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**Comparison of the distribution and formation of lamin A, emerin, LINC complex at the outer and inner surface of the nuclear envelope in contractile and proliferative pulmonary artery smooth muscle cells**

*by*

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**THE UNIVERSITY  
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## **Declaration**

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgement, the work presented is entirely my own.

Esmahan Durmaz

1 07/2021

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# Table of Contents

|                                                                                                                        |             |
|------------------------------------------------------------------------------------------------------------------------|-------------|
| <b>Declaration .....</b>                                                                                               | <b>I</b>    |
| <b>Acknowledgements .....</b>                                                                                          | <b>I</b>    |
| <b>List of Figures.....</b>                                                                                            | <b>IV</b>   |
| <b>List of Tables.....</b>                                                                                             | <b>IX</b>   |
| <b>Abbreviations .....</b>                                                                                             | <b>XI</b>   |
| <b>Abstract.....</b>                                                                                                   | <b>XIV</b>  |
| <b>Lay Summary .....</b>                                                                                               | <b>XVII</b> |
| <b>Introduction .....</b>                                                                                              | <b>1</b>    |
| <i>1.1 The phenotypes of contractile and synthetic SMC .....</i>                                                       | <i>3</i>    |
| <i>1.2 VSMC phenotypic switching .....</i>                                                                             | <i>4</i>    |
| <i>1.3 Gene expression in vascular smooth muscle cell phenotypic switching .....</i>                                   | <i>5</i>    |
| <i>1.5 Nuclear envelope proteins direct genome organisation .....</i>                                                  | <i>6</i>    |
| <i>1.6 The possible role of Ca<sup>2+</sup> flux by across the cell-wide web and nuclear invagination network.....</i> | <i>9</i>    |
| <i>1.7 Aim and Hypothesis .....</i>                                                                                    | <i>13</i>   |
| <b>Materials and Methods.....</b>                                                                                      | <b>15</b>   |
| <i>2.1 Pulmonary artery tissue dissection and identification .....</i>                                                 | <i>15</i>   |
| <i>2.2 Digestion protocol for the dissociation of PSMCs.....</i>                                                       | <i>16</i>   |
| <i>2.3 Acutely isolated cell fixation with methanol.....</i>                                                           | <i>17</i>   |
| <i>2.4 Immunocytochemistry.....</i>                                                                                    | <i>18</i>   |

|                                                                                                                                                                |           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| 2.5 Confocal imaging .....                                                                                                                                     | 21        |
| 2.6 Deconvolution and imaging processing .....                                                                                                                 | 23        |
| 2.6.1 Improving the resolution of the images by deconvolution .....                                                                                            | 23        |
| 2.7 3D reconstruction of confocal Z section.....                                                                                                               | 24        |
| 2.8 Data presentation and statistical analysis.....                                                                                                            | 26        |
| <b>RESULTS .....</b>                                                                                                                                           | <b>28</b> |
| 3.1 The formation and distribution of selected nuclear envelope proteins at the nuclear envelope of acutely isolated pulmonary artery smooth muscle cells..... | 28        |
| 3.1.1 Lamin A is widely distributed across the inner nuclear membrane and forms specific structures: invaginations and ridges.....                             | 28        |
| 3.1.2 The structures identified by A-type lamin is also placed on emerin labelling. ....                                                                       | 40        |
| 3.1.3 Nesprin does not surround across NE however, nesprin exists on the described specific structures .....                                                   | 50        |
| 3.1.4 SUN-2 and lamin A colocalized in the lamin A dense regions .....                                                                                         | 59        |
| 3.2 Cultured nucleus presented different morphological features from acutely isolated PASMC nucleus .....                                                      | 67        |
| 3.2.1. Lamin A labelling is formed by a variety of structures on D7PASMC surface. ....                                                                         | 68        |
| 3.2.2 Emerin labelling D7PASMC nucleus loss the determined structures on the surface of the AIPASMC. ....                                                      | 75        |
| 3.2.3 Nesprin puncta distributes across the periphery of the nucleus of proliferating pulmonary arterial myocytes cultured for 7 days. ....                    | 83        |
| 3.2.4 SUN-2 labelling stretches across the nucleus but concentrated in well-defined regions. ....                                                              | 87        |

|                                                                                                                                   |            |
|-----------------------------------------------------------------------------------------------------------------------------------|------------|
| 3.3 The outer structures on specific nuclear envelope proteins are colocalised with histone marks and BAF puncta. ....            | 95         |
| 3.3.1.1 H3K9me2 puncta attached at various points in the different structures on lamin A labelled nucleus in AIPASMC. ....        | 95         |
| 3.3.1.2 H3K9me2 and lamin A colocalization puncta were spread evenly through the D7PASMC nuclei .....                             | 99         |
| 3.3.2.1 BAF protein resides at particular points through the structures defined by emerin labelled acutely isolated nucleus. .... | 104        |
| 3.3.2.2 In contrast, emerin and BAF was presented as intertwined at 7 days cultured PASMC nucleus. ....                           | 107        |
| <b>Discussion.....</b>                                                                                                            | <b>111</b> |
| Possible functions of protein dense regions in contractile phenotype pulmonary artery smooth muscle cells.....                    | 111        |
| The possible functions of NE proteins at the synthetic phenotype.....                                                             | 115        |
| <i>Conclusion .....</i>                                                                                                           | <i>119</i> |
| <i>Limitations and future experiments .....</i>                                                                                   | <i>120</i> |
| <b>Appendix .....</b>                                                                                                             | <b>122</b> |
| <b>References.....</b>                                                                                                            | <b>132</b> |

## List of Figures:

|                                                                                                                               |          |
|-------------------------------------------------------------------------------------------------------------------------------|----------|
| <b>Figure 1. The illustration of SMC contraction mechanism, created by Biorender, was shown.....</b>                          | <b>2</b> |
| <b>Figure 2. The schematic illustration of a) contractile and b) synthetic SMCs, created by Biorender, was shown.....</b>     | <b>4</b> |
| <b>Figure 3. The schematic demonstration of nuclear envelope created by Biorender, including INM and ONM, was shown. ....</b> | <b>8</b> |

|                                                                                                                                                                   |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 4. Images taken by electron and confocal microscopy illustrates type 1 and type 2 nuclear invaginations as well as lamin A and H3K9me2 localizations. .... | 11 |
| Figure 5. The schematic illustration of tissue dissection process was shown.....                                                                                  | 16 |
| Figure 6. The spectrum of dye conjugated to secondary antibodies was shown.....                                                                                   | 21 |
| Figure 7. The schematic illustration of confocal microscopy was shown. ....                                                                                       | 22 |
| Figure 8. Confocal images of acutely isolated PASMC stained for emerin before (left) and after (right) deconvolution were shown.....                              | 24 |
| Figure 9. Deconvolved and 3D reconstructed images were shown.....                                                                                                 | 25 |
| Figure 10. The illustration of colocalization between two different dyes staining was produced by Imaris.....                                                     | 26 |
| Figure 11. Lamin A is a protein meshwork that lines through the inner nuclear membrane of PASMCs ..                                                               | 29 |
| Figure 12. Lamin A surrounds the entire inner nuclear membrane of AIPASMCs.....                                                                                   | 30 |
| Figure 13. The comparison of high and low threshold application on nucleus labelling for lamin A was shown.....                                                   | 32 |
| Figure 14. One example of lamin A labelling AIPASMC nucleus was shown.....                                                                                        | 33 |
| Figure 15. A type lamin labelling PASMC reveals three different types of ridges. ....                                                                             | 34 |
| Figure 16. Three different ridge types were described on lamin A labelling of acutely isolated PSMC.....                                                          | 35 |
| Figure 17. The half I-type ridge is found in every cell. ....                                                                                                     | 36 |

|                                                                                                                                     |    |
|-------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 18. Three different invaginations were identified on lamin A labelling PASMC nucleus.....                                    | 38 |
| Figure 19. The most frequently observed invagination is the blind invagination in lamin A labelled AIPASMC.....                     | 39 |
| Figure 20. The comparison of high, low threshold application and deconvolved image on nucleus labelling for emerin were shown. .... | 40 |
| Figure 21. Emerin labelled AIPASMC nucleus presented three different types of ridges.....                                           | 42 |
| Figure 22. Three types of ridges were observed on AIPSMC nucleus labelled for emerin.....                                           | 43 |
| Figure 23. Each cell studied contains half I-type and I-type ridges. ....                                                           | 44 |
| Figure 24. Three types of invaginations was observed on emerin labelled PASMC nuclei. ....                                          | 45 |
| Figure 25. Superficial invaginations were observed mostly.....                                                                      | 46 |
| Figure 26. Lamin A exists on the different types of ridges labelled by emerin.....                                                  | 47 |
| Figure 27. Lamin A and emerin colocalization shows diversity from one cell to another cell. ....                                    | 50 |
| Figure 28. The comparison of high and low threshold application on nucleus labelling for nesprin was shown.....                     | 51 |
| Figure 29. One example of nesprin labelling AIPASMC nucleus was shown.....                                                          | 52 |
| Figure 30. Nesprin labelling AIPASMC reveals two different types of ridges on the surface. ....                                     | 53 |
| Figure 31. Each cell studied contains branched and I-type ridges. ....                                                              | 55 |
| Figure 32. Two different types of the ridges were observed on the nesprin labelled AIPASMC nucleus .....                            | 56 |

|                                                                                                                                                 |           |
|-------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>Figure 33. Lamin A exists on the different types of ridges labelled by nesprin.....</b>                                                      | <b>56</b> |
| <b>Figure 34. Lamin A and nesprin colocalization shows diversity from one cell to another. ....</b>                                             | <b>58</b> |
| <b>Figure 35. One example of SUN-2 labelling AIPASMC was shown. ....</b>                                                                        | <b>59</b> |
| <b>Figure 36. SUN-2 labelling AIPASMC reveals three different types of ridges.....</b>                                                          | <b>61</b> |
| <b>Figure 37. Four types of ridges were observed on SUN 2 labelled AIPASMC nucleus. ....</b>                                                    | <b>62</b> |
| <b>Figure 38. The I-type ridge were observed on SUN-2 labelling mostly. .</b>                                                                   | <b>63</b> |
| <b>Figure 39. Lamin A exists on the different types of ridges labelled by SUN-2.....</b>                                                        | <b>64</b> |
| <b>Figure 40. Lamin A and SUN-2 colocalization shows diversity from one cell to another. ....</b>                                               | <b>65</b> |
| <b>Figure 41. The comparison of high and low threshold application on cultured PASMNC nucleus labelling for Lamin A was shown.....</b>          | <b>68</b> |
| <b>Figure 42. The most found structures on lamin A labelled 7 days cultured nucleus are O-type, I-type and irregular round shaped. ....</b>     | <b>71</b> |
| <b>Figure 43. Three types of structures were observed on lamin A labelled D7PASMNC nucleus: ....</b>                                            | <b>73</b> |
| <b>Figure 44. The most commonly found structure is O-type structures on lamin A labelled D7PASMNC nuclei. ....</b>                              | <b>73</b> |
| <b>Figure 45. The illustration of 3D skin application and deconvolved image on cultured PASMNC nucleus labelling for emerin were shown.....</b> | <b>76</b> |
| <b>Figure 46. The most found structures on emerin labelled 7 days cultured PASMNC nuclei are O and I shaped structures.....</b>                 | <b>77</b> |

|                                                                                                                 |    |
|-----------------------------------------------------------------------------------------------------------------|----|
| Figure 47. Two types of the structure were observed on emerin labelled D7PASM C nucleus .....                   | 78 |
| Figure 48. The number of O-type structure on emerin labelled cultured cells is more than I-type.....            | 79 |
| Figure 49. Lamin A exists on the different types of structures labelled by emerin.....                          | 80 |
| Figure 50. Lamin A and emerin colocalization on D7PASM C nucleus shows diversity from one cell to another. .... | 82 |
| Figure 51. One example of nesprin labelling PASM C nucleus cultured for 7 days were shown.....                  | 84 |
| Figure 52. The outer structure on nesprin labelled 7 days cultured PASM C nucleus is composed of puncta. ....   | 85 |
| Figure 53. Nesprin labelling surface is composed of puncta in different sizes.....                              | 86 |
| Figure 54. One example of SUN-2 labelling D7PASM C nucleus were shown.....                                      | 87 |
| Figure 55. The most found structures on SUN-2 labelled D7PASM C are O, I-type and globular structures. ....     | 88 |
| Figure 56. Three types of structures were observed on SUN-2 labelled D7PASM C nucleus .....                     | 89 |
| Figure 57. O-type structure was most frequently found structure on SUN-2 labelled D7PASM C.....                 | 90 |
| Figure 58. Lamin A exists on the different types of structures labelled by SUN-2.....                           | 91 |
| Figure 59. Lamin A and SUN-2 colocalization on D7PASM C nuclei shows diversity from one cell to another. ....   | 93 |
| Figure 60. The distribution of lamin A and H3K9me2 in AIPASM C nucleus was shown. ....                          | 95 |

|                                                                                                                                                            |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 61. The histone mark, H3K9me2, placed in the ridges through lamin A labelling AIPASMC nuclei. ....                                                  | 96  |
| Figure 62. The number of H3K9me2-lamin A puncta and areas of these puncta showed variety on co-labelled lamin A and H3K9me2 AIPASMC nucleus.....           | 98  |
| Figure 63. The distribution of lamin A and H3K9me2 in D7PASMC nucleus were shown. ....                                                                     | 99  |
| Figure 64. The place of where lamin A colocalization with H3K9me2 were detected distinct on D7PASMC nucleus from AIPASMC nucleus.....                      | 100 |
| Figure 65. H3K9me2 clusters around the raised surface of the O-type structure are formed by lamin A around the central hole.....                           | 102 |
| Figure 66. Lamin A and H3K9me2 colocalization decreased in D7PASMC nucleus.....                                                                            | 103 |
| Figure 67. The distribution of emerin and BAF in AIPASMC nucleus was shown. ....                                                                           | 105 |
| Figure 68. BAF protein is placed in the middle of the ridge labelled by emerin in AIPASMC nuclei.....                                                      | 106 |
| Figure 69. The distribution of emerin and BAF in D7PASMC nucleus was shown. ....                                                                           | 107 |
| Figure 70. The place where BAF colocalised with emerin were detected differently in 7 days cultured PASMC nucleus from acutely isolated PASMC nuclei. .... | 108 |
| Figure 71. The schematic illustration of AIPASMC(A) and D7PASMC(B) nuclei was shown. ....                                                                  | 120 |

## List of Tables

|                                                                                        |    |
|----------------------------------------------------------------------------------------|----|
| Table 1. The listed sequence-specific antibodies that were utilised in this study..... | 18 |
| Table 2. The table illustrated the secondary antibody information.....                 | 19 |

|                                                                                                                                      |            |
|--------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>Table 3. The table shows the comparison of the structure number, size and colocalization on proteins. ....</b>                    | <b>66</b>  |
| <b>Table 4. The table shows the comparison of the structure number, size and colocalization for proteins on D7PASMC nuclei .....</b> | <b>93</b>  |
| <b>Table 5. The table showed the comparison of colocalization for lamin A and H3K9me2 on AIPASMC and D7PASMC nuclei.....</b>         | <b>104</b> |
| <b>Table 6. The table showed the comparison of colocalization for lamin BAF and emerin on AIPASMC and D7PASMC nuclei.. ....</b>      | <b>110</b> |
| <b>Table 7. The ratio of ridges dimensions labelled by lamin A in AIPASMC to nucleus volume were shown.....</b>                      | <b>122</b> |
| <b>Table 8. The ratio of ridges dimensions labelled by emerin in AIPASMC to nucleus volume was shown .....</b>                       | <b>123</b> |
| <b>Table 9. The ratio of ridges dimensions labelled by SUN-2 in AIPASMC to nucleus volume was shown. ....</b>                        | <b>123</b> |
| <b>Table 10. The ratio of ridges dimensions labelled by nesprin in AIPASMC to nucleus volume was shown. ....</b>                     | <b>124</b> |
| <b>Table 11. The ratio of ridges dimensions labelled by lamin A in D7PASMC to nucleus volume was shown. ....</b>                     | <b>125</b> |
| <b>Table 12. The ratio of ridges dimensions labelled by emerin in D7PASMC to nucleus volume was shown. ....</b>                      | <b>127</b> |
| <b>Table 13. The ratio of ridges dimensions labelled by SUN-2 in D7PASMC to nucleus volume was shown. ....</b>                       | <b>130</b> |
| <b>Table 14. The ratio of ridges dimensions labelled by nesprin in D7PASMC to nucleus volume was shown. ....</b>                     | <b>131</b> |

# Abbreviations

|                                 |                                                                    |
|---------------------------------|--------------------------------------------------------------------|
| $\alpha$ -SMA                   | $\alpha$ -Smooth muscle actin                                      |
| AIPASMC<br>cell                 | Acutely isolated pulmonary artery smooth muscle<br>cell            |
| BAF                             | Barrier-to-autointegration factor                                  |
| BI                              | Blind invagination                                                 |
| $\text{Ca}^{2+}$                | Calcium                                                            |
| $[\text{Ca}^{2+}]_i$            | Intracellular calcium                                              |
| CREB<br>element-binding protein | Cyclic adenosine monophosphate response<br>element-binding protein |
| CRBP-1                          | Cellular retinol-binding protein-1                                 |
| CNN-1                           | h1-calponin                                                        |
| DAPI                            | 4',6-diamidino-2-phenylindole                                      |
| D7PASMC                         | Day 7 pulmonary artery smooth muscle cell                          |
| DNA                             | Deoxyribonucleic acid                                              |
| DTT                             | 1,4-Dithiothreitol                                                 |
| ECM                             | Extracellular matrix                                               |
| ER                              | Endoplasmic reticulum                                              |
| FOXO4                           | Forkhead box protein O4                                            |
| H3K9me2                         | Histone H3 dimethylated at lysine 9                                |

|              |                                           |
|--------------|-------------------------------------------|
| INM          | Inner nuclear membrane                    |
| IP3R         | Inositol 1,4,5-trisphosphate receptor     |
| KASH         | Klarsicht, Anc-1, Syne-1 homology         |
| KLF4         | Kruppel-like factor 4                     |
| LADs         | Lamina-associated domains                 |
| LEM          | LAP2-emerin-MAN1                          |
| LINC complex | Linker of nucleoskeleton and cytoskeleton |
| MHC          | Myosin heavy chain                        |
| MLH 1        | MutL homolog 1                            |
| NE           | Nuclear envelope                          |
| Nesprin      | Nuclear envelope spectrin repeat protein  |
| NPC          | Nuclear pore complex                      |
| NR           | Nucleoplasmic reticulum                   |
| ONM          | Outer nuclear membrane                    |
| PSS          | Physiological salt solution               |
| PASMC        | Pulmonary artery smooth muscle cell       |
| PDGF-A       | Platelet derived growth factor alfa       |
| PDGF         | Platelet derived growth factor            |
| PMCA1        | Plasma membrane calcium pump 1            |
| PSF          | Point Spread Function                     |

|                |                                                            |
|----------------|------------------------------------------------------------|
| PSS            | Physiological salt solution                                |
| RyR2           | Rayonidin receptor type 2                                  |
| S100A9         | S100 Calcium Binding Protein A9                            |
| SERCA2a<br>2a  | Sarco / endoplasmic reticulum $\text{Ca}^{2+}$ ATPase type |
| SM22- $\alpha$ | Smooth muscle 22 $\alpha$                                  |
| SMC            | Smooth muscle cell                                         |
| SRF            | Serum response factor                                      |
| SUN-2          | Sad1-UNC84                                                 |
| SI             | Superficial invagination                                   |
| TGF- $\beta$   | Transforming growth factor beta                            |
| TNF- $\alpha$  | Tumour necrosis factor alpha                               |
| TI             | Transnuclear invagination                                  |
| VSMC           | Vascular smooth muscle cell                                |

# Abstract

Smooth muscles found on the walls of hollow organs are composed of spindle-shaped cells and are known as Smooth Muscle Cells (SMCs). These cells contract involuntarily to perform their functions such as tissue oxygenation and blood pressure regulation. SMCs are able to adapt easily to environmental changes as a consequence considerable plasticity. Although, smooth muscles generally exist in quiescent and contractile states under physiological condition, in the conditions, under conditions of environmental changes such as hypertension, angiogenesis or vascular calcification, the contractile phenotype transforms into an actively proliferating, synthetic phenotype. This process is called phenotypic switching, which is a mechanism that underlies many severe diseases, including pulmonary hypertension and atherosclerosis. The phenotypic switching is the transition from a contractile to a synthetic phenotype. It is influenced by many factors including signalling pathways, biochemical compounds and proteins. To date, these factors and the characteristics of contractile and synthetic phenotype have been investigated. However, the role of nuclear membrane proteins on phenotypic switching has not been studied by researchers in much detail, compared to other factors.

Therefore, I hypothesised that nuclear envelope proteins may have an important role in phenotypic switching through interaction with particular chromatin marks in particular regions. To test this I focussed on the following nuclear envelope proteins: lamin A, emerin, SUN-2 (Sad1 And UNC84 Domain Containing 2) and nesprin (nuclear envelope spectrin repeat proteins). Lamin A and emerin have ability to interact with Barrier-to-autointegration factor (BAF) and histone 3 lysine 9 dimethylation (H3K9me2) that are in relation to the modulation of gene expression. Nesprin and SUN-2 are responsible for the communication between cytoskeleton and nucleus, which can transfer mechanical cue to lamin A and emerin affecting chromatin interaction. My aim was to determine whether these proteins in some way contribute to those signalling pathways that modulate gene expression during the switch in

phenotype, by modulating chromatin organisation. To test my hypothesis, I investigated the distribution and levels of these selected nuclear envelope proteins in acutely isolated pulmonary arterial myocytes and compare them to pulmonary arterial smooth muscle cells that had been cultured in a pro-proliferative medium for 7 days, which acted as a model for synthetic phenotype. The distribution of the nuclear membrane proteins detected in these two smooth muscle phenotypes was completely different. Moreover, the surface structures created by labelling these proteins were both diverse and phenotype-specific. In the contractile phenotype, for example, lamin covered the entire surface of the inner nuclear membrane, but also accumulated at higher density in identifiable structures, such as peripheral ridges, clefts and nuclear invaginations. As a result, although emerin formed similar structures, it was less uniform in distribution. The distribution of nesprin, on the other hand, was restricted to the periphery and SUN-2 was present at peripheral structures. Barrier-to-autointegration factor (BAF) and the histone mark H3K9me2 were found to be associated with emerin and lamin at the nuclear periphery, providing sites for chromatin attachment and gene repression. Remarkably, reconfiguration of the distribution and structural organisation of all proteins was observed in proliferating cells. For example, the periphery structures such as ridges and clefts as well as nuclear invaginations were not observed throughout the proliferating nuclei. Instead of these structures, lamin A accumulation was observed, which caused some indentations including O-type and I-type structures. As in the contractile phenotype, lamin A distribution covered the area underneath the nuclear envelope and emerin also showed similar pattern. However, the distribution of nesprin in proliferating nuclei was observed as puncta whereas SUN-2 was located on particular regions. Dependent on these changes in the distribution of proteins, alterations in their colocalization with BAF and histone mark, H3K9me2 was detected, which is that the association of lamin A and H3K9me2 spreads through the nuclei in proliferating nuclei, instead of located on particular structures. This colocalization regions was observed as different pattern in different ridges identified. Moreover, the colocalization between lamin A and H3K9me2

reduced from contractile to proliferative phenotype. Interestingly, BAF and emerin colocalization increased in proliferating phenotype, compared to contractile phenotype and the ridge specific distribution of colocalization puncta was also observed in this proteins association.

Overall, these results indicate that this redistribution of nuclear membrane proteins may correspond to an important role in SMC phenotypic switching, through gene expression modulation by changing chromatin organisation.

## Lay Summary

One of the cell types found in hollow organs, including arteries, airways, and the bladder, is the smooth muscle cells (SMCs) that provide tissue integrity and regulate, for example, blood flow by contracting and relaxing to narrow or widen blood vessels. SMCs exhibit plasticity in response to environmental inputs such as high blood pressure. Briefly, they change from one category (phenotype) to another (proliferating phenotype), by undergoing a phenotypic switch from a contractile to a proliferating and migratory smooth muscle types. This is associated with a change in the appearance (morphology) and the structure of the nucleus where gene expression is regulated. The evidence gathered from this study suggests that proteins in the nucleus might affect phenotypic switching. However, despite the possibility of this, there are only a limited number of studies to explore and explicate the protein functions in phenotypic switching.

Therefore, in this study, I investigated whether the redistribution of a selected group of nuclear proteins, which are known to be regulating gene expression, namely lamin A, emerin, nesprin (nuclear envelope spectrin repeat proteins) and SUN-2 (Sad1 And UNC84 Domain Containing 2), might occur during SMC proliferation. These proteins are known to respond to changes in environmental conditions and to undergo transformation in a way that changes gene expression. I discovered that these proteins aggregate at identifiable structures on nuclear membranes. That is, in comparison to contractile SMCs, these proteins can be redistributed in proliferation SMCs either driven by or driving changes in nuclear structure that may impact gene expression and, thus, coordinate phenotypic switching.

# Introduction

Smooth muscle cells (SMCs) are spindle-shaped, thin and elongated cells, with one nucleus in the cell centre (Shi et al., 2019). Interestingly, they are not terminally differentiated in contrast to other muscle cells (Worth et al., 2001). SMCs are found in the walls of all hollow organs, including intestines, blood vessels and the stomach. They fulfil various important tasks in these organs, such as the generation of a response to mechanical and chemical stimuli (Somlyo & Siegman, 2012). In particular, vascular smooth muscle cells (VSMCs), the key element of the vasculature, have critical physiological functions, including vasoconstriction and vasodilatation modulated by calcium signalling (Bacakova et al., 2018; Owens, 1995). Smooth muscle cells differ from cardiac and skeletal muscle cells in illustrating immense plasticity. In a pathological situation like injury, the phenomenon of “phenotypic switching” occurs, changing from contractile to synthetic phenotype, which is associated with identifiable changes in the shape of SMC nuclei. More specifically, the alteration in SMCs consists in the transition from a contractile phenotype, the defining features of which are quiescent, contractile and low proliferative states, into a synthetic phenotype characterised by high rates of proliferation and migration (Allahverdian et al., 2018). As an example of the changes that are triggered during the phenotypic switching process, pulmonary arterial hypertension leads to the transition from contractile to a highly proliferative synthetic SMC phenotype, resulting in the thickening of the arterial walls. The thickness in the arterial wall ultimately causes increased pulmonary arterial pressure, which affects the well-being of the patients (Wilson et al., 2015).

Additionally, the vascular smooth muscle contraction mechanism is modulated by intracellular calcium ( $[Ca^{2+}]_i$ ) signalling. Upon local increases in cytoplasmic calcium ( $Ca^{2+}$ ) concentration, myosin light chain kinase is activated by binding four  $Ca^{2+}$  to the binding regions on calmodulin, which results in the

phosphorylation of the light chain of myosin and generates the force for smooth muscle contraction (Wilson et al., 2002) (**see Figure 1**). Various mechanisms to maintain  $\text{Ca}^{2+}$  homeostasis are defined, including sodium ( $\text{Na}^+$ )/ $\text{Ca}^{2+}$  exchanger, plasma membrane  $\text{Ca}^{2+}$  pump 1 (PMCA1), ryanodine receptor type 2 (RyR2) and the Sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  adenosine triphosphatase type 2a (SERCA2a). Interestingly, the previous research into the transition from the contractile to non-contractile (synthetic) phenotype showed that it leads to down-regulation of  $\text{Na}^+$ / $\text{Ca}^{2+}$  exchanger, PMCA1, RyR2 and the SERCA2a. Therefore, these findings confirmed that  $[\text{Ca}^{2+}]_i$  has a role in excitation-transcription coupling in VSMCs, in addition to excitation-contraction coupling. Additionally, the  $\text{Ca}^{2+}$  transcription-dependent regulators, including serum response factor (SRF) and cyclic adenosine monophosphate response element binding protein (CREB), have been illustrated to transform VSMCs from contractile to synthetic phenotype (Matchkov et al., 2012).

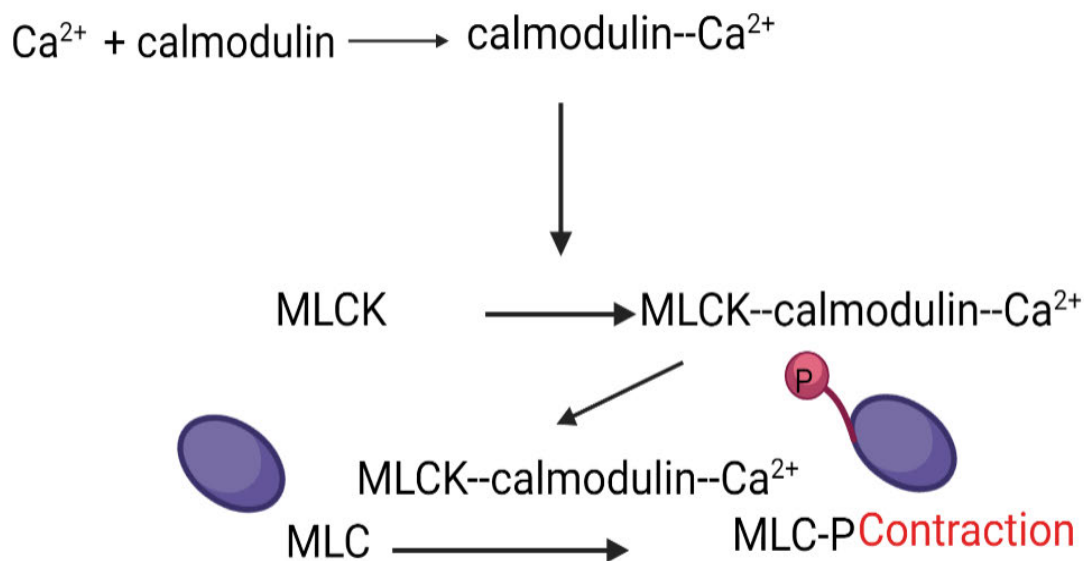


**Figure 1. The illustration of SMC contraction mechanism, created by Biorender, was shown.** Calmodulin binding with  $\text{Ca}^{2+}$  leads to activation of myosin light chain kinase (MLCK) that responsible for the phosphorylation of the myosin light chain (MLC) causing smooth muscle contraction (Hilgers & Webb, 2005).

Another significant aspect of phenotypic switching is that the morphology of the contractile phenotype differs completely from synthetic SMCs. The morphology of contractile SMCs is elongated and spindle-shaped, while the synthetic phenotype presents epithelioid or rhomboid morphology (Rensen et

al., 2007) (**see Figure 2**). The difference between the two phenotypes of SMCs is associated with changes in the nuclear protein distribution underneath the nuclear envelope (NE) and, thereby, may affect the gene expression. In this respect, recent studies on phenotypic switching have highlighted the need to investigate the role of nuclear envelope proteins in phenotypic switching (Qi et al., 2016). This chapter will provide a general overview of the relationship between SMC phenotypic switching, NE proteins and the mechanisms affecting phenotypic switching.

## 1.1 The phenotypes of contractile and synthetic SMC

It has been shown that there are diverse phenotypes of VSMCs, ranging from normal to pathogenic (Zhou et al., 2018). A more detailed account of the phenotypes of VSMC that may be presented during phenotypic switching is provided in the following section.

The quiescent or differentiated VSMCs, which are called the contractile phenotype, are responsible for blood flow with vasoconstriction and vasodilation functions in the arterial wall (Louis & Zahradka, 2010) with thick and thin myofilaments that fill the cytoplasm. SMCs are defined as elongated, spindle-shaped in appearance (Rensen et al., 2007) (**see Figure 2**). In addition to the ability to contract of the contractile proteins and cellular quiescence in SMCs also disappear during phenotypic switching.

In contrast to contractile SMCs, synthetic SMCs display more ovoid-shaped with an epithelioid structure (**Figure 2**). Synthetic SMCs present entirely different morphologies and proliferative and migratory properties (Rensen et al., 2007a) upon stimulation with growth factors and cytokines (Worth et al., 2001). Moreover, they include more organelles necessary for protein synthesis, such as ribosomes, rough endoplasmic reticulum and mitochondria, instead of contractile filaments. Additionally, the extracellular matrix (ECM) protein production is also increased in the synthetic phenotype (Beamish et al., 2010) (**Figure 2**). Unlike contractile SMCs, the synthetic phenotype is primarily found in the embryo, in young and growing organisms (Thyberg et

al., 1990) and, importantly, in the tissues that undergo intimal thickenings during repair and in disease, such as the formation of atherosclerosis plaques (Ang et al., 1990).

Considering the above-mentioned evidence, it seems that morphological differences depending on the phenotypic state display different functional characteristics (Halayko & Solway, 2001).

- |                                                     |                                                      |
|-----------------------------------------------------|------------------------------------------------------|
| ❖ Spindle shaped                                    | ❖ Rhomboidal shaped                                  |
| ❖ Differentiated                                    | ❖ Proliferative and migratory                        |
| ❖ Contractile apparatus                             | ❖ More organelles for protein synthesis              |
| ❖ Contractile proteins; SM-MHC, $\alpha$ -SMA, etc. | ❖ ECM proteins; osteopontin, Collagen I and II, etc. |

**Figure 2. The schematic illustration of a) contractile and b) synthetic SMCs, created by Biorender, was shown.** Contractile phenotype has contractile proteins and contractile phenotypic characteristics including spindle-shaped, differentiated, whereas synthetic phenotype is more rhomboidal-shaped with proliferative and migratory properties. Synthetic phenotype has more organelles for protein synthesis.

## 1.2 VSMC phenotypic switching

So far, numerous studies have suggested that during primary culture of enzymatically isolated SMCs, they transfer from a contractile to synthetic phenotype. Interestingly, SMCs maintain their contractile phenotype for up to 7 days in culture (Chamley-Campbell, 1979). However, when these SMCs are seeded at less density, they pass through switching to a synthetic phenotype at the end of variable time points (days) depending on the vessel type, species and age (Ang et al., 1990). Due to the advantages of in-vitro studies, such as

easy control of environmental factors, this system has a great valuable model for studies associated with SMC phenotypic switching.

### **1.3 Gene expression in vascular smooth muscle cell phenotypic switching**

A number of studies have shown that the phenotypic switching in SMCs leads to the downregulation of contractile markers and upregulation of the proliferation markers (Gomez & Owens, 2012; Owens et al., 2004). It has suggested that VSMC markers including alpha-smooth muscle actin ( $\alpha$ -SMA), smooth muscle 22-alpha (SM22- $\alpha$ ), smoothelin, myosin heavy chain (MHC) and h1-calponin (CNN-1) are highly expressed in the contractile state (Jaminon et al., 2019). However, MHC expression during maturation and the development of the mouse embryo are known as the most specific marker of contractile phenotype and found only in smooth muscles (Miano et al., 1994).

In the light of these investigations, it can be argued that transcriptional regulators are important for the phenotypic switching process. One of the transcriptional regulators is SRF that combines signals of two pathways: mitogen-activated protein kinase (MAPK) and actin. The other regulators, kruppel-like factor (KLF4) and forkhead box protein O4 peptide (FOXO4), also contribute to the process of phenotypic switching. In addition, signalling pathways, Rho-actin, tumour growth factor- $\beta$  can modulate SMC dedifferentiation (Frismantiene et al., 2018).

During phenotypic switching, the expression of contractile phenotype is steadily downregulated to a degree coincident with the increase in the expression of proliferation markers, which includes platelet derived growth factor alpha and cellular retinol-binding protein-1 (CRBP-1) (Rensen et al., 2007). The changes in smooth muscle cells cause the transition of contractile apparatus in favour of proliferative and migratory activity.

Additionally, biochemical compounds also regulate the process. For instance, platelet derived growth factor (PDGF) induces VSMC differentiation from

contractile to synthetic phenotype, downregulating  $\alpha$ -SMA, a contractile marker in aortic VSMCs. By contrast, tumour necrosis factor alpha and beta (TNF- $\alpha$  and  $\beta$ ) and angiotensin II contribute to the contractile, non-proliferative phenotype (Jaminon et al., 2019).

The regulators of phenotypic switching are, however, poorly understood and despite the investigation of the effects of the transcriptional regulators, hormonal factors and signalling pathways in phenotypic switching, there are a limited number of studies in relation to nuclear envelope proteins and their effects on the modulation of phenotypic switching.

## **1.5 Nuclear envelope proteins direct genome organisation**

The nuclear envelope is composed of a double phospholipid membrane and it surrounding the nucleus to separate deoxyribonucleic acid (DNA) from the cytoplasm (Watson, 1955). In the nuclear envelope, the perinuclear space lies between inner nuclear membrane (INM) and outer nuclear membrane (ONM) and is sized around 50 nm in width (Prunuske & Ullman, 2006). Additionally, INM is attached to the ONM by nuclear pores that provide trafficking from the cytosol to the nucleus and are embedded in the nuclear envelope and other large macromolecular assemblies (**Figure 3**). The flow from the cytosol to the nucleus by nuclear pore complex (NPC) includes the elements required for gene expression, DNA repair and chromatin organisation (Beck & Hurt, 2017).

For cell functions, the NE has a pivotal roles from communication by ion signalling to genome organisation (Starr, 2007). Additionally, nuclear functions, including gene expression, chromatin organisation and DNA metabolism, are modulated by INM associated proteins that comprise at least 70 individual proteins (Burke & Stewart, 2013; Hetzer, 2010). The nuclear envelope proteins are responsible for various tasks. The following part of this section describes in greater detail the NE proteins. Importantly, some of the nuclear envelope proteins affect the spatial organisation of chromatin that may in turn regulate gene expression (Van de Vosse et al., 2011). For example, the nuclear lamina, one of the nuclear envelope proteins, is placed underneath the

inner nuclear membrane as a protein meshwork that is approximately 15-20 nm thick (Gerace & Huber, 2012) **(see Figure 3)**. The meshwork is formed by two types of lamin, lamin A-type (lamin A, lamin C and lamin A $\Delta$ 10) and B-type lamin (lamin B1, lamin B2 and lamin B3). The lamina structure is unclear in somatic cells, owing to chromatin masking the nucleus side of the NE. Despite the filamentous structure of the somatic cell lamina, recent developments in high-resolution light microscopy have led to studies which have indicated that the A- and B-type lamins are form sperate, but interacting networks (Shimi et al., 2008).

Lamins, particularly lamin A, are responsible for maintaining nuclear shape and rigidity. As a result of this, stiff tissues such as bone and cartilage include more lamin A, and the cell differentiation may be affected by lamin A level and the stiffness of the extracellular environment (Burke & Stewart, 2013).

Another role of lamin A in nuclei is to provide anchoring points that determine the distribution of nuclear proteins by retaining INM proteins and concentrating proteins to the lamina. Furthermore, in chromatin organisation, two types of chromatins, heterochromatin and euchromatin, show different tendencies to locate in the nucleus, which are important to determine transcriptional activity. Heterochromatin, which is linked to repressive chromatin marks, has a propensity to be positioned at the nuclear periphery, while euchromatin, which is regarded as a transcriptionally active, concentrates in the interior of the nucleus (Bickmore, 2013). Additionally, it is considered that NPCs and chromatin are significant for the modulation of transcription. The nuclear lamina has a considerable task that affects chromatin organisation and conducts transcription and replication (Wilson & Foisner, 2010). In addition, nuclear lamina connects with a particular DNA sequences or histones and the nuclear pore complex (Raices & D'angelo, 2017) to regulate chromatin organisation (Torvaldson et al., 2015) **(Figure 3)**. Several studies associated on lamin A and chromatin have illustrated that A-type lamin connects to lamin-associated-domains on chromatin and therefore interacts the chromatin to

nuclear periphery, which leads to the suppression of gene expression (Harr & Reddy, 2016).

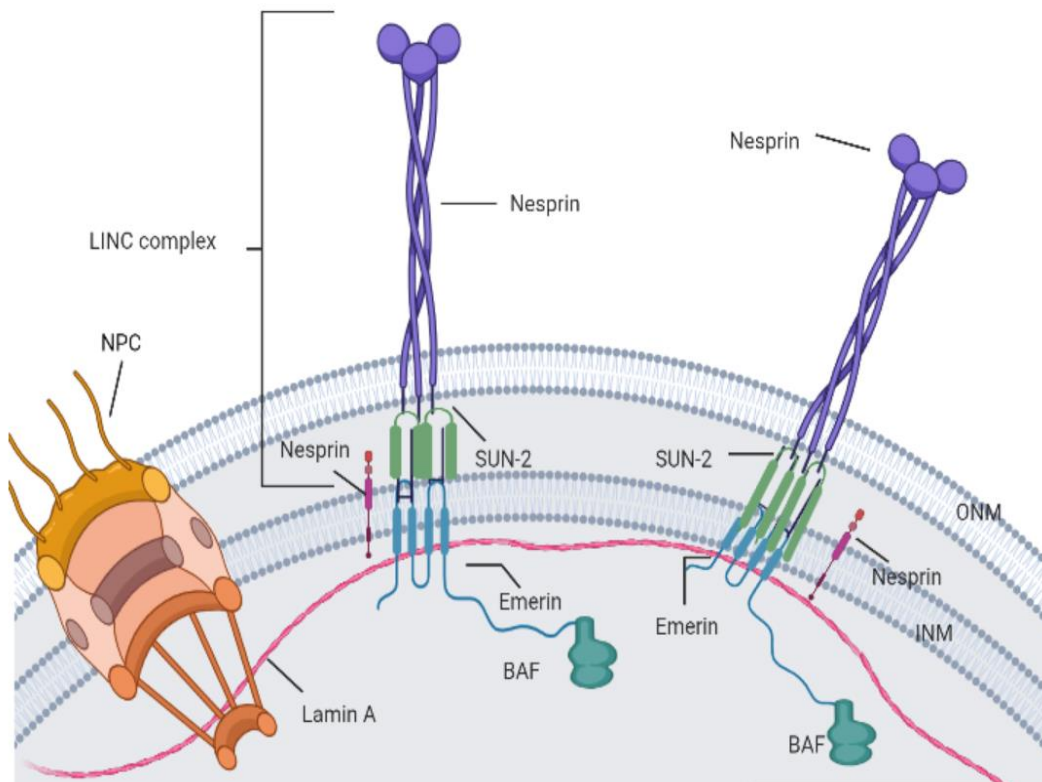


**Figure 3. The schematic demonstration of nuclear envelope created by Biorender, including INM and ONM, was shown.** NE is composed of two membranes, ONM and INM, bound with each other by NPC. Lamin A located underneath INM have interaction with emerlin that can bind BAF as well as SUN-2, one of the element of LINC complex binding with nesprin located in ONM.

