Expression and function of osteopontin variants in HCV-related liver disease and hepatocellular carcinoma

Renée Jade Phillips, B. Biotech (Hons)

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Appendix 1

Solutions

0.05 % PBS-T

0.05% PBS (v/v), Tween-20 (Sigma)

0.1 % PBS-T

0.1% PBS (v/v), Tween-20 (Sigma)

12% separating gel (SDS PAGE)

12% (w/v) acrylamide/bis-acrylamide (Sigma), 0.4 M Tris (pH 8.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (Sigma), 0.025% (w/v) TEMED (Sigma)

5% stacking gel (SDS PAGE)

12% (w/v) acrylamide/bis-acrylamide (Sigma), 0.13 M Tris (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (Sigma), 0.1% (w/v) TEMED (Sigma)

6 × Loading Dye (Agarose Gel Electrophoresis)

0.3% (w/v) bromophenol blue, 0.3% (w/v) xylene cyanol, 30% (v/v) glycerol

Gill's Haematoxylin

0.2% (w/v) haematoxylin (monohydrate), 935 μM NaIO4, 26.4 mM $Al_2(SO_4)_3$-18H_2O, 25% (v/v) ethylene glycol, 2% (v/v) glacial acetic acid

PBS (phosphate-buffered saline)

1.06 mM KH_2PO_4, 155.17 mM NaCl, 2.97mM Na_2HPO_4-7H_2O, pH 7.4

RIPA Buffer

PBS, 1% (w/v) NP-40, 5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS

SDS-PAGE running buffer

192 mM glycine, 25 mM Tris, 0.1% (w/v) SDS, pH 8.3

SDS-PAGE sample buffer (2×)

250 mM Tris (pH 6.8), 4% (w/v) SDS, 10% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.006% (w/v) bromophenol blue

TAE (Tris-acetate-EDTA)

