A comparison of proteins and their expression levels within different regions of multi-cellular spheroids (MCTS)

Kelly McMahon
The tumour microenvironment

- Due to inefficient blood supply within tumours a ‘microenvironment’ is established.

- This occurs between 150 and 200 µm from a blood vessel (BV).

- Characteristic features of this include:
  - Hypoxia
  - Acidic extracellular pH
  - Low nutrient status
  - High levels of catabolites
  - Low cell proliferation rates
  - Biochemical adaptations
  - Beyond a certain distance, necrosis or cell death occurs.
Comparison of spheroid versus tumour

Histology * ECM * Cell adhesion
Cell signaling * Response to therapy

(Adapted from Kunz-Schughart, 1999)
Spheroid Separation

(Sutherland, Mccredie and Inch, 1971)
Spheroid Separation
Spheroid Separation
Spheroid Separation

(Method adapted from Knowles and Phillips, 2001)
Validation of spheroid separation

Carbonic anhydrase- 9 (CAIX) staining
Validation of spheroid separation

Viable rim

Necrotic core

Viable rim + necrotic core

FACS Analysis – Viable rim and necrotic core cells
Proteomics approach

Cell lysis
- Sonication in the presence of lysis buffer
  - Protein isolation and purification: acetone precipitation
  - Protein concentration determination: Bradford assay

Reduction and Alkylation
- 50mM dithiothreitol (DTT) and 100mM iodoacetamide (IAA)
  - Trypsin Digestion
- 114, 115, 116, 117

ITRAQ

OffGel Fractionation

MALDI fraction collector

Dionex RP nano HPLC system

Bruker Ultraflex II MALDI-TOF-TOF

Agilent 6630 Accurate-Mass Q-TOF LC/MS

Agilent 1260 HPLC-Chip system
Proteomics results

Under and over expressed levels were those normalized ratio’s which deviated plus or minus 1 standard deviation from the mean for that reporter ion, respectively. (Proteins found differentially expressed with both instruments)
Glycolysis/Gluconeogenesis

Glycolysis

- glucose
  - glucose-6-phosphate
    - fructose-6-phosphate
      - fructose-1,6-diphosphate
        - glyceraldehyde-3-phosphate
          - 1,3-diphosphoglycerate
            - 3-phosphoglycerate
              - 2-phosphoglycerate
                - phosphoenolpyruvate
                  - Pyruvate

Gluconeogenesis

- S-acetyl-dihydriodippeptide
  - 2-(1-hydroxyethyl)thiamine diphosphate
    - EC 1.2.4.1 pyruvate dehydrogenase
      - EC 2.3.1.12 dihydrolipossylase-residue acetyltransferase
        - acetyl-CoA
        - EC 1.2.4.1 pyruvate dehydrogenase
          - EC 4.1.1.1 pyruvate decarboxylase
HS-1-associated protein X-1 (HAX-1)

- Localised HAX-1 staining in the spheroids viable rim.

<table>
<thead>
<tr>
<th></th>
<th>114/115 Intermediate region</th>
<th>114/116 Hypoxic region</th>
<th>114/117 Necrotic core</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>2.03</td>
<td>1.64</td>
<td>2.71</td>
</tr>
</tbody>
</table>

- Western blot analysis revealing a band in the viable rim sample only.

- These results both supporting the over expression of HAX-1 in the spheroids viable rim as first indicated by the proteomics analysis using iTRAQ.
Conclusions

• The ability to fractionate spheroids into different regions for proteomics analysis, has been demonstrated
• Well established markers for hypoxia were up-regulated in the hypoxic region
• Changes occurring in the necrotic core are currently under investigation
• Proteins not previously association with hypoxia or cancer were shown differentially expressed
Ongoing work

- Gene expression microarray analysis
- Continue validation of target proteins by Western blotting and immunohistochemistry
- Proceed with siRNA and functional assays for selected proteins
Acknowledgements

Chris Sutton
Roger Phillips
Sham Naal
Joachim Thiemann