Another important NE protein, emerlin, belongs to the LEM-domain (LAP2, emerin, MAN1) proteins family and it establishes NE-associated nucleoskeletal structure. It attaches itself to lamin and barrier-to-autointegration factor (BAF), which links DNA to emerlin and influences histone post-translational modifications (Burke & Stewart, 2013; Segura-Totten & Wilson, 2004; Shimi et al., 2004) (**Figure 3**). Furthermore, emerlin manages many processes that mediate ‘tethering’ of silent chromatin and stimulate force-sensitive genes (mechano-transduction signalling) (Lammerding et al., 2005). This information suggests that emerlin merges mechanical and other signalling inputs to mediate the modulation of gene expression (Berk et al., 2013).

The other protein complex connecting the nucleoplasm to the cytoskeleton is the linkers of the nucleoskeleton and cytoskeleton (LINC) complex has been suggested to transduce signals across the NE (Banerjee et al., 2014; Hieda, 2017; Kim et al., 2015). The components of the LINC complex are klarsicht-ANC-1-SYNE homology (KASH) domain proteins and sad1/UNC-84 homology (SUN) domain proteins. KASH domain proteins are located through the ONM and INM, whereas the transmembrane domain of SUN forms trimers in between the INM and ONM (**Figure 3**). KASH proteins are encoded by six genes in the human genome, including nuclear envelope spectrin-repeat proteins 1-4 (nesprin 1-4) with multiple isoforms (Hieda, 2017). Furthermore, some isoforms of KASH can connect with other inner nuclear proteins such as emerin and the nuclear lamina (Rothballer et al., 2013). Based on this information, it can be concluded that LINC complex influences cytoskeleton organisation and function, suggesting that it is a factor affecting the modulation of chromatin organisation (Burke & Stewart, 2013).

## **1.6 The possible role of $\text{Ca}^{2+}$ flux by across the cell-wide web and nuclear invagination network**

In some cell functions,  $\text{Ca}^{2+}$  is essential for intracellular information transfer, and nuclear calcium is regarded as one of the most powerful regulators in gene expression. The “nuclear calcium hypothesis” was first proposed by Bading (2000), suggesting that transcription activation is triggered by calcium entry into the cell nucleus in the neuron. In 2009, Zhang et al. demonstrated that the activation and repression of some genes, including 185 genes related to synaptic activity-regulated gene expression, required calcium. In addition, gene programmes are activated by nuclear calcium level, resulting in functional and structural changes in the cell (Wittmann et al., 2009). The other possible connection between calcium and transcription is that calcium signalling can modify the availability of transcription factors in the nucleus (Bading, 2013). Alterations in nuclear envelope structure, such as nuclear envelope infolding that is accepted to act as a perinuclear  $\text{Ca}^{2+}$  store, allow  $\text{Ca}^{2+}$  signalling to reach easily into the nucleoplasm, where it mediate the

production of highly localised  $\text{Ca}^{2+}$  signals to regulate gene expression (Bootman et al., 2009).

Cell nucleus morphology in the neuron is important in signal transmission, and in each neuron, its morphology can exhibit activity-dependent changes in shape. Moreover, these alterations in the nuclear shape influence intracellular functions and signal transmission, such as calcium dynamics. Queisser et al. (2011) proposed the phenomenon of “morphology modulation of nuclear calcium signals”, which suggests that nuclear infoldings have the ability to organise nuclear compartments resulting in various calcium patterns as well as variances in calcium signal frequency and amplitude (Queisser et al., 2011). Moreover, infoldings in the nuclear envelope that are related to transcriptional activities such as the phosphorylation process of histone H3 help to modulate gene expression (Wittmann et al., 2009a). As the structure of the nucleus can act to segregate functionally relevant subdomains, through nuclear infoldings mentioned above, was first articulated by Strouboulis and Wolfs (1996). It was shown that transcriptional and translational activities composed of DNA and ribonucleic acid (RNA) appear in specified regions, and interestingly, these subdomains were observed as not bounded by a membrane. In addition, the other possibility is that NE may include is the extensions created by the intranuclear membrane. In this regard, Bourgeois et al. (1979) defined nuclear invaginations in mammalian cells. By advanced the confocal microscopy, these extensions, which are called nuclear invaginations were observed in various cultured cell lines (Fricker et al., 1997a).

Additionally, one single transnuclear invagination has been observed in VSMCs grown in primary culture (Avedanian et al., 2011). It has been proposed that nuclear invaginations (Fricker et al., 1997) direct the  $\text{Ca}^{2+}$  signalling to the nucleus through cytosol extensions and genome regulation. Hence, NE invaginations might influence gene activation-repression. Additionally, it was suggested that nuclear infoldings elevated nuclear sensitivity to changes in calcium dynamics, with transcriptional activity being increased.

Nuclear invaginations are sized 0.2-0.5  $\mu\text{m}$  and include an outer and inner membrane (Fricker et al., 1997), which is found in various cells (Collings et al., 2000; Fischer et al., 2003; Malhas et al., 2011). This complex network formed by nuclear invaginations are known as the nucleoplasmic reticulum (NR) since their shape and function resembles to the endoplasmic reticulum (ER). Malhas et al. (2011) reported only two types of these, which are classified into 1) Type I, which are formed only by INM, and 2) Type II (tube-like), which are formed by INM and ONM (Malhas et al., 2011) (**Figure 4**). These two types of invaginations can be presented in the same cell and are independent of the cell cycle stage (Fricker et al., 1997b).

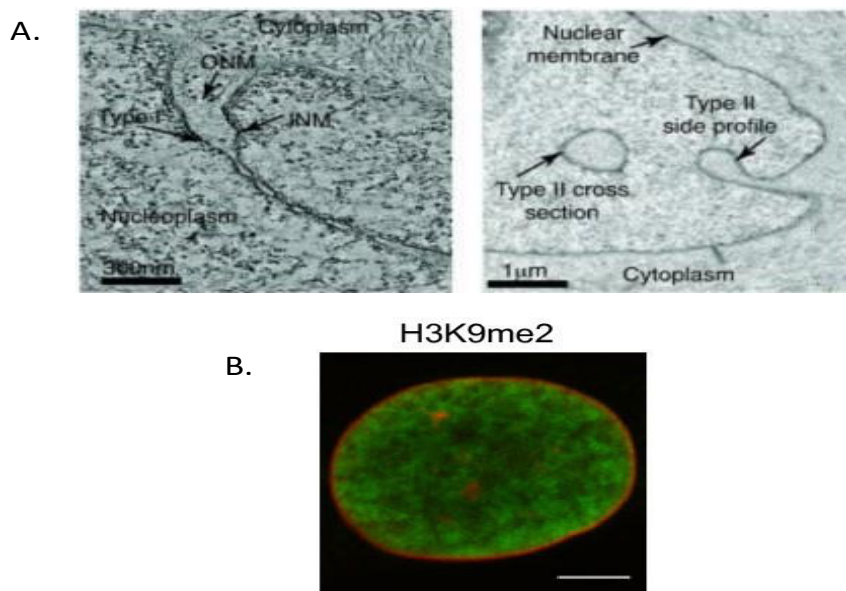


**Figure 4. Images taken by electron and confocal microscopy illustrates type 1 and type 2 nuclear invaginations as well as lamin A and H3K9me2 localizations. A)** Electron micrograph of mouse embryo fibroblast showing type 1 and type 2 nuclear invaginations. ONM: outer nuclear membrane, INM: Inner nuclear membrane (Malhas et al., 2011) was shown. **B)** The illustration of lamin A (red) and H3K9me2 (green) were shown in C2C12 myoblast nuclei by confocal microscopy (Wu & Yao, 2013).

As shown figure 4, nuclear invaginations extend into nucleoplasm to provide more separated and disparate network for delivery of  $\text{Ca}^{2+}$  signalling by SERCA and RyR1 located in ONM with subtypes BAF-emerin and lamin A-histone 3 lysine 9 dimethylation (H3K9me2). Recent papers have shown further evidence that  $\text{Ca}^{2+}$  movement into nuclear invaginations regulates

chromatin marks in differentiated cells, and that the modulation might be a result of protein-chromatin interactions (Duan et al., 2019). For example, Duan et al. (2019) shows that the inhibition of  $\text{Ca}^{2+}$  flux through RyRs leads to a decrease in the expression of S100 calcium binding protein A9 (S100A9) suppressed by BAF (Takama et al., 2013) and MutL homolog 1 (Mlh1) which is repressed by H3K9me2 activation located at the nuclear periphery (Sun et al., 2009) (**Figure 4**). Duan, 2019 illustrates that calcium flux across the ONM enters the cytoplasmic nano courses that is formed by sarcoplasmic reticulum membrane-membrane nanojunctions connecting distinct the communication wires though the entire cell in a cell-wide network. The other interesting point is that the cell-wide network is remodelled during the alteration to proliferation in culture, promoted proliferation and caused changes in RyR and inositol 1,4,5-trisphosphate (IP3R) expression, affecting  $\text{Ca}^{2+}$  flux as well as lamin A. The transition to proliferation leads to the loss in the lamin/emerin positive nuclear invagination, which is in support of the idea that nuclear invaginations have a role in the modulation of antiproliferative genes (Duan et al., 2019). Unlike the invagination studies on the effects of proliferation, there have in fact been limited studies on the nature of changes at the nuclear periphery, including the infoldings such as ridges due to proliferation.

The previous research (e.g., (House et al., 2008a)) recognises the critical role played by  $\text{Ca}^{2+}$  and the nuclear envelope in phenotypic switching. Additionally, it is now well established from a variety of studies (e.g., (Spin et al., 2010; Wittmann et al., 2009b)) that morphological changes in the nuclear envelope affect  $\text{Ca}^{2+}$  signalling and chromatin organisation. However, earlier studies offer limited explanations how nuclear envelope proteins associate with chromatin and can cause these results. For example, there is an important relationship between lamin A and morphological change in the nuclear envelope due to the ability of lamin A to modulate nuclear shape and its role in transcriptional regulation and chromatin organisation (Queisser et al., 2011). Moreover, the interaction between nesprin and SUN-2, which are responsible for mechanotransduction, may influence phenotypic switching. Therefore, this

indicates a need to understand nuclear envelope proteins during phenotypic switching.

Very little is currently known about the morphology of the nuclear envelope in contractile and synthetic phenotype. Morphological changes in the nuclear envelope, including nuclear invagination, affect the sensitivity of the nucleus to  $\text{Ca}^{2+}$  signalling and, thereby, gene transcription. Few studies (e.g., (Duan, 2018; Fedorova & Zink, 2008)) have investigated nuclear envelope morphology and its relationship with the proliferation and gene expression in any systematic way. As an example of this, Duan et al. (2019) illustrated that gene expression is regulated by  $\text{Ca}^{2+}$  flux into nuclear invaginations. Based on these facts, it can be argued that the structures on the nuclear envelope are important for nuclear calcium and transcriptional activity. Consequently, it is evident that much more investigations are needed to identify nuclear envelope morphology, for example, the investigation of ridges created by accumulation of proteins.

## **1.7 Aim and Hypothesis**

In this study, I aim to investigate how the nuclear envelope proteins' interactions with particular chromatin marks could contribute to gene expression. It has been shown that nuclear invaginations have an effect on the modulation of antiproliferative genes (Wang et al., 2015). Based this information, I hypothesized that the infoldings, including ridges on nuclear envelope, might affect specific chromatin marks and the nuclear envelope proteins, and, therefore, interactions between them. Particularly, I wish to examine whether the interaction of lamin A-H3K9me2 and emerin-BAF on these ridges created by lamin A and emerin and morphological features of these proteins might be sites for gene expression regulation. Additionally, LINC complex proteins may contribute to the modulation of gene expression by positioning in these regions where lamin A lines the INM proteins and may interact with these proteins.

In addition, it has been shown that the nuclear morphology of PASMNC changed during myocyte proliferation and the majority of nuclear invaginations was disappeared (Duan et al., 2019). Therefore, the association between nuclear envelope proteins with BAF and histone marks in ridges might be changed, impacting the phenotype characteristics of PASMNC nuclei through the regulation by these proteins of gene expression, perhaps mediated through their repressive association with chromatin in a manner modulated by LINC complex proteins.

# Materials and Methods

## 2.1 Pulmonary artery tissue dissection and identification

With the application of the regulations according to “the United Kingdom Animals (Scientific Procedures) Act 1986”, the animal tissue collections for experiments were carried out following a schedule 1 cull. The heart and lung blocks obtained from adult male Sprague Dawley (~300 g) were kept on ice in physiological salt solution (PSS) containing (mmol/L) 130 NaCl, 5.2 KCl, 1 MgCl<sub>2</sub>, 1.7 CaCl<sub>2</sub>, 10 glucose, and 10 HEPES, pH 7.4, which is isotonic solution with body fluid to keep the tissue live temporary and cold PSS reduces the tissue metabolism to maintain cell viability (Dabisch et al., 2004).

The protocol to dissect intrapulmonary arteries was performed under a dissection microscope (model PZM, World Precision Instruments, Sarasota FL, USA), using forceps and spring bow scissors (Duckworth & Kent, England, UK). At the starting point of the process, the organs were kept in cold PSS, one of the lung lobes was fixed onto a Sylgrad coated dissection dish with the interior face of lungs facing up. Then, the trachea was removed along the trachea's length, and the artery was determined as the parallel diaphanous tube that spans the underside of the trachea. The tissues outside of the artery and fat on the artery were entirely removed to reveal the artery, with minimum tissue damage. The artery was cleaned thoroughly and cut into small parts to ease dissociation. Then, the artery was placed on ice-cold PSS (see **Figure 5**).

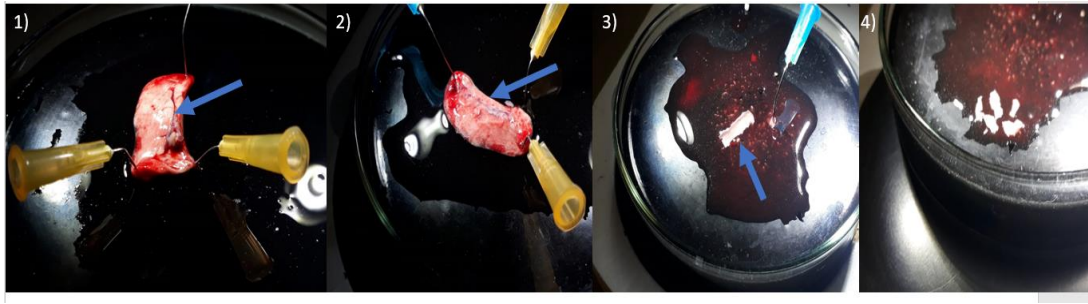


**Figure 5. Schematic illustration of the tissue dissection process is shown.** 1) A whole lobe of the lung pinned down by needles with the trachea (2) removed to reveal the pulmonary artery. 3) It was dissected from the lung lobe then, 4) cut into small sections.

## 2.2 Digestion protocol for the dissociation of PSMCs

Firstly, the tissues were submersed into a  $\text{Ca}^{2+}$  free bath solution containing 1.35 M NaCl, 50mM KCl, 0.1 M HEPES, 1mM  $\text{MgCl}_2$ , 10mM glucose and albumin, pH 7.4 as a buffer solution. HEPES was used to maintain physiological pH as well as glucose and albumin help maintain a stable osmolarity value (Pena & Dominguez, 2010). Due to the ability of calcium to promote cell adhesion and detailly, in digestion process, since  $\text{Ca}^{2+}$  provide stabilization of intracellular matrix,  $\text{Ca}^{2+}$  free solution was used to avoid cell accumulation (Wiesmann, 1999; Zhu et al., 2019). Moreover, an increase in the extracellular  $\text{Ca}^{2+}$  activates  $\text{Ca}^{2+}$  release mechanisms, including inositol 1,4,5-trisphosphate receptors (IP3R) on nuclear envelope (NE), and increase proliferation ( Lee et al., 2018; Resende et al., 2013), thereby bath solution with  $\text{Ca}^{2+}$  can influence  $\text{Ca}^{2+}$  stores in nuclear invaginations. By adding 1mg/ml papain, 0.8 mg/ml 1,4-Dithiothreitol (DTT) and 0.7 mg/ml bovine serum albumin to  $\text{Ca}^{2+}$  free PSS, the dissociation solution was prepared, in which arteries were placed for 15 minutes at 37°C. Papain is an enzyme that has proteolytic activity to split the peptide chain at basic amino acids, leucine or glycine. For cell isolation, papain was commonly utilised, especially as it allows to the isolation of rat VSMCs with high viability without the loss in the sensitivity of cells to stimuli. To fulfil the proteolytic function of papain, a free sulfhydryl group is required, and DTT can preserve the sulfhydryl group in a reduced state and thereby, it leads to papain activation (Bolzon & Cheung, 1989). For the next step, the tissues were washed three times in  $\text{Ca}^{2+}$  free PSS.

Subsequently, they were transferred into a pre-heated aliquot of bath solution at 37°C and then washed two times in fresh Ca<sup>2+</sup> free solution without enzymes. Finally, smooth muscle cells were isolated by gentle trituration with a fire-polished Pasteur pipette. The protocol for a fast digestion is able to isolate cells in an hour and these would be used in the same day to enhance the cell viability. Nonetheless, this protocol does not allow papain to penetrate the tissue which put the isolation efficiency in jeopardy.

### **2.3 Acutely isolated cell fixation with methanol**

After the isolation of SMCs, the cells were suspended in Ca<sup>2+</sup> free PSS with a density of cells 10<sup>5</sup>- 2x10<sup>5</sup> cells per ml. From the suspension, 100 µl was added to a 13 mm circular cover glass located in a 12-well plate and incubated for 45 minutes at room temperature for providing cell-slide attachment and spreading cells through the coverslip. Since the possibility that coating material such as poly L-lysine may lead to increase in unspecific staining, coverslips without coating were used in this study and instead of this, drying method was used before fixation. Then, ice-cold methanol (2 ml) was added to each suspension, and they were covered by parafilm to be stored at -20°C for 10 minutes. Alcohol fixatives acts through a denaturing proteins via the exclusion of the water, which is an extremely rapid fixation process, however it will lead to tissue shrinkage due to dehydration (Howat & Wilson, 2014). Commonly, it is accepted as less toxic compared to formaldehyde for studies of membrane-bound proteins.

## 2.4 Immunochemistry

| Antibody                        | Species           | Dilution | Immunogen                                                 | Company                                                       | Catalogue number |
|---------------------------------|-------------------|----------|-----------------------------------------------------------|---------------------------------------------------------------|------------------|
| Anti-Lamin A antibody           | Rabbit polyclonal | 1:200    | Residues 550 to the C-terminus of Human Lamin A           | Abcam                                                         | ab26300          |
| Anti-emerin antibody            | Rabbit polyclonal | 1:200    | Amino acid 3-254 of full-length Human emerin              | Santa Cruz                                                    | sc-15378         |
| Anti-BAF antibody               | Goat polyclonal   | 1:50     | C terminal residues of Human BAF                          | Santa Cruz                                                    | sc-9744          |
| Anti-Nesprin-1 antibody         | Mouse monoclonal  | 1:50     | Recombinant full-length nesprin-1- $\alpha$ proteins      | Gift from Dr. Eric Schirmer (Reference: NT Duong et al, 2014) | MANNES1E(8C3)    |
| Anti-dimethyl Histone H3 (Lys9) | Mouse monoclonal  | 1:200    | Amino acid 1-19 of human Histone H3, dimethylated on Lys9 | Millipore                                                     | 05-1249          |

**Table 1.** The listed sequence-specific antibodies that were utilised in this study. The table illustrated the primary antibody information, including the dilution factor, immunogen, animal origin species, company and commercial catalogue number (Duan, 2018).

After fixed by the methanol method described in section 2.3, samples were washed 3 times with Tris-buffered saline, with the following composition (mM) of 140 NaCl, 25 Tris-base, and 1.3 KCl pH 7.4. Tris-base is pH regulator and in this study it was used to maintain pH 7.4. The samples were then incubated with a blocking buffer, including a mixture of 1% bovine serum albumin, 10% donkey serum, 10% foetal bovine serum, and 0.1% Tween-20 in phosphate-buffered saline. The materials found in the blocking buffer, -foetal bovine serum and serum-, were used to avoid unspecific labelling that improves the signal. After that, cells were incubated overnight at 4°C in the dark with primary antibody diluted with blocking buffer by the dilution factors identified in Table 1. The next day, samples were washed using Tris-buffered saline with 0.1% Tween-20 three times to eliminate the remaining primary antibody.

| <b>Antibody name</b>                            | <b>Description</b> | <b>Dilution</b> | <b>Excitation wavelength</b> | <b>Emission wavelength</b> | <b>Supplier</b>          | <b>Catalogue number</b> |
|-------------------------------------------------|--------------------|-----------------|------------------------------|----------------------------|--------------------------|-------------------------|
| Donkey anti-Mouse IgG (H+L) Secondary Antibody  | Alexa Fluor 488    | 1:200           | 495                          | 519                        | Thermo Fisher Scientific | A21202                  |
| Donkey anti-Rabbit IgG (H+L) Secondary Antibody | Alexa Fluor 568    | 1:200           | 578                          | 603                        | Thermo Fisher Scientific | A10042                  |
| Donkey anti-Goat IgG (H+L) Secondary Antibody   | Alexa Fluor 647    | 1:100           | 650                          | 668                        | Thermo Fisher Scientific | A21447                  |

**Table 2.** The table illustrates the secondary antibody information, including the dilution factor, excitation and emission wavelength, company and commercial catalogue number (Duan, 2018).

The cells labelled by primary antibody were then exposed to the mixture of blocking buffer and secondary antibody conjugated with fluorescent molecules for 2 hours at room temperature. The secondary antibodies that were coupled to fluorescent molecules listed in Table 2 were raised against immunoglobulin host of the primary antibody, which provide specific targeting. For identification of nuclei, DAPI (4-6diamin-2-phenylindole 1Micog/ml; excitation 358nm, emission 461 nm) was applied to the coverslips. The samples were then washed three times in phosphate-buffered saline and phosphate-buffered saline containing Tween 20 that eliminates residual primary antibody. After that, it washed two times in phosphate-buffered saline. Finally, the coverslip was mounted on slides with anti-fading (2.4 g Mowiol 4–88, 6 g of glycerol, 2 ml of 0.2 M Tris-HCl, pH 8.5, 2.5% 1,4 diazabicyclo (2.2.2.) octane) to delay bleaching. The coverslips were sealed with translucent nail polisher and stored at 4°C. In Figure 6 below, The fluorescence spectra for excitation and the emission wavelengths of the secondary antibodies are shown. However, in fluorescence microscopy, fluorophore crosstalk can be a problem, which is described as overlapping excitation and emission wavelengths of two or more fluorescent dyes. Commonly, it can appear toward the blue in excitation and the red in emission. Therefore, fluorescence imaging that created by more than one dye should be firstly performed with the reddest (the longest wavelength peak emission) dye (*Confocal Microscopy - Spectral Bleed-Through Artifacts in Confocal Microscopy | Olympus LS, 2021.*).

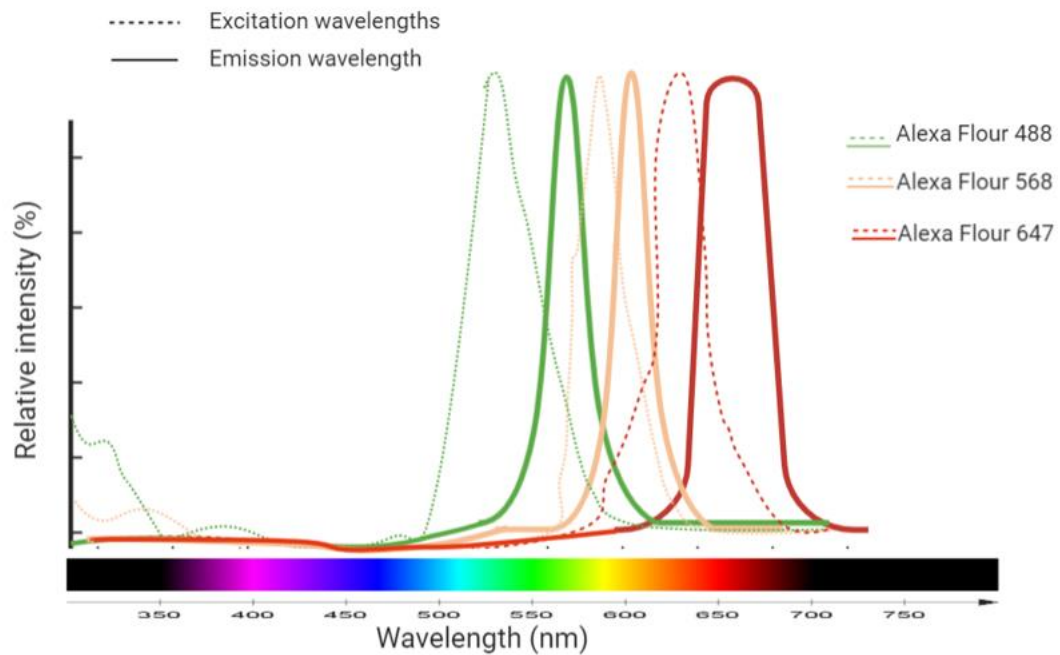


**Figure 6.** The spectrum of dye conjugated to secondary antibodies was shown. The excitation spectrum the absorption spectrum for fluorophore the acquire the excited state and the ground state is probed by excitation spectra.

## 2. 5 Confocal imaging

Confocal fluorescence microscopy is a technique that is specialised in visualising labelled protein distributions by antibody-conjugated fluorescent dye. The principle of confocal microscopy is to focus the laser light on the focal plane captured by the digital camera using pinhole apertures to avoid blurred images. Therefore, the out-of-focus lights can be blocked by this apparatus. As illustrated in Figure 7, the confocal microscope is composed of laser dichroic mirror, objective, detector and focal plane. The fluorophores within the sample are excited. They emit fluorescent light of another wavelength that will pass through the dichroic mirror and reach the second pinhole aperture for clear images. The light beam with a selected wavelength from the laser focuses on the small regions captured by the detector. The laser then scans the whole focal plane and illustrates an image gathered at each focal plane in the captured region.

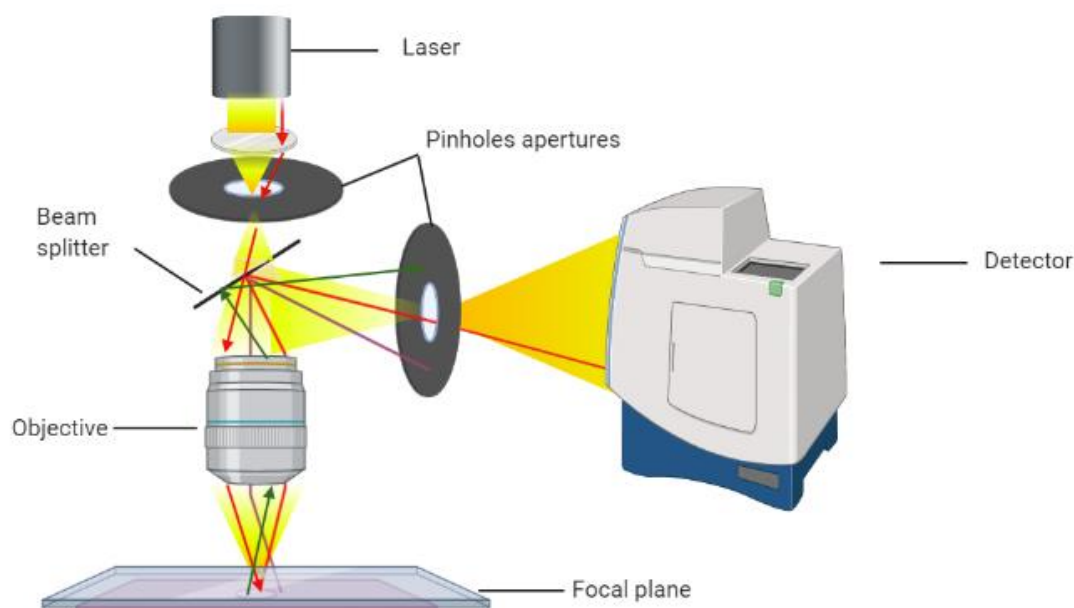


**Figure 7. Schematic illustration of confocal microscopy was shown.** Two pinholes in confocal microscopy permit the acquisition of a clearer images by blocking out-of-focus lights. First, a the laser beam passes through the first pinhole to the objective, then the collected lights on the focal plane is reflected on the beam splitter, directed to the second pinhole. After the second pinhole, detector captures the focusing lights. Adapted from (<https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts>)

Immunofluorescence imaging of labelled cells was conducted with a Nikon A1Rplus confocal system. The images were acquired at 22°C with sampling at a frequency of 0.5 Hz, via a Nikon Eclips Ti inverted microscope with a Nikon CFI plan Apo VC 60X,  $\lambda$ S DIC N2, 1.4 n.a oil immersion objective (Nikon Instruments Europe BV, Netherlands). Due to their different emission and excitation wavelengths, cells labelled with fluorescent secondary antibodies revealed the distribution of various proteins of interest and thus informed on the nuclear envelope structure.

Well-labelled cells were selected and visualised under a 60X oil immersion objective. The nuclei were magnified as possible and Z sections of the entire nuclei scanned with a step interval of 100-200 nm (Paddock, 2021, Retrieved from <https://www.microscopyu.com/techniques/confocal/introductory-confocal-concepts>)

Confocal microscopy conjugates the fluorescence signal from sample in a focal point by eliminating unfocused background, which provides a less blurred

image. With this property, it is superior to traditional wide-field fluorescence microscopy. Although super resolution microscopy is more successful with better resolution, confocal microscopy offers fast acquisition time to avoid bleaching, which is more efficient compared to super resolution microscopy (Vangindertael et al., 2018).

## 2.6 Deconvolution and imaging processing

### 2.6.1 Improving the resolution of the images by deconvolution

The Point Spread Function (PSF) is the fundamental principle in microscopy. The PSF is described as the spreading of fluorescence coming from a single point. The integration of PSFs from different positions can create any images, which is called convolution. All obtained images were degraded by convolution, which is described by the convolution equation;

$$g = f * h$$

The image  $g$  arises from both the real light sources  $f$  and PSF  $h$ . To compute this, I used the Huygens Essentials software.

Images with higher resolution can be acquired by deconvolution, which means reverse convolution. With known value  $g$  and calculation of PSF  $h$ , the real light source  $f$  can be assumed. The implication of deconvolution will increase the resolution up to 100 -150 nm, improving background noise. In this study, I used Huygens Essential software (Scientific Volume Imaging BV, Netherlands) to view infoldings on nuclear envelope ([Huygens Deconvolution | Scientific Volume Imaging](#), 2021). The images before and after deconvolution were shown in Figure 8 below.

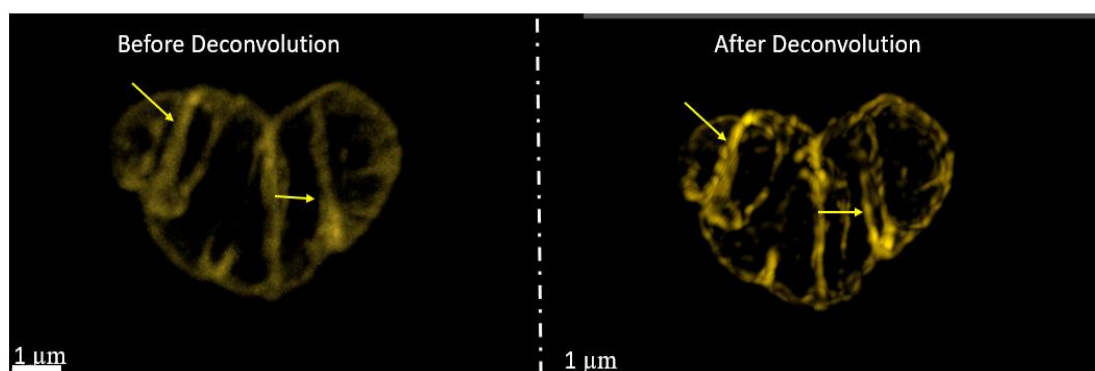


**Figure 8.** Confocal images of acutely isolated PASMC stained for emerin before (left) and after (right) deconvolution were shown. Two examples of ridges were illustrated by two arrows to demonstrate the improvement in the resolution.

## 2.7 3D reconstruction of confocal Z section

With confocal microscopy, Z sections are taken from acutely isolated or cultured PASMC with fluorescent labelling as described in section 2.4 and applied deconvolution. Aided by the increased resolution, these deconvolved images were transferred into IMARIS 9.3.1 (Bitplane, Oxford Instruments, UK) that makes 3D reconstructions with a digital skin to allow further detailed analysis of co-labelled proteins (**Figure 9**). IMARIS provides an opportunity for visualization and interpretation of data objectives in 3D. The unique feature of IMARIS software provides that the particular object can be selected by adjustment of object's intensity profile by eliminating unwanted signals (Spiegelhalter et al., 2010). The adjustment can be made by brightness changes that alters the displayed intensity value and to refer the linearity relationship between actual image concentration value and presented image brightness, gamma was defined which range from 0.1 to 5.0 in IMARIS and raising the gamma will lead to brighter image (*Optimal Image Characteristics | Display Adjustment Window- Oxford Instruments, 2011*). To investigate structures such as nuclear invagination and ridges, the dim background is removed by the increasing threshold. Because the dense voxels are where a higher concentration of the labelling and which may show specific structure. In contrast to this, scientists argue that the dye dense regions may be created as a results of epitope masking, given that the epitopes are more accessible in particular nuclear regions (Tunnah et al., 2005). Additionally, low threshold

was used for description of nucleus general view, which is selected as possible as composed of whole nuclear shaped labelling excluding background (Fogarty et al., 2013).

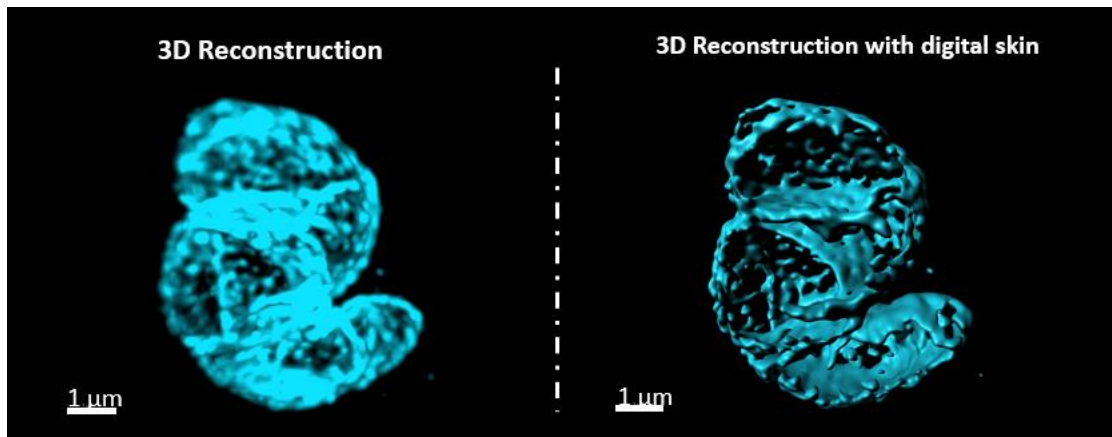


**Figure 9. Deconvolved and 3D reconstructed images were shown.** 3D reconstruction of a series of confocal Z-stacks showing emerin (cyan) labelled nucleus of an acutely isolated pulmonary arterial smooth muscle cell was shown without (left) and with digital skin (right).

In the experiments labelling with two or more dyes for samples, the colocalization between them can be calculated by IMARIS (in Figure 10) and this will present a measurement for colocalization volume and Pearson's correlation coefficient. The colocalization volume is referred to the comparison of the volumes of one labelling colocalization with total volume of labelling, which reveals the proportion of staining volume that colocalized with another labelling. The Pearson's correlation coefficient gives the linear correlation between two labelling: the value is between +1 and -1, in which +1 stands for positive linear correlation, 0 is accounted for no linear correlation and lastly, -1 represents total negative linear correlation.

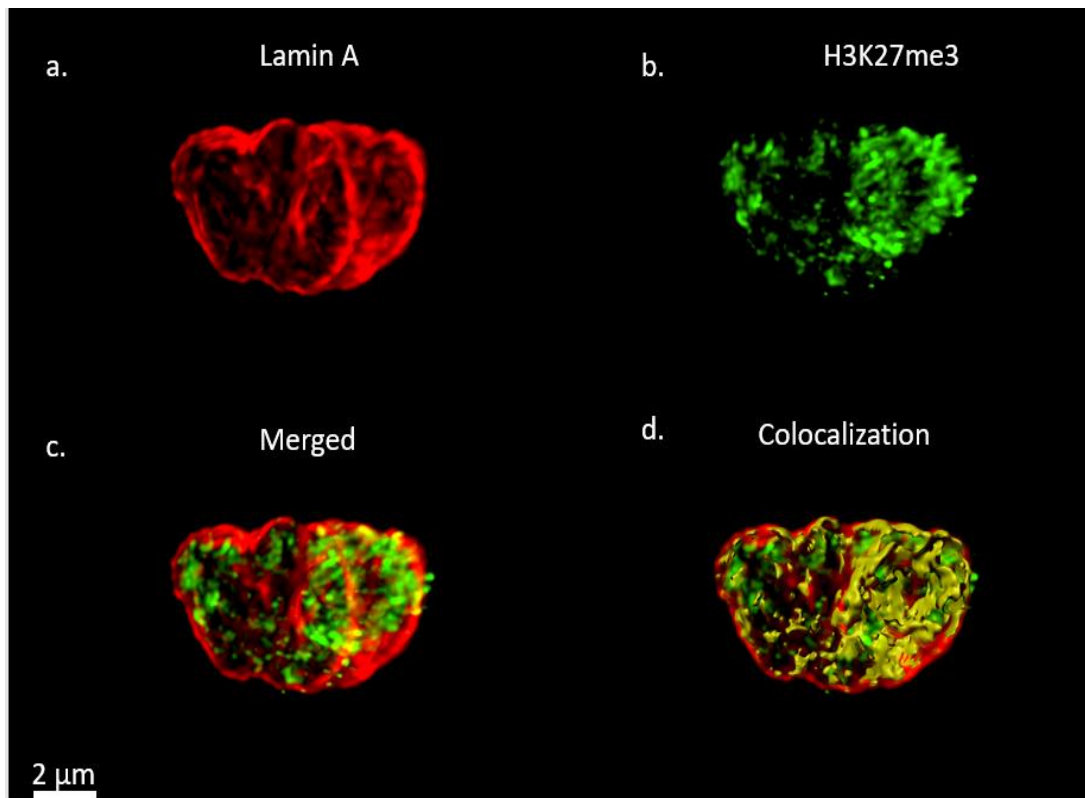


**Figure 10.** The illustration of colocalization between two different dyes staining was produced by *Imaris*.. An AIPASMC nucleus was labelled for lamin A (red,a) and H3K27me3 (green,b). The red and green staining merged as illustrated in c without digital skin, the colocalization for lamin A and H3K27me3 was analysed and shown in the yellow channel (colocalization channel) with digital skin (d).

## 2.8 Data presentation and statistical analysis

Statistics were displayed as the mean  $\pm$  SEM for 'n' experiments. The analysis, comparison and figure production were performed by using GraphPad 9.0.0 and Microsoft Excel 2019. When assessing two datasets, t-test was performed. In the event of evaluating between three or more groups of data, the alterations of datasets were examined via the one-way variance test (in GraphPad 9.0.0). There would be a number of independent variables when the data sets included similar variances as well as showing the normal distribution. To compare the data set composed of one independent variable, the analysis was performed via one-way ANOVA with a Tukey or holm-sidak multiple comparisons test. With regards to the data sets including two independent variables, the two-way ANOVA followed by a Tukey's multiple comparisons test was used to compare these groups. Conversely, when the data sets include considerably distinct

variances, Kruskal-Wallis test was used for the analysis while the paired data sets were compared via the Friedman test followed by Dunn's multiple comparison. Probability values less than 0.05 were considered to be statistically significant.

# RESULTS

This project investigated the morphological differences between the nuclei of acutely isolated contractile pulmonary arterial smooth muscle cells (AIPASMC) and pulmonary arterial smooth muscle cells that had proliferated in culture for 7 days (D7PASMC). To this end, I have compared the distribution at the nuclear periphery and along the nuclear envelope invaginations of nuclear envelope proteins including lamin A, emerin, nuclear envelope spectrin repeat proteins (nesprin) and Sad1 and UNC domain-2 (SUN2) proteins, , that are targeted to either the outer or inner nuclear membrane and have been proposed to play a role in gene expression regulation.

The aim of this project is to reveal how the structural organisation of nuclear envelope proteins changes when pulmonary arterial smooth muscle cells switch phenotype from contractile to proliferating cells. In order to achieve this, I analysed 3D images of nuclei labelled for lamin A, emerin, nesprin 1 and SUN 2 as well as the interactions between these proteins. I characterised the nature of the structures formed by these proteins at the periphery of the nucleus and at the nuclear invaginations. I found out that due to the dramatic reorganisation of these proteins at the nuclear periphery and the loss of nuclear invaginations in proliferating D7PASMC, the number and types of structures at nuclear proteins labelled in AIPASMC and proliferating D7PASMC varied markedly.

## **3.1 The formation and distribution of selected nuclear envelope proteins at the nuclear envelope of acutely isolated pulmonary artery smooth muscle cells**

### **3.1.1 Lamin A is widely distributed across the inner nuclear membrane and forms specific structures: invaginations and ridges**

Under environmental stimuli and metabolic stresses, smooth muscle cells undergo morphological changes dependent on their function, affecting signal transduction, and transcriptional activity whereas the nuclear morphology

influences signal processing. Using computational modelling, it has been suggested that the increases and decreases in the nuclear surface area lead to changes in calcium ( $\text{Ca}^{2+}$ ) dynamics. There are two different observations, which support the view that the existence of morphological modulation has effects on  $\text{Ca}^{2+}$  signalling. These observations are as follows: (1) nuclear sensitivity for  $\text{Ca}^{2+}$  signalling increases as a result of the rise in the number of nuclear pore complexes dependent on the nuclear surface area increased by nuclear infoldings; and (2) induced alterations in the morphology of the nuclear envelope, such as nuclear infoldings, affect changes in the distribution and/or function of lamin A, a key protein located underneath the nuclear envelope with a role in transcriptional activity (Torvaldson et al., 2015; Wittmann et al., 2009a).

In this study, I will investigate the morphological changes at the nuclear envelope that are induced during the switch from contractile to proliferating pulmonary artery smooth muscle cells (PASMC) phenotype. In order to achieve this, I will first explore the distribution of lamin A in acutely isolated, differentiated contractile smooth muscles, for which, I hypothesize, that it may adjust chromatin association and thus gene expression during the switch to a proliferative phenotype.

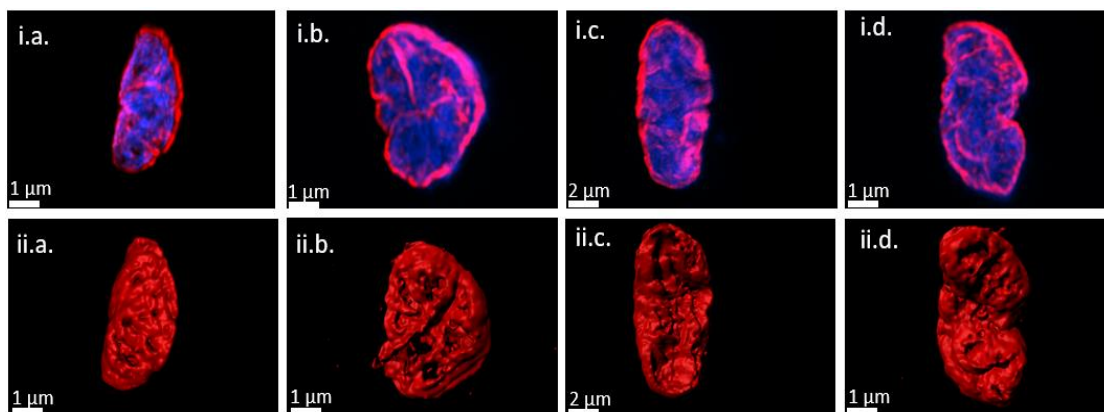


**Figure 11. Lamin A is a protein meshwork that lines through the inner nuclear membrane of PASMCs.** i.a) Lamin A (red) was co-labelled with DAPI (blue) in the first, (i.b) second, (i.c) third and (i.d) fourth PASMC nucleus. Then, the 3D skin was applied on deconvolved Z stacks of confocal images through the (ii.a) first, (ii.b) second, (ii.c) third and (ii.d) forth PASMC nucleus labelled for lamin A.

It is known that lamin A covers the entire inner surface of the inner nuclear membrane, forming a continuous meshwork from which one can map the morphological pattern of the nuclear envelope (Sapra et al., 2020). I first decided to investigate the entire boundary of lamin A meshwork in AIPASMC to understand general characteristics of lamin A labelling in AIPASMC. It covers the nucleoplasmic surface of the inner nuclear membrane, identified by the DNA marker, DAPI (blue), which was confirmed by the confocal images of 4 different nuclei of acutely isolated pulmonary artery smooth muscle cells (AIPASMC) labelled for lamin A (red) (**Figure 11 i. a, b, c & d**). Moreover, it is apparent from figure 11 i.a, b, c and d that A-type lamin labelling was found in the entire boundary of the inner nuclear membrane (INM), illustrated by 3D skin application with the detection threshold set low in order to detect all the staining. Since the low threshold application in 3D images detected both strong and weak staining of lamin A, I used it to show all lamin A staining to illustrate the entire boundary of lamin A labelling (**Figure 11 ii. a, b, c, d**).

Moreover, the 3D application to different nuclei illustrated that each nucleus differs in shape, and they ranged from nearly spherical to elongated. In addition to this, the 3D skin application revealed the uneven form of lamin A labelling more clearly (**Figure 11 ii. a, b, c, d**). Thus, I was able to confirm that the lamin A meshwork forms showed diversity, as discussed in the earlier studies (Fischer et al., 2003; Skinner & Johnson, 2017). Then, I investigated all corner of lamin A labelling (**Figure 12**).

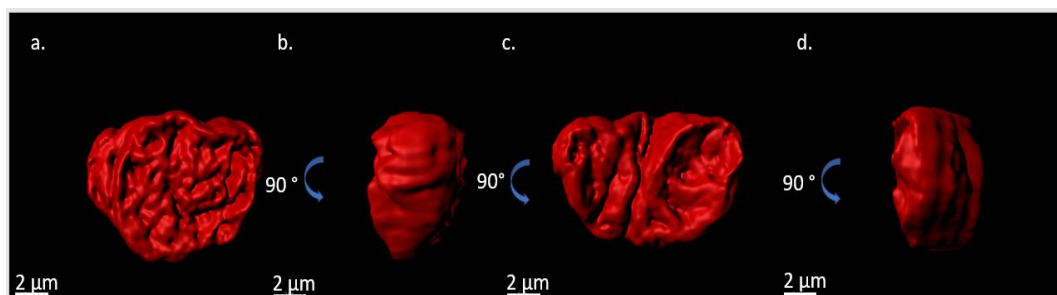


**Figure 12. Lamin A surrounds the entire inner nuclear membrane of AIPASMCs.** a.) AIPASMC nucleus was labelled for A-type lamin(**red**), b.) turned 90° anticlockwise, c.) rotated one more time 90° anticlockwise and lastly, turned 90° anticlockwise to show the all sides.

One major issue in early lamin A staining morphology research concerns the suggestion that the lamin A staining morphology is rounded and regular. However, the irregular and crumpled shapes are supported by more recent publications on different cells including neuron and human bone marrow stromal cells (Dorland et al., 2019; Wittmann et al., 2009). Consistent with these recent papers, I also observed crumpled and irregular shapes of lamin A staining by 3D skin application (n=24) (**Figure 12 a, n=24**). In keeping with this suggestion, the “crumpled” surface of the nucleus outlined by lamin A was observed from different aspects, through being rotated by 90° in every stage (**Figure 12 b,c,d**). This investigation showed that the nucleus labelled by lamin A included the indentations on different angles of the surface (**Figure 12 c**). Moreover, tiny holes were observed that were spread across various sides of the AIPASMC nucleus labelled for lamin A in figure 12.a and c. The literature exploring lamin A suggests meshwork, it surrounds the AIPASMC nucleus. Therefore, possible reasons related to this observation are; first; nuclear pore complex (NPC) or nuclear infoldings of the nucleus are labelled by lamin A (Malhas et al., 2011; Shah et al., 1998; Stiekema et al., 2020) , and secondly, the limitations of the immunostaining technique that I used such as epitope masking or antibody accessibility (Tunnah et al., 2005) or thirdly, lamin A negative, emerin positive nuclear invaginations identified by Duan (Duan, 2018).

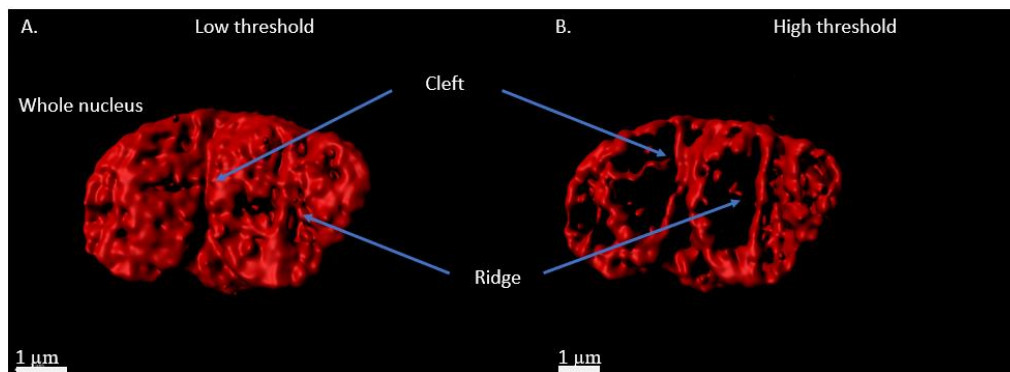


**Figure 13. The comparison of high and low threshold application on nucleus labelling for lamin A was shown.**

(A) Digital skin in low detection threshold was applied lamin A labelling AIPASMC nucleus (**red**).

(B) As in (A), but it was shown with higher threshold application. Arrows showed cleft and ridge.

Since the characteristics, particularly morphological, of lamin A distribution may affect chromatin spatial organization, by using low threshold application to show the all nucleus outlined by lamin A, I decided to investigate the crumpled surface of the PASMNC nucleus outlined by lamin A. As a result, I detected the indentations on PASMNC nuclei labelled by lamin A, particularly on the clefts and ridges. This is in accordance with previous studies on nuclear envelope morphology, which also showed the clefts and ridges on the nuclear envelope of smooth muscle cells (Wamhoff et al., 2002) (**Figure 13 A**). Here, it is necessary to clarify what is meant by clefts. As discussed in the previous studies (Wittmann et al., 2009b), clefts refer to a partial division located between two close membranes. In addition to the clefts, I detected linear lamin A dense areas around the lamin A staining via the application of a high threshold by removing less dense lamin A staining (**Figure 13 B**). The linear regions here could suggest that lamin A accumulates in higher density in certain regions of the INM, perhaps, where labelling on two adjacent INM surfaces are brought together in a manner that confocal microscopy does not have the resolution to separate, such as with infoldings (clefts) and out folding (ridges) (**Figure 13 A&B**). Additionally, there are some limitation about imaging including autofluorescence of the tissue and issues about the marker used for labelling the cell (Mitchell et al., 2019). Related to this, in this study high threshold eliminates where the lamin signal is weaker to investigate the

structures, but higher lamin A concentration might be reasoned by to the epitopes being more accessible in these particular nuclear regions due to epitope masking.

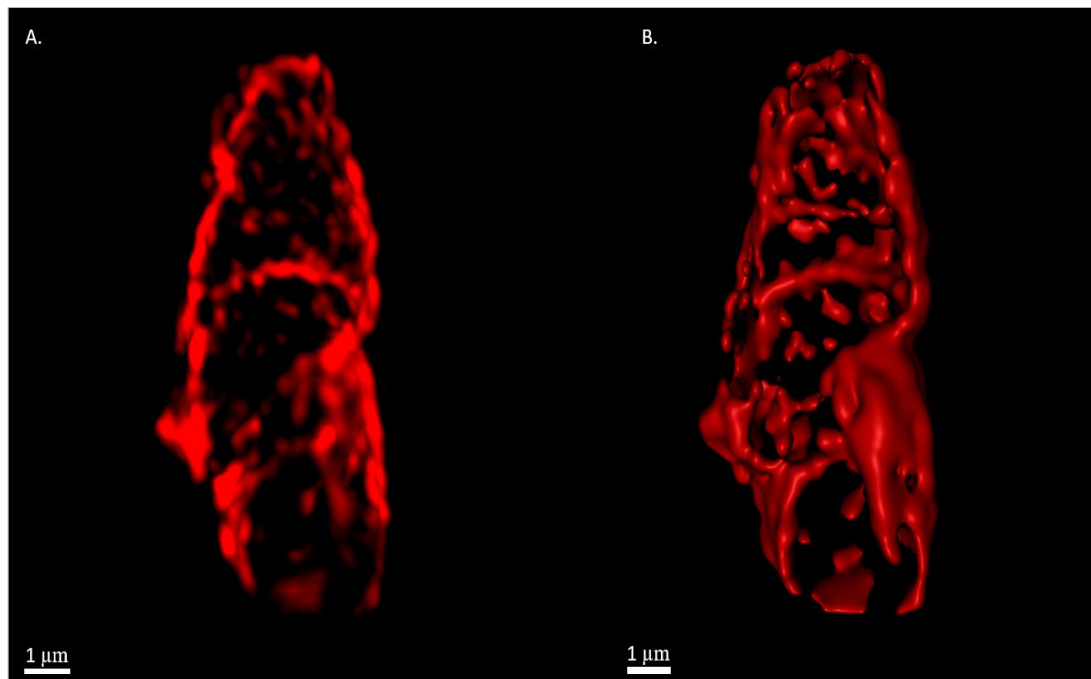


**Figure 14.** One example of lamin A labelling of a AIPASMC nucleus was shown. A) A deconvolved confocal images of the AIPASMC nucleus labelled for lamin A (red) was demonstrated and, B) digital skin software was used on the 3D reconstructed images of A) for the detailed view with high threshold application.

After acquiring a clearer view with the high threshold application, it was necessary to explore the deconvolved confocal image of the lamin A meshwork for testing the accuracy of 3D skin application, and in parallel, the high threshold 3D application, low-density fluorescence areas were removed and leaving only sites with high densities of labelling remained to determine structure. In figure 14.B, the indentations were revealed following by the deconvolution of the image of the cell (**Figure 14 A**). To date, although clefts and ridges on the surface of the nuclear envelope that are defined by lamin A staining can be seen in published images (Duan, 2018), a detailed description of their architecture and any attempt at classifications are lacking. Therefore, I tried to classify the ridges, determine their average number per cell as well as the dimensions of these regions of high density labelling, which may be

important given that lamin A holds association points for chromatin and the shape diversity may offer specific function in ridges.

In the pages that follow, it will be argued that clefts and ridges occur in variable shapes, after which I will carry out an in-depth investigation to classify architectural form, because nature form is often related to function (**Figure 15**).

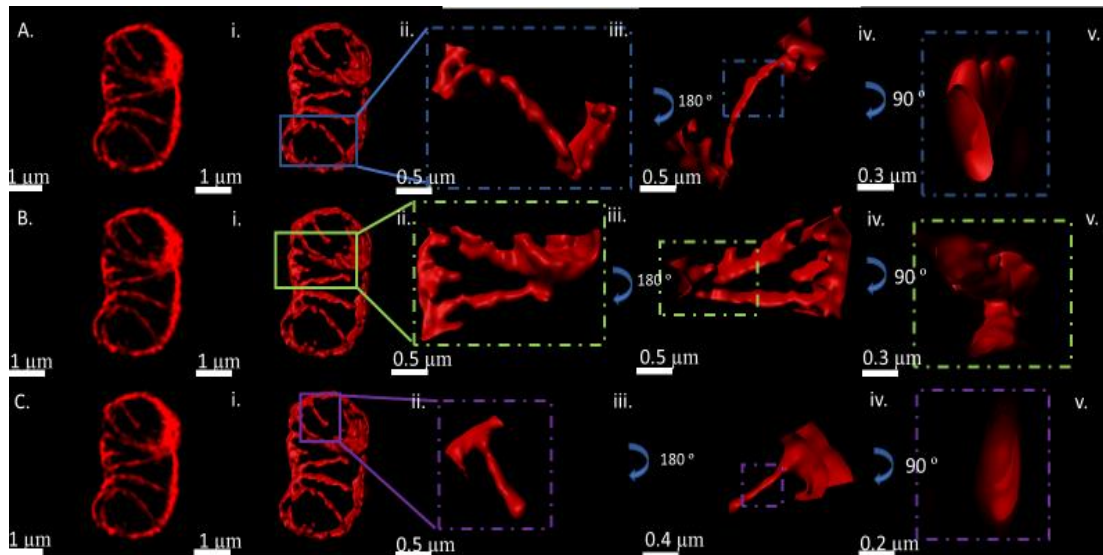


**Figure 15. A type lamin labelling of AIPASMC reveals three different types of ridges.** A) (i) Deconvolved Z-stacks of confocal images with lamin A labelling in (red) of a AIPASMC nucleus was demonstrated. (ii) 3D reconstructed lamin A labelling of a AIPASMC nucleus (iii) was cut from the marked area to show I shape ridge. (iv) Image was rotated 180° showing inside of nucleus, (v) cutting from the blue dashed box and the section rotated 90° anticlockwise.

B) As in (A), however, V shape ridge was illustrated.

C) As in (A), however, half I shape ridge was illustrated.

After the rough description, the in-depth investigation revealed that commonly spread through the nuclear envelope labelled by lamin A are a variety of the ridge forms through the nuclear envelope: I-shaped, V-shaped and half I-shaped. To classify the ridges, these structures were indicated by the coloured boxes and separately magnified. In doing so, I aimed to investigate them in more detail to be able to get more information about the characteristics of lamin A distribution. By categorising them based on morphology, I firstly determined

the I-type ridge shaped like a stick stretching through the nuclear envelope's width, illustrating a uniform distribution of lamin A throughout the ridge (**Figure 15 A.iii**). Following this, I investigated the internal surface of ridge view facing the nucleoplasm that showed a slight ledge by turning 180°, which is the surface (**Figure 15 A.iv**).

By applying the same process, I detected the V-shaped ridge that is two-branched arranged on the same point (**Figure 15 B.iii**) with a similar appearance: chopped from the marked area by green box and magnified. Similar to the I-type ridge, its surface facing nucleoplasm showed tiny protrusion (**Figure 15 B.iv**), acquired by turning it 180°. Thirdly, I determined a half I-shaped ridge with starting point from the edge and which did not bind anywhere and ended in the middle of the width of the nucleus labelled with lamin A (**Figure 15 C.iii**). Additionally, I observed that the half of I-type ridge interior facing nucleoplasm was the same as the INM facing surface in appearance (**Figure 15 C.iv**) when rotated 180°. These results suggest that the different shapes are highly likely to form through the nuclear envelope. Moreover, each may confer a unique architecture on the surface facing the nucleoplasm. To clarify the diversity of structures, I magnified and merged the three most common ridge types in the same image.

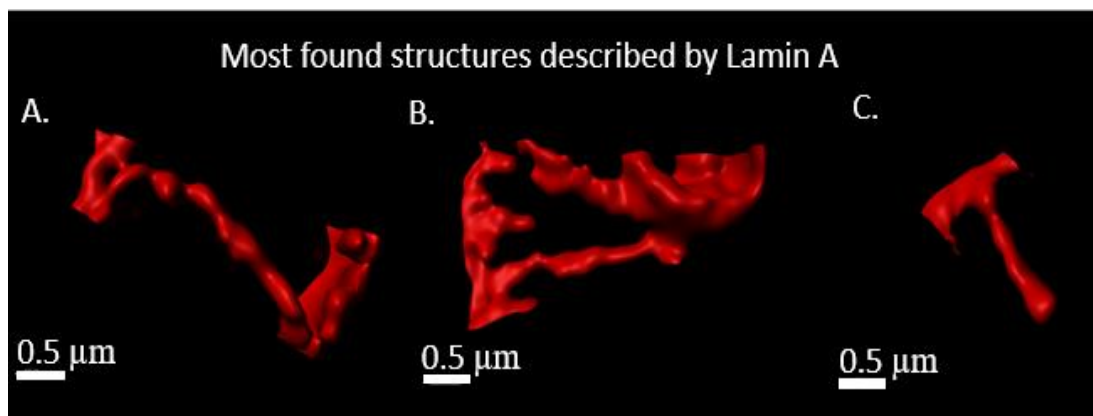


**Figure 16.** Three different ridge types were described on lamin A labelling of acutely isolated PSMC. A) I-type ridge, B) V-type ridge and C) half I-type ridge are shown.

The results in this chapter indicate that there appears to be various structures on lamin A labelling. The most commonly observed structures are the I-type, V-type and half I-type ridge (**Figure 16**), which may have a particular distribution. The exciting point is that the structures are entirely different in shape, and notably, the structural diversity may drive different localisations of the lamin A in the nuclear protein complex. Next, a statistical analysis was employed to understand the distribution of the structures in each cell (see below).

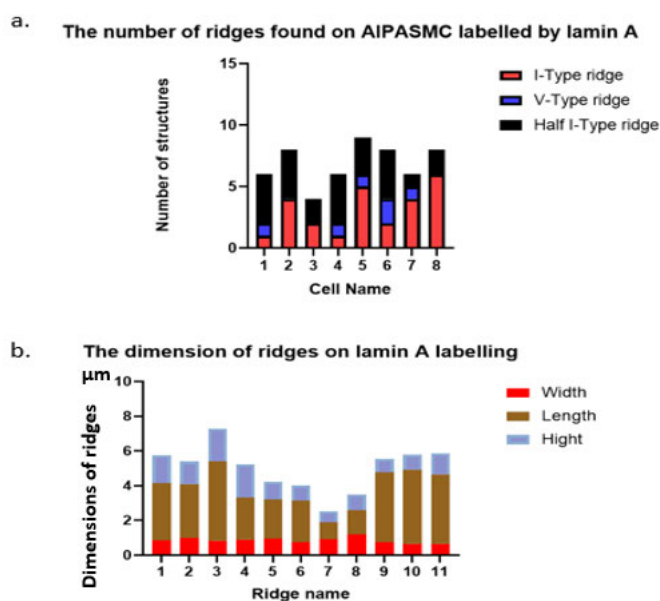


**Figure 17. The half I-type ridge is found in every cell.**

- The bar chart demonstrating the number of structures identified by lamin A labelling. Each bar represents a cell. The numbers are presented as mean±SEM, n=8. I-type (**red**), V-type (**blue**) and half I-type ridge (**black**) are shown.
- The bar chart showing the dimensions of ridges upon the lamin A labelling. The width (**red**), length (**brown**) and height (**blue**) of clefts are differentiated by different colours. The dimensions are presented as mean±SEM, n=11.

As reported previously, the ridges outlined by lamin A were observed in every nucleus studied (Duan, 2018), and I detected that the total number of ridges outlined by lamin A in cells studied ranges from 9 to 4. In order to gain an

understanding of the relationship between the distinct types of ridges, I plotted their numbers in a bar chart Figure 17, which presents the number of ridges found in each cell and suggests that the highest number of ridges in each nucleus is for the I-type ridge with  $3.12 \pm 0.66$  (**red**,  $n=8$ ). Interestingly, the V-type ridge was not observed in each cell and had lowest number of ridges with  $0.75 \pm 0.25$  (**blue**,  $n=8$ ) on average (**Figure 17 A**). The confirmation of the linear regression analysis showed a poor correlation between each type of ridge and others ( $R^2 = 0.1615$  for I-type versus V-type ridges,  $R^2 = 0.2573$  for I-type versus half-I type). The regression analysis results showed that the three different ridges might be independently formed in each cell given.

Despite a large body of research on the investigation of ridge numbers in the literature, more specific features like the dimensions of ridges and ridge number for each ridge type have been observed for the first time. By investigating randomly selected ridges from different cells, I identified the sizes of each type of ridge in height (blue), lengths (brown) and width (red) for I, half-I and one of the branches of branched ridges, which appeared to vary among the variety of ridges (**Figure 17 B**). On average, the width is  $0.86 \pm 0.15 \mu\text{m}$  (mean  $\pm$  SEM,  $n=11$ ) (**Figure 17 B**), while the mean length of the ridges is  $2.97 \pm 1.18 \mu\text{m}$  (**Figure 17 B**). To provide more accurate comparison, I calculated the ratio between dimensions and nucleus volume (dimension ( $\mu\text{m}$ )/nucleus volume ( $\mu\text{m}^3$ ); width;  $0.03 \pm 0.02$ , length  $0.07 \pm 0.03$  and height  $0.02 \pm 0.002$ , which is showed that which ridge dimension belong to which cell (Appendix Table.7).

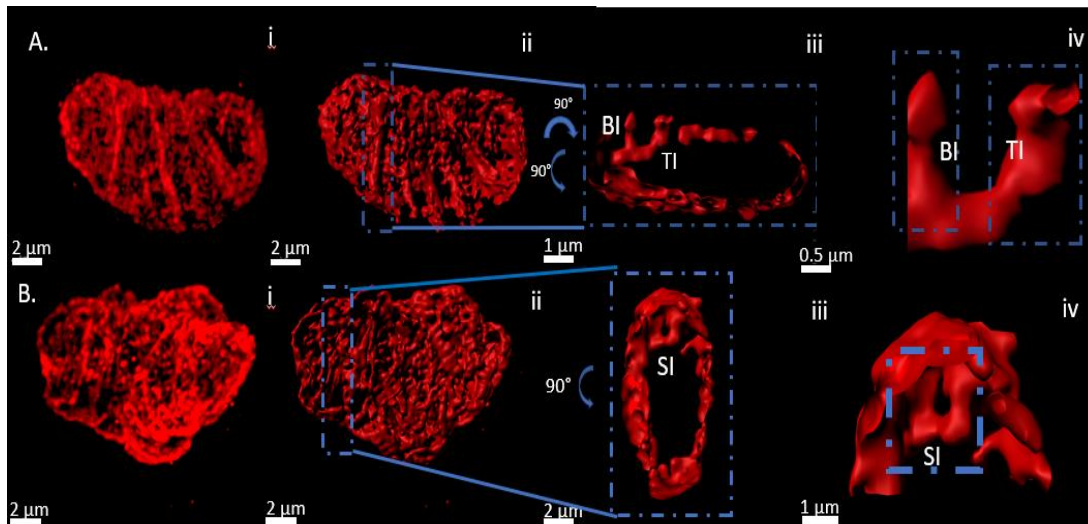


**Figure 18. Three different invaginations were identified by lamin A labelling of AIPASMC nucleus.**

**A)** (i) A deconvolved confocal images for the PASMC nucleus is labelled for lamin A (red). (ii) 3D reconstructed image of (i) (iii) cut from the blue box, rotated 90° to downwards and 90° clockwise and (iv) magnified image of transnuclear invagination (TI) and blind invagination (BI).

**B)** (i) A deconvolved confocal image for the AIPASMC nucleus is labelled for lamin A (red). (ii) 3D reconstructed images of (i), (iii) cut from the blue box and rotated 90° anticlockwise and lastly, (iv) magnified image of superficial invagination (SI).

Therefore, the investigation of the lamin A inner surface facing nucleoplasm is as important as outer surface facing INM. Since I investigated the different structures on the outer surface of lamin A staining, I expected that lamin A labelling's inner surface facing nucleoplasm might show different structures in parallel to the previous data (Wittmann et al., 2009b). In view of the fact that interior structures facing nucleoplasm might provide lamin A function (Cohen, 1999; Ikegami et al., 2020), I cut out a particular location indicated by the dashed blue box (**Figure 18 A & B.ii**) and rotated 90° to explore the nucleus' internal structures as magnification (**Figure 18 A & B.iii**). Similar to the earlier research, I have also demonstrated three types of invaginations based on morphology (Duan, 2018): (1) transnuclear invagination forming a two-point connection to the periphery of the nucleus (**Figure 18 A.iii**), (2) blind invagination that ends in the middle of the nucleus (**Figure 18 A.iii**) and (3) superficial invaginations that is formed as closed-rounded (**Figure 18 B.iii**). The images were then magnified to the clearer illustrations separately (**Figure**

**18 A&B iv).** The differences in the formation and distribution among them were revealed and more obviously after magnification. The results strongly suggested that the lamin A lined the entire inner surface facing nucleoplasm of each different type of invagination.

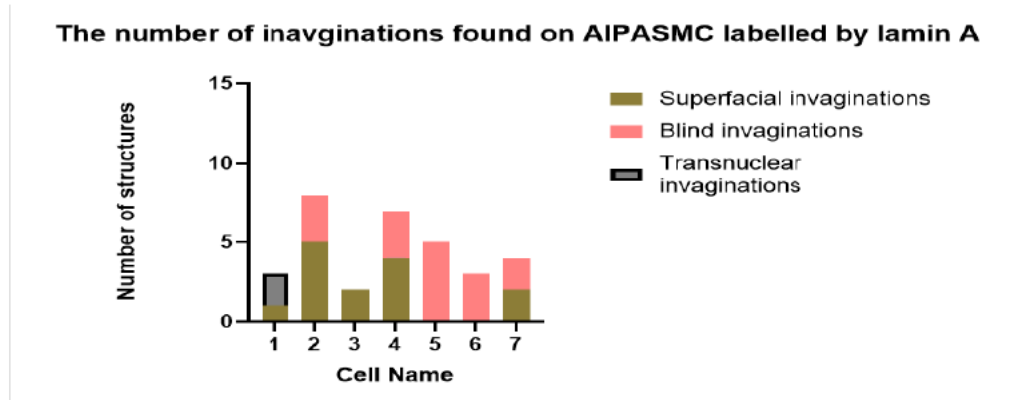


**Figure 19.** The most commonly observed invagination in AIPASMC is of the blind type. The bar chart demonstrates the number of invaginations identified by lamin A labelling. Each bar represents a cell,  $n=8$ . The superficial (green), blind (pink) and transnuclear invaginations (gray) are shown.

After the determination of structural diversity, the numbers of invaginations were counted to understand the distribution of the invagination types in a cell. The average numbers of each structure (**Figure 19**) illustrated that the most prevalent structure was the blind invagination, whose number was  $2.28 \pm 0.68$  per nucleus with suggesting a numerical superiority of blind invaginations compared to others ( $p=0.0607141$ ). It was observed that the second most common structure was the superficial invagination with a  $2 \pm 0.72$  average per nucleus. Interestingly, a transnuclear invagination was observed in significantly fewer instances than the others with  $0.28 \pm 0.28$ . Moreover, the number of the nuclear invaginations shows their diversity. The fact that some cells have many invaginations, while some others have few, and some types of invaginations were not observed in all cells shows that the nuclear invaginations were dynamic structures, as discusses in the earlier research. Thus, the previous research has already reported invaginations found on most of the cells but not all (Duan, 2018).

### 3.1.2 The structures identified by A-type lamin are also highlighted by emerin labelling.

It has been shown that nuclear envelope proteins have a role in the  $\text{Ca}^{2+}$  signalling processes (Wittmann et al., 2009). Thus, the investigation of lamin A offers various structures such as nuclear invaginations. In the previous data, it has been demonstrated that emerin contributes to the modulation of nuclear invaginations and  $\text{Ca}^{2+}$  transience (Shimojima et al., 2017). Moreover, scientists defined nuclear invaginations formed by only emerin labelling, without lamin A (Duan, 2018). More interestingly, emerin can bind to BAF, which has a direct association with DNA (Berk et al., 2014). Due to lamin A's and emerin's localisation in the inner nuclear protein complex (Dubińska-Magiera et al., 2019), I hypothesized that emerin might show a similar pattern to lamin A in appearance. In this section, I describe emerin's structural organisation in acutely isolated, differentiated contractile pulmonary artery smooth muscle cells.

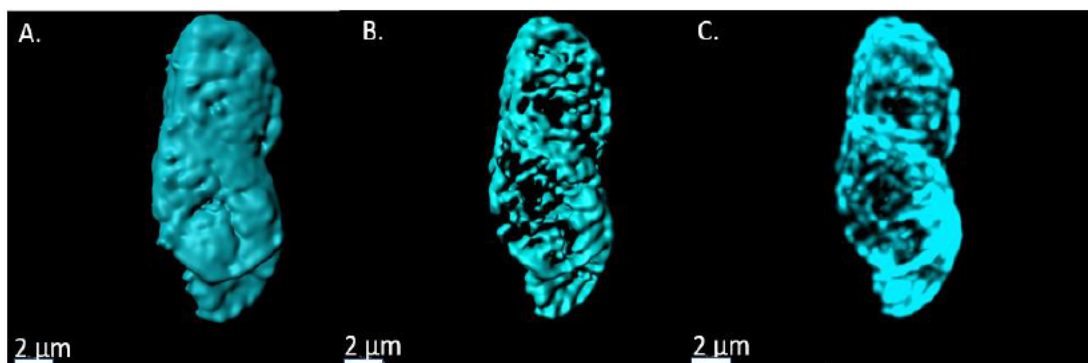


**Figure 20.** Comparison of high, low threshold application and deconvolved image on nucleus labelled for emerin is shown. Emerin labelling of APASMC nucleus (cyan) at low detection threshold (A), digital skin application to illustrate detailed structures. (B) As in (A), but a higher threshold application was applied to the deconvolved image. C) As in (A), but deconvolved confocal image was shown.

To describe the distribution of emerin throughout the nucleus, I was able to differentiate areas of dense emerin labelling relative to more diffuse emerin labelling by applying the low threshold, which depicts the entire boundary of emerin labelling, and then the high threshold, which shows only the emerin-dense areas (**Figure 20**). The examination of 3D reconstruction of z stacks

revealed the general view of a nuclear envelope shape composing many indentations without holes (**Figure 20.A**). Consequently, I determined whether the certified boundary of emerin labelling resembles lamin A labelling. After obtaining a general overview of the distribution of emerin, I created a digital surface skin by using the high threshold to remove "noise" within the labelling. The high-density emerin labelling formed on discrete ridges that in some cases, spanned the entire width of the nuclear envelope (**Figure 20 B**). I previously explained the indentations referring to ridges and clefts on the surface outlined by lamin A; and, interestingly, the high threshold application also indicated this structure in this nucleus labelled by emerin (**Figure 20 B**). Then, I tested whether the digital skin coincide with the rough image before 3D application. It demonstrated that emerin labelling was irregular and crumpled in appearance, rather than being smooth, flat and uniform in distribution (**Figure 20 C**). Therefore, the findings showed that the 3D skin application presented similar results to the deconvolved image.

As a result, it was evident that emerin labelling displayed a similar appearance to lamin A labelling. Therefore, emerin seemed to have a similar distribution in nuclear protein complex with lamin A. To test this possibility, I explored the structural diversity on the surface of emerin labelled AIPASMC in more detail and the detailed description of the emerin surface was firstly produced.

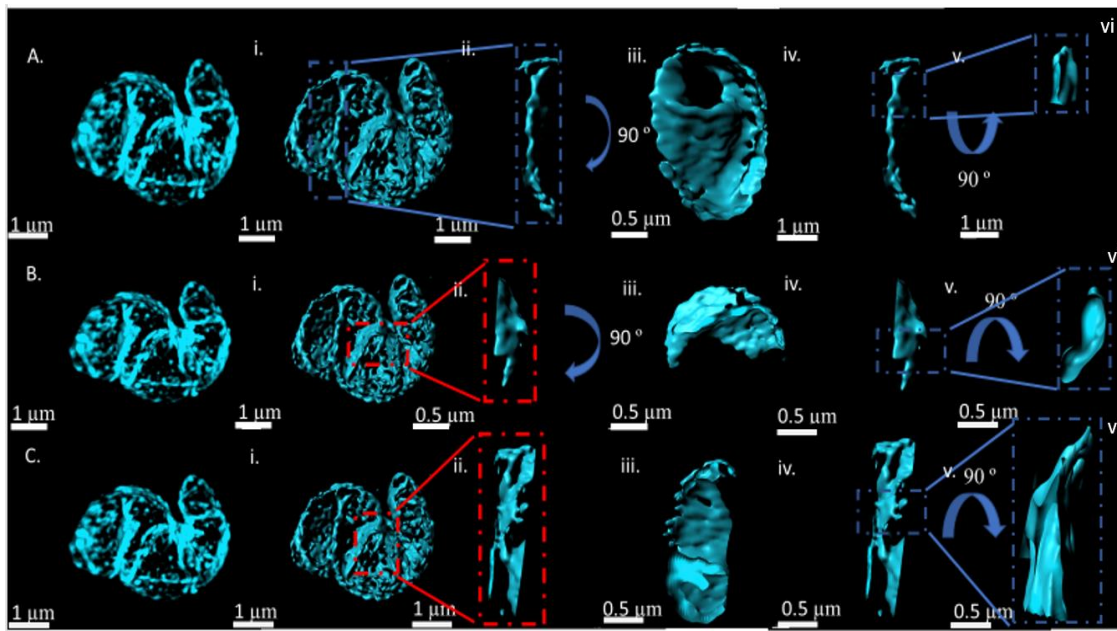


**Figure 21. Emerin labelled AIPASMC nucleus presented three different types of ridges.**

A) (i) Deconvolved Z-stacks of confocal images with emerin labelling in **(cyan)** of a AIPASMC nucleus was demonstrated. (ii) 3D reconstructed emerin labelling of a AIPASMC nucleus (iii) was cut from the marked area showing I-shape ridge. (iv) Image was rotated 90° showing inside of nucleus (v) cutting from the blue dashed box, and (vi) the cross-section rotated 90° upwards.

B) As in (A), however, half I-shaped ridge is illustrated.

C) As in (A), however branched ridge is illustrated.

Forms very often inform on the function related to tethering of lamin A, so I decided to classify the structures formed by emerin surface facing nuclear envelope. It was evident that there was a wide variety of ridges formed by higher density emerin labelling, which differed in shapes. Firstly, I cut the particular areas on AIPASMC areas of interest, and then I magnified these specific structures (**Figure 21**). This in-depth analysis of the outer structures displayed three types of ridges defined by a high density of emerin labelling, which had a consistent appearance across multiple cells (**Figure 21**): (1) I-shaped ridge extended across the width of the nucleus (**Figure 21 A iii**). Then, I rotated the I-shaped ridges 90° to investigate the interior surface of this ridge, (**Figure 21 A.iv**). At the internal surface that faces the nucleoplasm, a mirror image of this ridge was also evident (**Figure 21 A.iv**). (2) Half I-shaped ridges

(**Figure 21 B.iii**), which does not continue the entire breadth of the nucleus, exempts this feature, similar to the I-type ridge described previously (**Figure 21 B.iii**). Here, only a partial ridge was evident at the inner surface. The features were better described as similar to a tiny ledge, visualised by 90° rotation of this ridge (**Figure 21 B.iv**). (3) A branching ridge of irregular shapes (**Figure 21 C.iii**) were detected, with each branch point presenting distinct width, length and volume, and the nature of branching varying from one cell's nucleus to the next (**Figure 21 C.iii**). Moreover, rotating this structural form 90° was mirrored at the inner surface facing nucleoplasm (**Figure 21 C.iv**). The most striking result of this investigation is the remarkable similarity in the lamin A surface structures. To compare the similarities, I showed the ridges after removing another labelling around these ridges.

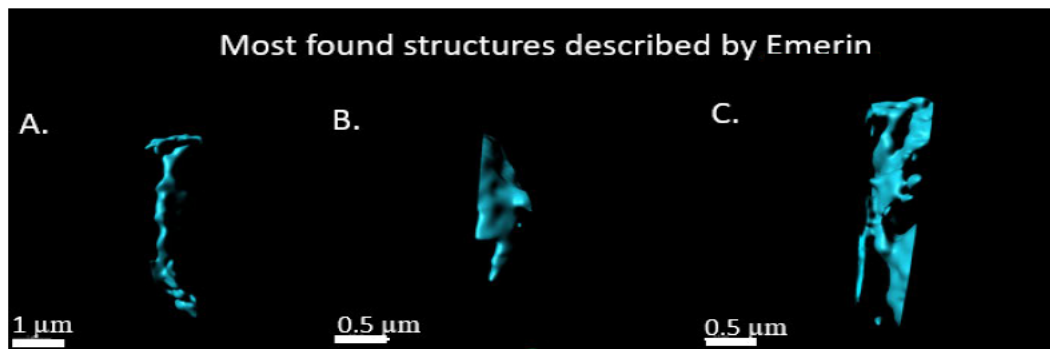


**Figure 22.** Three types of ridges were observed on a AIPSMC nucleus labelled for emerin: A) I-type B) half I-type ridge and C) branched ridge are shown.

In summary, I discovered three types of ridges defined by emerin; I-type, half I-type and branched ridge. These structures were also evident with A-type lamin, albeit with slight differences (**Figure 22 A-C**). Interestingly, the V-type ridge identified by lamin A was quite different from the branched ridge lined by emerin, in terms of the branch appearance and where the branches split (**Figure 16 B&22 C**). Also, similar to branched ridges outlined by emerin, I have rarely detected the ridges on the surface created by lamin A. Consequently, the view of the surface outlined by emerin is very similar to lamin A labelling surface. Due to this, my findings support the previous

publications in cardiac and muscle cells that proposed that lamin A and emerin distribution in nuclear protein complex is related to each other (Holaska, 2008).