40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA

Trypan Blue

0.4% (w/v) Trypan Blue (Sigma), 0.81% (w/v) NaCl, 0.06% (w/v) KH_2PO_4, pH 7.2

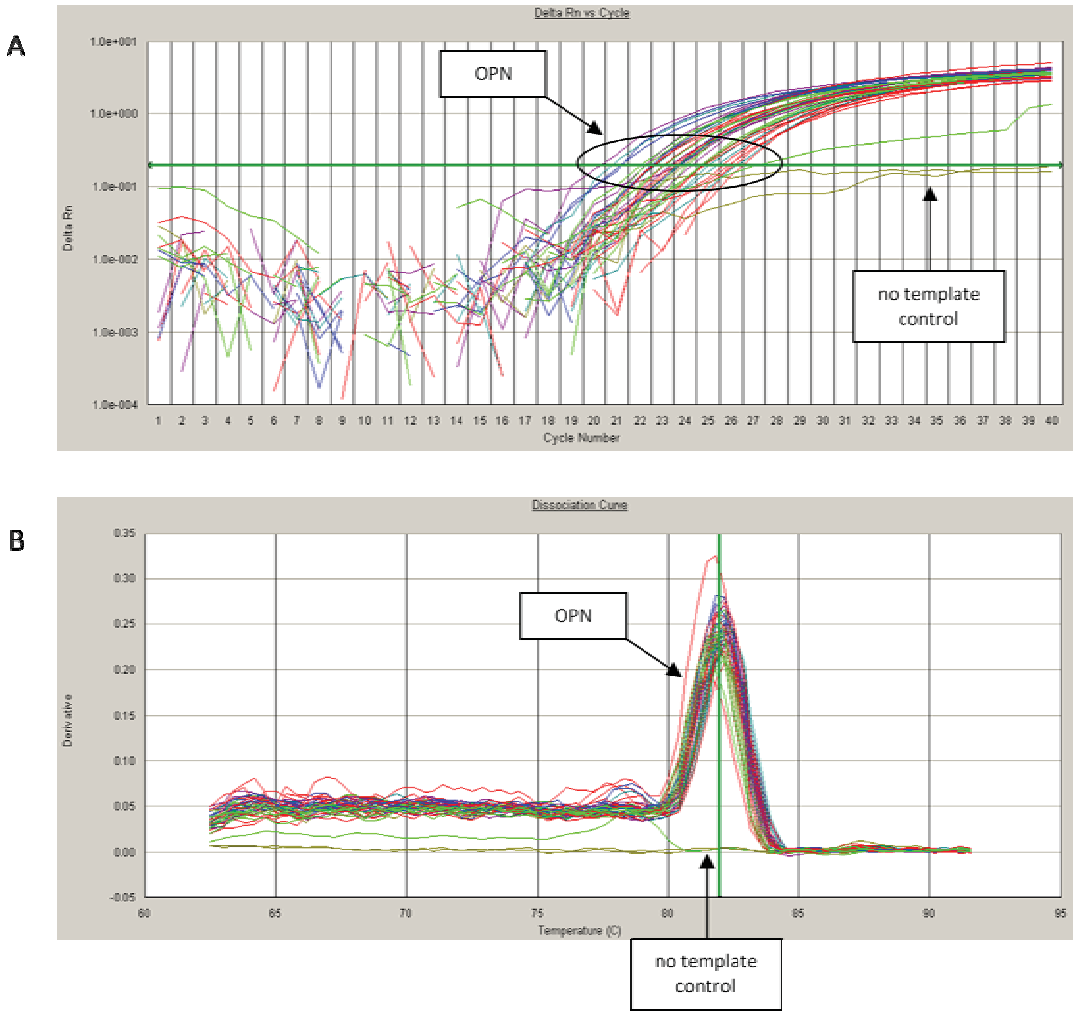
Trypsin-EDTA

0.25% (w/v) Trypsin, 0.53 mM EDTA-4Na

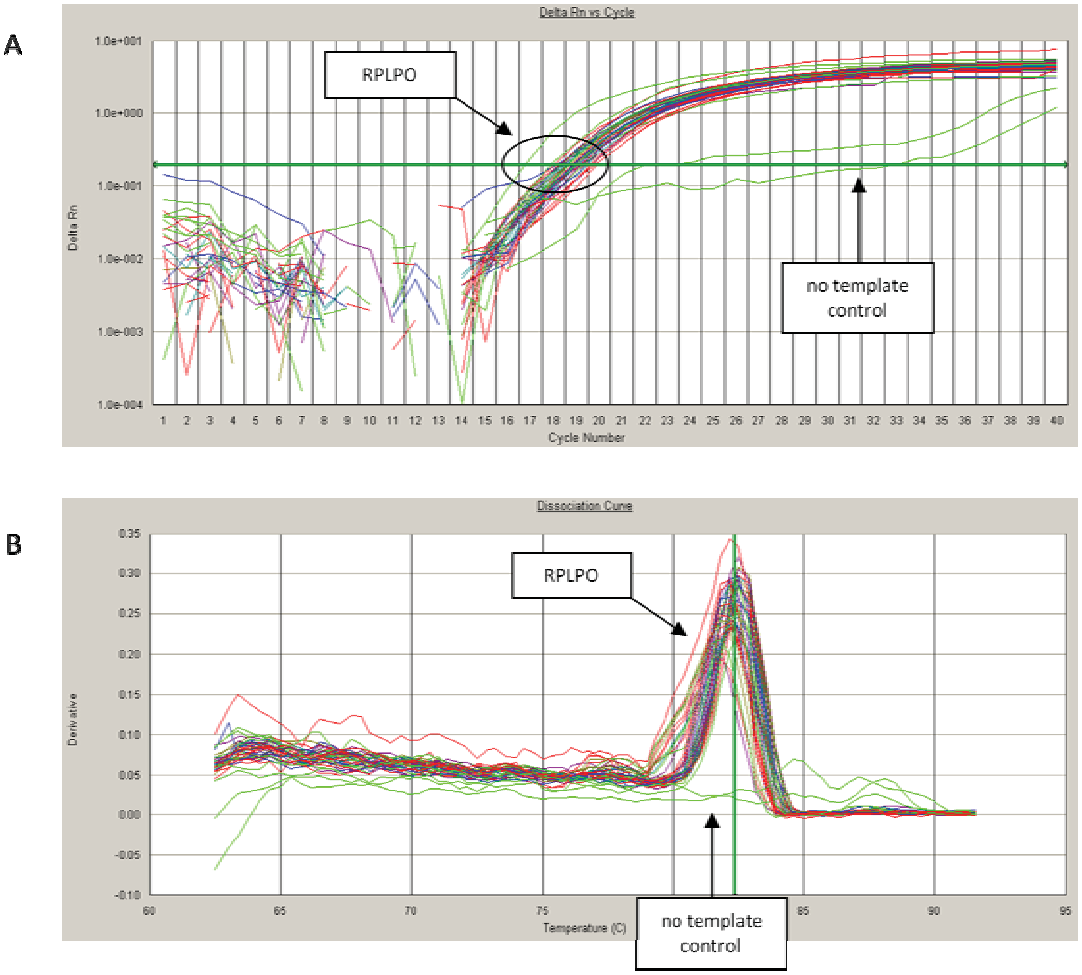
Western transfer buffer

25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3

Appendix 2



OPN amplification (A) and melt (B) curves from real-time quantitative RT-PCR on HCV-infected patient liver samples. The fixed threshold of 0.2 is shown by the horizontal green line (A) and indicates the cycle number at which each sample crosses the threshold. The green vertical line (B) indicates the observed melt temperature.



RPLPO amplification (A) and melt (B) curves from real-time quantitative RT-PCR on HCV-infected patient liver samples. The fixed threshold of 0.2 is shown by the horizontal green line (A) and indicates the cycle number at which each sample crosses the threshold. The green vertical line (B) indicates the observed melt temperature.

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Amendments to thesis based on reviewers comments

Examiner 1.

Page 68, line 2: reference to Figure 3.4 should be Figure 3.5: Figure 3.4" reference will be changed to "Figure 3.5"

Fig 4.15 and 4.16 missing (after page 97): Figures 4.15 and 4.16 will be inserted between pages 97 and 98

Page 98, fourth line from bottom: "OPN specific bands" should be "CD44 specific bands" : "OPN" should read "CD44"

Examiner 2

Chapter 1

Pg 29: Mention which parts of the protein are lacking in OPN-B and -C due to the missing exons: The following should be inserted on page 29. "The lack of exon 5 in OPN-B results in the loss of a conserved casein kinase II phosphorylation sequence (SSEE), and subsequently two serine phosphorylation sites, whereas the lack of exon 4 in OPN-C results in the loss of a single serine phosphorylation site (PDPS) and a transglutaminase (QKQ; refer to Figure 1.14)." *Current text then continues (pg 29 line 4):* "Very little investigation has been done into the differential roles of the two alternatively spliced variants and the significance of themissing exons to their structure and function; this is addressed in part in this thesis."

Have stated that Ras regulates CD44, so Ras should be included in Fig 1.17: The thesis states that Ras regulates CD44 (pg 26 third line from bottom), however Fig 1.17 and the accompanying text (pg 32 line 7) refer to pathways *downstream* of the OPN/CD44 interaction. No upstream pathways are shown or mentioned in the text relating to the figure. As Ras regulates this pathway upstream of the OPN/CD44 interaction, we don't think it would suit the figure or the accompanying text to include it.

Chapter 4

EIA usually needs 5ug/ml Ab on bottom layer for optimal results; I used a maximum of 3ug/ml – this should be discussed: Examiner doesn't provide any references for this statement. References I have found mention using 1-10 ug/ml as optimal (Abcam, Thermo Scientific, BD ELISA methods), so 3 ug/ml is within this range. *Add this new paragraph to the end of section 4.2.3 (pg 86):* "The fact that secreted OPN could not be detected in naïve Huh-7 cells may imply that the OPN ELISA developed during this study does not possess sufficient specificity. Validated ELISA methods from scientific companies suggest a capture antibody concentration of 1-10 μg/ml (Abcam, Thermo Scientific, Becton Dickinson); the maximum used in this study was 3 μg/ml. Therefore, further optimisation of the ELISA method using higher concentrations of capture

antibody may increase specificity and allow for detection of lower OPN concentrations in samples such as naïve Huh-7 conditioned media."

OPN-C might be misfolded which could explain why Ab doesn't bind in ELISA (poor conformation of the epitope). Could the missing part of OPN-C (due to missing exon) cause misfolding? *Add the following ststement on pg 86 sixth line from bottom.* "It is possible that the loss of exon 4 in OPN-C could cause misfolding of the protein, which may result in poor confirmation of the antibody binding epitope and therefore a failure of the antibody to recognise the protein.

Chapter 5

OPN may not have been detected in mouse serum due to the serum blocking the ELISA. The following statement should be added to page 115. "Human OPN levels in serum have been successfully monitored by other groups using standard ELISA methods (Kadkol et al 2006). However, the blood coagulation that occurs during serum preparation can result in cleavage of OPN by thrombin. Thrombin-cleaved OPN is not recognised by many anti-OPN antibodies; the antibodies used in this study make no mention of whether they recognise the thrombin cleaved form. Therefore, it remains possible that serum OPN has been cleaved by thrombin and therefore may not been detected by our ELISA method. Future experiments should utilise plasma rather than serum, as the use of the anti-coagulant EDTA in plasma preparation prevents thrombin cleavage of OPN."

Pg 119: Claims of increased tumor vasculature in OPN-expressing tumors should be supported by data (images) and be confirmed by CD31 staining. Claims about proliferation should be supported by ki67 and p21 stains. VEGF stains should have been done. We agree that this would enhance the thesis but time precluded these experiments. The follloewing statement will be added to Ch5 discussion: "Tumor histology revealed a higher proportion of Huh-7 cells undergoing mitosis in tumors expressing each of the OPN variants compared to control Huh-7/mock tumors. The observation of increased cell division in OPN expressing tumors correlates with our *in vitro* observations of increased cellular proliferation in hepatoma cells expressing OPN variants, however the suggested increase in proliferation could be confirmed by immunohistochemical staining with anti-proliferative markers such as ki67 or p21 or anti-angiogenesis markers such as VEGF. Also observed was a visual increase in vasculature in OPN expressing tumors. This could be due to increased blood supply simply as a consequence of tumor growth or possibly due to the angiogenic potential previously described for OPN. Whilst this observation suggests an OPN-mediated increase in tumor vasculature, further confirmation is required and could be obtained through immunohistochemical enumeration of tumor vasculature with an anti-CD31 antibody and subsequent measurement of microvessel density."

Chapter 6

Data is insufficient to label OPN as a biomarker, there are too many mentions of "biomarker" and The need to do blinded trials and measure rates of false positives/negatives should be mentioned.

At no stage do we suggest OPN is a biomarker but suggest that our data is indicative of more studies to investigate its potential.

Current text (pg 133 line 9):
"Our studies suggest that OPN may be a biomarker of HCV-related liver disease and HCC..."
Replace with:
"Our preliminary studies suggest a potential for OPN as a biomarker of HCV-related liver disease and HCC..."
Current text (pg 135 fifth line from bottom):
"... we then investigated whether OPN could be used as a biomarker of earlier stages of damage in HBV-induced liver disease."
Replace with:
"... we then investigated whether OPN was significantly expressed during earlier stages of damage in HBV-induced liver disease."
Current text (pg 137 bottom line):
"This suggests a potential role for OPN as a diagnostic biomarker for not only HCC but..."
Replace with:
"This preliminary data incorporating a small sample set indicates a potential future role for OPN in diagnosis of not only HCC but..."
Current text (pg 142 line 3):
"However, further research is required to fully investigate the diagnostic potential of OPN."
Then add:
"Investigations into OPN expression performed in this chapter should be expanded to include much larger samples sizes in the hope of identifying significant correlations between OPN expression and disease stage in a wide range of liver diseases, and should include liver conditions not discussed in this study (such as NASH, autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis). Ideally these studies should be performed blind to minimise bias. Rates of false positive and false negative results should also be determined. From these studies, ..."
Current text continues (pg 142 line 4):
"calculation of the specificity and sensitivity..."
Add this statement at the end of pg 142:
"Large-scale longitudinal studies must then be performed on individuals with liver disease from the very early stages of disease to fully investigate the effectiveness of OPN as a disease biomarker."

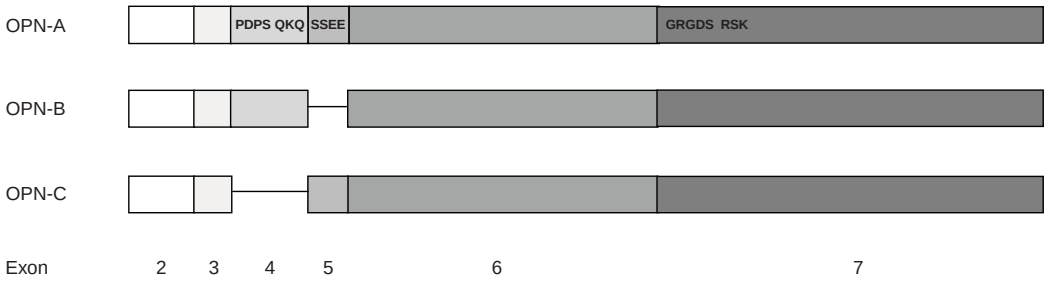

Figure 1.16 The three splice variants of OPN. OPN-A represents the full length protein, whilst OPN-B and OPN-C each lack an exon (5 and 4 respectively). The lack of these exons results in the loss of either one serine phosphorylation site (PDPS) and a transglutaminase cross-linker (QKQ; exon 4) or two serine phosphorylation sites (SSEE; exon 5). GRGDS represents the signal sequence and RSK the thrombin cleavage site.

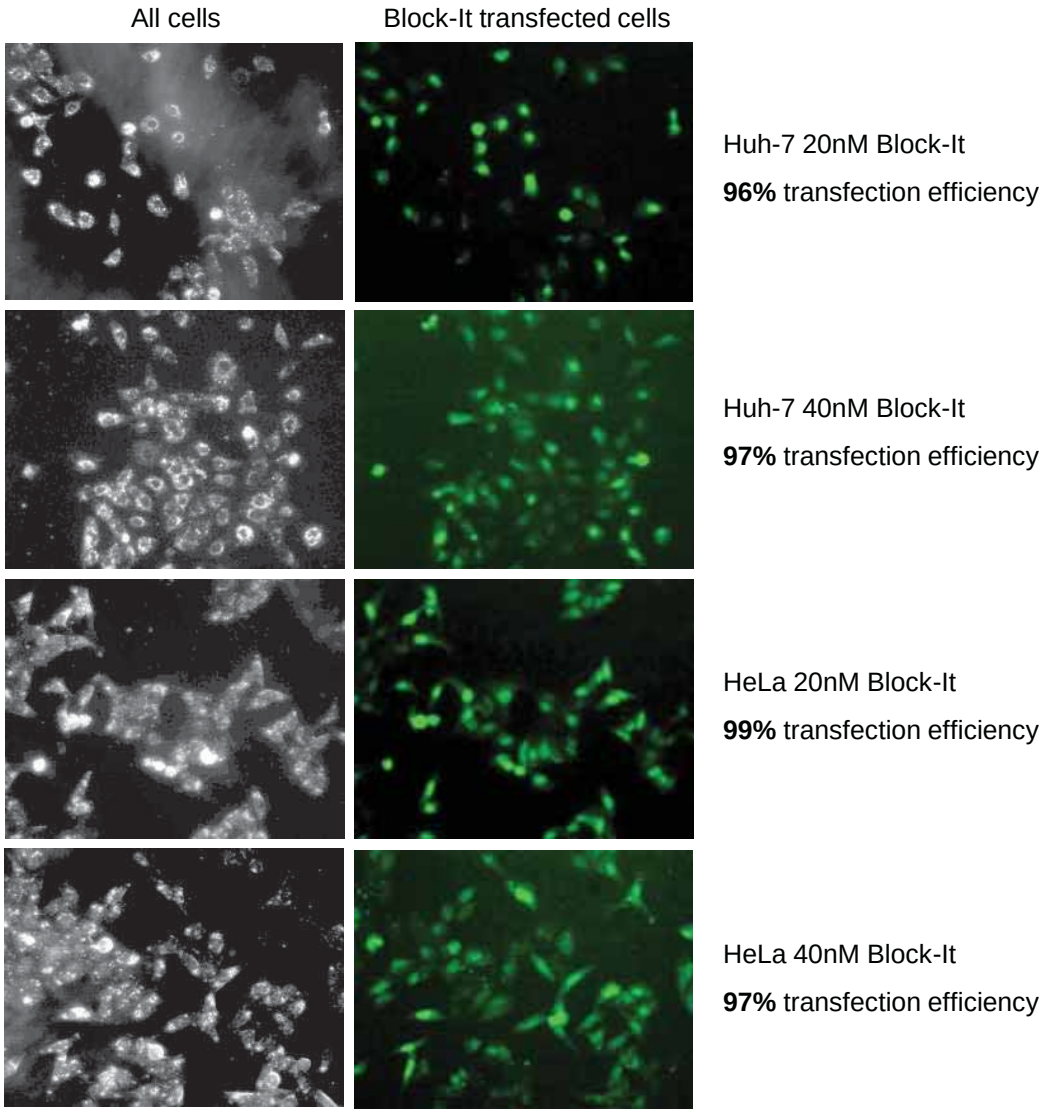

Figure 4.15 Transfection of BLOCK-iT™ Fluorescent Oligo into Huh-7 and HeLa cells allowed for the calculation of transfection efficiency of each cell line when using the standard reverse transfection method of siRNA transfection. BLOCK-iT™ Fluorescent Oligo emits its own fluorescent signal, and transfection efficiency was calculated by visual calculation of fluorescent cells compared to total cells in a field of view. Transfection with 8 nM BLOCK-iT™ gave less than 20% transfection efficiency in both cell lines (data not shown). Transfection with both 20 nM and 40 nM gave greater than 95% transfection in both cell lines.

NOTE:
This figure is included in the print copy of the
thesis held in the University of Adelaide Library.

Figure 4.16 Sequence location of the three CD44 siRNA oligonucleotides received from Invitrogen. siRNA 1 and 3 sequences bind within the constant (c) exons of CD44 and siRNA 2 binds at the junction between constant exons 1 and 2; therefore all siRNA sequences should minimise expression of all CD44 isoforms (adapted from Liu and Jiang 2006).