

**Title:** The Role of Exosomes in Uveal Melanoma

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**Background:**

Uveal melanoma (UM) is the most common primary intraocular tumour in adults. In the UK, approx. 500-600 new cases are diagnosed every year. Despite a success rate above 98% for the control of the local primary tumour, ~50% of patients develop refractory and ultimately fatal metastatic disease, predominantly in the liver. An individual's risk of developing metastasis can be interpolated by combining clinical, histopathological and genetic parameters from the primary tumour, as well as age and gender of the patient.

As UM dissemination is solely haematogenous, our group has focused on the search for blood-borne biomarkers as a dynamic measure of the development of micro-metastatic disease. Recent evidence in the literature suggests that small membrane vesicles, known as exosomes, are secreted by tumour cells into the blood and represent a valuable source of molecular biomarkers. Exosomes are small membrane vesicles (30-100 nm) formed during fusion of multi-vesicular bodies (MVB) with the plasma membrane. Exosomes have been shown to transport and transfer a range of molecules between cells, including proteins, lipids and genetic material, eliciting tumour promoting effects both locally and at distant sites.

**Aims of the PhD thesis:**

Examine the role of exosomes in primary UM

Examine metastatic the microenvironment of metastatic UM

**Objectives:**

**Year 1**

1. Bioinformatic analyses of a proteomic dataset of the UM secretome\*.
2. Compare and optimise exosomal isolation techniques: differential ultracentrifugation isolation (DUI) and polymeric precipitation (PP).

**Year 1/2**

3. Optimise the ultracentrifugation technique for the isolation of exosomes from UM; with particular attention to the purity of vesicles isolated while maintaining/improving vesicle yield.
4. Optimise methods for the characterisation of vesicles isolated by ultracentrifugation, these techniques include:
  - 4.1. Western Blot.
  - 4.2. Optimise Nanosight detection, quantification and size distribution profiling of extracellular vesicles.
  - 4.3. Transmission Electron Microscopy.

**Year 2/3**

5. Co-culture UM cell lines with hepatic stellate cells, and examine the secretome.
6. Examine metastatic UM, correlating stages of metastasis with stages of fibrosis and hepatic stellate cell activation.
7. Complete writing thesis and submit; Defend thesis at viva.

**Results:**

**Year 1**

1. Bioinformatic analyses of a proteomic dataset of the UM secretome completed and included in manuscript published in Oncotarget 2016 (see below).
2. DUI chosen as the most applicable exosome isolation technique due to lower amounts of contaminating protein.

**Year 1/2**

3. Exosomes consistently isolated from UM cell line culture media at a high yield, and were analysed for purity.
4. Optimisation of characterisation techniques completed
  - 4.1. Optimisation of western blotting achieved.  
Several exosomal and non-exosomal proteins were in whole cell lysates from UM cell lines; the sensitivity of the exosome lysates varied due to low protein yields.
  - 4.2. Extracellular vesicles from all UM cell lines were profiled consistently using the Nanosight. Size distribution curves have been generated for a number UM cell lines and this instrument is being used for the optimisation of the DUI protocol.
  - 4.3. A Technology Directorate voucher from the UoL enabled the use transmission electron microscope. Isolated UM exosomes were visualised using EM.

### **Year 2/3**

5. UM cell lines with defined immune- and molecular profiles were co-cultured with hepatic stellate cells, and the culture media examined for the secretome. This was compared to that of primary UM cells. Due to the rarity and difficulty in obtaining fresh metastatic UM cells, it was not possible to culture mUM, and examine the secretome.
6. Metastatic UM samples were obtained from the Liverpool Ocular Oncology Biobank. These were taken from consented patients, who had undergone liver surgery at the Aintree University Hospital. These were stained using a range of conventional and immunohistochemical stains to determine the degree and extent of fibrosis, and the activation of the Hepatic stellate cells. These slides were scanned in using a Leica/Aperio whole slide scanner and analysed using ImageJ. The degree of fibrosis was quantified and compared with the stage of metastatic disease.
7. Viva held January 2019. Revisions nearly complete. Following re-submission, writing up of scientific papers planned (see below).

### **Conclusions:**

Our group has demonstrated that UM cells secrete a large number of proteins and a large dataset was generated comparing the secreted proteome of normal choroidal melanocytes with UM at high and low risk of metastatic progression. Functional enrichment was performed to assess the signalling pathways associated with the UM secretome. This demonstrated association with cell growth survival and migration, as well as canonical signalling pathways linked with metastatic progression to the liver, such as mTOR signalling, RhoA signalling and hepatic fibrosis/stellate cell activation. A large component of exosomal proteins was also identified in the UM secretome. Following the pathway analysis, the level and pattern of hepatic fibrosis was assessed in metastatic UM. Histological and immunohistochemical analysis demonstrated frequent fibrosis associated with hepatic stellate cell activation. Two distinct patterns of fibrosis presented; fibrotic collagen deposition surrounding the tumour and collagen loops within the tumours. To further assess the role of exosomes in UM progression and liver metastases, methods were developed for the isolation and characterisation of UM exosomes. Extracellular vesicles in the size range of exosomes were isolated and had characteristic morphology and protein presentation. Exosomes isolated from a panel of UM cell lines by UC were subjected to proteomic analysis by liquid chromatography tandem mass spectrometry. The resulting dataset was analysed with several bioinformatic tools, and demonstrated an association with mTOR and GTPase signalling. PUM exosomes were associated with a more aggressive phenotype than MUM exosomes, as were GNA11-mutant UM exosomes compared with those from GNAQ-mutant UM. The research herein provides the rationale for further investigating UM exosomal signalling and the role of hepatic fibrosis in mUM.

**How Closely Have the Original Aims been Met:**

The two above aims have been met. The work has generated further questions, which would be interesting to address using functional studies.

**Outputs (including meeting abstracts, oral presentations, original papers, review articles) from the study in which the Pathological Society has been acknowledged:**

I have presented data from my ongoing studies at the following meetings/conferences:

- NCRI Conference, Liverpool, November 2015.
- University of Liverpool Exosome Workshop, organised by the Liverpool Ocular Oncology Research Group ([www.loorg.org](http://www.loorg.org)) and the Liverpool Lung Cancer research group.
- Institute of Translational Medicine research days, 2016, 2017 and 2018, at the University of Liverpool.
- European Ophthalmic Oncology Group meeting, Athens 2016.

Angi M, Kalirai H, **Prendergast S**, Simpson D, Hammond DE, Madigan MC, Beynon RJ, Coupland SE. *In-depth proteomic profiling of the uveal melanoma secretome*. *Oncotarget*. 2016 Aug 2;7(31):49623-49635. doi: 10.18632/oncotarget.10418.

**Publications planned:** x 3 – a) technical paper addressing improved methods for exosome isolation; b) the extracellular matrix associated with UM; c) UM exosomal content and their roles in signalling pathways.

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