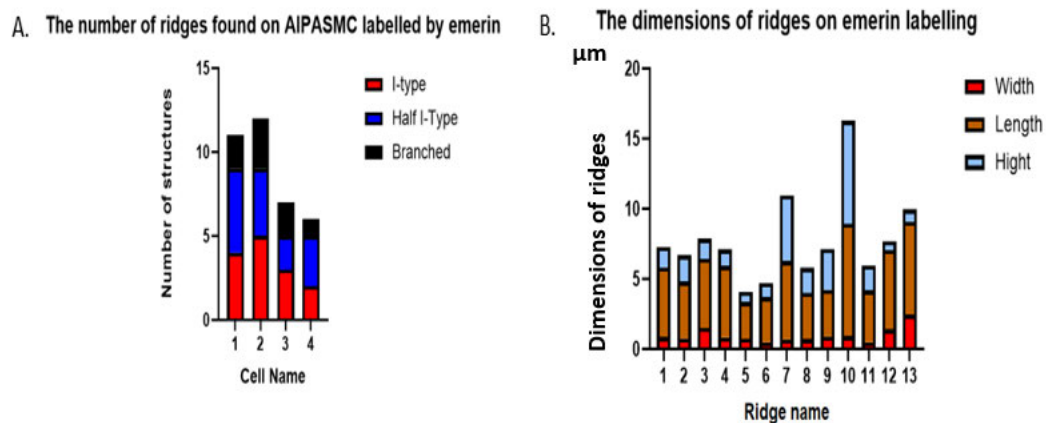


**Figure 23. Each cell studied contains half I-type and I-type ridges.** A) Bar chart demonstrates the number of structures identified by emerin labelling. Each bar represents a cell (n=4). I-type(**red**), half I-type ridge(**blue**) and branched (**black**) ridge are shown. B) Bar chart illustrates the dimensions of ridges (n=13): the width (**red**), length (**brown**) and height (**blue**) of clefts are differentiated by different colours.

Interestingly, the I-type and half I-type ridges were observed in each cell given with an average of  $3.5 \pm 0.64$  for both ridge types and lastly, the branched ridge was detected on these cells in the lowest number with  $2.0 \pm 0.4$  (n=4) (**Figure 23 A**). The linear regression analysis showed that there was a strong relationship between the numbers of some ridges ( $R^2 = 0.36$  for the I-type versus half I-type ridges, and  $R^2=0.9$  for I-type versus branched ridges). Therefore, the number of each type of the ridges in each cell might be regular, not random.

Following the determination of the surface characteristics, an examination of the ridge dimensions was required to assess the emerin distribution in protein complexes. For the ridges selected randomly from 4 cells, the dimensions illustrated the variance between the ridges (n=6). The average width for clefts was  $0.97 \pm 0.53 \mu\text{m}$ . Additionally, the length varies was tremendously large, compared to the width, with  $4.7 \pm 1.54 \mu\text{m}$ . Lastly, the height of cleft was  $2.11 \pm 1.9 \mu\text{m}$  on average (**Figure 23 B**). The ridges were selected from different

cells and therefore, the comparison with each other and also lamin A ridges, might not be accurate. To solve the problem, I calculated the ratio of ridge dimension/nucleus volume, which was for width, length and height,  $0.02 \pm 0.01$ ,  $0.06 \pm 0.003$  and  $0.03 \pm 0.001$ , respectively (Appendix, Table 8). Compared the lamin A labelled ridge dimensions, they are very similar. Therefore, lamin A and emerin might realise their functions by these ridges.

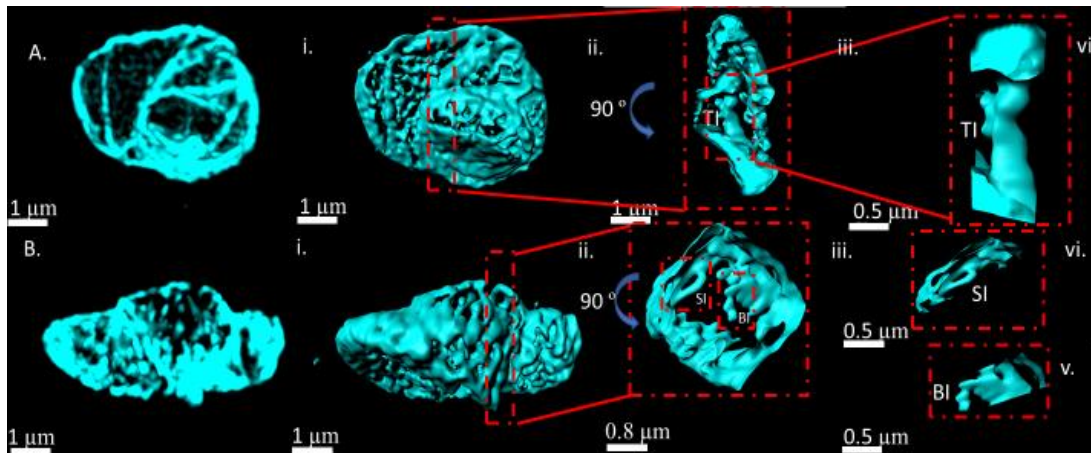


**Figure 24. Three types of invaginations was observed on emerin labelled PASM nuclei.**

- A)** (i) Deconvolved confocal images for the AIPASMC nucleus labelled for emerin (cyan) was shown and (ii) 3D reconstructed applied image of (i), (iii) was cut from the red box and rotated 90°. (iv) It was magnified image of transnuclear invagination (TI).
- B)** As in (A), however superficial invagination (SI) and blind invagination (BI) were illustrated.

Based on structural features, it is highly likely that emerin occurs in different conformations at protein complexes, and therefore the differences may represent a specific function. Previous studies that have explored the role of emerin in nuclear invaginations, demonstrated that emerin regulates nuclear invaginations (e.g., Shimojima et al., 2017). Moreover, sole emerin positive and lamin A negative nuclear invaginations has been reported (Duan, 2018). In line with these findings, I decided to determine the different invaginations labelled by emerin according to shape. To achieve this, I cut the AIPASMC nucleus labelled by emerin for specific region led by a red box and then rotated it 90° to examine the section's interior surface. First of all, I detected that the

internal and outer structures were quite similar to the structures mentioned earlier on the outer surface on lamin A labelling, including ridges, and interior structures, and looked like lamin A labelling interior structures, such as blind invagination. As discussed earlier, the nuclear invaginations can extend deep into the nucleus and away from the nuclear envelope's perimeter, which potentially associate with chromatins (**Figure 18**). Similar to the previous studies, I identified three types of invaginations: namely, transnuclear, superficial and blind (Duan, 2018). Transnuclear invaginations were obtained by cutting out the red box and were magnified in Figure 24 A.ii, which seem to cross the cell's entire width, forming a tube-like structure that connects two opposing surfaces of the nuclear envelope (**Figure 24 A.iii**). In contrast, the blind invaginations position into the nucleus, and do not connect to the INM at any other point than the entry point at which they arise (**Figure 24 B.iii**). The other structure reported at the nuclear interior structure is the superficial invagination, which appears like a ring (**Figure 24 B.iii**). As a result, the emerlin distribution in the nucleus display a variety of forms, and determining the number of each invagination may provide more details about their distribution of them in the nuclear protein complex.

**The number of invagination found on AIPASMC labelled by emerlin**

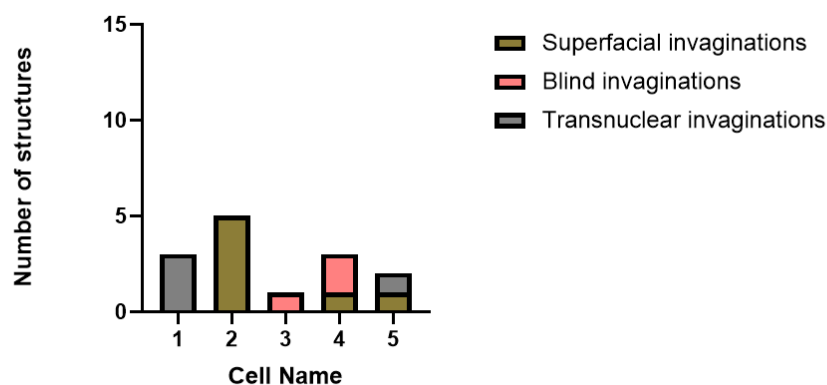


**Figure 25. Superficial invaginations were observed mostly in AIPASMC.** The bar chart demonstrates the number of structures identified by emerlin labelling. Each bar represents a cell, and superficial invagination (**green**), blind invagination (**pink**) and transnuclear invagination (**gray**) are presented (n=5).

The most frequently found invagination type is superficial invagination at AIPASMC with  $1.4 \pm 0.92$  (mean  $\pm$  SEM,  $n=5$ ). In contrast, blind invagination was observed the lowest number on emerin labelled AIPASMC with  $0.6 \pm 0.4$ . Lastly, I observed that the average number of transnuclear invaginations was  $0.8 \pm 0.58$  (**Figure 25**). Compared to the lamin A invagination number data, the number of nuclear invaginations is less for lamin A labelled nuclear invaginations, however it is not significant statistically ( $p=0.599787$  for superficial invagination,  $p=0.0807807$  for blind invagination and  $p=0.372952$  for transnuclear invagination) and the most common invagination type is different from lamin A. Therefore, the question regarding lamin A and emerin association was raised as to how to target these proteins to surface structures. With regards to this question, the lamin A and emerin was investigated to explain how lamin A and emerin formed in the outer structures like ridges. The association of lamin A and emerin was previously investigated through the nuclear invaginations before; however, this investigation of the outer structures including ridges was performed for the first time.

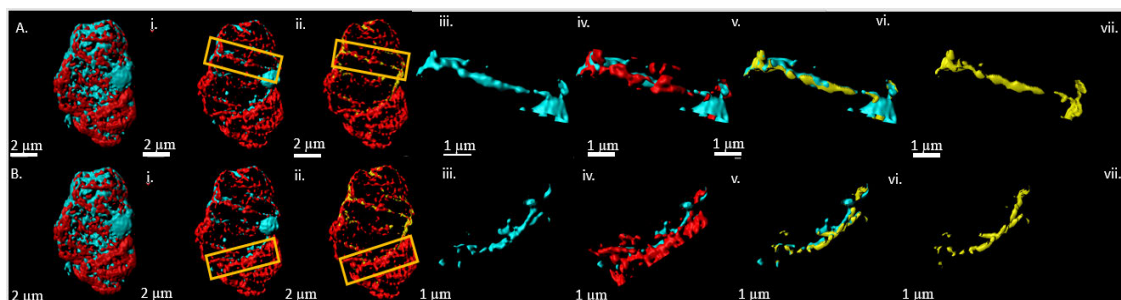


**Figure 26. Lamin A exists on the different types of ridges labelled by emerin.**

- A) (i) The low threshold 3D application of emerin (**cyan**) co-labelled with lamin A (**red**) and (ii) the high threshold 3D application are illustrated. (iii) Lamin A with colocalization channel was shown (**yellow**), (iv) cutting from the marked area by yellow box and magnified to show I-type ridge labelled by emerin. (v) The emerin labelled I-type ridge co-labelled with lamin A, (vi) colocalization channel and (vii) only colocalization channel are shown.
- B) (i) The low threshold 3D application of emerin co-labelled with lamin A and (ii) the high threshold 3D application are illustrated. (iii) Lamin A showed with colocalization channel (iv) cut from the marked area by yellow box and magnified to show branched ridge labelled by emerin. (v) The emerin labelled branched ridge co-labelled with lamin A, (vi) with colocalization channel (yellow) and (vii) only colocalization channel are shown.

Interestingly, the outer surface facing nuclear envelope structures labelled by emerin appeared to be in common with lamin A labelled surface structures, which was obtained by a high threshold application showing emerin dense regions and specific structures. Therefore, this investigation demonstrated not only that emerin and lamin A might work collaboratively but also their function by working together. In order to gain new insights regarding the distribution of emerin and lamin A in the same cell, I, first, investigated the entire nucleus of emerin and lamin A co-labelled AIPASMC at low threshold. This investigation revealed that lamin A labelling covered the whole nucleus, while the emerin labelling colocalized with lamin A existed on particular areas (**Figure 26 A.i**). Then, by removing the noise via high threshold application, I determined more certain structures like ridges, illustrating that these structures colocalised with lamin A at most of the ridges (**Figure 26 A.ii**). Given that I observed a similar conformation for emerin to lamin A, the emerin staining might be targeted to the structures defined by lamin A strategically. To test this possibility, I performed a detailed investigation for the structures, which showed that the colocalization channel created for lamin A and emerin colocalization was partially located in the ridges outlined by lamin A labelling (**Figure 26 A.iii**). I cut out the particular regions illustrating the I-type ridges that I identified before (**Figure 26 A.iv**). It was clear that the lamin A staining existed through the ridges outlined by emerin; however, lamin A did not cover the ridges drawn by emerin entirely (**Figure 26 A.v**). In sum, I investigated the colocalised region on the I-type ridge created by emerin. As a result, my data showed that colocalization of lamin A and emerin was appeared on some particular areas on the emerin labelled ridge (**Figure 26 A.vi**). Then, I illustrated the colocalization staining solely to highlight the colocalization region (**Figure 26 A.vii**).

The other structure observed on the outer surface of AIPASMC nucleus is a branched ridge that was observed by emerin labelling. Following the same process used in the investigation of I-type ridges, I cut out the branched structure from the whole nucleus and magnified it (**Figure 26 B.iv**). Then, I studied it co-labelled images for lamin A and emerin and showed that it

covered the upper face of the ridge in most places (**Figure 26 B.v**). To display the colocalised areas on the emerin labelled branched ridges more clearly, I demonstrated the emerin labelled ridges in the colocalization channel, which show that the lamin A staining covered the ridges in more places than the I-type ridge colocalization areas (**Figure 26 B.vi**). Lastly, I explored the colocalization channel solely to explain the colocalization between these two proteins clearly. This investigation revealed that the colocalization regions appeared similar to emerin labelling which means that emerin and lamin A colocalized through most places of the branched ridges created by emerin labelling (**Figure 26 B.vii**).

Consequently, as might expect, emerin and lamin A colocalized in certain regions particularly for ridges. In line with this, it was observed that the colocalization regions was located at most places of the ridges outlined by emerin. Therefore, lamin A and emerin may be positioned strategically and the colocalization between lamin A and emerin might be meaningful. To explain this colocalization more clearly, I evaluated the colocalization results by using Pearson's coefficient correlation, which showed positive association between two proteins with a value of  $0.63 \pm 0.06$  (**Figure 27 B**). Moreover, the percentage of colocalized volume of emerin with lamin A was determined as  $3.63 \pm 1.41$  (**Figure 27 A**). Previous data showed that emerin can directly bind lamin A and its localization was dependent on lamin A (Vaughan et al., 2001). Hence, the studies showed that emerin was located on lamin A labelled nuclear invaginations (Shimojima et al., 2017). However, the colocalization of lamin A and emerin at lamin A as well as emerin dense regions have remained as under-researched; thus, this study will provide a new insights gained from these earlier studies of emerin and lamin A colocalization.

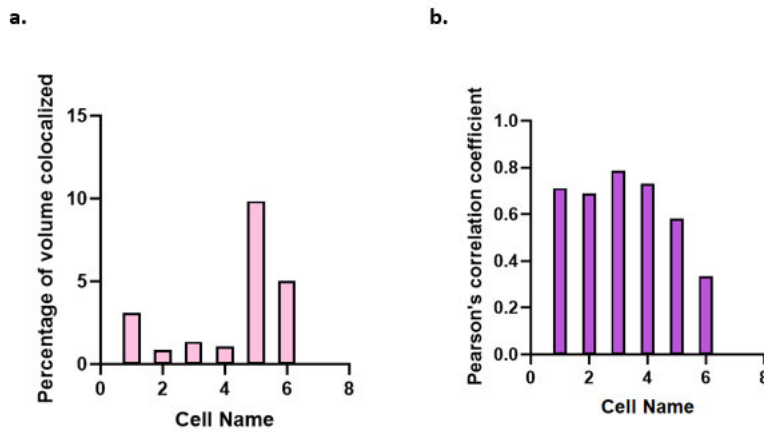


**Figure 27. Lamin A and emerin colocalization shows diversity from one cell to another cell.**

- A) Percentage of volume lamin A colocalization (pink) with emerin in AIPASMCs nuclei are illustrated in the bar charts (mean $\pm$ SEM, n=6).
- B) Lamin A and emerin colocalization (purple) are showed in the bar chart, by using Pearson's correlation coefficient (mean $\pm$ SEM, n=6)

### 3.1.3 Nesprin does not surround across NE however, nesprin exists on the described specific structures

In previous studies, it has been shown that shorter nesprin-1 at INM interact with lamin A and emerin (Mislow et al., 2002). Moreover, the interaction between lamin A, emerin and shorter nesprin 1 provide support to stabilize nuclear lamina and the modulation of nuclear intermembrane spacing (Warren, 2005; Zhang et al., 2009). Existing research illustrated the colocalization between nesprin as well as lamin A and emerin in SMCs (Mislow et al., 2002). Moreover, nesprin-1-giant provides communication between cytoplasm and nucleoplasm by creating organizational/structural/localization alteration (Stroud et al., 2014). However, there is limited information about its colocalization with lamin A in literature on nesprin, which is required to investigate lamin A and nesprin association in detail. Moreover, the investigation of LINC complex on PASCs was very limited. For that, I have investigated AIPASMC nuclei labelled by antibody for giant and shorter nesprin-1 at INM and this antibody recognises both the shorter and giant nesprin-1.

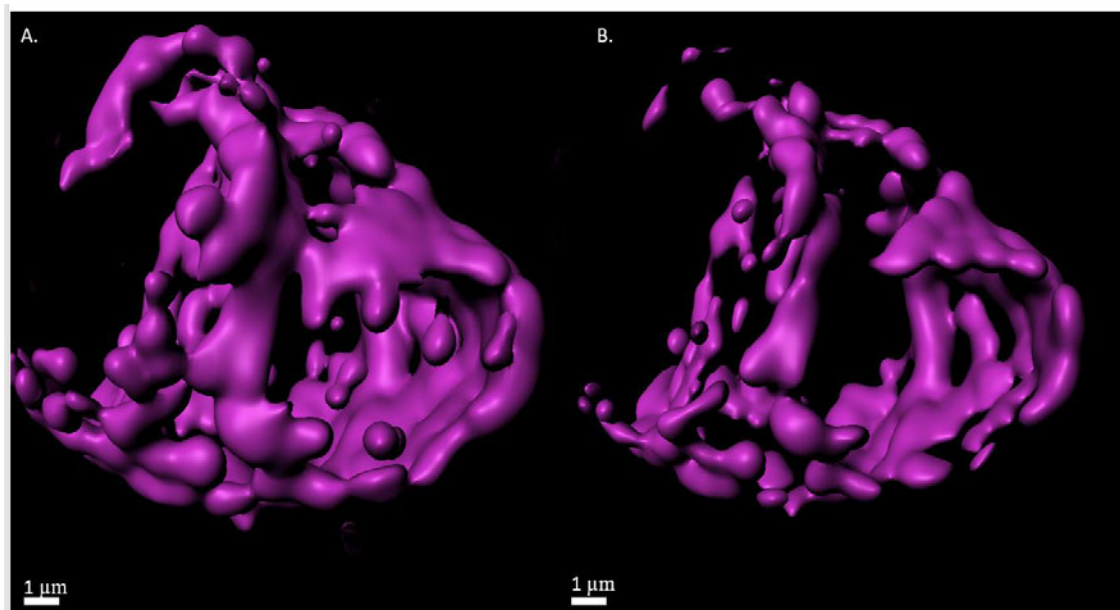


**Figure 28.** The comparison of high and low threshold application on nucleus labelled for nesprin was shown. (A) Nesprin labelled AIPASMC nucleus (**purple**) was shown at low detection threshold and (B) high threshold application.

Regarding the relationship between nesprin and nuclear processes, the localization of nesprin might be important. For the investigation of the nesprin distribution, I tried to understand the pattern and shape of the entire nesprin staining in the nuclear envelope. To achieve this, I applied the low threshold that detected not only nesprin staining dense regions, but also weakly stained regions. This investigation illustrated that nesprin labelling was located at the particular areas of NE, rather than spreading across the entire nucleus periphery (**Figure 28 A**). The antibody used in this study, MANNES1E, might cause this result, which is also proposed that nesprin distribution was not even in the previous studies (e.g., Holt et al., 2016, 2019). Then, I increased the threshold to show only nesprin dense regions, which revealed that the nesprin dense lines were similar to the ridges defined by other proteins (**Figure 28.B**) spanning across the nucleus's width (**Figure 28.B**).

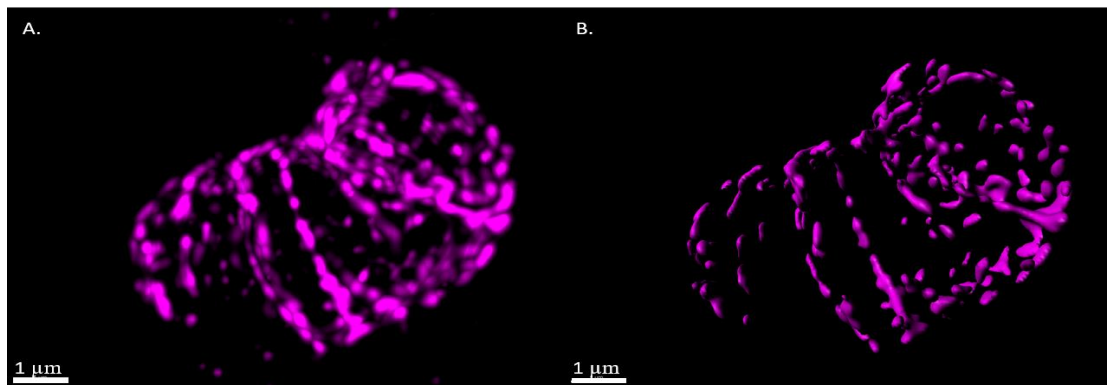


**Figure 29.** One example of nesprin labelling of the AIPASMC nucleus was shown. A) Deconvolved confocal images of the AIPASMC nucleus labelled for nesprin (**purple**) was demonstrated. B) Digital skin software was used on the 3D reconstructed images from A) to better define the illustrated structures.

Following these exciting results, I examined the deconvolved confocal image to verify the conformity with the 3D skin (**Figure 29 A**). I demonstrated that unlike other NE proteins observed previously, nesprin did not exist on the whole periphery and did not depict the periphery's entire boundary in the deconvolved image (**Figure 29 A**). Then, I applied 3D skin for the same AIPASMC nucleus labelled by nesprin, which revealed that nesprin labelling as an elongated, non-uniform and curved structure in appearance (**Figure 29 B**). Thus, by applying 3D skin, I noted that nesprin labelling intensified as linear structures (**Figure 29 B**). Moreover, the 3D application on different nuclei displayed that nesprin labelling of AIPASMC nuclei did not yield a uniform view, either (**Figure 28 & 29**). Thus, the next question must be about how the surface is shaped and how diverse the surface structure is morphologically. The previous research findings into nesprin on the structures labelled by lamin A have shown that nesprin is found on nuclear invaginations (Duan, 2018). Therefore, the investigation of the relationship between nesprin labelling and the ridges will be meaningful for gaining further understanding of nesprin distribution in the inner nuclear protein complex.

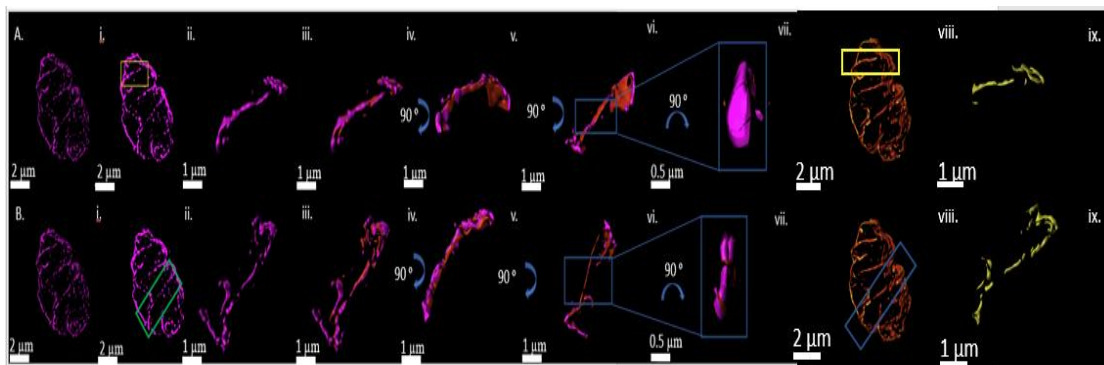


**Figure 30. Nesprin labelling AIPASMC reveals two different types of ridges on the surface.**

A) (i) Deconvolved Z-stacks of confocal images with nesprin labelling (purple) of a AIPASMC nucleus was illustrated. (ii) 3D reconstructed nesprin labelling of a AIPASMC nucleus of (i), (iii) cut from the marked area showing I shape ridge (iv) co labelled with lamin A. (v) Image was rotated 90° clockwise showing inside of nucleus (vi) cutting from the blue box and the cross-section was rotated 90° downwards, (vii) cut from the marked area. Then, lamin A and colocalization channel was illustrated and cut from the marked area to show ridge with only colocalization channel.

B) As in (A), however a branched ridge was illustrated.

In order to explain the distribution of nesprin which may affect lamin A distribution differently by each ridge type, I cut the ridges of interest outlined by the boxes indicated by a different colour, and then, I magnified these structures in order to investigate them in detail (**Figure 30 A.ii-iii & B.ii-iii**). I identified the two most common types of ridges. Firstly, I observed the I-type ridge with similar properties to the lamin A labelling I-type ridge that I determined previously (**Figure 30 A.iii**). It was a thin, smooth and straight structure that crossed the entire width of NE (**Figure 30 A.iii**). Moreover, I looked at the I-type ridge on the surface of AIPASMC nucleus co-labelled for nesprin and lamin A to examine nesprin distribution in the nuclear organisation. I observed that lamin A extended across the ridge outlined by nesprin (**Figure 30 A.iv**). Interestingly, it was found through the lamin A labelled ridge, which looks like the two proteins, nesprin and lamin A, which covered through the ridge on the nuclear envelope. As a result, I supposed that nesprin and lamin A association might be distributed through the I-type ridge equally. Additionally, its interior surface facing nucleoplasm that may important for

chromatin association obtained through being rotated 90° showed a very flat structure through the ridges, rather than a big protrusion. It was similar to the internal lamin A labelled I-type ridge, which I defined as a small protuberance, unlike emerin I-type ridge (**Figure 30 A.v**). Subsequently, nesprin association with lamin A was examined, which demonstrated exist as a lamin A through the ridge outlined by nesprin on the interior surface (**Figure 30 A.vi**). Moreover, the lamin A and colocalization channel was shown, revealing that lamin A colocalized with nesprin through the nucleus (**Figure 30 A.viii**). To understand their colocalization on the ridge, I cut ridge from marked area and it confirmed that lamin A and nesprin colocalization depicted the ridge outlined by nesprin (**Figure 30 A.ix**).

Then, I described the other interesting structure outlined by nesprin, namely, the branched ridge that split into two branches with dashed structure (**Figure 30 B.iii**). The result from the co-labelled nesprin AIPASMC nucleus with lamin A was that nesprin labelling colocalised with lamin A through the ridge partially (**Figure 30 B.vi**). By turning it 90°, I revealed that the inside was also a tiny overhang, similar to the the interior view of I-type ridge as I described earlier, except for the fact that it did not colocalized the ridge interior surface facing nucleoplasm completely (**Figure 30 B.v**). Moreover, I cut the double stained ridge and rotated it 90° downward. As a result, I found that lamin A and nesprin labelling appeared intertwined (**Figure 30 B.vii**). Moreover, the lamin A and colocalization channel was shown, revealing that lamin A colocalized with nesprin through the nucleus (**Figure 30 B.viii**), and by cutting from the marked area shows colocalization between them at most place through branched ridge (**Figure 30 B.ix**). Thus, it could be concluded that the nesprin labelling might be distributed on the lamin A ridges strategically, supporting the view that nesprin aided to stabilize nuclear lamina (Mislow et al., 2002; Zhang et al., 2009). More interestingly, this investigation has shown the nesprin-1- $\alpha$  role in  $\text{Ca}^{2+}$  processing and the modulation of RyR has been shown, which may be in line with lamin A and nesprin-1 association (Pare et al., 2005). Moreover, shorter nesprin-1- $\alpha$  is mostly expressed in cardiac and skeletal cells

(Puckelwartz et al., 2009); however, in this study, lamin A and nesprin-1- $\alpha$  association was detected in PASMNC nuclei.

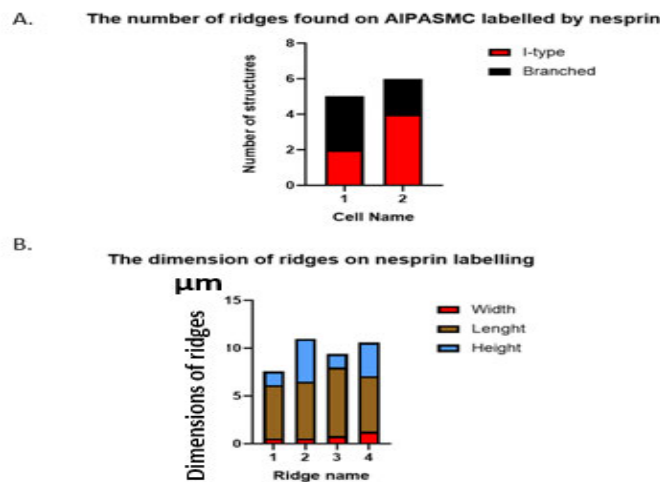


**Figure 31. Each cell studied contains branched and I-type ridges.** A) The bar chart demonstrates the number of structures identified by nesprin labelling. Each bar represents a cell ( $n=2$ ). I-type(**red**) and branched (**black**) were shown. B) The bar chart illustrated the dimensions. Each bar represents a ridge ( $n=4$ ) as well as height (**blue**), length (**brown**) and width (**red**) are shown.

For more detailed characterisation of the ridges, I analysed the number of ridges stained by nesprin, which displayed the numbering  $3.0 \pm 1.0$  for I-type ridges per nucleus and  $2.5 \pm 0.5$  for branched ridges (**Figure 31 A**).

As I noted earlier that the diameter of nesprin labelled ridges varied along with the nucleus for each nucleus. The length was on average  $6.11 \pm 0.36 \mu\text{m}$ , while the height of the ridge was  $2.72 \pm 0.77 \mu\text{m}$ . Lastly, the ridge's width on the nesprin labelled nucleus was  $0.80 \pm 0.16 \mu\text{m}$  (**Figure 31 B**). The dimensions of ridges might be change from cell to cell, which leads to incorrect comparison. To eliminate the possibility, I calculated the ratio of ridges dimensions to nucleus volume, which was for width, length and height,  $0.1 \pm 0$ ,  $0.01 \pm 0.01$  and  $0.01 \pm 0.02$ , respectively (Appendix, Table 10). Compared to other proteins investigated, the dimensions of ridges labelled by nesprin is tiny.

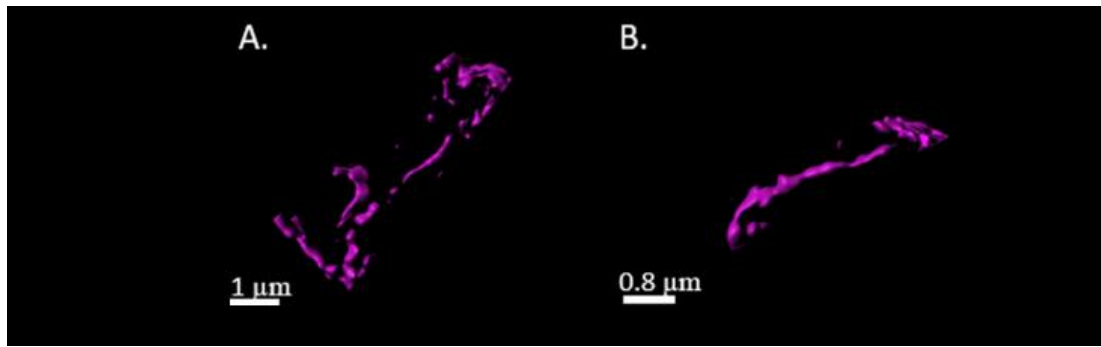


**Figure 32.** Two different types of the ridges were observed on the nesprin labelled AIPASMC nucleus, which are A) branched ridge and B) I-type ridge.

In reviewing the outer structures on nesprin, two distinct structures were defined; branched and I-type ridges. I-type ridge characteristics here were the same as in I-type ridges outlined by other proteins (**Figure 32 A & B**). Moreover, previously, I detected particular structures on the surface of emerin and lamin A labelled AIPASMC nuclei. These common structures for each protein might work collaboratively, which might offer more detailed insight into nesprin binding to lamin A (Mislow et al., 2002).

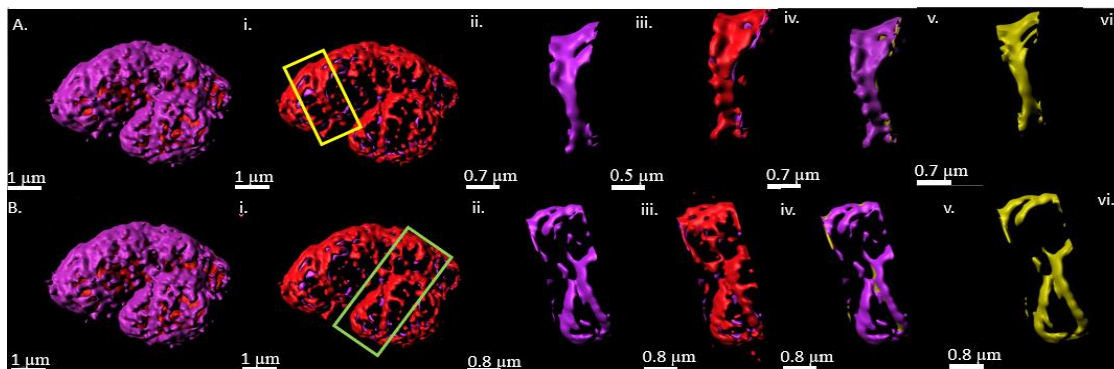


**Figure 33.** Lamin A exists on the different types of ridges labelled by nesprin.

- A) (i) The low threshold 3D skin was applied to co-labelled nesprin with lamin A image and ii) the high threshold 3D application iii) cut from the marked area by yellow box and magnified to show I-type ridge labelled by nesprin. iv) The nesprin labelled I-type ridge co-labelled with lamin A, (v) with colocalization channel with nesprin (**yellow/purple**) and vi) colocalization channel only (yellow) are shown.
- B) (i) The low threshold 3D skin was applied to co-labelled nesprin with lamin A image and ii) the high threshold 3D application iii) cut from the marked area by yellow box and magnified to show branched ridge labelled by nesprin. iv) The nesprin labelled branched ridge co-labelled with

*lamin A, (v) with colocalization channel with nesprin (yellow/purple) and vi) colocalization channel only(yellow) were shown.*

It has been observed that the impairments in lamin and nesprin association causes important diseases, which proves the importance of lamin A and nesprin association. Noting the similar structures for nesprin labelling to lamin A, I decided to investigate the double labelled AIPASMC nucleus for lamin A and nesprin. Firstly, in order to clarify the entire labelling of lamin A and nesprin, I demonstrated that the whole PASMC nucleus co-labelled for lamin A and nesprin with a low threshold application, which could detect the whole staining of lamin A and nesprin via capturing weak and strong staining represented for two proteins taking the same form. However, it was evident that nesprin labelling was not complete and located in particular regions **(Figure 33 A.i)**. Secondly, I applied a high threshold that showed only structures for this co-labelled lamin A and nesprin AIPASMC nucleus. It revealed that the particular structures identified in dense regions of nesprin colocalized with the lamin A labelling after removing the less specific staining. It showed that lamin A existed only on nesprin labelling, but it presented within the ridges and out of the ridge in areas labelled by nesprin **(Figure 33 A.ii)**. Thirdly, I cut the defined ridges labelled by nesprin, in case the structures might influence lamin A localization differently or might have different functions in the nuclear protein complex. Then, the ridges were magnified to examine for the lamin A and nesprin co-labelling on the ridges **(Figure 33 A.iii)**. This showed that lamin A colocalized with the entire nesprin labelled I-type ridge and they were very similar in appearance **(Figure 33 A.iv)**. To show the nesprin and lamin A colocalization more clearly, I improved the image by showing the colocalization channel and nesprin, which demonstrated that the colocalization covered the entire I-type ridge labelled by nesprin **(Figure 33 A.v)**. Finally, for support this view I showed only the colocalization channel, confirming the results of a nesprin and lamin A co-labelled image **(Figure 33 A.vi)**.

In a similar process, the branched ridge outlining by nesprin was demonstrated, **(Figure 33 B.iii)** and its co-labelling with lamin A was shown,

which revealed that the nesprin labelled ridge colocalized with lamin A at many points through the ridge (**Figure 33 B.iv**). Then, I showed the nesprin labelling and colocalization channel together to clarify the colocalization of these two proteins (**Figure 33 B.v**). It was evident that lamin A labelling colocalized through the branched ridge labelled by nesprin. Moreover, lamin A staining exists outside the nesprin labelling through the ridges, confirmed by Figure 33.B.iv. Therefore, it seems possible that nesprin may connect with the lamin A in particular regions of branched ridges.

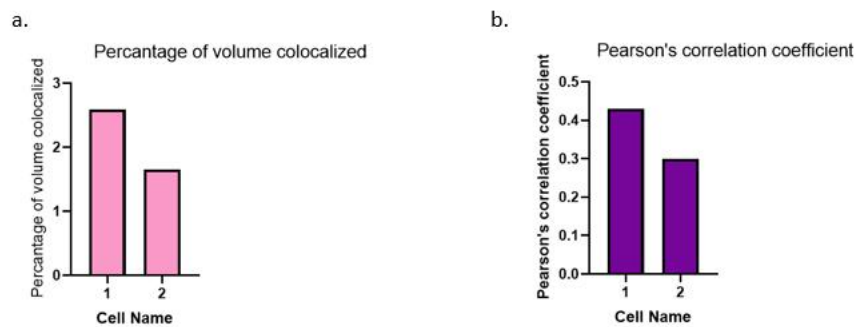


**Figure 34. Lamin A and nesprin colocalization shows diversity from one cell to another.**

A) Percentage of volume lamin A colocalization (*pink*) with nesprin in AIPASMCs nuclei is illustrated in the a bar charts (mean $\pm$ SEM, n=2).

B) Lamin A and nesprin colocalization (*purple*) is shown in the bar chart, by using Pearson's correlation coefficient (mean  $\pm$ SEM, n=2)

Lastly, I investigated lamin A and nesprin colocalization, which showed that Pearson's correlation coefficient was  $0.36\pm0.06$  (**Figure 34.B**) and the percentage of colocalized volume of nesprin with lamin A was  $2.12\pm0.47$  (**Figure 34.A**).

To conclude, it is evident that the relationship between nesprin and lamin A exists on the ridges, and more importantly, nesprin occupies particular locations throughout the lamin A labelling.

### 3.1.4 SUN-2 and lamin A colocalized in the lamin A dense regions

As stated previously, lamin A is a regulator for the localization of SUN-2, which is another LINC complex protein and SUN-2 provides a support for the regulation of the size and shape of the NE, and the signalling processes through the NE (Matsumoto et al., 2015; Sun et al., 2018). Moreover, recent studies illustrate that SUN-2 and lamin A association sustains a chromatin repressive effect (Sun et al., 2018). After gaining information about three important proteins, lamin A, emerin and nesprin, I decided to examine the SUN-2 distribution in the nuclear protein complex.

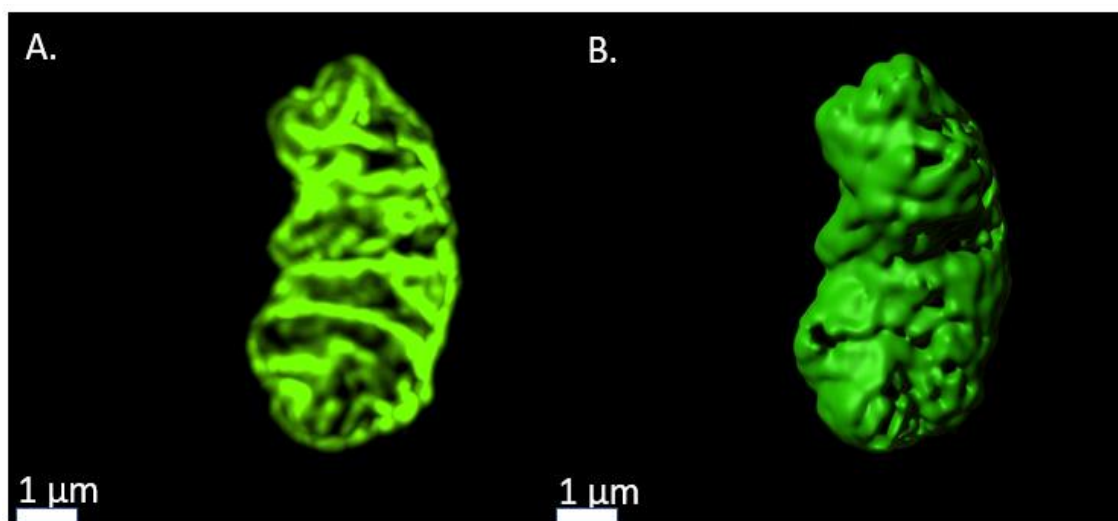


**Figure 35.** One example of SUN-2 labelling AIPASMC was shown.

A) Deconvolved confocal images for the AIPASMC nucleus labelled for SUN-2 (**green**) was illustrated with B) digital skin software to better define the illustrated surface structures.

Firstly, I defined the SUN-2 labelling, which showed the shape of the AIPASMC nucleus outlined by SUN-2 labelling was irregular, elongated and kidney-shaped with ridges and clefts in the deconvolved image (**Figure 35 A**). To compare the rough image from confocal image with 3D skin application for the same nucleus, I investigated the deconvolved image, illustrating how the SUN-2 protein labelling looked like and which properties showed similarities or diversity with the other nuclear envelope proteins studied. Supported by 3D skin application to provide better illustration, the SUN-2 labelled AIPASMC

nucleus showed the same features as its rough image obtained by confocal (**Figure 35 B**). Moreover, SUN-2 labelling illustrated similar patterns as other inner nuclear proteins, namely, lamin A and emerin, showing nuclei that were elongated and crumpled in appearance.

