOVA p-value of $3.58 \times 10^{-12}$, n = 8 for each value

* "-" indicates unavailable values
Discussion:

The abundance of Nampt expression is quantifiable through immunostaining and digital processing with microscopy images. Immunostaining of Nampt results in a brown hue, which is difficult to isolate when the image is represented by the RGB colour model when it is taken by a digital camera, image processing allows for resolving the area of expression. Quantifying the area of staining allows one to remove the variable of human perception when comparing relative expression\(^9\). For example, there is a visible difference in the expression in the aortic endothelium and aortic media in Figure 1b. Applying a value for the relative expression, such as the area fraction of expression, removes deviation in the results based on perception.

The conversion of image model from RGB to HBS, isolates the brown hue of the positive Nampt expression in immunostained tissue. In the RGB model, the brown hue is detected in all slices, red, green and blue, as it is composed of the combination of the three different light sources. Converting the image to HSB in a digital image processing program, such as ImageJ, allows for the isolation of the pure brown hue in the hue stack when thresholding for the specified colour. Image model conversion has been previously applied to detect the DAB brown hue in various protein expressions, such as the nuclear MYC protein\(^5\). The same technique is also used to isolate the areas that lack expression, thresholding for a different hue, as was done to determine the epidermal growth factor receptor expression\(^2\).

The localized level of Nampt expression has not been previously quantified in embryonic tissue. There is a general decrease in the level of expression of the enzyme as the mouse embryo develops, as shown in Figure 2 and Table 1, and an overall variation in the abundance in tissue sections with the highest area fraction in the adult kidney. There is the exception of the stomach tissue as expression in the epithelium does not vary significantly and there is a large increase in the expression in the lamina propria. This could be due to the fact that at the later stages of development, 18.5 days of the mice fetus right before birth\(^10\), there is more metabolic and regulatory development in the stomach media in
preparation for digestion after birth. This allows for further explorations into the determining factors that affect the expression of Nampt.

There are different methods and approaches to quantifying the expression of immunostaining. Including a larger sample size would define an average value of expression with higher accuracy. Other factors that should be expanded to finding the area fraction of expression of Nampt, would be to include a larger sample size in the mice at each developmental stage. There are variations in each mouse on the levels of protein expression and incorporating different mice and a larger sample size will aid in the accuracy of expression of Nampt at each stage. To understand the progression during embryonic and fetal development as well as maturation to the adult mouse, more stages are to be incorporated. A similar system of quantifying immunostaining has been automated\(^\text{12}\), which includes turning the thresholded image into a matrix in processing programs such as Matlab, allowing for higher specificity than the area fraction of expression\(^\text{3}\). This process would allow for a larger sample size including mice variation and an increase in the number of developmental stages.

Defining expression of Nampt through localizing the area of staining is a potential replacement or efficient precursor to molecular techniques in quantifying protein expression. Immunostaining allows for the localization of staining in specific areas of interests, especially in the embryo\(^\text{10}\). Embryonic mice tissues are differentiated in areas in the order of hundreds of microns and are difficult to isolate when performing protein analysis. If it is possible to isolate the heart at 11.5 days as shown in Table 1, there would be approximately \(1.25 \times 10^5 \, \mu\text{m}^3\) of differentiated tissue, not a sufficient sample size per embryo to perform protein analysis. Shearing the whole embryo would not allow for specified, relative expression in the various regions of tissue. Techniques such as Western blotting would be used to verify the abundance of expression in larger tissue sections in the adult tissue or embryonic mice at later developmental stages such as 18.5 days\(^\text{10}\).
There are disadvantages to immunostaining of the cells and quantifying protein expression through area fraction. In the immunostaining process for Nampt, the high temperature and pressure degrades the brain tissue, thus delicate tissue types cannot be studied with this method. The DAB oxidization reaction varies with each procedure, thus the amount of brown stain will vary regardless of the consistency of the protein between samples. The sections of tissue are to be stained all at once to allow for consistency in the antibody binding and DAB oxidization. For a larger sample size, it is unrealistic to perform all the staining at once. There is also the possibility of variation at the light microscopy level when focusing, as there is a depth at which the brown stain is most prominent. When quantifying the level of expression through area fraction, the relative value does not take into account the intensity of the brown hue. For example, in Figure 1d, the brown hue is more intense at the nucleus compared to the cytoplasm but that would not be taken into account when quantifying area fraction of the stain. The staining in the cytoplasm is more dispersed and the area fraction would take that into account versus the actual intensity of the stain.

The quantification of Nampt in wild type mice allows for a basis in future experimentation in the PBEF gene knockout in specified tissue to study pathologies related to NAD concentrations in the cell. Nampt regulates the cell’s supply of NAD and thus has an effect in the cell signalling effects that could lead to obesity or diabetes in an individual, excess proliferation leading to tumour growth, or apoptosis of the cell before maturation. It is associated with cell division cycle protein 42, which regulates the rate of cell proliferation, enabling a reduction or increase in proliferation depending on NAD concentration. Nampt expression control could lead to human vascular smooth muscle cell migration, which stimulates edge protrusion. The relative abundance of Nampt in various tissues is a precursor to acquiring the complete effects of the enzyme on the cell and surrounding tissues.
Conclusion:

A digital analysis of immunostained tissue can be used to compare Nampt expression between embryonic and adult tissue. Image processing through ImageJ allows for the isolation of the brown hue of the positively expressed tissue to quantify the area fraction of expression. There is variation in the expression of Nampt in differentiated tissue types over the embryonic development stages of the mouse. There is a significant variation and decrease in Nampt expression in the vascular tissue.

References: