Shilpa’s Olink Data Analysis Report

Approach

There were quite a lot of different conditions for these experiments so I decided to mainly make use of ANOVAs to help disentangle these conditions and find the true drivers for differential protein expression. The main aims going in were:

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| **Aim** | **Variable Name** | **Conditions** |
| Determine the effect of media fat and sugar concentration on inflammatory protein release in each cell type | Media | MO – Media Only  LFLS – Low Fat, Low Sugar  HFHS – High Fat, High Sugar |
| Determine the differences in inflammatory protein release across the cell types | Cell Origin | HO – Hepatocyte Only  H – Hepatocytes in co-culture  L – Endothelial cells in co-culture |
| To determine if there is any effect of fatty acid composition on inflammatory protein release | Fat | O – OPLA  P – POLA |

Early analysis showed that Fat composition had very minimal effect and so it was separated from Media variable. A final aim was also to examine any Media : Cell Origin interactions where cell types responded differently to changes in Media composition.

Methods

Data was kindly provided by Shilpa 😊, manipulated on Excel and analysed using the “OlinkAnalyze” package on R version 4.2.3. Firstly, to account for proteins which were present in the serum that was added to the cell media, an average was taken from samples of stock media and subtracted from our sample data for all proteins. All samples passed the Olink QC however some samples were below the limits of detection for the assay. Where >50% of the samples in an assay were below this limit the entire assay was removed, otherwise all other samples and assays were included.

For analysis of variables with multiple levels or analysis of multiple variables at once, one-way or two-way ANOVAs were performed (respectively) across all assays and used the Benjamini & Hochberg (1995) False Discovery Rate (FDR) method to adjust for multiple comparisons. Significant assays then underwent post-hoc pairwise T tests using the Tukey correction for multiple comparisons. For comparison of variables with two levels, Welch 2-sample t-tests were performed using the same FDR method to adjust for multiple comparisons. Significance was set to p < 0.05.

Results

**Principle Component Analysis (PCA)**

PCA plots were generated using the entire dataset based on relative protein concentrations. Principle component 1 (PC1) clearly identified as corresponding to Cell Origin variable and accounted for 43.38% of the total data variance (*fig.1*). Principle component 2 (PC2) was identified as Media variable and accounted for 20.96% of total data variance. (*fig. 2*).

**Cell Origin**

This variable had the biggest effect on the dataset and was investigated first. A two-way ANOVA of variables Media and Cell Origin showed that 96% of proteins measured had Cell Origin as a significant main effect whereas only 82% had Media as a significant main effect. Of these, the top ten most significant hits had the same pattern of protein expression with hepatocyte monoculture being highest, followed by hepatocyte co-culture and then endothelial cell co-culture (*fig.3*).

To assess the impact of co-culture on inflammatory protein expression a T test was carried out between hepatocytes in monoculture compared to co-culture (*fig.4*) which confirmed that hepatocytes monoculture released higher amounts of inflammatory cytokines measured however there were some which were higher expressed in the co-culture condition (CCL23, MMP-1, MMP-10, TRANCE, OPG, CXCL11, MCP-1 and CXCL1). Most of these proteins upregulated in the co-culture condition have been associated with endothelial migration and angiogenesis (PMID: 16378600, PMID: 11741951 and PMID: 31350844).

To examine the cytokine expression or hepatocytes compared to endothelial cells a T test between hepatocytes and endothelial cells both in the co-culture condition was performed (*fig.5*). As seen in the ANOVA top hits, hepatocytes had a significantly higher expression of the proteins measured compared to endothelial cells. However a few proteins were increased in endothelial cells compared to hepatocytes (CD40, PD-L1, IL-17A, OPG and IL8).

**Media**

Examination of the differentially expressed proteins for Media showed two different patterns of MO > LFLS > HFHS or HFHS > LFLS > MO as illustrated by the top 10 significant proteins in *fig. 6*. Top proteins which increased with media fat and sugar concentration were 4EBP-1, CASP-8, FGF-19, STAMPBP, ST1A1, ADA, AXIN1 and TRANCE. Many of these proteins appear to be linked to hormonal and/or metabolic triggers.

To analyse the expression of NAFLD-associated cytokines in these cells, data from Dragan’s NAFLD patients was compared to SOS and FAMOUS baseline visits (*fig.7*). 65% of the proteins measured were significantly upregulated in NAFLD compared to control, with none downregulated, and a list was made of the top significant proteins (adjusted p-value < 0.01). When T tests were performed comparing HFHS to media only and LFLS using only these top significant NAFLD-associated proteins (*fig. 8 and 9*). Whilst many of these proteins were still significantly differently expressed between the conditions there was no apparent skew towards one condition or another.

**Cell Origin : Media Interaction**

The two-way ANOVA between Cell Origin and Media also showed several instances of Cell Origin : Media interactions. Those between hepatocytes in monoculture and co-culture are shown in *fig.10 and 11*. These are often differences where proteins from one Cell Origin change with Media but do not from the other Cell Origin. For example, FGF-21 (one of the top hits) appears to decrease with increasing fat and sugar in the media for the monoculture but doesn’t change (or even increases) in the co-culture. Similarly, MMP-10 also decreases with increasing fat and sugar in the media in monoculture but doesn’t change (at least not as substantially) in the co-culture condition.

Cell Origin : Media interaction between hepatocytes and endothelial cells are shown in *fig. 12* and again they show a diverging in responses to the Media conditions between the two cell types.

**Fat**

Fat had no significant difference on protein expression across LFLS and HFHS in all Cell Origins. However, a two-way ANOVA where Cell Origin was taken into account showed that FGF-21 expression was significantly increased in response to POLA compared to OPLA in the media (*fig. 13*). All other proteins were still not significantly differentially expressed.

**Media effects within each Cell Origin**

The results described above were checked by analysis within each Cell Origin and similar results were found. For example, the proteins which were most significantly affected by Media in hepatocyte monoculture (*fig. 14*) were similar to those across all Cell Origins affected by Media.

Conclusions

Hepatocytes release more inflammatory cytokines than endothelial cells and hepatocytes in a co-culture with endothelial cells have lower cytokine release than hepatocytes in a monoculture. However, hepatocytes in co-culture do upregulate the expression of some proteins and these are often associated with angiogenesis and endothelial cell migration.

Whilst almost all proteins are differentially expressed between Cell Origins, Media also has a large effect on protein expression. Proteins were generally either up- or down-regulated with increasing fat and sugar in the media in a dose-dependent manner. However, increasing media fat and sugar did not selectively upregulate NAFLD-associated proteins potentially suggesting a lack of model specificity in the context of NAFLD inflammatory markers. OPLA and POLA had no effect on inflammatory protein expression except for with FGF-21 which was upregulated in POLA compared to OPLA.

Challenges of this analysis was trying to extract meaningful information from the list of proteins that were differentially expressed as there were not enough for conventional gene ontology or pathway enrichment analysis. This meant that in many comparisons proteins associated with certain pathways would be both up and down regulated and there was little ability to determine the greater effect.

Although the comparison between Cell Origin was very useful and informative, hopefully analysis of more “aggressive” NAFLD *in vitro* models may show a clearer increase in inflammatory protein release in response to the Media conditions.