Overall, these results suggested that SUN-2 labelled AIPASMC nuclei had a similar appearance as with the other proteins studied. Previously, scientists showed that SUN-2 interacts with other proteins in a docking site and in the same publication, it has been demonstrated that SUN-2 is located in the microtubule network (e.g., Wang et al., 2006). Based on this information, SUN-2 dense regions may refer to important function collaborated with other proteins. Moreover, the SUN-2 dense regions may be similar to those for lamin A, which can be identified using a high threshold application. To test the possibilities that lamin A and SUN-2 may associate within SUN-2 dense regions, I applied a high threshold and identified SUN-2 dense regions.

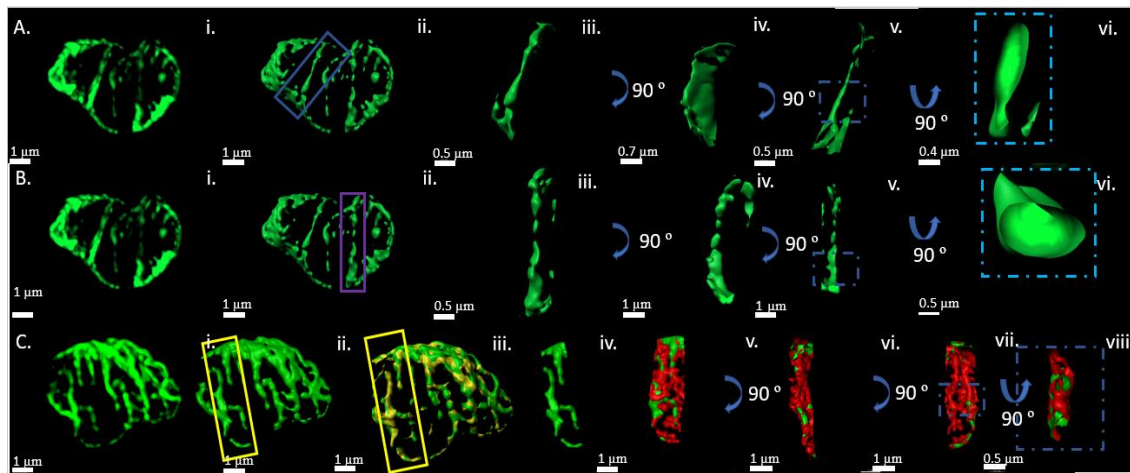


**Figure 36. SUN-2 labelling AIPASMC reveals three different types of ridges.**

A) (i) Deconvolved Z-stacks of confocal images with SUN-2 labelling in **(green)** of a AIPASMC nucleus in high threshold was shown. (ii) 3D reconstructed SUN-2 labelling of a AIPASMC nucleus (iii) cut from the marked area, showing I shape ridge. (iv) Image was rotated 90° showing inside of nucleus and (v) rotated 90° clockwise, (vi) cutting from the blue dashed box. The cross-section rotated 90° upwards.

B) As in (A), but dashed I-type ridge was illustrated

C) (i) Deconvolved Z-stacks of confocal images with SUN-2 labelling **(green)** in a AIPASMC was illustrated at high threshold application. (ii) 3D reconstructed SUN-2 labelling of a AIPASMC nucleus and (iii) SUN-2 labelling with colocalization channel (yellow) for SUN-2 and lamin A (red) (iv) cut from the marked area to show branched and half-I type ridge (iv) co-labelled labelling with lamin A. (v) Image was rotated 90° showing inside of nucleus (vi) cut from the blue dashed box and the cross-section rotated 90° anticlockwise.

The crumpled surface of SUN-2 labelled AIPASMC nucleus includes different ridges, revealed by high threshold application to determine SUN-2 dense regions. To uncover the ridge types which may show different effects on the other nuclear envelope proteins and nuclear processes, I cut from the PASM nuclei labelled by SUN-2 the particular areas outlined by the boxes and magnified it to explore the ridges' characteristics (**Figure 36 A.ii-iii & B.ii-iii & C.ii-iii**). Then, I classified the most frequently found ridges. (1) I detected the I-type ridge that spans through the nucleus's width, as defined in the other selected proteins (**Figure 36 A.iii**). Besides, similar to emerin labelled I-type ridges, the inside of the I-type ridge facing nucleoplasm which may lamin A binding point stretched into the nucleus' centre, which was obtained through

90° rotation (**Figure 36 A.iv**). (2) I detected a dashed I-type ridge that seemed like an I-type ridge; however, it consisted of dashes through the ridge (**Figure 36 B.iii**). Intriguingly, the ridge's interior was different from the I-type ridges' (**Figure 36 A.iv**), which shows a slight elevation, rather than being a projection to the centre of the nucleus (**Figure 36 B.iv**). (3) I detected the branches that divided into two arms from the middle of the ridge, and (4) the half I-type ridges located opposite to each other (**Figure 36 C.iv**). Specifically, the half I-shaped ridge can be depicted as long, thin, smooth and it does not extend through the width of the nucleus labelled by SUN-2, which is similar to the half I-type ridge defined in lamin A labelled PASMNC nuclei in section 3.1.1 (**Figure 36 C.iv**). Subsequently, to understand the colocalization of lamin A and SUN-2, which has an important role in regulating the subcellular localisation of nuclear envelope proteins, I explored co-labelling of SUN-2 with lamin A in AIPASMC (Mislow et al., 2002). The lamin A and SUN-2 colocalization was observed through some parts of the two ridges, the half-I and the branched ridge (**Figure 36 C.v**). The particular ridge, when turned to display the interior of the co-labelled ridge, showed that the colocalization continued with lamin A labelling in the inner surface facing lamin A, which may be very important in view of the ability of lamin A to regulate chromatin organization (**Figure 36 C.v&vi**). Moreover, the interior surface showed lamin A labelling, which can be explained by the location of the proteins. For instance, SUN-2 is an INM-located protein and lamin A is underlying the INM (Chen et al., 2019).

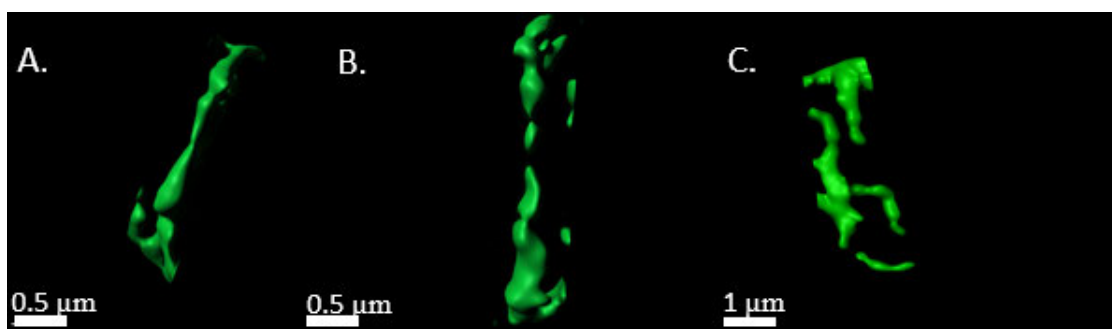


**Figure 37. Four types of ridges were observed on SUN 2 labelled AIPASMC nucleus. A) I-type ridge, B) dashed ridge, C) branched ridge and half-I type ridges are shown.**

Summarily, I have detected four types of ridges based on the SUN-2 labelling; I-type ridge, dashed ridge, branched ridge and half-I ridges (**Figure 37**). Some of these were observed on lamin A labelled ridges. Therefore, SUN-2 would seem to be purposely positioned on the lamin A labelled ridges.

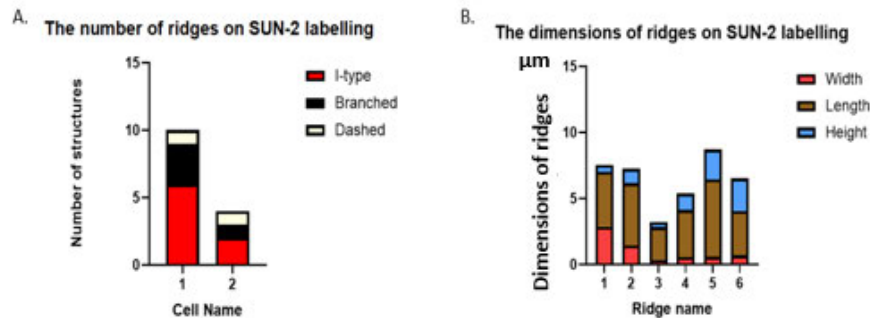


**Figure 38. The I-type ridge was observed most often by SUN-2 labelling mostly.**

A) The bar chart shows the number of ridges with SUN-2 labelling and each bar shows a cell (n=2). The I-type ridge (red), branched ridge (black) and dashed ridge (cream) are shown.

B) The sizes of ridges on SUN-2 labelled AIPASMC nuclei are shown. Each bar represents a ridge (n=6). The width (blue), length (orange) and height (grey) of clefts are discriminated by colour.

To acquire detailed information on the structures and gain an understanding of the distribution of the ridges through the nucleus, I investigated the number in which these structures occurred. This investigation illustrated that the I-type ridge was observed with the highest number of the ridges on SUN-2 labelled AIPASMC nuclei with  $4.0 \pm 2.0$ , and following that, the branched ridge was coming as the second highest number with  $2.0 \pm 1.0$  (**Figure 38 A**). Subsequently, I explored the dimensions of ridges. The interesting point of these results was that unlike the other proteins I studied, the height of the ridges was higher than their width, with  $1.34 \pm 0.35 \mu\text{m}$  and  $1.08 \pm 0.38 \mu\text{m}$  respectively. The length of ridges labelled by SUN-2 was  $6.11 \pm 0.36 \mu\text{m}$  (**Figure 38 B**). I selected these ridges randomly, which may cause incorrect comparison. Therefore, I calculated the ratio of ridge dimensions to nucleus

volume, which was for width, length and height,  $0.03 \pm 0.01$ ,  $0.06 \pm 0.01$  and  $0.02 \pm 0.01$ , respectively (Appendix, Table 9)

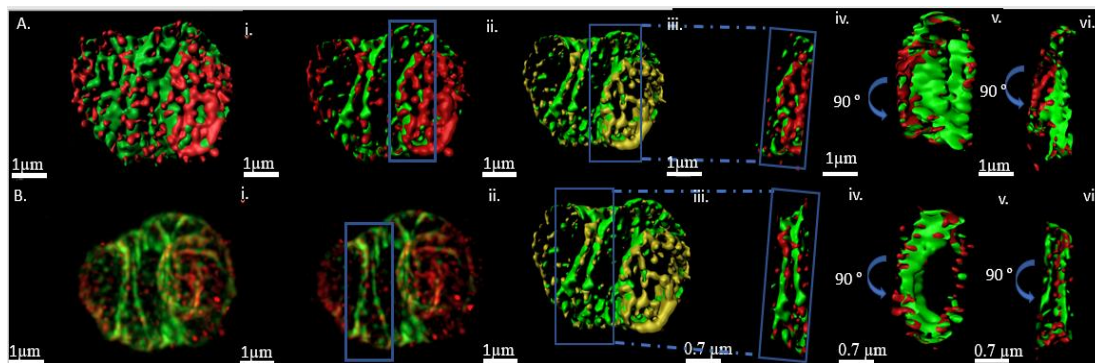


**Figure 39. Lamin A exists on the different types of ridges labelled by SUN-2.**

- A) (i) The low threshold 3D application of co-labelled SUN-2 with lamin A was shown. ii) The high threshold 3D application and iii) SUN-2 with colocalization channel shown and iv) cut from the area marked by blue box to show dashed ridge labelled by SUN-2. v) The SUN-2 labelled dashed ridge was turned 90° anticlockwise, and (vi) rotated once more by 90° in the same direction to show the ridge interior.
- B) As in (A), but a branched ridge was illustrated.

In lamin A and SUN-2 double staining, I recorded lamin A fluorescence as spotty, which might be caused by epitope masking (Chen et al., 2011). In the previous data, SUN-2 was observed just at the entry point of the invaginations and it did not detect invaginations extending towards the nuclear centre (Duan, 2018). Therefore, the different pattern for lamin A/SUN-2 positive ridges from the other proteins' associations might be expected, for example, the structure belonging to surface, not invaginations might show fully colocalization. To investigate this probability, I decided to evaluate the co-labelled nucleus to understand the distribution of SUN-2 on lamin A meshwork. To show the general view with all the staining of SUN-2 and lamin A distribution through the nucleus, I used the low threshold, which demonstrated that SUN-2 spread through the particular area of lamin A staining (**Figure 39 A.i**). Then, I applied the high threshold to remove the noise and determine structures. This process illustrated that the SUN-2 labelling was allocated on the ridges outlined by A-type lamin partially, but it filled the gaps between ridges at some points (**Figure**

**39 A.ii).** Then, I investigated SUN-2 and colocalization channel of lamin A and SUN-2, which illustrated that lamin A and SUN-2 colocalized on the SUN-2 dense regions such as dashed and branched ridges (**Figure 39 A.iii**). Moreover, I observed ridges outlined by SUN2 that were covered by lamin A partially through the ridge (**Figure 39 A.iv**). The interior of this ridge facing nucleus showed that SUN2 existed through the lamin A labelling (**Figure 39 A.vi**). Then, I showed the deconvolved images of the whole nucleus with low and high threshold (**Figure 39 B.i&ii**), which confirmed the 3D images. The Figure 39 B.iv showed that the other ridge type was the V-type that colocalized with lamin A through the V-type ridge. Following this, the interior surface of the ridge facing nucleus showed that lamin A staining located at the entire ridge (**Figure 39 B.v**).

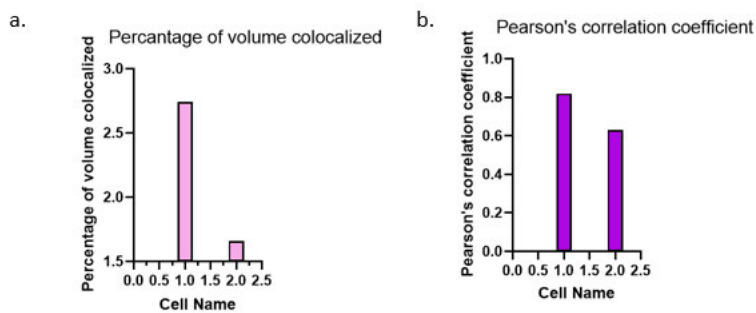


**Figure 40. Lamin A and SUN-2 colocalization shows diversity from one cell to another.**

A) Percentage of volume lamin A colocalization (**pink**) with SUN-2 in AIPASMCs nuclei are illustrated in the bar chart. Each bar shows a cell ( $n=2$ , mean  $\pm$  SEM).

B) Lamin A and SUN-2 colocalization (**purple**) is shown in the bar chart, by using Pearson's correlation coefficient. Each bar shows a cell ( $n=2$ , mean  $\pm$  SEM)

Considering this information, to understand the correlation between these two proteins, I calculated the Pearson's correlation coefficient, which was  $0.29 \pm 0.09$  (**Figure 40 B**), as well as the percentage of colocalized volume of SUN-2 with lamin A, which was  $1.18 \pm 0.35$  (**Figure 40 A**).

|    |               |               |                    |              |                |              |
|----|---------------|---------------|--------------------|--------------|----------------|--------------|
| A. | Protein       | I-Type Ridges | Half I-Type Ridges | V-Type Ridge | Branched Ridge | Dashed Ridge |
|    | Lamin (n=8)   | 3.12±0.66     | 3±0.42             | 0.75±0.25    | 0              | 0            |
|    | Emerin (n=4)  | 3.5±1.29      | 3.5±1.29           | 0            | 2±0.81         | 0            |
|    | Nesprin (n=2) | 3±1           | 0                  | 0            | 2.5±0.5        | 0            |
|    | SUN-2 (n=2)   | 4±2           | 0                  | 0            | 2±1            | 1            |

|    |                |           |           |           |
|----|----------------|-----------|-----------|-----------|
| B. | Protein        | Width     | Length    | Height    |
|    | Lamin A (n=11) | 0.86±0.04 | 2.97±0.35 | 1.17±0.13 |
|    | Emerin (n=13)  | 0.97±0.14 | 4.70±1.54 | 2.11±0.52 |
|    | Nesprin (n=4)  | 0.80±0.16 | 6.11±0.36 | 2.72±0.77 |
|    | SUN-2 (n=6)    | 1.08±0.38 | 4.02±0.48 | 1.34±0.35 |

|    |                                  |                                   |                                  |
|----|----------------------------------|-----------------------------------|----------------------------------|
| C. | Colocalized protein with lamin A | Pearson's correlation coefficient | Percentage of colocalized volume |
|    | Emerin                           | 0.63±0.06                         | 3.53±1.41                        |
|    | Nesprin                          | 0.06±0.14                         | 0.075±0.03                       |
|    | SUN-2                            | 0.29±0.09                         | 1.18±0.06                        |

**Table 3. The table shows the comparison of the structure number, size and colocalization on proteins.** A) The structure distribution on four different proteins including lamin A, emerin, nesprin and SUN-2, B) the dimensions of the ridges defined by these four proteins and lastly, the colocalization volume and Pearson's correlation coefficient for emerin, nesprin and SUN-2 and lamin A are shown.

Lastly, the table 3 shows the structure number, dimensions and the percentage of colocalization volume of proteins to compare these proteins' characteristics. As shown in the table 3 A, I-type ridge was observed in the nuclear envelope labelled for all proteins. Moreover, the I-type ridges on SUN-2 labelled AIPASMC nucleus are the most numerous, but not significantly different from others ( $p=0.923316$ , one-way ANOVA). In addition to this, half I-type ridges were observed only on emerin and lamin A labelled AIPASMC, and the number of half-I ridges on AIPASMC labelled by emerin was not significantly higher than for lamin labelled ridges ( $p=0.520018$ , t-test). The branched ridge was not detected significantly more on nesprin than on SUN-2 ( $p=0.698455$ , t-test) and lastly, the dashed ridge was observed only on SUN-2 (**Table 3 A**).

In the table 3.B, the average dimensions of the ridges labelled by different proteins were shown; however, the comparison of these values was not possible, as they were obtained from differently sized cells. That is, as the cells might have different sizes, the comparison of their dimensions might provide incorrect information.

Finally, I showed the Pearson's correlation coefficient results for lamin A and the other proteins studied (Table 3.C). The linear regression analysis verified the poor correlation among them ( $R^2=0.008836$   $\hat{Y}=0.6628-0.08385X$ , Lamin/emerin versus Lamin/SUN-2 and  $R^2=0.008836$   $\hat{Y}=0.6712-0.545X$ , Lamin/emerin versus Lamin/nesprin). Additionally, the percentages of the colocalized volume was calculated and tested regarding the relationship between them. It showed that lamin A and emerin colocalization volume was the highest amongst other proteins, though, not significantly different from others ( $p=0.344798$ , one-way ANOVA followed by Tukey HSD).

### **3.2 Cultured nucleus presented different morphological features from acutely isolated PASMC nucleus**

In response to proliferative cues (e.g., serum and growth factors), the contractile phenotype of smooth muscle cells transform into the proliferative phenotype, resulting in a reduction in the differentiated cell markers and the upregulation of proliferation markers (Rensen et al., 2007). Moreover, proliferation is associated with loss of nuclear invaginations, and thus associated pathways of  $Ca^{2+}$  flux into the nuclear envelope lumen between the ONM and INM and outwards across the ONM into cytoplasmic nanocourses demarcated by nuclear invaginations. Proliferation is also associated with loss of the nuclear buffer barrier which restricts direct entry of calcium into the nucleoplasm of differentiated contractile pulmonary arterial myocytes (Duan, 2018).

To investigate phenotypic switching in SMCs, I selected the cells which are enzymatically isolated SMCs in primary culture containing 10% of fetal bovine serum. In response to growth factors, these cells showed proliferative and migratory properties and the phenotypic properties changed in the nucleus from contractile to proliferative dependent on SMCs culture conditions (Chamley-Campbell et al., 1979). Based on previous knowledge, I hypothesized that phenotypical switching might cause significant morphological changes in the nuclear envelope as well as on the

distribution of nuclear envelope proteins. I decided to explore this possibility by comparing AIPASMC and D7PASMC.

### 3.2.1. Lamin A labelling is formed by a variety of structures on D7PASMC surface.

To investigate the similarities and differences between D7PASMC and AIPASMC nuclei, I, firstly, explored lamin A distribution, as before, which one would expect to cover the entire inner surface of the inner nuclear membrane, which provides mechanical stability to the nuclear envelope and fulfil the tasks such as directing chromatin attachment (Dechat et al., 2008). Importantly, as discussed previously, it has a role in the modulation of the nuclear architecture that is significant given that it has the ability to regulate chromatin organization (Takizawa & Meshorer, 2008)

In order to explain morphological alterations on lamin A labelled PASMC nucleus after phenotypic switching, I described the phenotypic features of lamin A labelling in 7 days cultured PASMC nucleus. Because, lamin A surface facing nucleoplasm included regions to provide histone association, while the surface of this protein facing INM could interact with LINC complex proteins (Janin & Gache, 2018; Shevelyov & Ulianov, 2019).

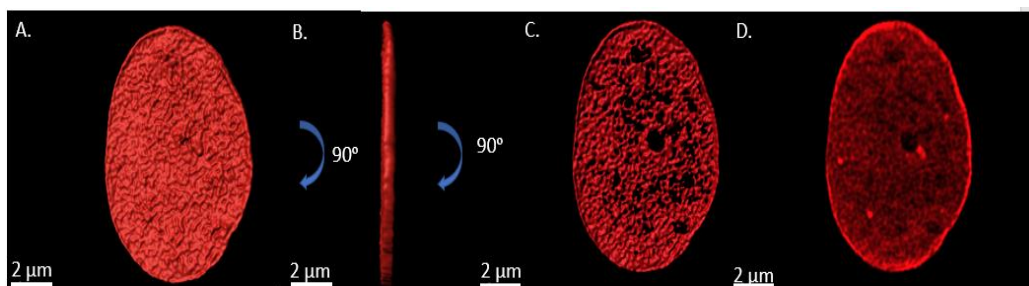


**Figure 41. The comparison of high and low threshold application on cultured PASMC nucleus labelling for Lamin A was shown.** (A) Low detection threshold with digital skin was applied Lamin A labelling on D7PASMC nucleus (red), B) rotated 90° and C) turned 90° but with higher threshold application. D) Lastly, a deconvolved confocal images for (C) is shown.

First, I examined the D7PASMC nuclei labelled by lamin A surface facing INM with a low threshold application to demonstrate all details relating to labelling across the entire INM, and to describe the general aspect of the

surface of lamin A labelling in D7PASMC nuclei. In accordance with the previous studies, it is apparent from Figure 41 A that the lamin A net-like meshwork structure is more elliptical, which is quite different from the AIPASMC nucleus (Chamley-Campbell, 1979; Thakar et al., 2009). Furthermore, the lamin A labelled D7PASMC nuclei were flattened and latticelike structure, rather than rugby ball like lamin A labelling in AIPASMC nucleus, as shown in Figure 41. B, which was obtained by 90° rotation of D7PASMC labelled by lamin A.

As reported by others, the folds and wrinkles on the lamin A labelled AIPASMC which are referred to as lamin A dense regions, such as ridges and clefts in section 3.1, were not observed on the surface of lamin A labelled D7PASMC nuclei (Duan, 2018). Moreover, it was evident that the lamin A labelling was not uniform throughout the D7PASMC nucleus, as proposed in the previous studies on cultured pulmonary arterial myocytes (D7PASMC) and different cells including fibroblasts, cardiac progenitor cells (e.g., Duan, 2018; Lavrushkina et al., 2019) (**Figure 41.A**). The cause of this non-uniformity could be caused by the epitope masking due to the antibodies used in this study (Dyer et al., 1997), or it might be related to the antibody accessibility at different points (Tunnah et al., 2005.). However, both of these possibilities seem unlikely. Alternatively, this non-uniformity consisted of special structures and small holes revealed by using high threshold application to define lamin A dense regions on lamin A D7PASMC (**Figure 41.C**), the architecture which might be governed by the presence of nuclear pore complexes as stated in literature (Maeshima et al., 2006). However, the application of a different approaches including super resolution methods and different antibody staining control for epitope masking, may provide more accurate results (Tunnah et al., 2005).

It was clear that the D7PASMC nuclei labelling did not display some of the structures detected on AIPASMC nuclei, such as ridges, which suggests that the appearance is in a more symmetrical and net-like structure (**Figure**

**41 A).** All D7PASMCs studied showed the typical elliptical shape, which was flat and thinner than AIPASMC nuclei (n=13).

After obtaining a general overview, I tested the coincidence of 3D skin application, and examined the deconvolved image, which in cultured D7PASMC lamin A labelled was thinner, ovoid and larger, rather than elongated and spindle-shaped like AIPASMC nuclei (**Figure 41 D**). Moreover, it emphasized the net-like structure, composed of small holes (**Figure 41 D**), which illustrated that the deconvolved image was identical with the 3D skin application and it appeared to have the standard appearance of lamin A labelled D7PASMC that is the same in different nuclei from D7PASMC labelled with lamin A. As stated above, the rough description of lamin A labelled cultured nuclei was studied previously; however, the classification of the structures on lamin A dense regions would be explored here for the first time, which might be important due to the nuclear shape effects on the nuclear processes (Skinner & Johnson, 2017). More importantly, each different structure on the nucleus might have a special function in the nuclear processes.

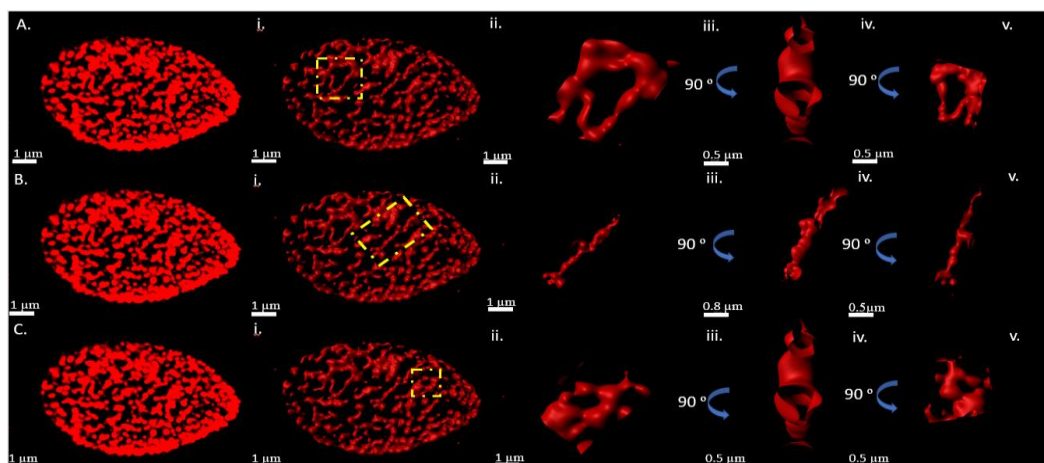


**Figure 42. The most prevalent structures on lamin A labelled 7 days cultured nucleus are O-type, I-type and irregular round shaped.**

A) (i) Deconvolved Z-stacks of confocal images with lamin A labelling in (red) of a D7PASMC nucleus was demonstrated. (ii) 3D reconstructed lamin A labelling of a D7PASMC nucleus cultured for 7 days (iii) cut from the marked area showing irregular rounded structure. (iv) Image was rotated 90° showing inside of nucleus and (v) turned 90° upwards, (v) cutting from the middle and rotating 90° upwards.

B) As in (A), but I-shaped structure was illustrated.

C) As in (A), but O-shaped structure was illustrated.

After the description of lamin A labelled D7PASMC nuclei, I suggested that the structures on the surface of D7PASMC nuclei labelled by antibodies against A-type lamin in different cells (n=4), might be located strategically, as the surface facing INM has regions where LINC complexes "bind". In order to assess the morphological changes from AIPASMC to D7PASMC labelled by lamin A, I selected the most common structures on the surface. Then, I cut and magnified them from the marked areas by different colours through applying a high threshold to show the structures created by accumulation on lamin A (**Figure 42 A.i-ii-iii&B.i-ii-iii&C.i-ii-iii**). In doing so, I illustrated the inner surface facing nucleoplasm of lamin A. As shown in Figure 42, the outer surface had a significant number of tiny bumps, utterly different from ridges. Considering that substantial changes on the surface may represent different lamin A function, I categorised the structures commonly found on the lamin A meshwork among multiple cells: (1) an irregular round-shaped form shaped like a circle with smooth convex boundaries (**Figure 42 A.iii**). Then, I turned the structure to investigate the

structure's inner surface facing nucleoplasm. This investigation revealed that it had a flat and smooth surface on the inner face of the irregular rounded shape (**Figure 42.A.iv**). Drawing on these findings, the question was raised that the structure's back might be the same view of the irregular round-shaped structure. To investigate it, I rotated the surface one more time, which resulted in the same surface with the irregular round-shaped (**Figure 42 A.v**). (2) Secondly, I identified that the I-type structure was long, rough and thin (**Figure 42 B.iii**) obtained by cutting a particular region referring to the I-type structure and magnifying it for more detailed investigation. In the following step, I turned the structure 90° to show characteristics of the inner surface that determined chromatin associations, which revealed that the inner surface was rough, irregular and flattened (**Figure 42 B.iv**). With this view, I decided to rotate the surface one more time to demonstrate detail of the back face of the structure (**Figure 42 B.v**). (3) Lastly, I described the O-type structure as a small and regular circular shape by cutting and magnifying a section from the specific area of the image accounting for the O-type structure and magnifying it (**Figure 42 C.iii**). I continued with the inner surface examination of the O-type structure by rotating 90°, which showed its a flat and smooth structure. Then, I turned it 90° again to examine this structure's surface facing INM characteristics, demonstrating the same structure as the O-type structure (**Figure 42 C.iv**). Interestingly, I did not observe nuclear invagination throughout my investigation for the inner surface of the A-type lamin labelled D7PASM C nucleus (**Figure 42.A & B & C.iv**). However, the evidence of single transnuclear invagination that was likely lamin negative in D7PASM C was observed in live cell staining studies through using endoplasmic reticulum tracker (Duan et al., 2019).

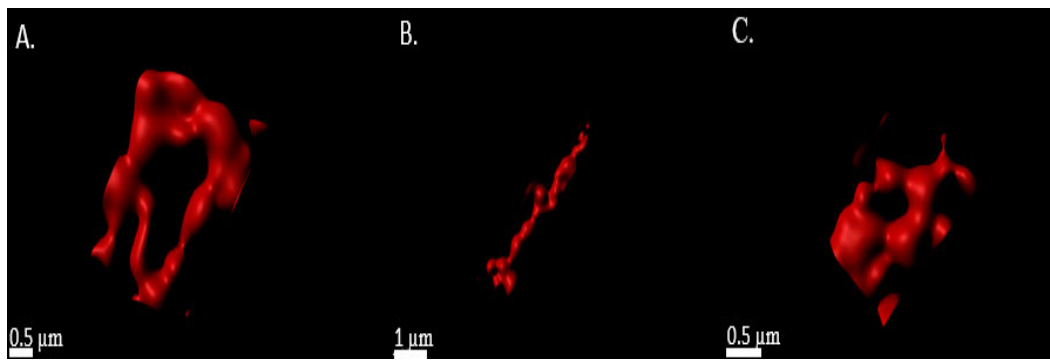


**Figure 43.** Three types of structures were observed on lamin A labelled D7PASC nucleus: A) irregular rounded shape, B) I-type, and C) rounded structures are shown.

To conclude, I have determined three types of structures on lamin A labelled D7PASC; irregular rounded shape, I-type and O-type structures (**Figure 43**). The structures were found widely on D7PASC labelled for lamin A. The most striking point was that the morphology of a D7PASC nucleus labelled for lamin A differed significantly from the morphological features of the AIPASC nucleus, which may support the idea that lamin A is distributed strategically in the PASC nucleus. Furthermore, the structures were diverse throughout the nucleus; therefore, it is necessary to examine the numbers of these structures on the D7PASC nuclei.

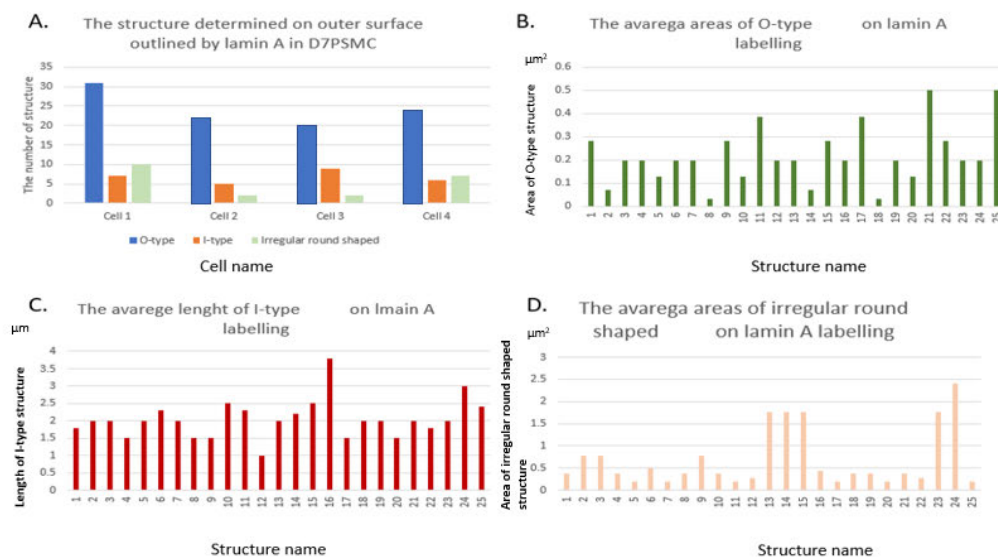


**Figure 44.** The most commonly found structure is the O-type structures on lamin A labelled D7PASC nuclei. A) The number of the outer structures defined by A-type lamin in each cell is demonstrated in the stacked bar chart, with the O-type structure in blue, I-type structure in orange, irregular rounded structure in water green. Each set of 3 bar represents a cell. (n=4) B) the average

*surface area of O-type structure (n=25), C) the average length of the I-type structure and D) the average area of irregular round-shaped structure (n=25) were shown.*

On average, I identified  $24.75 \pm 2.21$  O-type structures in each cell (mean $\pm$ SEM, n=4); however, there were fewer irregular round-shape structures with  $5.25 \pm 1.97$  than O-type numbers for that labelling. From the chart, it can be seen that a mean of  $6.75 \pm 0.85$  I-type structures were found in each cell (**Figure 44 A**); however, there were no statistically significant differences between the group means as determined by one-way ANOVA (p= 0.579067), so could be significant with more cells. Moreover, linear regression analysis showed that there was a very weak relationship between O-type and I-type structure numbers ( $R^2=0.00001302$ ). However, a very strong relationship between O-type and irregular round-shaped structure was observed ( $R^2=0.8395$ ). As a result, O-type structure may have a similar or related function to irregular rounded shaped structure in the D7PASMNC nuclei.

To add more details to this argument, I identified the region occupied by these different structures through selecting them randomly from different cells whose sizes vary among the structures. This investigation suggested that the average areas of O-type structures which correspond to the hole size of lamin A dense region shaped in “O” were identified as  $0.21 \pm 0.02 \mu\text{m}^2$  (n=25) (**Figure 44.B**). In contrast, the irregular shaped structures average area was identified with  $0.68 \pm 0.13 \mu\text{m}^2$  (n=25) (**Figure 44.D**). In addition, the average length of I-type structures was determined as  $2.04 \pm 0.11 \mu\text{m}$  (n=25) (**Figure 44.C**). Since the selection was done from different cells, I calculated the ratio of dimension or area/nucleus volume. For O-type, I-type and irregular shaped structures, they are  $0.001 \pm 0.0002$ ,  $0.02 \pm 0.001$  and lastly,  $0.01 \pm 0.001$ , respectively (Appendix, Table 11). Consequently, I examined that the O-type structure represented a tiny region, whereas the number of O-type structure is the highest among them. The interesting point is that nuclear lamina is composed of filaments sized 10 nm; however, my results suggest that the structure sizes are more than this value, for example, I-type structure length is  $2.04 \pm 0.11 \mu\text{m}$ , which might

be caused by microscopy technique used in this study (Turgay & Medalia, 2017; Vangindertael et al., 2018). Lamin A filaments come together to form bundles, which may be segregated by interactions with other proteins and affected by charge differences across INM (i.e., calcium load between INM and ONM). In one of the studies on lamin A filaments, it was proposed that the length of lamin A filaments can change in response to electrostatic interaction with N-terminal and C-terminal region on lamin A (Makarov et al., 2019). Higher sizes in my results compared to literature might be explained by this kind of studies.

### **3.2.2 Emerin labelling of D7PASMNC nuclei indicates the loss of structures determined on the surface of the AIPASMNC.**

It has been shown that the phenotypic characteristics of A-type lamin labelled D7PASMNC were dissimilar from AIPASMNC nuclei in appearance. In this case, the other nuclear proteins including emerin, nesprin and SUN-2 may be affected by the changes in the lamin A morphology, since these proteins interact with lamin A (Haque et al., 2010a; Mislow et al., 2002; Sakaki et al., 2001). As one of these proteins, the distribution of emerin is important in the modulation of the gene expression, given that it affects the connection points of heterochromatin and BAF (Bengtsson & Wilson, 2004). As the next step, I explored the emerin labelling's morphological characteristics at D7PASMNC.

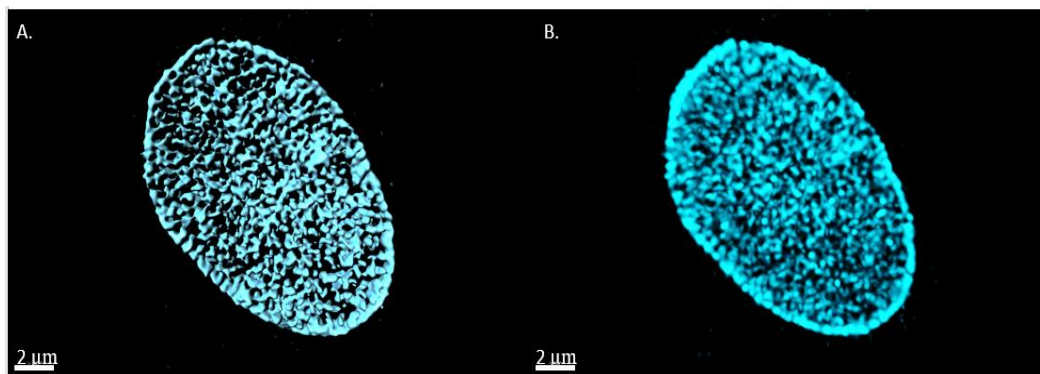


**Figure 45. Illustration of 3D skin application and deconvolved image of a cultured PASMC nucleus labelled for emerin were shown. (A)** Digital skin was applied emerin labelling D7PASMC nucleus (cyan) to illustrated detailed structures.

*B) As in (A), but a deconvolved image without digital skin were illustrated.*

The first set of analysis concerned examining the general aspect of an emerin labelled D7PASMC nucleus, in which the elliptical boundary of emerin labelling on a cultured PASMC nucleus's surface was found evident (**Figure 45.A**). I applied the low threshold, which allows to include less dense emerin staining. It illustrated a more smooth and flat surface of emerin labelled D7PASMC than AIPASMC nucleus outlined by emerin (**Figure 45.A**). This is similar to lamin A distribution in D7PASMC nucleus, and in this regard, it coincides with the literature on emerin localization being dependent on lamin A (Vaughan et al., 2001). It seems that it includes the regions in which there is a higher density of emerin exists (**Figure 45.A**).

Following the investigation, I examined the deconvolved confocal images in which confirmed in the cultured emerin labelling structures' diversity and displayed massive differences between day 0 and day 7 PASMC nuclei labelled by emerin (**Figure 45.B**). Moreover, the examination of both deconvolved and 3D skin applied images of the D7PASMC nuclei outlined by emerin confirmed the accuracy of the 3D skin application by showing the existence of specific structures on the surface of D7PASMC nucleus labelled by emerin.

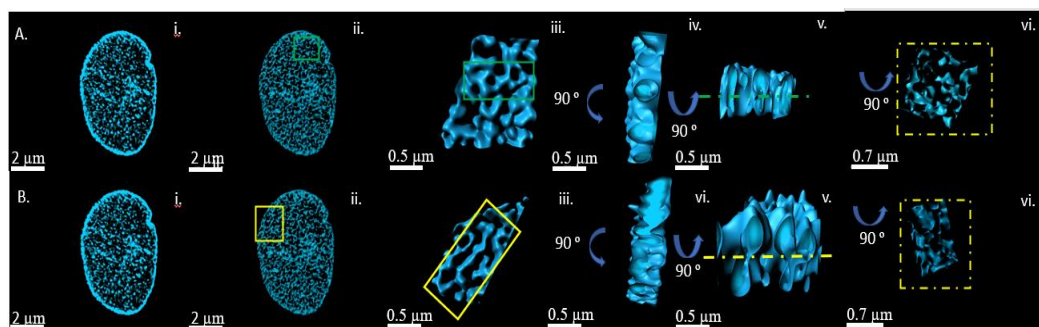


**Figure 46. The most found structures on emerin labelled 7 days cultured PASC nuclei are O and I shaped structures.**

A) (i) Deconvolved Z-stacks of confocal images with emerin labelling (**cyan**) in a D7PASC nucleus was demonstrated. (ii) 3D reconstructed emerin labelling of a D7PASC nucleus cultured for 7 days (iii) was cut from the marked area showing O-formed structure. (iv) Image was rotated 90° showing the other side of nucleus and (v) rotated 90° upwards (v) cutting from the middle and turned 90° upwards.

B) As in (A), but an I-shaped structure were shown.

Accordingly, I investigated another D7PASC nucleus outlined by emerin. Interestingly, emerin, consistent with lamin A formed a flattened and oval structure, which I think, is representative of the shape of the INM and thus of the nucleus. This is different from what is seen for AIPASC emerin labelling. Furthermore, this nuclear appearance was almost the same as the previous D7PASC nuclei labelled by emerin. Therefore, I supposed that the synthetic phenotype is characterised by emerin illustrated standard shape in cell, in which a regularly shaped nuclear rim, was revealed as in osteoblast and fibroblast nuclei in dermal tissue (Lüke et al., 2008). In this instance, my observation points out to the structural differences between the surfaces of the contractile and synthetic phenotype of PASC nuclei with emerin labelling. In parallel to this, the D7PASC labelled by emerin showed granular roughness, unlike any ridges or clefts on the surface of D7PASC nuclei drawn by emerin (**Figure 46 A.i & ii**). Following this, on this roughness surface of emerin labelled D7PASC, I found that the various surface structures, which were obtained through cutting the particular structures from this nucleus and magnifying them, were further subcategorised into two groups: O and I-type (**Figure 46 A.iii-B.iii**). This subcategorization might be important, as the structures might be

responsible for different functions: (1) I observed that the O-type structure was the most frequently found form described as symmetrical and closed-rounded (**Figure 46 A.iii**). I turned the structure 90° to understand the shape of the structure. When viewed it from its side, it showed a finely compacted structure (Lee et al., 2001) (**Figure 46 A.iv**). Then, by turning it 90° and cutting it in the middle, I examined the structure's interior that can be characterised as irregular, thinner and misshapen (**Figure 46 A.v&vi**). (2) Applying the same techniques to the I-type structure, I defined I-type structures in the shape of short and narrow tubes (**Figure 46 B.iii**). Similarly, I turned the structure showing that the inside of this structure facing nucleoplasm was similar to O-type, which had an uneven shape with tiny indentations and protrusions (**Figure 46 B.vi**). Mainly, I examined the similarity of structures on the emerin surface of D7PASMNC nuclei to A-type labelling for D7PASMNC nuclei.

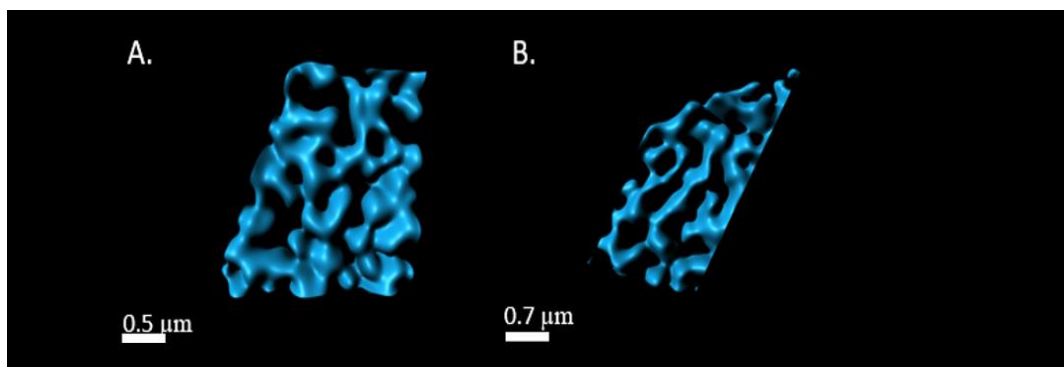


**Figure 47.** Two types of the structure were observed on emerin labelled D7PASMNC nucleus, which are A) O-type and B) I-type structures.

In conclusion, I have detected two different types of structures on emerin labelled D7PASMNC nuclei: O and I-type structure (**Figure 47**). The structures formed the outer surface outlined by emerin. Interestingly, I also observed these structures on the surface of lamin A labelling for D7PASMNC nucleus. Additionally, it has been shown that their inner surfaces are very similar to the lamin A labelled structures on D7PASMNC nucleus. Moreover, the structures are thinner and merged on the opposing surfaces. This is quite different from AIPASMNC nucleus labelled by emerin that looks like rugby ball shaped, and there is a space between the opposite surfaces

outlined by emerin. As a consequence, the structures defined on the surface of emerin labelled D7PASMNC nuclei may be located strategically given that the emerin can interact with lamin A, nesprin and BAF (Mislow et al., 2002; Montes De Oca et al., 2005). Next, I questioned the areas occupied by these structures and how many structures were placed on the surface of each emerin labelled D7PASMNC nucleus.

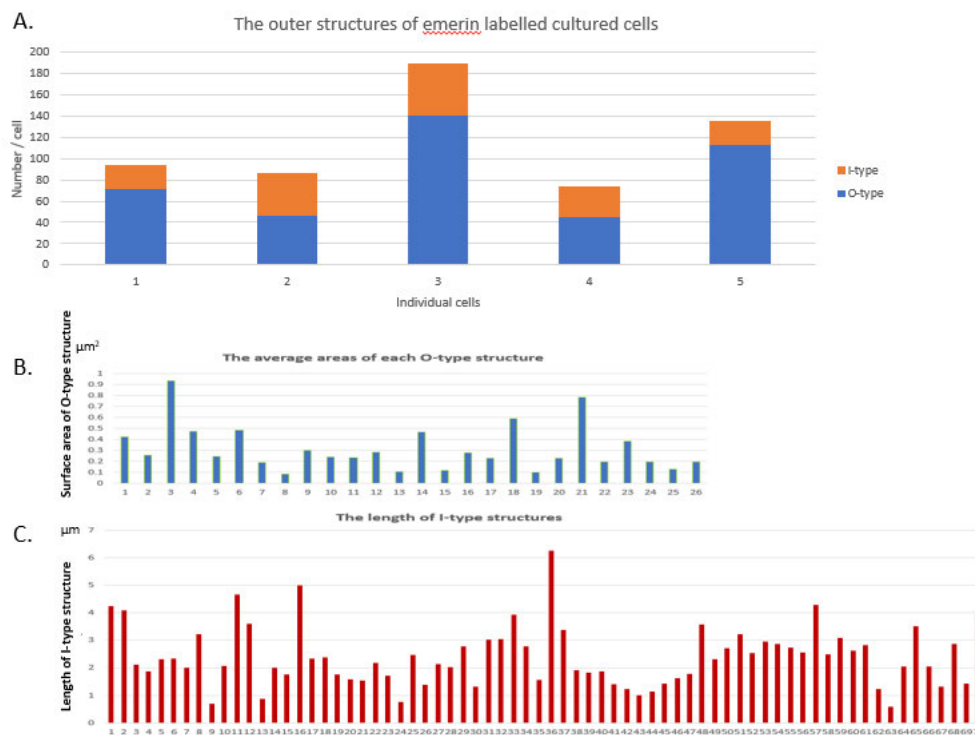


**Figure 48. The number of O-type structure on emerin labelled cultured cells outnumber I-type.** A) The number of two different structures which are I-type (orange) and O-type (blue) was shown on emerin labelled day 7 PASMNC nuclei (n=5). B) On average O-type area (blue) (n=26), C) the length of I-type structure (red) (n=70) are differentiated by different colours

For the detailed investigation of the different structures, I examined the structure number and their sizes. Intriguingly, as shown in Figure 48 A, O-type structures were found over two times more, compared to I-type on the emerin labelled structures (mean  $\pm$  SEM: O-type structures  $83 \pm 18.84$ ; I-type  $32.8 \pm 11.41$ ; n =5) (**Figure 48.A**). Additionally, the difference between them is statistically significant ( $p=0.0214829$ ). Interestingly, the O-type structure is the most frequently found structure on A-type lamin labelled D7PASMNC as well, which may be because emerin's localization being dependent on lamin A localization (Wu et al., 2014). Therefore, the O-type

structure might serve an important function for both proteins; however, the numbers of this structure on the surface of lamin A labelled for D7PASMNC nucleus are lower than that of emerins.

After obtaining these findings, I continued with measuring the areas occupied by these structures dimensions to evaluate these structure distributions. This showed that the O-type structure average surface area was determined as  $0.31 \pm 0.04 \mu\text{m}^2$  ( $n=26$ ) (**Figure 48.B**). In contrast, the I-type length as one of the structures I had subcategorized was  $2.40 \pm 0.12 \mu\text{m}$  ( $n=70$ ) (**Figure 48.C**). The structures were selected randomly from cells. To provide clearer comparison, I calculated the ratio of dimension or area of structure/nucleus volume which showed for O-type and I-type that  $0.002 \pm 0.0003$  and  $0.02 \pm 0.0001$  (Appendix, Table 12). These two values showed diversity among the structures (**Figure 48 B&C**). Moreover, these measurements were very similar to the structures created lamin A labelled D7PASMNC nucleus.

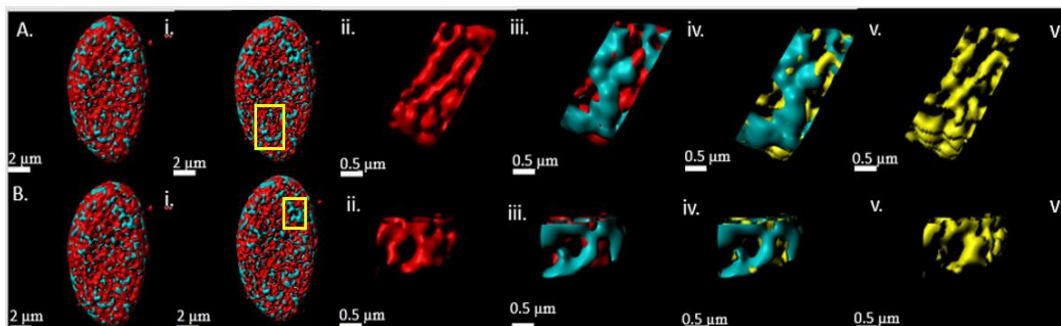


**Figure 49. Lamin A exists on the different types of structures labelled by emerins.**

A) (i) The low and (ii) the high threshold 3D application of co-labelled emerins (**cyan**) with lamin A (**red**) (iii) cut from the marked area by coloured box and magnified to show I-type structure labelled by lamin A. (iv) The emerins labelled I-type structure co-labelled with lamin A, (v) with colocalization channel (**yellow**) and (vi) only colocalization channel were shown.

B) As in (A), but an O-type structure was illustrated.

As explained above, the distribution of emerins is very similar to lamin A, which is consistent with the findings of the earlier studies in the literature (e.g., Vaughan et al., 2001). Taking these facts into consideration, it can be argued that these structures may refer to different functions collaboratively or separately. After the investigation of the emerins surface in D7PASMNC, I

decided to explore emerin in the lamin A meshwork. For that, I first investigated the low threshold 3D digital skin applied image, which allowed me to show general view of the emerin and lamin A assembly by displaying all spots stained by lamin A and emerin. It was then evident that the emerin labelling and lamin A overlapped throughout the D7PASMNC nucleus (**Figure 49 A & B.i**). Subsequently, I showed the regions that accumulated lamin A and emerin on the same nucleus via the high threshold application. It demonstrated that emerin labelling spread widely on lamin A surface merged by lamin A and covered the lamin A surface (**Figure 49 A&B.ii**). To investigate the colocalization of the structures defined previously with emerin or lamin A, I examined co-labelled D7PASMNC for lamin A and emerin, which might provide evidence for whether these two proteins distributed strategically or not. Firstly, I cut the I-type structure outlined by lamin A and magnified it (**Figure 49 A.iii**). Then, I colocalised this structure with the emerin labelling (**Figure 49 A.iv**), which showed that the I-type structure labelled by lamin A was filled by emerin staining. Early examples of research into the lamin A and emerin association suggests that these two proteins are homogeneously localized through the nucleus (Taranum et al., 2012). Conversely, Duan (2018) has reported structures on an emerin AIPASMNC nucleus without lamin A. Moreover, recent studies have illustrated an emerin-prelamin A interaction in human fibroblast (Capanni et al., 2009), adding to lamin A. My findings complies with Duan's (ibid.) observations, which showed that emerin and lamin A are distributed differently on I-type structure labelled by lamin A. Lastly, I emphasised the colocalization regions by showing only the colocalization channel (**Figure 49 A.vi**). With regards to lamin A and emerin colocalization channel, it showed lamin A colocalized with emerin through the I-type structure. Therefore, it can be postulated that emerin might not have formed in the I-type structure.

Secondly, I defined the O-type structure labelled by emerin and lamin A co-labelling as a second structure. I identified the O-type structure outlined by lamin A, as shown in section 3.2.1 (**Figure 49 B.iii**). Then, I investigated

the lamin A and emerlin colocalization on the O-type structure where the emerlin labelling did not fill the structure. Interestingly, it formed the same structure in the same localization with lamin A (**Figure 49 B.vi**). With regards to this, I investigated the emerlin and colocalization channel, which showed the colocalization regions, which outlined the O-type structure (**Figure 49 B.v**). The last images also highlighted the colocalization regions and showed that the O-type structure might be formed by both proteins (**Figure 49 B.vi**). This finding is consistent with the findings of the earlier studies that showed that lamin A and emerlin were distributed evenly (Taranum et al., 2012).

Consequently, it was evident that there were two different colocalization forms for the defined structures on D7PASMNC labelled by lamin A and emerlin. One of them is showing the emerlin labelling on the I-type structure outlined by lamin A and the other one is that the emerlin and lamin A labelling formed in edges of the O-type structure collaboratively. The different colocalization forms may support the idea that each structure on the surface of emerlin labelled D7PASMNC nucleus may realize different functions.

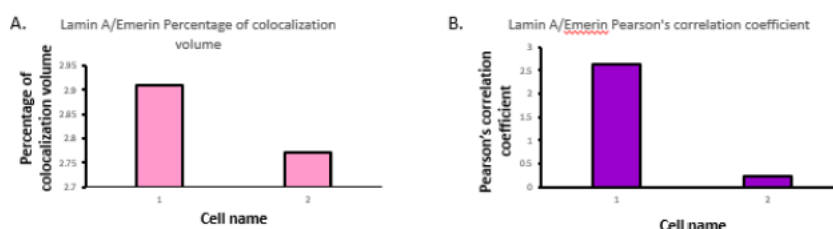


**Figure 50. Lamin A and emerlin colocalization on D7PASMNC nucleus shows diversity from one cell to another.**

- A) Percentage of volume lamin A colocalization (**pink**) with emerlin in D7PASMNCs nuclei are illustrated in bar charts. Each bar shows a cell ( $n=2$ ,  $\text{mean} \pm \text{SEM}$ ).
- B) Lamin A and emerlin colocalization (**purple**) is shown in the bar chart, by using Pearson's correlation coefficient. Each bar shows a cell ( $n=2$ ,  $\text{mean} \pm \text{SEM}$ )

As shown in Figure 49, lamin A and emerlin illustrated interesting colocalization patterns, which illustrates diversity depending on the structure outlined by

lamin A. To understand the colocalization between these two proteins, I showed the percentage of colocalized volume of emerin with lamin A ( $2.84 \pm 0.05$ ) and Pearson's correlation coefficient, which are respectively ( $0.24 \pm 0.01$ ) (**Figure 50**). Compared to lamin A and emerin colocalization in AIPASMC nuclei, the percentage of colocalized volume was reduced in D7PASMC nuclei ( $p=0.795877$ ). In line with this, Pearson's correlation coefficient value in proliferating cells was lower than in AIPASMC nuclei ( $p=0.0175815$ ). Together, these results provide important insights regarding the lamin A and emerin colocalization in AIPASMC and D7PASMC, which results in decrease in colocalization of these two proteins in AIPASMC and D7PASMC. As mentioned above, it can be postulated that perhaps emerin and lamin A association may serve different functions in these cell types.

### **3.2.3 Nesprin puncta distributes across the periphery of the nucleus of proliferating pulmonary arterial myocytes cultured for 7 days.**

Following the exciting findings related to lamin A and emerin on D7PASMC nucleus, alterations in nesprin distribution might be expected due to the ability of nesprin to bind emerin and lamin A directly (Mislow et al., 2002). Moreover, recent studies have shown that the role of nesprin-1- $\alpha$  in nuclear processes is to control the nuclear architecture contributing to realize the correct nuclear function (Sur-Erdem et al., 2020). To assess the distribution and shape of nesprin in the D7PASMC nucleus, I investigated the deconvolved and 3D skin image of the nesprin labelled D7PASMC nucleus as the first step.

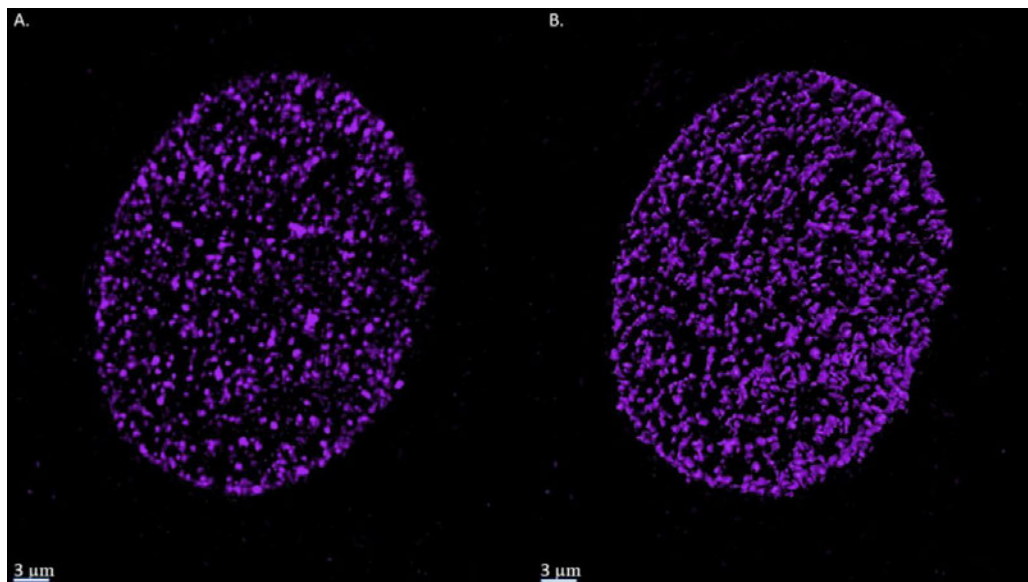


**Figure 51.** One example of nesprin labelling PASM nuclei cultured for 7 days were shown. A) A deconvolved confocal image for the D7PASM nuclei labelled for nesprin (purple) were demonstrated. B) Digital skin software was used to the 3D reconstructed the image in A) to better define the structures.

Initially, to understand nesprin distribution in the general view, I explored the whole nucleus of nesprin labelled D7PASM, illustrated by an oval and regular shaped. This was similar to the other important nuclear envelope proteins labelling for D7PASM, which was also shown in fibroblasts in the literature (e.g., Taranum et al., 2012) (**Figure 51.A**). In line with lamin A and emerin distribution in D7PASM nuclei, it had the plane and puncta shape, which was different from AIPASM nesprin labelling that was not located through the nucleus. However, there was a difference in the other NE proteins, which nesprin labelling was composed of puncta, instead of specific structures such as O-type or I-type structures (**Figure 51.A**). Then, I applied 3D skin to show the nucleus more distinctly, which appeared as a rough surface created by nesprin labelling (**Figure 51.B**). The interesting nesprin distribution on D7PASM nuclei was highlighted by 3D skin application. Then, I did a closer inspection of the interesting composition of the nesprin labelling by cutting it from a random place and magnifying this region. This surface created by nesprin labelling in D7PASM nuclei was also reported in literature (Duan, 2018).

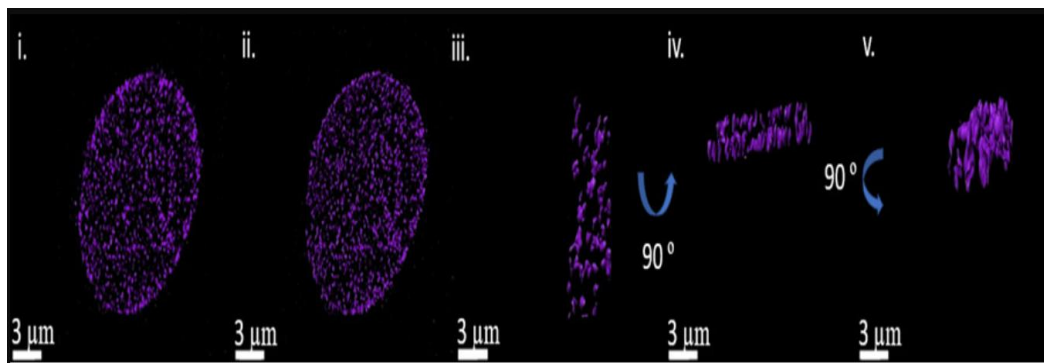


**Figure 52.** The outer structure on nesprin labelled 7 days cultured PASM nucleus is composed of puncta.

(i) A deconvolved Z-stack confocal images with nesprin labelling (**purple**) of a D7PASM nucleus were shown. (ii) 3D reconstructed nesprin labelling of a D7PASM nucleus cultured for 7 days (iii) cut from the marked area, showing puncta. (iv) This image was rotated 90° showing inside of nucleus and (iv) one more time rotated 90° anticlockwise (v).

In accordance with the previous cell, I observed the same appearance in the second D7PASM nucleus labelled by nesprin (**Figure 52 i**) and the 3D skin application highlighted the same distribution of nesprin labelling in cultured PASM nuclei for 7 days (**Figure 52 ii**). Since each structure may serve a function, I investigated this regular shaped nuclei closely by cutting it from random places. Intriguingly, I observed that the surface structures were created by small puncta, rather than definable forms were determined for the other proteins studied (**Figure 52 iii**). Besides, most of the puncta did not connect with each other, and they showed a uniform distribution across the D7PASM nucleus, instead of being dense in particular areas like AIPASM nucleus (**Figure 52 iii**). By turning the nesprin labelling nucleus 90° anticlockwise, I explored the interior view facing nucleoplasm, which might provide the connection point for lamin A (Mislow et al., 2002). It demonstrated a flat and pressed structure (**Figure 52 iv**) similar to the other specific nuclear envelope proteins in D7PASM nucleus images, albeit, composed of the puncta. Additionally, it was then necessary to investigate the other side of the piece of D7PASM labelled by nesprin to understand the distribution of nesprin via turning it 90° to the right, which was same as the previous view in Figure 52.iv (**Figure 52 v**).

Consequently, these findings suggested that the nesprin distribution pattern in the D7PASMNC was entirely different from lamin A and emerin. It was also evident that nesprin labelling was composed of uniform structures in contrast to lamin A and emerin labelling, which showed diversity in the structures that constituted of these protein labelling in the D7PASMNC nucleus. Based on these results, it is highly likely that nesprin dynamic in the D7PASMNC nucleus is completely different and seems to employ this function on lamin A and emerin. Therefore, examining the number of these puncta and the region for one punctum may aid to understand the nesprin distribution more clearly.

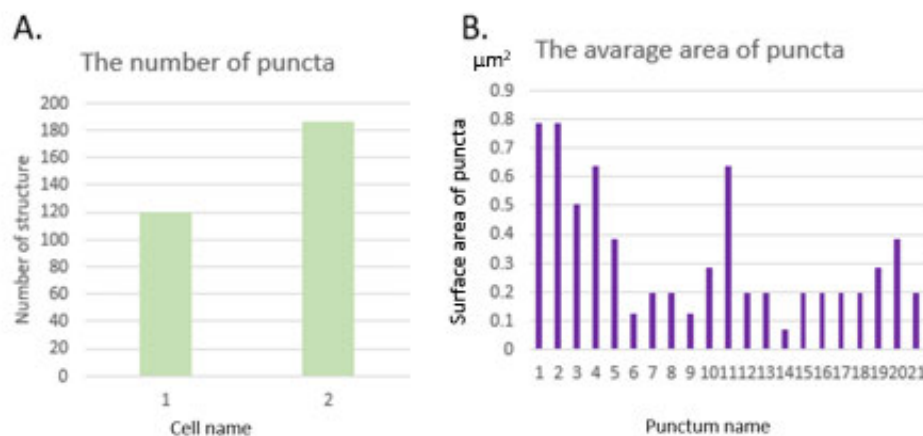


**Figure 53. Nesprin labelling surface is composed of puncta in different sizes.** A) The number of puncta (water green) on nesprin labelled day 7 PASMNCs (n=2), and B) on average puncta area (purple) stained by nesprin were shown (n=21).

For that, I counted the number of puncta, which revealed that the average number of puncta for each cell was  $153.5 \pm 33.5$  on the nesprin labelling D7PASMNC nucleus (n=2) (**Figure 53 A**). Furthermore, I assessed the areas of each puncta, which were defined as on average  $0.32 \pm 0.47 \mu\text{m}^2$  (n=21) (**Figure 53 B**). These puncta was selected randomly from cells. To eliminate nucleus volume differences, I calculated the ratio of puncta area to nucleus volume, which was  $0.01 \pm 0.002$  (Appendix, Table 14).

### 3.2.4 SUN-2 labelling stretches across the nucleus but concentrated in well-defined regions.

SUN 2, another key element of LINC complex, employs a bridge between the giant nesprin and lamin A, which mediates communication between cytoskeleton and nucleus. Therefore, the distribution and formation of SUN-2 were characterised after investigating the distribution of other NE proteins distribution on the surface of D7PAMSC, in which demonstrated that these proteins' localization affects each other (Chen et al., 2019). Therefore, I investigated the general distribution of SUN-2 and this investigation revealed the similarities with lamin A and emerin labelling on D7PAMSC nucleus.

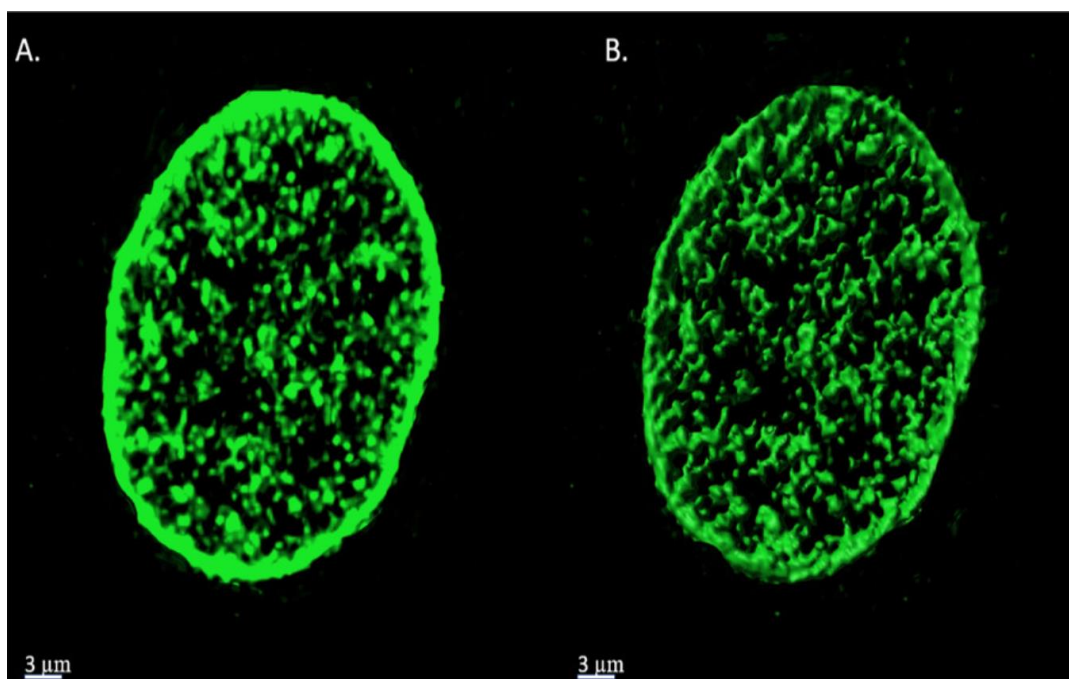


**Figure 54.** One example of a SUN-2 labelling D7PAMSC nucleus were shown. A) A deconvolved confocal images for the D7PAMSC nucleus was labelled for SUN-2 (**green**). B) Digital skin software was used on the 3D reconstructed image from A) to better define the structures.

To assess the distribution of SUN-2, I first investigated the deconvolved image that illustrated an uncovered, ovoid and smooth shaped of SUN-2 staining on the D7PAMSC nucleus (**Figure 54 A**). However, for SUN-2, I observed a scattered distribution which resembled to the fragments of the net-like structures observed for lamin A and emerin labelled D7PAMSC

nuclei. Following 3D skin application (**Figure 54 B**), it was more evident that SUN-2 concentrated in certain places, such as at the centre and periphery of nucleus labelled for SUN-2, may be selected randomly. Accordingly, the labelling looks like partial lamin A labelling, which means that similar structures exist on SUN-2 labelling in D7PASMNC nucleus to lamin A, but not in all parts of the nucleus surface: i.e. SUN-2 is present on some structures identified as emerin and lamin A positive, but not others. In contrast this, SUN-2 covered the nuclear periphery similar to lamin A and emerin in AIPASMNC. Therefore, SUN-2 may be located at particular regions and may be colocalised with lamin A in these regions. To address this possibility, I investigated these special structures on the SUN-2 labelled D7PASMNC nuclei, which may also be related to the different protein function.

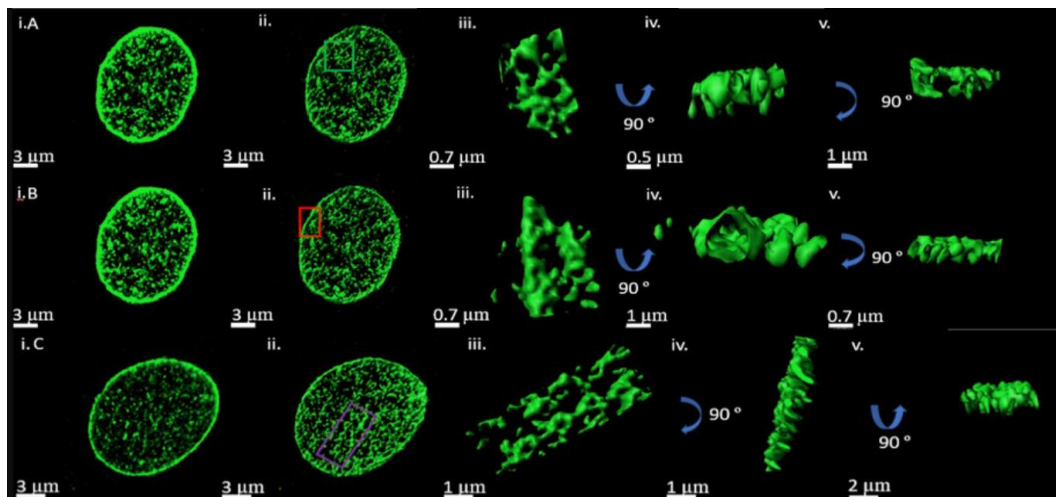


**Figure 55.** The most found structures on SUN-2 labelled D7PASMNC are O, I-type and globular structures.

A.(i) A deconvolved Z-stack confocal images was labelled for SUN-2 (**green**) in a D7PASMNC nucleus. (ii) 3D reconstructed SUN-2 labelling of a D7PASMNC nucleus cultured for 7 days (iii) cut from the marked area showing O-type structure. (iv) Image was rotated 90° showing inside of nucleus and (v) one more time rotated 90° downwards.

B. As in (A), but I-type structures were illustrated.

C. As in (A), but globular structures were illustrated.

To classify the structures that might fulfil different tasks, I cut the regions accounting for these structures, and then magnified them. On the surface

of the nuclei, various structures were depicted by SUN-2 staining showed by deconvolved images (**Figure 55 A & B & C.i**) and 3D skin applied images (**Figure 55 A & B & C.ii**). In the literature on SUN-2 labelled nucleus, various uniformity was reported in fibroblasts nuclei, which is very parallel to my results on PSMCs (Hoffman et al., 2020). I identified three types of structures on the D7PSMC nucleus. Similar to lamin A and emerin, the most apparent structures revealed by SUN-2 labelling were O and I-type structures. The O-type structure was rounded and symmetrical in shape, as obtained by a cut and magnified from the area marked by a green box (**Figure 55 A.iii**). To show the interior facing lamin A of SUN-2 labelling that offers anchoring point to lamin A, I turned the structure 90° to view it from different angles. The structure's horizontal and back view acquired by rotating it 90° one more time was also similar to lamin A and emerin day 7 labelled nuclei, as it was flat, pressed, and thin (**Figure 55 A.iv and A.v**). By using the same technique, I defined the I-type structures, which were long and thin placed mostly at the periphery of the nucleus outlined by SUN-2 (**Figure 55 B.iii**). Then, I investigated the interior face due to the same reason with O-type structure, illustrating the mirror image of O-types' after being turned 90° (**Figure 55 B.iv**). The interior face was flat and similar to the upper face (**Figure 55 B.iv**). Lastly, the globular shaped structure was identified as a chain composed of nodules binding to each other (**Figure 55 C.iii**). The other sides of this interesting structure were obtained by turning it 90°. It was observed that they were flat and thin (**Figure 55 C.iv&v**), which was very similar to the other structures defined by SUN-2.

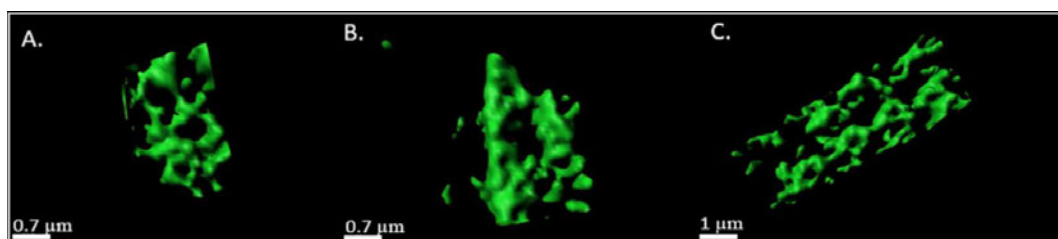


**Figure 56.** Three types of structures were observed on SUN-2 labelled D7PSMC nucleus, which were A) rounded shape, B) I-type and C) globular structures.

As a result, I have determined 3 types of structures on SUN-2 labelling (**Figure 56**). The first two of them were similar to the structures described on emerin labelling, but I have not detected the last structure, globular structure, on the other proteins' labelling studied (**Figure 56.C**). Therefore, these two structures may be related to the other important nuclear envelope proteins in terms of realizing their function. Moreover, since the distribution of SUN-2 is dependent on lamin A, it may tether at these structures (Liang et al., 2011b). Additionally, SUN-2 appears to label all categories of structures identified by lamin A and emerin labelling. However, it does not label these structures uniformly, which could mean that another as yet unidentified binding partner is required to couple SUN-2 to emerin/lamin A.

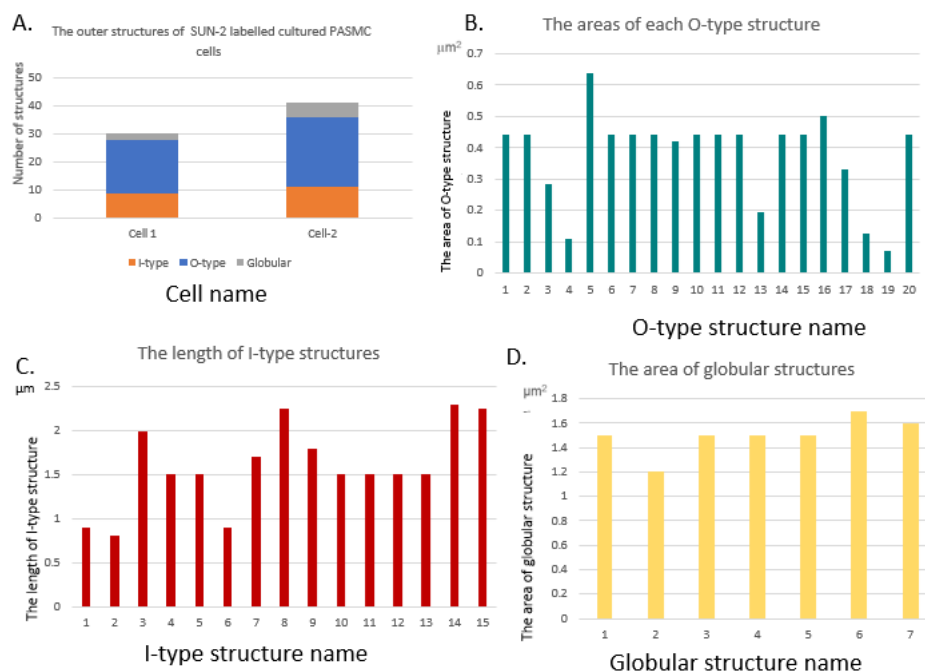


**Figure 57. O-type structure was most frequently found structure on SUN-2 labelled D7PASM C.** A) The bar chart shows the number of the three different structures, which are I-type (orange), O-type (blue) and globular (grey) structures on SUN-2 labelled day 7 PASM C nuclei, (n=2). B) On average, O-type area (green), n=20, C) the length of I-type (red), (n=15) and C) average area of globular structure (yellow) are differed by different colours, (n=7).

For the detailed investigation on SUN-2 labelled D7PASM C nucleus, the structure number and sizes were analysed. As shown in Figure 57.A, the O-type structure occurred frequently on the D7PASM C nucleus illustrated

with SUN-2 labelling, with  $22 \pm 3$  on average,  $n=2$ . Conversely, the globular type structure was considerably less than the other outer structures with a mean of  $3.5 \pm 1.5$ ,  $n=2$ . The I-type structure was relatively frequent, with only  $10 \pm 1$ ,  $n=2$  occurred in each SUN-2 labelled D7PASM nucleus (**Figure 57 A**). The values belonging to these structures are significantly different from each other ( $p=0.0165777$ ).

The sizes of these structures in the D7PASM nucleus could provide insights into the complex process. Thus, looking at the measurements of the outer structures on nucleus labelling for SUN-2, the O-type structure areas were determined as  $0.376 \pm 0.03 \mu\text{m}^2$ ,  $n=20$  (**Figure 57 B**) and the length of the I-type structures was measured as an average of  $1.59 \pm 0.12 \mu\text{m}$ ,  $n=15$  (**Figure 57 C**). Lastly, globular structures were examined as  $1.5 \pm 0.12 \mu\text{m}^2$ ,  $n=7$ , which shows that the area of the globular structure occupied was larger than O-type (**Figure 57 D**). The structures to measure was selected randomly from cells. To eliminate nuclear differences, I calculated the ratio of dimension or area to nucleus volume. For O-type, I-type and globular type structures, they are  $0.004 \pm 0.0003$ ,  $0.02 \pm 0.001$  and  $0.02 \pm 0.001$  respectively (Appendix, Table 13). Since the lamin A and SUN-2 association regulate diverse functions in nucleus (Matsumoto et al., 2015b) and the N-terminal domain of SUN-2 has affinity to bind with lamin A (Haque et al., 2010a), gaining an understanding of how the structures defined by SUN-2 on lamin A labelling positioned might be important. Therefore, I investigated co-labelled lamin A and SUN-2 D7PASM nucleus, which might be selected as the co-association of binding partners.

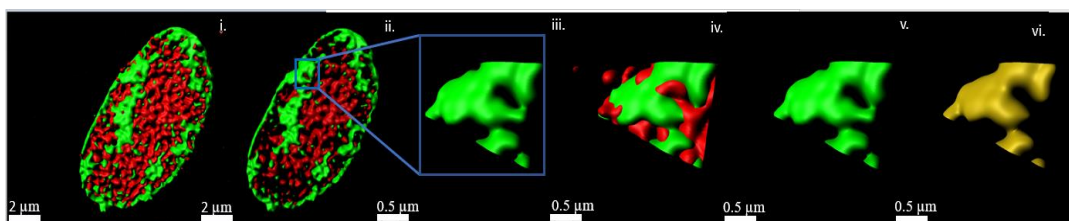


**Figure 58. Lamin A exists on the different types of structures labelled by SUN-2.**

- A) (i) The low and (ii) the high threshold 3D application of co-labelled SUN-2 (**green**) with lamin A (**red**) (iii) cut from the marked area by blue box and magnified to show O-type structure labelled by SUN-2. (iv) The SUN-2 labelled O-type structure co-labelled with lamin A, (v) with colocalization channel (**yellow**) and (vi) only colocalization channel is shown.

After determining surface structures on SUN-2 labelling, I inspected the co-labelling D7PASMNC nucleus for SUN-2 and lamin A. Given the fact that SUN-2 and lamin A association serve as a bridge between cytoskeleton and nucleus, the distribution of these two proteins may affect cellular process (Liang et al., 2011a). Using a low threshold to indicate whole staining belonging to lamin A and SUN-2 association, I observed that SUN-2 was localised in specific areas of the periphery of lamin A labelled D7PASMNC nucleus (**Figure 58 i**). Then, I tested the high threshold application for the same D7PASMNC nucleus, which showed lamin A and SUN-2 as accumulated together in highly defined regions but not in other regions. This further supports my proposal that SUN-2 was present on all types of the specific structures formed by lamin A labelling of D7PASMNC nucleus, but did not co-label all of these specific structures (**Figure 58 ii**). Subsequently, I cut the structure defined on D7PASMNC nucleus labelled by SUN-2 and magnified it to show this structure better (**Figure 58 iii**). To understand the colocalization lamin A and SUN-2, this structure was colocalized with lamin A, which showed that lamin A did not formed as O-type structure in that structure created by SUN-2 labelling (**Figure 58 iv**). It is different from this structure labelled by emerin. Therefore, I investigated the colocalization channel and SUN-2, which demonstrated that SUN-2 covered this structure outlined by lamin A (**Figure 58 v**). As the last part, I illustrated the colocalization solely, which demonstrated that the O-type structure was shaped by both lamin A and SUN-2 (**Figure 58 vi**). Therefore, the difference between emerin labelled and SUN-2 labelled O-type structures may suggest that these proteins have different functions.

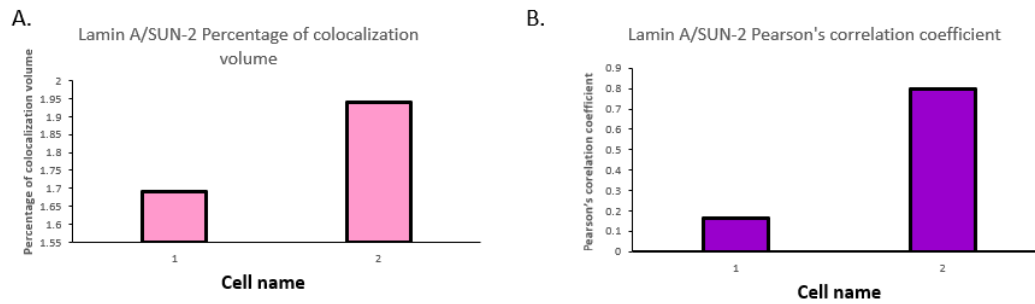


**Figure 59. Lamin A and SUN-2 colocalization on D7PASMNC nuclei shows diversity from one cell to another.**

- A) Percentage of volume lamin A colocalization (**pink**) with SUN-2 in D7PASMNCs nuclei is illustrated in a bar chart. Each bar shows a cell (n=2, mean  $\pm$  SEM).
- B) Lamin A and SUN-2 colocalization (**purple**) is shown in a bar chart, by using Pearson's correlation coefficient. Each bar shows a cell (n=2, mean  $\pm$  SEM)

To understand the colocalization between lamin A and SUN-2 statistically, I showed the percentages of the colocalized volume of SUN-2 with lamin A and Pearson's correlation coefficient for lamin A and SUN-2, which are  $1.81 \pm 0.08$  and  $0.4819 \pm 0.2247$  respectively, as shown in Figure 59.

**A. The number of structures found on D7PASMNC nucleus labelled by proteins**

| Proteins      | O-type structure | I-type structure | Puncta           | Globular structure | Irregular rounded structure |
|---------------|------------------|------------------|------------------|--------------------|-----------------------------|
| Lamin A (n=4) | 24.75 $\pm$ 2.21 | 6.75 $\pm$ 0.85  | 0                | 0                  | 5.25 $\pm$ 1.97             |
| Emerin (n=5)  | 83 $\pm$ 18.84   | 32.8 $\pm$ 11.41 | 0                | 0                  | 0                           |
| Nesprin (n=2) | 0                | 0                | 153.5 $\pm$ 33.5 | 0                  | 0                           |
| SUN-2 (n=2)   | 22 $\pm$ 3       | 10 $\pm$ 1       | 0                | 3.5 $\pm$ 1.5      | 0                           |

**B. Dimensions of structures detected in D7PASMNC nucleus labelled by different proteins**

| Proteins | Area of O-type structure ( $\mu\text{m}^2$ ) | Area of globular/puncta/irregular rounded shaped structure ( $\mu\text{m}^2$ ) | Length of I-type structure ( $\mu\text{m}$ ) |
|----------|----------------------------------------------|--------------------------------------------------------------------------------|----------------------------------------------|
| Lamin A  | 0.21 $\pm$ 0.02 (n=25)                       | 0.68 $\pm$ 0.13 (n=25)                                                         | 2.04 $\pm$ 0.11 (n=25)                       |
| Emerin   | 0.312 $\pm$ 0.04 (n=26)                      | ---                                                                            | 2.40 $\pm$ 0.12 (n=70)                       |
| Nesprin  | ---                                          | 0.322 $\pm$ 0.47 (n=21)                                                        | ---                                          |
| SUN-2    | 0.376 $\pm$ 0.032 (n=20)                     | 1.5 $\pm$ 0.12 (n=7)                                                           | 1.59 $\pm$ 0.12 (n=15)                       |

**C. Colocalized proteins with lamin A**

| Colocalized proteins with lamin A | Pearson's correlation coefficient | Percentage of colocalization volume |
|-----------------------------------|-----------------------------------|-------------------------------------|
| Emerin                            | 0.2412 $\pm$ 0.01503              | 2.84 $\pm$ 0.495                    |
| SUN-2                             | 0.4819 $\pm$ 0.2247               | 1.815 $\pm$ 0.0884                  |

**Table 4. The table shows the comparison between the structure numbers, sizes and colocalizations for proteins on D7PASMNC nuclei . A) The structure distribution on four different proteins including lamin A, emerin, nesprin and SUN-2 on D7PASMNC nuclei, B) the dimensions of the structures defined by these four proteins and lastly, C) the colocalization volume and Pearson's correlation coefficient of emerin, nesprin as well as SUN-2 and lamin A are shown.**

In summary, these results suggested that all studied proteins excluding nesprin show similar patterns of distribution across the nuclear envelope and include similar structures on the surface D7PASMNC labelled by these three proteins. With regards to the distribution of these structures, O-type structures was reported mostly on emerin labelled D7PASMNC nucleus with  $83 \pm 18.84$ , compared to the other protein labelled nuclei and significantly different from others statistically ( $p=0.0196254$ , one-way ANOVA, followed by Tukey HSD) (**Table 4 A**). However, the least value was observed on D7PASMNC nucleus labelled by SUN-2 with  $22 \pm 3$  (**Table 4 A**). The other most common structure among proteins labelled D7PASMNC nuclei was I-type structure, which was  $32.8 \pm 11.41$  on emerin labelled D7PASMNC nuclei and the value related to these structures was significantly different from each other ( $p=0.00313920$ , one-way ANOVA following by Tukey HSD test) (**Table 4 A**). In addition to these, some structures observed were special for only one protein, which were globular structure on SUN-2, irregular rounded structure on lamin A and puncta on nesprin labelled D7PASMNC nucleus with  $3.5 \pm 1.5$ ,  $5.25 \pm 1.97$  and  $3.5 \pm 1.5$  respectively (**Table 4 A**).

As the possibility exists that these structures may provide different functions, I showed the regions occupied by these structures (**Table 4 B**). However, as the structures were obtained from different cells in size, the comparison between the measurements for each structure does not seem to be possible.

Lastly, I presented colocalization of emerin as well as SUN-2 and lamin A on D7PASMNC nuclei. In doing so, I suggest that the percentage of the volume of emerin colocalized with lamin A co-labelled D7PASMNC nucleus was higher than the percentage volume of SUN-2 colocalized with lamin A in D7PASMNC nuclei with were  $2.84 \pm 0.49$  and  $1.81 \pm 0.80$  respectively, which is statistically significant ( $p=0.00962717$ ). Moreover, the assessment of Pearson's correlation coefficient for these two types labelling pattern shows similar results to the percentage of colocalized volume, as emerin/lamin A is higher than SUN-2/lamin A ( $p=0.397090$ ), (**Table 4.C**).

### 3.3 The outer structures on specific nuclear envelope proteins are colocalised with histone marks and BAF puncta.

#### 3.3.1.1 H3K9me2 puncta attached at various points in the different structures on lamin A labelled nuclei in AIPASMC.

I have described three different ridges on AIPASMC labelled by lamin A. This led to an alternative proposal that these ridges might offer attachment points for chromatin through LADs (lamin-associated domains), coincident with the well-known fact that Lamin A presents association sites for chromatin domains and modulatory proteins (Poleshko et al., 2019). I thought of investigating whether lamin A sites associating with H3K9me2 on these structure defined, which is important for transcriptional silencing (Shevelyov & Ulianov, 2019). The investigation could be important to provide an understanding of the role of lamin A in transcriptional regulation.

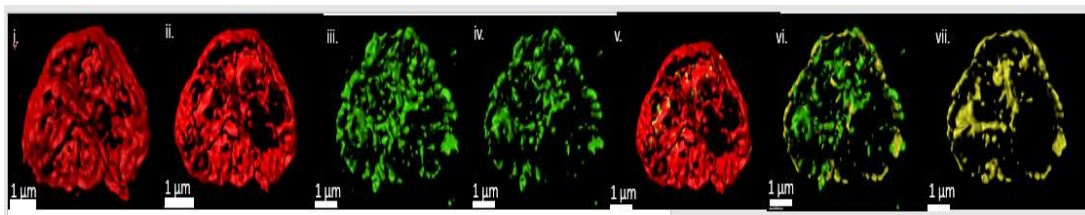


**Figure 60.** The distribution of lamin A and H3K9me2 in AIPASMC nucleus was shown. i) A deconvolved Z-stacks of confocal images with lamin A were applied a low and ii) a high threshold 3D skin. iii) A deconvolved Z-stacks of confocal images with H3K9me2 were applied a low and iv) a high threshold 3D skin, additionally v) lamin A and colocalization channel for lamin A and H3K9me2 in high threshold was shown. vi) H3K9me2 and colocalization channel for lamin A and H3K9me2 in high threshold, and lastly vii) colocalization channel only with 3D skin application were illustrated.

Firstly, I identified the lamin A labelled AIPASMC nucleus surface characteristics described as in section 3.1.1 (**Figure 60 i&ii**). Then, I defined the H3K9me2 staining in the AIPASMC nucleus through a low threshold application, which showed that the H3K9me2 existed on only defined structure (**Figure 60 iii**). Then, I applied the high threshold for the H3K9me2 staining, which seemed to be composed of puncta or small structures (**Figure 60 iv**). Then, colocalization channel for lamin A and H3K9me2 were showed with lamin A labelling. To explore where H3K9me2

was located in lamin A distribution, this image showed the colocalization puncta through the lamin A labelling (**Figure 60 v**). The colocalization regions resided at some places on H3K9me2 which may have specific function (**Figure 60 vi**). Lastly, I demonstrated only the colocalization channel (**Figure 60 vii**), which underlined the colocalized areas. As a result of this, it was clear that H3K9me2 was located in the lamin A labelled important region identified in section 3.1.1. The findings correlate with the previous publications investigating lamin A-H3K9me2 distribution in AIPASMC nucleus (Duan, 2018). Following this, I anticipated that H3K9me2 might bind each ridge type created by lamin A differently, depending on their functional specifications.

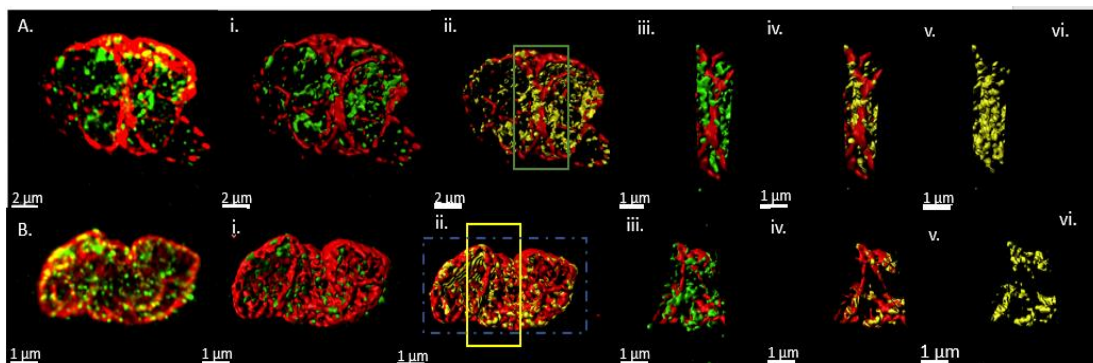


**Figure 61.** The histone mark, H3K9me2, located in the ridges through lamin A labelling AIPASMC nuclei.

A) (i) A deconvolved Z-stacks of confocal images of lamin A (**red**) labelling with H3K9me2 (**green**) of a AIPASMC nucleus was demonstrated with (ii) 3D reconstruction and (iii) the 3D reconstruction of lamin A with the colocalization channel (**yellow**) for lamin A and H3K9me2 was shown. (iv) The I-shaped ridge was cut from the marked area, showing lamin A with H3K9me2 labelling and (v) with colocalization channel. (vi) Only, the colocalization channel was finally shown.

B) (i) A deconvolved Z-stacks of confocal images of lamin A (**red**) labelling with H3K9me2 (**green**) of a AIPASMC nucleus was demonstrated with (ii) 3D reconstruction and (iii) the 3D reconstruction of lamin A with the colocalization channel for lamin A and H3K9me2 was shown. (iv) The V-shaped ridge was cut from the marked area and sliced through the middle of nucleus to show more clearly, illustrating lamin A with H3K9me2 labelling and with (v) colocalization channel. (vi) Only the colocalization channel was finally shown.

To test my expectation, I, initially, investigated the AIPASMC nucleus co-labelled by lamin A and H3K9me2, suggesting that the distribution of H3K9me2 puncta was different for each types of ridges labelled by lamin A (**Figure 61 A.i&ii**). The image of lamin A and colocalization channel showed that lamin A and H3K9me2 colocalization were spread through the

nucleus, however the puncta belonging to the colocalization channel for lamin A and H3K9me2 seem to gather across the ridge (**Figure 61 A.iii**). In the image obtained by cut from the marked area, it was clearly demonstrated that H3K9me2 puncta were located in the middle of I-type ridge (**Figure 61 A.iv**). Then, I showed the lamin A and colocalization channel through the ridge to illustrate where colocalization puncta are found on the lamin A labelled ridge (**Figure 61 A.v**), affirming the H3K9me2 localization in the I-type ridge. Finally, I emphasised the colocalization pattern by showing only colocalization channel (**Figure 61 A.vi**). The colocalization puncta seemed to merge or be located very closely to each other, as it appeared in the middle of the ridge.

Another AIPASMC nucleus was explored to show distribution of H3K9me2 and lamin A. It was very similar to the previous nucleus, but had a different kind of ridge (**Figure 61 B.i&ii&iii**). The image Figure 61 B.iii was obtained by slicing through the nucleus vertically from the marked area to show the specific ridge. In contrast to I-type ridge, V-type ridge illustrated different phenomena. Accordingly, H3K9me2 puncta on V-type lamin labelled ridge were located at particular points on the ridge, which suggesting that H3K9me2 is presented at the attachment points of the ridge along with the nucleus intensely (**Figure 61 B.iv**). Then, I showed the colocalization channel for lamin A and H3K9me2 with lamin A labelling and viewed the colocalization channel located in the ridges' attachment points to present a clearer view for lamin A and H3K9me2 colocalization (**Figure 61 B.v**). Lastly, I showed only the colocalization channel to highlight colocalization between them, which illustrated the colocalization puncta localization more clearly (**Figure 61 B.vi**).

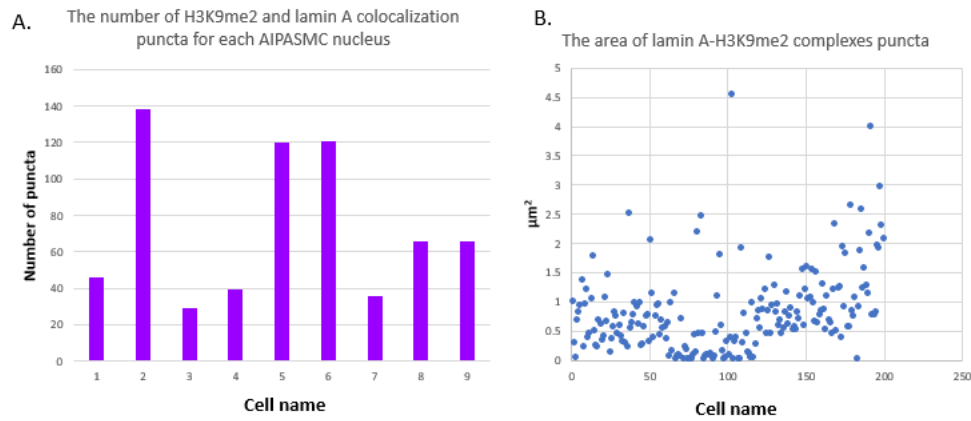


**Figure 62. The number of H3K9me2-lamin A puncta and areas of these puncta showed variety on co-labelled lamin A and H3K9me2 AIPASMC nucleus.** A) The number of puncta (purple) that represents lamin A and H3K9me2 colocalization puncta on H3K9me2-lamin A co-labelling nucleus was showed, and B) on average area of puncta (blue) were illustrated.

Due to the nuclear architecture effects on the chromatin localization and the cells capability to independently modulate such interaction, H3K9me2 and lamin A colocalization puncta may provide information about the regulatory mechanisms of chromatin activation (Poleshko et al., 2019). For each co-labelled AIPASMC nucleus, the number of colocalization puncta created by the colocalization of lamin A with H3K9me2 was  $73.44 \pm 13.95$  in each cell,  $n=9$ . The distribution of colocalization puncta varied from cell to cell (**Figure 62 A**). Strikingly, the areas of the dots representing colocalization of lamin A with H3K9me2 was  $0.79 \pm 0.04 \mu\text{m}^2$ , and the diameters ranged from  $6.44 \mu\text{m}^2 \sim 4.54 \mu\text{m}^2$  (**Figure 62 B**). In literature, the size of lamin filaments is composed of 10 nm (Turgay & Medalia, 2017). Moreover, the total area of puncta for each cell varied between 58,50 and  $27,47 \mu\text{m}^2$ . Therefore, the total square microns of puncta on a per cell basis was not similar. A possible explanation for this might concern the resolution of confocal microscopy used in this study due to its ability to catch reflectance imaging (Vangindertael et al., 2018).

### 3.3.1.2 H3K9me2 and lamin A colocalization puncta were spread evenly through the D7PASCN nuclei

Since the lamin A labelling on the AIPASCN and D7PASCN nucleus illustrated different patterns, I projected that the H3K9me2 and lamin A colocalization in D7PASCN pattern that might be different from AIPASCN nucleus.

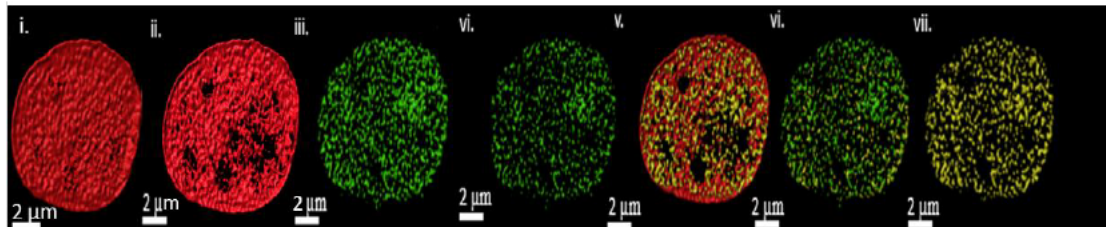


**Figure 63.** The distribution of lamin A and H3K9me2 in D7PASCN nucleus were shown. i) A deconvolved Z-stacks of confocal images with lamin A were applied a low and ii) a high threshold 3D skin. iii) A deconvolved Z-stacks of confocal images with H3K9me2 were applied a low and iv) a high threshold 3D skin. v) The colocalization channel for lamin A and H3K9me2 were shown with lamin A in high threshold, vi) with H3K9me2 and lastly, vii) only colocalization channel with 3D skin application.

To examine the possibility, I compared the lamin A labelled D7PASCN at low and high threshold application, as identified in section 3.2.1 (**Figure 63 i & ii**). Then, I investigated the H3K9me2 labelling on the D7PASCN, which showed that this labelling was composed of puncta. However, these puncta were not accumulated in the specific areas (**Figure 63 iii**), as observed by using low threshold. Then, I applied a high threshold for H3K9me2 labelling which showed the H3K9me2 puncta spread over the nucleus (**Figure 63 iv**). Subsequently, the colocalization channel and lamin A proved that H3K9me2 staining dispersed across the lamin A labelling (**Figure 63 v**). Then, I demonstrated the colocalization channel with H3K9me2, which revealed that in the colocalization puncta covering the H3K9me2 labelling, and there were some H3K9me2 puncta that was not colocalized with lamin A (**Figure 63 vi**). Lastly, only the colocalization channel was shown to highlight the puncta's distribution through the D7PASCN nucleus (**Figure 63 vii**).

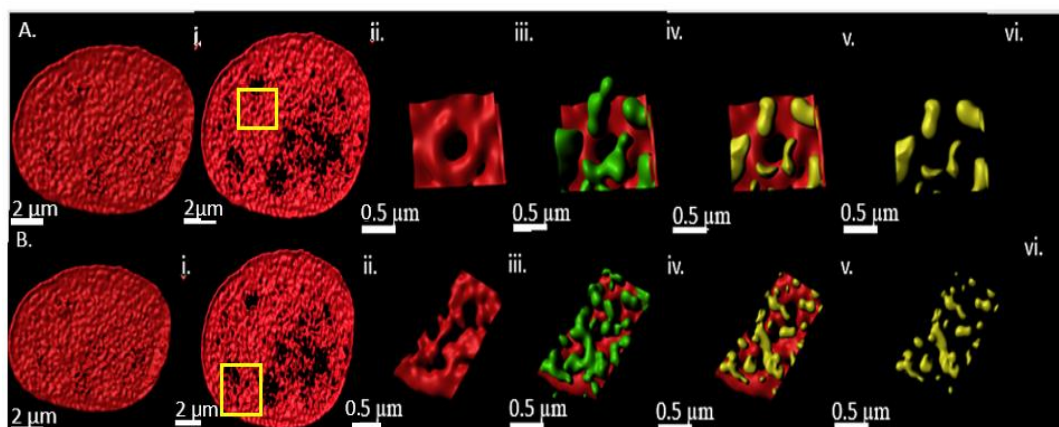


**Figure 64. Locations where lamin A colocalization with H3K9me2 were detected are distinct on the D7PASMC nucleus from the AIPASMC nucleus.**

A) (i) A deconvolved Z-stacks of confocal images of lamin A (**red**) were applied with 3D skin at a low and (ii) high threshold. (iii) The O-type structure was cut from the marked area and magnified, (iv) shown with H3K9me2 labelling (**green**) in D7PASMC nucleus and (v) colocalization channel (yellow) for H3K9me2 and lamin A. (vi) Lastly, only the colocalization channel was shown.

B) As in (A), but I-shaped structure was shown.

Due to the possibility of the specific function of the structures detected on lamin A in D7PASMC, further investigation into lamin A labelled structures with H3K9me2 puncta was required. To observe them, I, first, showed the lamin A distribution on D7PASMC nuclei and lamin A labelled O-type structures, as described in section 3.2.1 (**Figure 64 A.i & ii & iii**). Then, the localisation of the H3K9me2 puncta on the lamin A labelling showed that the H3K9me2 did not fill into the lamin A labelled O-type structure and it presented just little patches in shaped as associated with lamin A (**Figure 64 A.iv**). Adding to this, the lamin A and H3K9me2 colocalization was demonstrated with lamin A labelling to make the colocalization regions on the lamin A labelled O-type structure clear. It was evident then that the colocalization puncta located at the tiny region at the edge of the lamin A labelled O-type structure (**Figure 64 A.v**). Then, I showed the colocalization puncta solely to emphasize the colocalization regions. This investigation illustrated that it included many puncta (**Figure 64 A.vi**).

The other structure defined by lamin A labelling for D7PASMC nucleus, I-type structure, was investigated to understand if there was any difference

among the structures identified on lamin A labelled D7PASMNC nucleus in terms of H3K9me2 association. To clarify that, I demonstrated the I-type structure as defined in section 3.2.1 (**Figure 64 B.iii**). With regards to the H3K9me2 colocalization points on lamin A explanation, I colocalised the lamin A labelling and H3K9me2, which showed the H3K9me2 staining presented partially in the I-type structure (**Figure 64 B.iv**). The region at which lamin A and H3K9me2 colocalised, represented similar view of the co-labelled image for H3K9me2 and lamin A (**Figure 64 B.v**). Then, the colocalization channel was shown solely, which suggested that the H3K9me2 was represented at the edge of the I-type structure (**Figure 64 B.vi**).

Consequently, lamin A and H3K9me2 colocalization regions showed different patterns on the lamin A labelled structures for D7PASMNC nucleus, due to lamin A distribution in proliferative nuclei. Considering that, this can be postulated that H3K9me2 and lamin A association in D7PASMNC may have different functions from AIPASMNC structures. Moreover, I did not observe any nuclear invaginations through the nucleus in D7PASMNCs labelled by lamin A. This results might be caused by the alterations on the lamin A distribution and epitope masking. Since physical interaction of the lamin A and chromatin might cause epitope masking in tail and rod of lamin A, it is also possible for lamin A and H3K9me2 association (Dyer et al., 1997). However, in previous studies on H3K9me2 and lamin A association, it has been showed tiny number of invagination, likely 1-2 invagination per cell in small number of cell, in proliferating nuclei but chromatin and lamin A association was not observed on these structures and these invaginations suggested that may not be responsible for gene regulation (Duan, 2018).

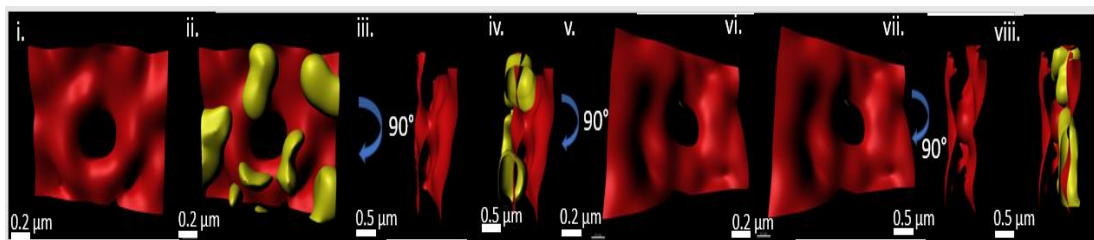


**Figure 65.** H3K9me2 clusters around the raised surface of the O-type structure are formed by lamin A around the central hole. i) The O-type structure was illustrated with (ii) colocalization channel. (iii) To investigate from different angles, the structure with only lamin A labelling was turned by 90° (iv) and was shown with colocalization channel. Once more time the structure was turned 90° and showed with (v) and without (vi) colocalization channel. Lastly, the another side obtained by image rotation 90° (vii) without and (viii) with colocalization channel was shown.

One of the structures investigated was O-type, which showed interesting colocalization with H3K9me2. It seems that H3K9me2 clusters around the raised surface of the O-type structure formed by lamin A around the central hole. This appears to indicate that the surface shape of lamin A guides H3K9me2 binding. Therefore, I carried out a closer investigation of O-type structure through higher magnification versions of the O-type structure and colocalization channel with lamin A labelling at different angles (**Figure 65**). Initially, I showed the O-type structure labelled by lamin A, which displayed characteristics as described in the previous figure (**Figure 65i&64.iii**). Following that, I conducted an investigation of the colocalization channel and lamin A labelling in order to determine where colocalization puncta located, revealed that colocalization puncta were located around the hole (**Figure 65.ii**). The image without colocalization channel was turned 90° to view it from another side of O-type structure. This demonstrated the flattened view of the O-type structure (**Figure 65.iii**). For the purpose of getting the illustration of lamin A and H3K9me2 localization, the same side after the rotation of it 90° was shown with and without colocalization channel. This process demonstrated that the channel the colocalization puncta sit in the lamin A labelling (**Figure 65.iv**), which may be caused for being at the periphery of the H3K9me2 location (Poleshko et al., 2019) and H3K9me2 might be retained by lamin A in D7PASM. Moreover, the inside view also supported the results obtained by the image rotation with and without colocalization (**Figure 65.v&vi**). Lastly, the image illustration obtained through turning it one more step showed that H3K9me2 and lamin

A seemed like intertwined (**Figure 65.vii**). However, there are some possibilities which may cause the results such as epitope masking, antibody accessibility and some drawbacks of the microscopy used as explained in detail in section 3.1.1.

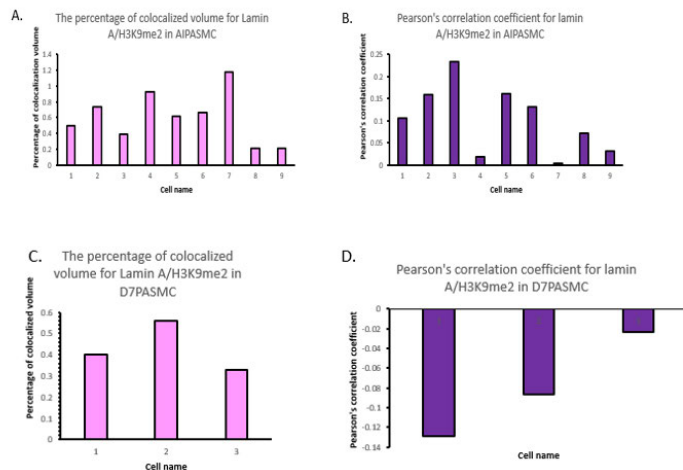


**Figure 66. Lamin A and H3K9me2 colocalization decreased in D7PASMC nucleus.**

- A.** The percentage of volume lamin A colocalization (**pink**) with H3K9me2 in AIPASMCs nuclei is illustrated in the bar charts. Each bar shows a cell ( $n=9$ , mean  $\pm$  SEM).
- B.** Lamin A and H3K9me2 colocalization (**purple**) in AIPASMC is shown in the bar chart, by using Pearson's correlation coefficient. Each bar shows a cell ( $n=9$ , mean  $\pm$  SEM)
- C.** The percentage of volume lamin A colocalization (**pink**) with H3K9me2 in D7PASMCs nuclei is illustrated in bar charts. Each bar shows a cell ( $n=3$ , mean  $\pm$  SEM).
- D.** Lamin A and H3K9me2 colocalization (**purple**) in D7PASMC is shown in the bar chart, by using Pearson's correlation coefficient. Each bar shows a cell ( $n=3$ , mean  $\pm$  SEM)

To explain the colocalization differences between AIPASMC and D7PASMC, I, initially, showed the percentage of colocalized volume of H3K9me2 with lamin A and Pearson's correlation coefficient, which are  $0.60 \pm 0.10$  and  $0.10 \pm 0.02$  respectively (**Figure 66 A&B**). After acquiring the colocalization results in AIPASMC for lamin A and H3K9me2, I calculated them in D7PASMC nuclei that revealed the percentage of colocalized volume of H3K9me2 with lamin A and Pearson's correlation coefficient as  $0.43 \pm 0.05$  and  $-0.08 \pm 0.02$  respectively (**Figure 66 C&D**). As highlighted in the table 6, the percentage of both colocalized volume decreased

significantly ( $p=0.0175063$ ), while it declined in Pearson's correlation, which was also reported in D7PASMNC nuclei ( $p= 0.00250614$ ).

| Cell types    | Percentage of colocalized volume                                                                  | Pearson's colocalization coefficient                                                                 |
|---------------|---------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
| AIPASMC (n=9) | $0.60 \pm 0.10$                                                                                   | $0.10 \pm 0.02$                                                                                      |
| D7PASMC (n=3) | $0.43 \pm 0.05$  | $-0.08 \pm 0.02$  |

**Table 5.** The table showed the comparison of colocalization for lamin A and H3K9me2 on AIPASMC and D7PASMC nuclei. The colocalization volume and Pearson's correlation coefficient of lamin A and H3K9me2 are illustrated.

Consequently, the results in this chapter report on lamin A and H3K9me2 association in acutely isolated and for 7 days cultured PASMNC nuclei. It is evident that there is a remarkable change between lamin A labelling in these two types of cells. Accordingly, the association of lamin A and H3K9me2 is affected by this change, and H3K9me2 is spread more evenly in D7PASMNC than AIPASMC. Specifically, since the structures detected on AIPASMC disappeared on D7PASMNC labelled by lamin A, H3K9me2 association changed from bringing together particular regions to spreading though nucleus, which are emphasised by the colocalization channel on lamin A labelling. Significantly, the colocalization between them changed after 7 days of cultured application, which may relate to changes in gene expression. The next section, therefore, moves on to discuss another important protein, emerin, that binds BAF and their association.

### 3.3.2.1 BAF protein resides at particular points through the structures defined on emerin labelled acutely isolated nucleus.

As indicated above, I have determined significant changes between acutely isolated to 7 days cultured pulmonary artery smooth muscle cell nuclei. Moreover, it has been demonstrated above that the alterations observed were similar for lamin A and emerin labelled D7PASMNC nuclei. This led to an alternative proposal that emerin-BAF interaction might illustrate differences in AIPASMC and D7PASMNC nuclei, as the association of emerin and BAF acts like a bridge between DNA and nuclear envelope proteins, such as emerin and lamin A (Samson et al., 2018). Moreover, the

changes in emerin and BAF association are important in that they might be strategically positioned to coordinate alterations in gene expression. Eventually, I evaluated the BAF-emerin colocalization in both acutely isolated and day 7 PASMNC nucleus due to these reasons mentioned above.

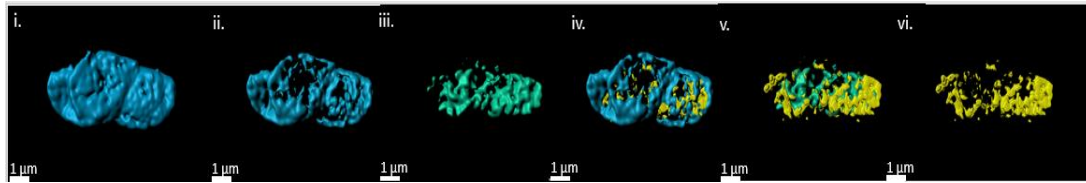


**Figure 67.** *The distribution of emerin and BAF in AIPASMC nucleus was shown. i) A deconvolved Z-stacks of confocal images with emerin (cyan) were applied a low and ii) a high threshold 3D skin. iii) A deconvolved Z-stacks of confocal images with BAF (pistachio green) was applied with 3D skin and iv) the co-localization channel with emerin and v) BAF. Lastly, vi) the only colocalization channel was shown.*

The nature of emerin distribution in AIPASMC nuclei was described in section 3.1.2 (**Figure 67 i & ii**). The distribution of BAF that could bind to emerin was observed in the middle of the nuclei in AIPASMC (**Figure 67 iii**). In a study exploring the association between BAF and emerin, it has been shown that BAF was localized in the core region of cells during telophase in HeLa cells (Haraguchi et al., 2001). Therefore, the assembly might be related to the cell cycle phase. Then, emerin and colocalization channel was showed in a view of emerin and BAF colocalization, which suggests that the colocalization regions spread through the nuclei (**Figure 67.iv**). Then, the colocalization channel and BAF was observed, which resulted in the colocalization puncta through the BAF labelling as concentrating in some points (**Figure 67.v**). Lastly, I showed only colocalization channel to explain the colocalization between them more clearly (**Figure 67.vi**), which revealed that colocalization puncta were brought together in some places.

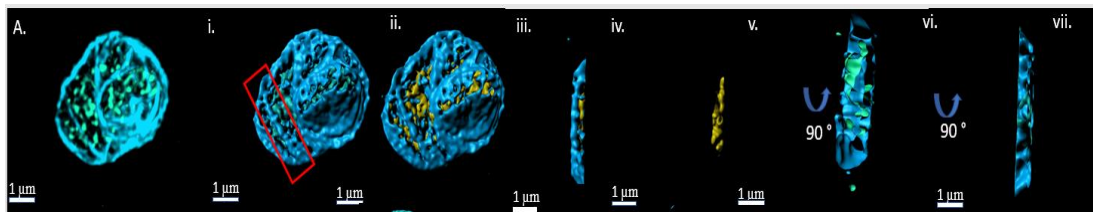


**Figure 68. BAF protein is placed in the middle of the ridge labelled by emerin in AIPASMC nuclei.** (i) A deconvolved Z-stacks of confocal images with emerin (cyan) labelling with BAF (pistachio green) in an AIPASMC nucleus was shown. (ii) 3D reconstructed emerin labelling at high threshold was applied for an acutely isolated PASC nucleus and (iii) emerin with colocalization channel (yellow) for emerin and BAF (iv) cut from the marked area showing I-type ridge with colocalization channel and (v) the only colocalization channel for BAF were shown. (vi) Emerin image rotated 90° anticlockwise as well as (vii) rotated 90° one more time to show inside of ridge.

As the possibility exists that the structures determined on AIPASMC nuclei labelled by emerin might represent a different function for cell processes, I decided to investigate the structures with BAF protein to understand emerin and BAF machinery, which binds DNA directly (Segura-Totten & Wilson, 2004). As shown in the previous part through deconvolved images, it was evident that emerin labelling was bulging and it rumbled on the surface of AIPASMC nucleus, and BAF was placed in the middle of the nucleus covered by emerin (Haraguchi et al., 2001) (**Figure 68 A.i & ii**). Regarding to BAF, this showed more clearly that BAF protein seemed to be confined to emerin labelled D7PASC nucleus via 3D skin application at high threshold for a better view (**Figure 68 A.i&ii**). This affirms the image showing emerin and the colocalization channel for emerin and BAF. Along with that, BAF and emerin colocalized in different regions through the AIPASMC nucleus, which do not refer to particular regions (**Figure 68 A.iii**). Then, I did the detailed investigation of the interesting view that included different outer structures like ridges. As stated before, the structures might serve an important function and show different associations with emerin. Firstly, I observed the I-type ridge outlined by emerin that colocalize with BAF (**Figure 68 A.iv**) by cutting this specific structure from the whole nucleus. As a results of this investigation, there was no BAF labelling on the outer surface of the I-type emerin labelled nucleus (**Figure 68 A.iv**), which was coincident with the findings stated in the previous literature (e.g., Morris, 2001). Then, in order to illustrate where BAF tethers with emerin through the ridge, I showed the colocalization

channel for BAF and emerlin, which revealed that BAF and emerlin colocalization concatenated in the middle of this particular structure (**Figure 68 A.v**). Following that, to understand BAF and emerlin association more clearly, I showed only the colocalization channel, which accumulated in the centre point of nucleus (**Figure 68 A.vi**). In order to illustrate the colocalized points across the I-type ridge, I turned this I-type ridge 90° anticlockwise, through which I gained a view of some colocalization points with the BAF (**Figure 68 A.vi**). Lastly, I turned the I-type ridge 90° one more time, which highlighted that BAF and emerlin colocalized at some points (**Figure 68 A.vii**).

### 3.3.2.2 In contrast, emerlin and BAF was presented as intertwined at 7 days cultured PASC nucleus.

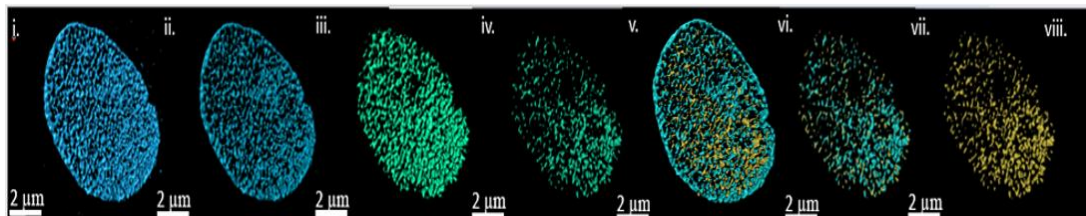


**Figure 69. The distribution of emerlin and BAF in D7PASC nucleus was shown.** i) A deconvolved Z-stacks of confocal images with emerlin (cyan) were applied at low and ii) at high threshold 3D skin. iii) A deconvolved Z-stacks of confocal images with BAF (pistachio green) was applied at low and iv) at high threshold 3D skin and v) the co-localization channel with emerlin, and vi) BAF. Lastly, vii) the only colocalization channel were shown.

Considering that emerlin morphology was changed after 7 days culture application, its associations with proteins might be affected by this alteration. To investigate this, I explored the distribution and formation on the D7PASC nucleus with low and high threshold, as presented in section 3.2.2, which again showed the main view of emerlin labelled D7PASC nucleus (**Figure 69 i&ii**). After revealing the structures of the emerlin, I have investigated BAF distribution with a low threshold application that detected all staining belonging to BAF and showed the overall view of BAF. The figure 69 iii illustrated that the distribution of BAF was even but not uniform, and it included tiny indentation similar to other proteins studied (**Figure 69 iii**), rather than being localized to a specific region. By using high threshold

to remove the excess labelling, I emphasized the structures and some gaps existed on the D7PASMNC nucleus labelled by BAF (**Figure 69 iv**). The colocalization of emerin and BAF formation was investigated with low and high threshold, which postulated the idea that BAF was woven on to the surface of emerin that is exposed at the edge of holes in day 7 PASMNCs nuclei (**Figure 69 vi-vii**). This has also been previously shown by Duan (2018). After showing colocalization of BAF and emerin, to show the colocalization more clearly, I illustrated only the colocalization channel, which was very similar to the BAF formation and distribution (**Figure 69 viii**). This indicates that emerin and BAF colocalized very highly in D7PASMNC.

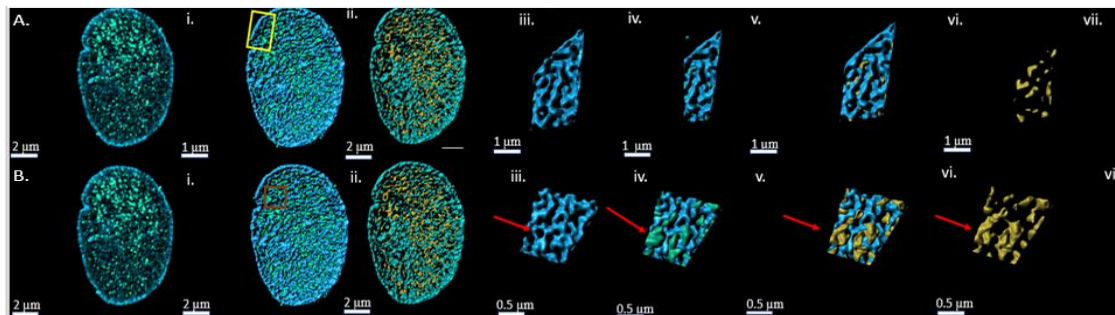


**Figure 70. The place where BAF colocalised with emerin were detected differently in 7 days cultured PASMNC nucleus from acutely isolated PASMNC nuclei.**

A) (i) A deconvolved Z-stacks confocal images with emerin (**cyan**) labelling with BAF (**pistachio green**) in a D7PASMNC nucleus was demonstrated. (ii) 3D reconstructed emerin labelling of a 7 days cultured PASMNC nucleus and (iii) with colocalization channel (**yellow**) were shown, then (iv) it cut from the marked area showing I-type structure, (v) the same structure with BAF and (vi) colocalization channel. Lastly the only colocalization channel for BAF and emerin were showed.

B) With regards to (B), however O-shaped structure was illustrated and O-type structure was shown by arrows in different panels.

Similarly to changes in the general aspect of co-labelled emerin and BAF PASMNC nucleus, it was distinct in the specific structures outlined by emerin and BAF association defined in day 7 labelled PASMNC nucleus, which appeared to be a sandwich-like structure. As shown section 3.2.2, emerin labelling for D7PASMNC was flattened and more even than AIPASMNC nucleus labelled by emerin. I showed that BAF appears as if caught in a emerin cage in the D7PASMNC nuclei co-labelled for emerin with BAF,

confirmed by the application of low threshold showing all staining for BAF and emerin (**Figure 70 A.i**), and by the high threshold application showing just intense stained regions (**Figure 70 A.ii**). Results were very similar to H3K9me2/lamin A association and might suggest the same model for emerin and BAF, which indicated that the surface shape of lamin A guides H3K9me2 binding. It was further illustrated that BAF and emerin colocalized puncta, seen in the image that showed emerin and the colocalization channel for emerin and BAF (**Figure 70 A.iii**). Due to the alterations on the general aspect, I expected the changes in BAF and emerin association in specific structures described in section 3.2.2, which might be important given that the association mediated gene expression (Samson et al., 2018). To provide a closer inspection, I-type structure were first analysed, which was defined in section 3.2.2 in detail (**Figure 70 A.iii&iv**). For exploring the BAF and emerin colocalization, I colocalised emerin with BAF by showing only this structure, which suggested that emerin and BAF meshed the opposite of AIPASMC nuclei (**Figure 70 A.v**). The image of the colocalization channel and emerin confirmed this pattern for BAF-emerin association on D7PASMC nuclei, in which BAF was located at some points of emerin labelled I-type structure (**Figure 70 A.vi**). Finally, I showed only colocalization channel to illustrate that BAF was found in the holes created by emerin and was composed of just puncta, rather than being a structure joint puncta (**Figure 70 A.vii**). This could be the case in acutely isolated and proliferating cells, where there is a break in the distribution of emerin across the INM: i.e., an open edge of emerin is revealed to which BAF may bind. Perhaps more edges for binding may be presented in proliferating cells.

Secondly, the same features were investigated on the O-type structures, as defined in section 3.2.2 (**Figure 70 B.iv**). The exploration of the emerin and BAF co-labelling illustrated that BAF filled in the round holes on D7PASMC nucleus showed by arrow (**Figure 70 B.v**), which was acquired by cutting it from the red box to determine BAF localization on this particular structure. The image that showed emerin and colocalization channel for

emerin and BAF revealed that the colocalization channel was found on the these structures, which explains colocalization on this emerin structure **(Figure 70 B.vi)**. Closer inspection of the complex structure on the image of colocalization channel and emerin, it is observed that the combination of emerin and BAF, created a surface in day 7 cultured PASMNC nucleus **(Figure 70 B.vii)**.

Consequently, there is a difference between the emerin and BAF assembly in the AIPASMC and D7PASMNC nuclei, which is highly likely to be caused by the changes in emerin labelling after 7 days culture application. In AIPASMC nuclei, the colocalization distribution is mostly revealed as a continues structure **(Figure 68 A.iii)**, whereas the colocalization channel in D7PASMNC composed of puncta, in parallel to emerin distribution in D7PASMNC **(Figure 70 B.iii)**. Therefore, the colocalization for emerin and BAF may be directed by emerin.

| Cell types     | Percentage of colocalized volume                                                               | Pearson's corelation coefficient                                                                 |
|----------------|------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| AIPASMC (n=2)  | 0.45±0.01                                                                                      | 0.05±0.02                                                                                        |
| D7PASMNC (n=2) | 11.46±1.21  | 0.06±0.001  |

**Table 6.** The table showed the comparison of colocalization for lamin BAF and emerin on AIPASMC and D7PASMNC nuclei. The colocalization volume and Pearson's correlation coefficient of emerin and BAF were illustrated (mean ±SEM).

To show different colocalization for emerin and BAF in AIPASMC and D7PASMNC, the percentage of BAF colocalized volume with emerin were calculated for both cell types, which are 0.45±0.011 and 11.46±1.21 respectively **(Table 6)**. As illustrated in table 6, the percentage of colocalized volume in AIPASMC is less than D7PASMNC (p=0.0119163).

Another important value for colocalization, Pearson's correlation coefficient in AIPASMC was analysed as 0.05±0.02, whereas in D7PASMNC, it was 0.06±0.001 **(Table 6)**, which revealed that in parallel to percentage of colocalized volume, Pearson's correlation coefficient increased, and was not statistically significant (p=0.868990).

# Discussion

## Possible functions of protein dense regions in contractile phenotype pulmonary artery smooth muscle cells

In this study, I reported how nuclear envelope proteins affect chromatin organization in contractile and proliferating phenotype, which may influence the gene expression. This study illustrated that the ridges in pulmonary artery smooth muscle cell (PASMC) provide association point for chromatin, and thus contribute to the modulation of gene expression. I have demonstrated that in acutely isolated smooth muscle cells, the distribution of lamin A is elongated, bulging and crumpled composed of infoldings, which covered the underneath of the nuclear envelope. Several reports have shown that the interactions of inner nuclear membrane (INM) proteins with chromatin provide contributions to the concentric areas of gene expression (Rowley & Corces, 2018). More importantly, Malhas et al. (2011) showed that the chromatin clusters was found close to the nuclear invaginations. Following the research about chromatin and nuclear invaginations, histone 3 lysine 9 dimethylation (H3K9me2) rich invaginations was detected on nuclei labelled by lamin A antibody (Duan et al., 2019). Along with invaginations, some clefts and ridges were observed on nuclear envelope created by lamin A labelling. Previously, the clefts were described as different from tubular structures (Wittmann et al., 2009a). Depending on the information stated above, the structures such as ridges and clefts might aid the lamin A function, the organizer of chromosome architecture. However, there is no information associated with the detailed characterisation of these structures including ridges in literature. I have identified 3 types of ridges labelled by lamin A based on their shape, including I-type, V-type and half I-type ridges. The morphological differences between them might cause the alteration concentric zones of chromatin due to lamin A association with heterochromatins through the lamina-associated domains (LADs) (Briand

& Collas, 2020). The I-type structure was described as tubular ridge by Duan (2018), however more detailed investigation about the ridges remained un-researched. Briefly, the ridges may be a part of the nuclear network suggested by Duan (ibid.), which is a protein-dependent chromatin modulation, supporting the regulation of gene transcription. Turning now to the nuclear invaginations, I investigated three types of nuclear invaginations in acutely isolated pulmonary artery smooth muscle cells (AIPASMC) in a variety of numbers among the cells. In line with the previous findings, this study has revealed that nuclear invaginations are dynamic structures (Duan, 2018; Fricker et al., 1997b).

The results of this study indicate that emerin distribution was very similar to lamin A distribution through the nucleus confirming the interaction of lamin A and emerin (Vaughan et al., 2001). Moreover, the surface characteristics are also very similar to those of proteins, which include some infoldings and invaginations on the surface. Manilal et al. (1998) examined the spots on emerin labelling, which could be the nuclear invaginations. Twenty years later, after observing it in defects in nuclear invagination in case of mutations in genes encoding emerin, it has been proven that emerin had an important role in the nuclear invaginations (Shimojima et al., 2017). Finally, emerin positive nuclear invaginations were observed by Duan (2018) and it was stated that these structures covered by barrier-to-autointegration factor (BAF) and emerin colocalization puncta. These findings are encouraging to investigate emerin dense regions through the nucleus, which was termed as ridges in section 3.1.1. My data showed that there are 3 types of ridges labelled by emerin through the nuclear envelope, which are I-type, half I-type and branched ridges. Importantly, two of them were also observed in lamin A labelled nucleus, which may suggest the view that these structures may belong to lamin A-emerin-BAF complex (Samson et al., 2018). Due to the lamin A negative/emerin positive nuclear invagination observed in the previous literature, the ridges were observed with lamin A labelling. However, my findings showed that there was no lamin A negative ridges

through the nucleus labelled by emerin. Moreover, the number of these two ridges was very similar for each protein labelling on average. Therefore, these structures may be responsible for emerin functions that work with lamin A collaboratively such as organisation of lamin A and BAF (Samson et al., 2018). More interestingly, my results indicate that lamin A is colocalized with emerin through the ridges outlined by emerin.

The linker of nucleoskeleton and cytoskeleton (LINC) complex is defined as a bridge between cytoskeleton and lamin A and have sensitivity to myosin-dependent cellular tension for the creation of changes in nuclear shape (Bouzid et al., 2019). Additionally, shorter nuclear envelope spectrin-repeat proteins-1 (nesprin-1) can interact with lamin A. Also, giant nesprin-1 can bind Sad1 and UNC84 homology (SUN-2) and particularly, this isoform of nesprin was responsible for the transmission of cellular tension (Holt et al., 2019). Taking all functions of nesprin and interaction with other nuclear envelope proteins into consideration, the possibility appeared that nesprin might create a lamin A, nesprin and emerin complex to fulfil its function (Warren, 2005). In this study, the nature of nesprin-1 morphology and nesprin/lamin A interaction in terms of morphology have been handled in detail, which showed the nesprin distribution being localized in particular areas, rather than through all of the nuclear envelope. In previous studies (e.g., Holt et al., 2016) examining the staining created by nesprin antibody, MANNES1E that recognises both nesprin shorter and giant. Due to this property, it showed both shorter and giant nesprin localization and nesprin labelling was observed as uneven distribution (Holt et al., 2019). Therefore, my results may be also caused by antibody. However, in my study, I showed the nesprin localized on ridges labelled by lamin A, and therefore, nesprin localization in a particular region might be explained by possible its role in ridge formation. These two isoforms of nesprin, namely, shorter and giant, are located at INM and ONM respectively. While shorter nesprin mediates structural support with lamin A to nuclear envelope, giant nesprin is composed of LINC complex with SUN-2 to provide communication from cytoskeleton to nucleus (Morris & Randles, 2010). However, I observed

similar nesprin dense regions through the nesprin labelling, which showed that I-type and branched ridges are very similar to emerin labelled ridges. This finding supports the hypothesis that nesprin created a nesprin-lamin A and emerin complex with lamin A and emerin to transmit the cellular tension (Warren, 2005). Moreover, for many times, lamin A and nesprin-1 associations proved, however, that there was no findings about the fact that nesprin-1 can bind to chromatins. Therefore, the nesprin may affect chromatin architecture indirectly by affecting lamin A distribution. Lamin A and nesprin-1 colocalization was observed through these ridges in this study and the number of these structures was very similar with that of emerin and lamin A. Considering that, these structures may mediate the results of lamin A and nesprin colocalization. Another LINC complex protein is SUN-2 that acts as a bridge between lamin A and nesprin (Matsumoto et al., 2015). This study confirms the nuclear rim-like pattern of SUN-2 localization (Hodzic et al., 2004). Moreover, very similar to lamin A and emerin distribution, it outlined similar ridges by SUN-2 dense regions in similar numbers, which also supports the presence of a complex comprising of lamin A, emerin, nesprin and SUN-2 created by these ridges (Warren, 2005). Moreover, some studies associated with nesprin-1 showed that the absence of lamin A caused the mislocalization of nesprin and in the event of mutations in encoding nesprin, defects in nuclear morphology was observed (Morris & Randles, 2010; Zhang et al., 2009). However, in the loss in SUN-2, lamin A distribution was not affected. In addition, whether there is a direct or indirect interaction of lamin A and SUN-2 is not clear and it has been illustrated that lamin A is necessary for the organization of SUN-2 localization, which can be considered as one contribution to SUN-2 organisation in the literature (Crisp et al., 2006). Furthermore, SUN-2 labelling was observed in all types of structures described by lamin A/emerin, but not in uniform. This result provides supporting evidence from the previous observations, which suggests the existence of a binding partner for lamin A and SUN2. Moreover, studies on SUN-2 localization explained that no alteration in SUN-2 localization was

observed in the case of the emerin-null fibroblasts (Haque et al., 2010b). Therefore, a complex for these proteins like the possibility of nesprin-lamin A-emerin complex mentioned above may not be conceivable in my results. In contrast, the colocalization results between lamin A/SUN-2 are more than that of lamin A/nesprin colocalization based on my results, which may show the existence of the closer functional connection of lamin A/SUN-2 than lamin A/nesprin. In accordance with this result, the existence of a function realised by SUN-2/lamin A association, which is not dependent on emerin, may be postulated. This can be supported by the fact that N terminus of SUN-2 has affinity to bind lamin A (Haque et al., 2010b).

Moreover, lamin A and SUN-2 association was observed through the ridges outlined by lamin A according to SUN-2 localization, which was INM facing surface of lamin A (Chen et al., 2019). It was observed that lamin A might support SUN-2 function by this association in this particular region including mechanotransduction or SUN-2 perhaps affects lamin A through this specific region to regulate nuclear shape (Bouzid et al., 2019; Hoffman et al., 2020).

### **The possible functions of NE proteins at the synthetic phenotype**

In this part, primary culture of enzymatically isolated smooth muscle cells in the presence of serum was investigated, which resulted in the phenotypic switching that was studied in the previous research (e.g., Chamley-Campbell & Campbell, 1981; Duan, 2018). The previous studies (e.g., Rensen et al., 2007b) have suggested that contractile and synthetic phenotype shows different cell characteristics and nucleus morphology. In parallel to these studies, I observed that the nuclear envelope proteins, particularly the ones supporting nuclear architecture, change completely in cultured for 7 days PASMC. For example, the ridges that were observed on the surface of lamin A in AIPASMC were not detected in day 7 pulmonary artery smooth muscle cell (D7PASMC), which affected chromatin organization and other nuclear envelope proteins that could bind lamin A. Instead of the ridges, the distribution of A-type lamin in D7PASMC

has various structures including O, I-type and irregular rounded shaped structures. Additionally, there were not any invaginations detected in the cells studied. Interestingly, the O-type structures were observed as the most commonly found structures, which suggests a potentially significant role for O-type structures in synthetic phenotype. Moreover, other studies showed that  $\text{Ca}^{2+}$  handling alters in proliferating phenotype, and lamin A expression is regulated based on the cellular tension (González-Cruz et al., 2018; House et al., 2008b). Therefore, the change in the lamin A after phenotypic switching might be caused by alteration in the mechanical properties.

Additionally, emerin reconfiguration showed very similar patterns with lamin A, including O-type and I-type structures. In the previous studies, it has been shown that the expression of lamin A and emerin after phenotypic switching was repressed, which suggests that lamin A and emerin may have similar roles in phenotypic switching (Qi et al., 2016). Moreover, others have showed that the ratio between the lamin isoforms changed (e.g., González-Cruz et al., 2018). This can be the explanation for the disappearance of infoldings and nuclear invaginations in proliferating phenotype, since these structures increases the surface area (Wittmann et al., 2009) that may be affected by the amount of lamin A. In order to provide an explanation for this, the investigation of other lamin isoforms might be performed in comparison with the contractile and proliferating phenotype in future studies.

In contrast to the emerin, the distribution of SUN-2 labelling on the surface of D7PASMNC nucleus is localised on restricted area, which may be strategically, and the O-type structure is the most commonly found structure on the SUN-2 surface, which are also found in lamin A. Therefore, these proteins could mediate their function by this structure in proliferating phenotype.

Nesprin labelling is composed of puncta that is very likely to be strategically formed in D7PASMNC nucleus, which is significantly different from the other proteins. The antibody used in this study can recognise both shorter nesprin and nesprin giant that is located to ONM and interacting with cytoskeleton proteins (Rajgor & Shanahan, 2013). Moreover, giant nesprin is formed as LINC complex with SUN-2 to provide communication between cytoskeleton and the interior of nucleus, such as through signalling process. In addition, it has been proven that nesprin has a role in the  $\text{Ca}^{2+}$ /calmodulin molecular trafficking (Kelkar et al., 2015) and in the interaction between ryanodine receptors (RyR) and nesprin-1- $\alpha$  (Pare et al., 2005). Therefore, the interesting pattern of nesprin labelling in the D7PASMNC nucleus may be related to the changes in the  $\text{Ca}^{2+}$  channels after phenotypic switching or this distribution might be affected by  $\text{Ca}^{2+}$  signalling.

One limitation of this study is that the experiments were performed on only some of INM proteins and nesprin. However, the investigation of how cytoskeleton proteins including actin interact with nesprin might be beneficial in the specific regions defined in contractile and proliferative phenotype. The disruption of LINC complex proteins results in changes in actomyosin activity. More specifically, it has been shown that nesprin disruption enhances actomyosin activity in skeletal muscle progenitor and endothelial cells (Ahmed & Warren, 2018). Moreover, in a study investigating actin and phenotypic switching, it was reported that actin polymerization was considerably reduced in proliferating phenotype (Alajbegovic et al., 2017). Therefore, these alterations may be the cause or results of changes in the distribution of the relationship between nesprin and actin filaments. The other possibility is that nesprin can transform into different isoforms, but not SUN-2 (Randles et al., 2010), which may cause the view of composed of puncta.

### **The lamin A and emerin dense regions presents anchoring zones for chromatin, caused by protein-chromatin complex**

Co-labelling for lamin A and H3K9me2 was revealed that the colocalization puncta was located through the lamin A accumulated regions, which may

explain why lamin A is dense at these regions and more importantly, that lamin A may mediate transcriptional regulation by interacting chromatin through the LADs (Burke & Stewart, 2013). In addition, the LAD location associated with gene repression was preferentially near to the nuclear lamina, which can be considered as the supporting evidence for the view of the strategically localization of lamin A dense regions (Kind et al., 2013; van Steensel & Belmont, 2017). Therefore, the ridges may regulate gene expression dynamically. Furthermore, it has been suggested that LADs are lined with nuclear lamina in relation to gene suppression, but intermittently (van Steensel & Belmont, 2017), which also supports strategically positioning of chromatin through the ridges. Accordingly, colocalization puncta for lamin A and H3K9me2 in different model was observed in the specific regions defined as regions, which are classified based on morphology in this study. According to one model associated with nuclear lamina and histone, nuclear lamina holds enzymatic activities that mediates histone modification (van Steensel & Belmont, 2017), which may be the explanation for the absence of morphologically different regions. More interestingly, in proliferating nuclei, the distribution of H3K9me2 in PSMC nuclei changed according to the changes in lamin A morphology. It was suggested that the distribution of H3K9me2 switched from concentrated on specific regions in contractile phenotype to distributed evenly in proliferating phenotype, which coincides with the findings in the previous studies (e.g., Salzberg et al., 2017). As mentioned above, lamin A accumulated regions were defined in different shapes and one of them, namely, O-type structure, localized with H3K9me2, which suggests that lamin A structure are guided to H3K9me2 distribution. Lastly, colocalization value for these proteins was reduced, which may demonstrate that lamin A-H3K9me2 interaction related to gene repression was decreased, which may be caused by the decrease in lamin A expression in proliferative cell that showed in vascular smooth muscle cell proliferation induced by cyclic stretch application (Qi et al., 2016). In previous study (Duan et al., 2019), by blocking  $\text{Ca}^{2+}$  flux in PSMCs, BAF and H3K9me2 related genes (S100A9 and Mlh1, respectively) was investigated, resulting in decrease in both gene expression.

Moreover, changes in  $\text{Ca}^{2+}$  flux in proliferating nuclei was showed as parallel with the loss of nuclear invagination (Duan, 2018). I also observed that ridges disappeared in proliferating nuclei, therefore ridges may be related to  $\text{Ca}^{2+}$  flux. Additionally, lamin A and H3K9me2 association changes in ridges in contractile and proliferative nuclei may show the existence of ridges role in gene modulation.

Emerin has regions to bind with BAF protein, a DNA-linking protein (Lee et al., 2001), which facilitates either repression or enhances gene expression (Takama et al., 2013). My investigation showed that emerin and BAF protein in AIPASMC cell bond at specific outer membrane structures described as ridges, but it did not localized at the specific regions. In contrast to lamin A and H3K9me2, the interesting point is that BAF-emerin colocalization was increased in proliferating nucleus, which cannot be clearly concluded due to the different effects of BAF in genes.

## Conclusion

It was proposed before that the inner nuclear proteins have roles in the modulation of gene expression via nuclear invagination (Duan et al., 2019), which interacts with the inner nuclear proteins H3K9me2 and BAF in this structure. My study proposes new specific association regions, ridges, for H3K9me2 and BAF, which might be a regulator for the association of chromatins and BAF. The colocalizations of BAF and emerin as well as H3K9me2 and lamin A changed in proliferating cell. For more explanation into INM proteins role in gene regulation via specific chromatin marks, I postulated a model (**Figure 71**) showing that chromatin marks interact with INM membrane proteins differently in cells under two different conditions.

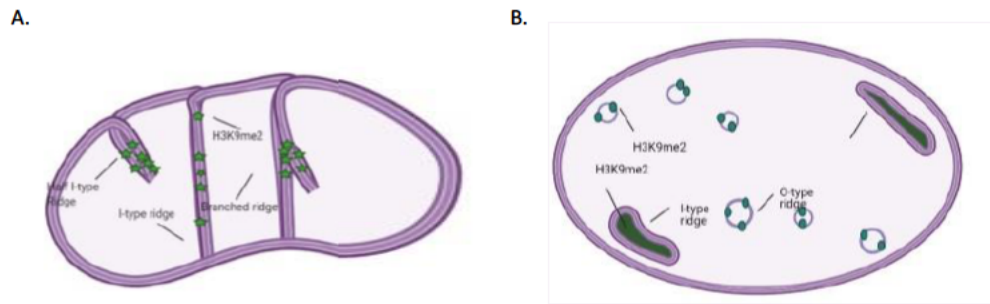


**Figure 71. The schematic illustration of AIPASMC(A) and D7PASMC(B) nuclei was shown. H3K9me2 and BAF was associated with the inner nuclear membrane proteins in ridges and classification of the ridges showed different association models for this associations.**

## Limitations and future experiments

The common disadvantages of lamin A co-labelling is epitope masking, in which other staining can be overlapped on the lamin A surface (Tunnah et al., 2005). For nesprin antibody, it recognises two isoforms of nesprin and the usage of isoform specific antibody for nesprin giant or shorter might be more beneficial (Holt et al., 2019). Moreover, alcohol fixation method, which cause cell shrinkage, was used in this study (Panzacchi et al., 2019). Confocal microscopy is not as good as super-resolution microscopy in the investigation of cellular dynamics and the structure investigated in this study might be explored via better resolution (Vangindertael et al., 2018). The sample size is very limited in this study, particularly for LINC complex proteins and their colabelization with lamin A, which can affect statistical results. The other important thing is that the association with BAF and emerin might change depending on cell cycle. Therefore, the investigation of this interaction in each cell phase might be more advantageous.

The previous studies mentioned Lamin A-emerin and BAF complex. Thus, triple labelling of them might give more information about the ridges' functions (Samson et al., 2018). In this study, I investigated only LINC complex and inner nuclear membrane proteins. However, their interaction with cytoskeleton proteins in contractile and proliferating phenotype can provide further information about the modulation of phenotypic switching. Additionally, other histone marks, particularly H3K27me2, and other lamins might be studied in

contractile and proliferative phenotype, since lamins can switch to different isoforms (González-Cruz et al., 2018). Moreover, ridges identified in this study might be investigated through live  $\text{Ca}^{2+}$  imaging to understand the relationship between  $\text{Ca}^{2+}$  signalling and the lamin A accumulated regions.

Additionally, the other factors causing phenotypic switching might be investigated in line with the specific structures such as cell ageing, phosphorylation and mechanical force.

## Appendix

**Table 7. The ratio of ridges dimensions labelled by lamin A in AIPASMC to nucleus volume were shown.**

|       | Width( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Length( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Height( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) |   | Nucleus<br>volume<br>( $\mu\text{m}^3$ ) |
|-------|-----------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|---|------------------------------------------|
|       | 0.013                                                           | 0.005                                                            | 0.026                                                            | } | 63.3                                     |
|       | 0.016                                                           | 0.049                                                            | 0.02                                                             |   |                                          |
|       | 0.013                                                           | 0.072                                                            | 0.03                                                             |   |                                          |
|       | 0.014                                                           | 0.38                                                             | 0.03                                                             |   |                                          |
|       | 0.1896                                                          | 0.039                                                            | 0.017                                                            | } | 58                                       |
|       | 0.016                                                           | 0.041                                                            | 0.015                                                            |   |                                          |
|       | 0.0094                                                          | 0.016                                                            | 0.027                                                            |   |                                          |
|       | 0.0112                                                          | 0.0241                                                           | 0.027                                                            |   |                                          |
|       | 0.009                                                           | 0.054                                                            | 0.01                                                             | } | 79.3                                     |
|       | 0.001                                                           | 0.06                                                             | 0.012                                                            | } | 72.3                                     |
|       | 0.001                                                           | 0.056                                                            | 0.017                                                            |   |                                          |
| Mean  | 0.02665                                                         | 0.07237                                                          | 0.021                                                            |   |                                          |
| S.E.M | 0.01561                                                         | 0.02989                                                          | 0.002093                                                         |   |                                          |

**Table 8. The ratio of ridges dimensions labelled by emerlin in AIPASMC to nucleus volume was shown**

|       | Width( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Length( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Height( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) |   | Nucleus<br>volume<br>( $\mu\text{m}^3$ ) |
|-------|-----------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|---|------------------------------------------|
|       | 0.01                                                            | 0.05                                                             | 0.02                                                             | } | 80.3                                     |
|       | 0.02                                                            | 0.07                                                             | 0.01                                                             |   |                                          |
|       | 0.03                                                            | 0.08                                                             | 0.01                                                             |   |                                          |
|       | 0.011                                                           | 0.071                                                            | 0.02                                                             | } | 70.4                                     |
|       | 0.01                                                            | 0.058                                                            | 0.03                                                             |   |                                          |
|       | 0.021                                                           | 0.07                                                             | 0.02                                                             |   |                                          |
|       | 0.012                                                           | 0.072                                                            | 0.02                                                             | } | 67.8                                     |
|       | 0.01                                                            | 0.04                                                             | 0.01                                                             |   |                                          |
|       | 0.08                                                            | 0.05                                                             | 0.02                                                             |   |                                          |
|       | 0.01                                                            | 0.06                                                             | 0.05                                                             | } | 90.6                                     |
|       | 0.01                                                            | 0.04                                                             | 0.02                                                             |   |                                          |
|       | 0.01                                                            | 0.04                                                             | 0.03                                                             |   |                                          |
|       | 0.01                                                            | 0.08                                                             | 0.08                                                             | } |                                          |
| Mean  | 0.01877                                                         | 0.06008                                                          | 0.02615                                                          |   |                                          |
| S.E.M | 0.005175                                                        | 0.003974                                                         | 0.005164                                                         |   |                                          |

**Table 9. The ratio of ridges dimensions labelled by SUN-2 in AIPASMC to nucleus volume was shown.**

|       | Width ( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Length( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Height( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) |   | Nucleus<br>volume<br>( $\mu\text{m}^3$ ) |
|-------|------------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|---|------------------------------------------|
|       | 0.045                                                            | 0.07                                                             | 0.01                                                             | } | 63.5                                     |
|       | 0.02                                                             | 0.07                                                             | 0.02                                                             |   |                                          |
|       | 0.01                                                             | 0.04                                                             | 0.01                                                             |   |                                          |
|       | 0.09                                                             | 0.06                                                             | 0.02                                                             |   |                                          |
|       | 0.01                                                             | 0.1                                                              | 0.04                                                             | } | 57.7                                     |
|       | 0.01                                                             | 0.06                                                             | 0.04                                                             |   |                                          |
| Mean  | 0.03083                                                          | 0.06667                                                          | 0.02333                                                          |   |                                          |
| S.E.M | 0.01193                                                          | 0.007328                                                         | 0.005092                                                         |   |                                          |

**Table 10. The ratio of ridges dimensions labelled by nesprin in AIPASMC to nucleus volume was shown.**

|       | Width( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Length( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Height( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) |   | Nucleus<br>volume<br>( $\mu\text{m}^3$ ) |
|-------|-----------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|---|------------------------------------------|
|       | 0.01                                                            | 0.08                                                             | 0.02                                                             | } | 71.9                                     |
|       | 0.01                                                            | 0.08                                                             | 0.06                                                             |   |                                          |
|       | 0.01                                                            | 0.1                                                              | 0.02                                                             |   |                                          |
|       | 0.01                                                            | 0.07                                                             | 0.04                                                             | } | 86                                       |
| Mean  | 0.1                                                             | 0.0825                                                           | 0.035                                                            |   |                                          |
| S.E.M | 0                                                               | 0.005447                                                         | 0.008292                                                         |   |                                          |
| S.D.  | 0                                                               | 0.01089                                                          | 0.01658                                                          |   |                                          |

1

**Table 11. The ratio of ridges dimensions labelled by lamin A in D7PASMC to nucleus volume was shown.**

| O-type structure area( $\mu\text{m}^2$ )/nucleus volume( $\mu\text{m}^3$ ) | I-type structure length ( $\mu\text{m}$ )/nucleus volume( $\mu\text{m}^3$ ) | Irregular shaped structure area( $\mu\text{m}^2$ )/nucleus volume( $\mu\text{m}^3$ ) | Nucleus volume ( $\mu\text{m}^3$ ) |
|----------------------------------------------------------------------------|-----------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------------------------------------|
| 0.002642                                                                   | 0.016822                                                                    | 0.003597                                                                             | 107                                |
| 0.000661                                                                   | 0.018692                                                                    | 0.00734                                                                              |                                    |
| 0.001835                                                                   | 0.018692                                                                    | 0.00734                                                                              |                                    |
| 0.001835                                                                   | 0.014019                                                                    | 0.003597                                                                             |                                    |
| 0.001174                                                                   | 0.018692                                                                    | 0.001835                                                                             |                                    |
| 0.001835                                                                   | 0.021495                                                                    | 0.004698                                                                             | 107                                |
| 0.001835                                                                   | 0.018692                                                                    | 0.001835                                                                             |                                    |
| 0.000294                                                                   | 0.014019                                                                    | 0.003597                                                                             |                                    |
| 0.002642                                                                   | 0.014019                                                                    | 0.00734                                                                              |                                    |
| 0.001174                                                                   | 0.023364                                                                    | 0.003597                                                                             |                                    |
| 0.003597                                                                   | 0.021495                                                                    | 0.001835                                                                             | 112                                |
| 0.001835                                                                   | 0.009346                                                                    | 0.002642                                                                             |                                    |
| 0.001835                                                                   | 0.018692                                                                    | 0.016515                                                                             |                                    |
| 0.000661                                                                   | 0.020561                                                                    | 0.016515                                                                             |                                    |
| 0.002642                                                                   | 0.023364                                                                    | 0.016515                                                                             |                                    |
| 0.001753                                                                   | 0.033929                                                                    | 0.003945                                                                             | 125                                |
| 0.003436                                                                   | 0.013393                                                                    | 0.001753                                                                             |                                    |
| 0.00028                                                                    | 0.017857                                                                    | 0.003436                                                                             |                                    |
| 0.001753                                                                   | 0.017857                                                                    | 0.003436                                                                             |                                    |
| 0.001122                                                                   | 0.013393                                                                    | 0.001753                                                                             |                                    |
| 0.004021                                                                   | 0.016                                                                       | 0.003079                                                                             | 125                                |
| 0.002262                                                                   | 0.0144                                                                      | 0.002262                                                                             |                                    |
| 0.001571                                                                   | 0.016                                                                       | 0.014137                                                                             |                                    |
| 0.001571                                                                   | 0.024                                                                       | 0.019242                                                                             |                                    |

|       |          |          |          |   |     |
|-------|----------|----------|----------|---|-----|
|       | 0.004021 | 0.0192   | 0.001571 | } | 125 |
| Mean  | 0.001931 | 0.01832  | 0.006137 |   |     |
| S.E.M | 0.000205 | 0.000952 | 0.001104 |   |     |

**Table 12. The ratio of ridges dimensions labelled by emerlin in D7PASM to nucleus volume was shown.**

| Nucleus<br>volume( $\mu\text{m}^3$ ) | O-type<br>area( $\mu\text{m}^2$ )/nucleus<br>volume( $\mu\text{m}^3$ ) | I-type<br>length<br>nucleus<br>volume( $\mu\text{m}^3$ ) | structure<br>( $\mu\text{m}$ )/<br>nucleus<br>volume( $\mu\text{m}^3$ ) | Nucleus<br>volume( $\mu\text{m}^3$ ) |
|--------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------------|--------------------------------------|
| 175                                  | {                                                                      | 0.002418                                                 | 0.017257                                                                | {                                    |
|                                      |                                                                        | 0.001443                                                 | 0.017429                                                                |                                      |
|                                      |                                                                        | 0.005332                                                 | 0.022514                                                                |                                      |
| 175                                  | {                                                                      | 0.00271                                                  | 0.015829                                                                | {                                    |
|                                      |                                                                        | 0.001377                                                 | 0.008971                                                                |                                      |
|                                      |                                                                        | 0.002773                                                 | 0.035714                                                                |                                      |
|                                      |                                                                        | 0.001091                                                 | 0.019257                                                                |                                      |
|                                      |                                                                        | 0.000486                                                 | 0.010971                                                                |                                      |
|                                      |                                                                        | 0.001697                                                 | 0.0104                                                                  |                                      |
|                                      |                                                                        | 0.001363                                                 | 0.010686                                                                |                                      |
|                                      |                                                                        | 0.001898                                                 | 0.008                                                                   |                                      |
|                                      |                                                                        | 0.000699                                                 | 0.006971                                                                |                                      |
|                                      |                                                                        | 0.00313                                                  | 0.005714                                                                |                                      |
| 148                                  | {                                                                      | 0.000791                                                 | 0.006457                                                                | {                                    |
|                                      |                                                                        | 0.00186                                                  | 0.008171                                                                |                                      |
|                                      |                                                                        | 0.001547                                                 | 0.009257                                                                |                                      |
|                                      |                                                                        | 0.003971                                                 | 0.010229                                                                |                                      |
|                                      |                                                                        | 0.000661                                                 | 0.028649                                                                |                                      |
| 151                                  | {                                                                      | 0.003892                                                 | 0.027568                                                                | {                                    |
|                                      |                                                                        | 0.000648                                                 | 0.014257                                                                |                                      |
|                                      |                                                                        | 0.001511                                                 | 0.012568                                                                |                                      |
|                                      |                                                                        | 0.005201                                                 | 0.015608                                                                |                                      |
| 151                                  | {                                                                      | 0.0013                                                   | 0.015743                                                                | {                                    |
|                                      |                                                                        | 0.002549                                                 | 0.013581                                                                |                                      |
|                                      |                                                                        | 0.0013                                                   | 0.021757                                                                |                                      |
| 151                                  | {                                                                      | 0.000832                                                 | 0.00475                                                                 | {                                    |

|       |          |          |     |
|-------|----------|----------|-----|
|       | 0.0013   | 0.013986 | 148 |
| Mean  | 0.001992 | 0.031486 |     |
| S.E.M | 0.000251 | 0.024257 |     |
|       |          | 0.005946 |     |
|       |          | 0.013581 |     |
|       |          | 0.011959 |     |
|       |          | 0.033784 |     |
|       |          | 0.015811 |     |
|       |          | 0.016149 |     |
|       |          | 0.011959 |     |
|       |          | 0.010676 |     |
|       |          | 0.010405 |     |
|       |          | 0.01473  |     |
|       |          | 0.011622 |     |
|       |          | 0.005128 |     |
|       |          | 0.016622 | 151 |
|       |          | 0.009324 |     |
|       |          | 0.014392 |     |
|       |          | 0.013649 |     |
|       |          | 0.018716 |     |
|       |          | 0.008851 |     |
|       |          | 0.023642 |     |
|       |          | 0.015298 |     |
|       |          | 0.018013 |     |
|       |          | 0.021325 |     |
|       |          | 0.016755 |     |
|       |          | 0.019603 |     |
|       |          | 0.019007 |     |
|       |          | 0.018146 |     |
|       |          | 0.016887 |     |
|       |          | 0.028344 |     |
|       |          | 0.016424 |     |

|       |          |   |     |
|-------|----------|---|-----|
|       | 0.020397 | } | 151 |
|       | 0.017351 |   |     |
|       | 0.018675 |   |     |
|       | 0.008079 |   |     |
|       | 0.003821 |   |     |
|       | 0.01351  |   |     |
|       | 0.023311 |   |     |
|       | 0.01351  |   |     |
|       | 0.008675 |   |     |
|       | 0.01894  |   |     |
|       | 0.009404 |   |     |
|       | 0.026291 |   |     |
| Mean  | 0.01553  |   |     |
| S.E.M | 0.000831 |   |     |

**Table 13. The ratio of ridges dimensions labelled by SUN-2 in D7PASC to nucleus volume was shown.**

|       | O-type structure area( $\mu\text{m}^2$ )/nucleus volume( $\mu\text{m}^3$ ) | I-type structure length ( $\mu\text{m}$ )/nucleus volume( $\mu\text{m}^3$ ) | Globular structure area( $\mu\text{m}^2$ )/nucleus volume( $\mu\text{m}^3$ ) | Nucleus volume ( $\mu\text{m}^3$ ) |
|-------|----------------------------------------------------------------------------|-----------------------------------------------------------------------------|------------------------------------------------------------------------------|------------------------------------|
| 95.3  | 0.004617                                                                   | 0.008395                                                                    | 0.01574                                                                      | 95.3                               |
|       | 0.004617                                                                   | 0.007345                                                                    | 0.012592                                                                     |                                    |
|       | 0.003043                                                                   | 0.020986                                                                    | 0.01574                                                                      |                                    |
|       | 0.001154                                                                   | 0.01574                                                                     | 0.01574                                                                      |                                    |
|       | 0.006611                                                                   | 0.01574                                                                     | 0.01574                                                                      |                                    |
|       | 0.004512                                                                   | 0.008395                                                                    | 0.017838                                                                     |                                    |
|       | 0.004512                                                                   | 0.017838                                                                    | 0.016789                                                                     |                                    |
|       | 0.004512                                                                   | 0.024134                                                                    | 0.01574                                                                      |                                    |
| 102   | 0.004302                                                                   | 0.018888                                                                    | 0.000561                                                                     | Mean                               |
|       | 0.004512                                                                   | 0.014706                                                                    |                                                                              |                                    |
|       | 0.004216                                                                   | 0.014706                                                                    |                                                                              |                                    |
|       | 0.004216                                                                   | 0.014706                                                                    |                                                                              |                                    |
|       | 0.001863                                                                   | 0.014706                                                                    |                                                                              |                                    |
|       | 0.004216                                                                   | 0.022549                                                                    |                                                                              |                                    |
|       | 0.004216                                                                   | 0.021569                                                                    |                                                                              |                                    |
|       | 0.005                                                                      | 0.01603                                                                     |                                                                              |                                    |
| 102   | 0.003235                                                                   | 0.001289                                                                    |                                                                              | S.E.M                              |
|       | 0.001176                                                                   |                                                                             |                                                                              |                                    |
|       | 0.006863                                                                   |                                                                             |                                                                              |                                    |
| Mean  | 0.004216                                                                   |                                                                             |                                                                              |                                    |
| S.E.M | 0.00408                                                                    |                                                                             |                                                                              |                                    |
|       | 0.000317                                                                   |                                                                             |                                                                              |                                    |

**Table 14. The ratio of ridges dimensions labelled by nesprin in D7PASMC to nucleus volume was shown.**

|       | Puncta<br>area( $\mu\text{m}^2$ )/nucleus<br>volume( $\mu\text{m}^3$ ) | Nucleus<br>volume( $\mu\text{m}^3$ ) |
|-------|------------------------------------------------------------------------|--------------------------------------|
|       | 0.008142                                                               | 95.3                                 |
|       | 0.007933                                                               |                                      |
|       | 0.006785                                                               |                                      |
|       | 0.005219                                                               |                                      |
|       | 0.003967                                                               |                                      |
|       | 0.001775                                                               |                                      |
|       | 0.002088                                                               | 102                                  |
|       | 0.002088                                                               |                                      |
|       | 0.001275                                                               |                                      |
|       | 0.002843                                                               |                                      |
|       | 0.006176                                                               |                                      |
|       | 0.001961                                                               | 102                                  |
|       | 0.001961                                                               |                                      |
|       | 0.008824                                                               |                                      |
|       | 0.001961                                                               |                                      |
|       | 0.001961                                                               |                                      |
|       | 0.001961                                                               |                                      |
|       | 0.037255                                                               | 102                                  |
|       | 0.028431                                                               |                                      |
|       | 0.001961                                                               |                                      |
| Mean  | 0.006728                                                               |                                      |
| S.E.M | 0.002043                                                               |                                      |

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