New approaches for non-invasive embryo quality assessment

Tours, France

April 11-12 2008

Embryology in the era of proteomics

Roger Sturmey
Department of Biology
THE UNIVERSITY of York
rgs102@york.ac.uk

- Background
- Definitions
- Methods
- Specific challenges
- Progress
- The next phase…

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Context – *The UK perspective*

- Average age at which UK women have their first child is ~30
- One in 8 UK couples subfertile
- 40% of hospital gynaecology workload is in treatment of subfertility
- 30 000+ cycles of IVF per annum (~£3000; ~€4000)
- One in 80 UK babies is now conceived by IVF
- 'Take-home baby rate' – approx. 23%

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Multiple births

- High risk of multiple births associated with ART
  - *Multiple embryos transferred*
- US take-home baby rate is 30%, but more embryos are transferred leading to a higher multiple birth rate
- Annual cost of caring for IVF multiple births in Europe and the US exceeds $1.3 billon!
- '*Multiple births are now the biggest challenge facing assisted reproductive technology*
  Adamson & Baker (2004) *Fertil Steril* **81**, 517

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- Still birth and neonatal deaths per thousand birth events

Singleton	9.9
Twin	43.8
Triplet	59.6

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Move toward Single Embryo Transfer (SET)

Requires methods to select 'best' embryos

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Ideal criteria for an embryo test

- Non invasive
- Sensitive
  - Distinguish between individual embryos
- Simple
- Objective
- Robust
- Consistent
- Reliable
- Provide diagnostic information additional to embryo morphology

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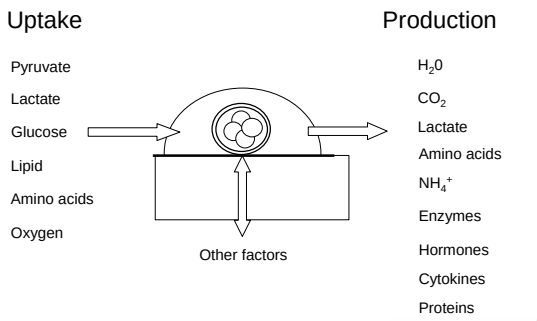
Factors which may form the basis of a non-invasive test of embryo viability



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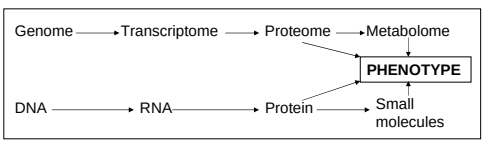
The Proteome

- Entire protein complement within a cell, arising from translation of the genome
  - Isoforms
  - PTMs



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Proteomics defined

… the large-scale study of proteins, particularly their structures and functions.
(Wikipedia)

The comprehensive study of proteins and their functions.
plan2005.cancer.gov/glossary.html

An emerging field of science that focuses on the multitude of tasks assigned to proteins churned out by our genes.
ww.mercksource.com/ppdocs/us/cns/harvard-health-reports/MerckSHR-healthyliving092906/sections/glossary.htm

The study of the proteome. Any global analysis of changes in the quantities and post-translational modifications of all the proteins in cells taking genome sequence as the starting point. ...
www.proteome.med.umich.edu/what_we_do/glossary.html

A branch of biotechnology concerned with applying the techniques of molecular biology, biochemistry, and genetics to analyzing the structure, function and interactions of the proteins produced by the genes of a particular cell, tissue, or organism, with organizing the information in databases ...
www.tripathimaging.com/patients/glossary.html

…is the study of the set of proteins produced (expressed) by an organism, tissue, or cell, and the changes in protein expression patterns in health and disease, or in different environments and conditions.
www.esainc.com/resources/glossary

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Can use proteomics for 3 types of biological question;

1. Generation of protein-protein linkage maps
2. Annotation and correct identification of genomic DNA sequence
3. 'Quantitative' analysis of protein expression as a function of cellular state/function

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Uses

- Identification of BIOMARKERS
  - "…hallmarks for the physiological status of the cell/organism at any given time and change during the progression of disease."
- Ideal biomarker will be
  - disease specific
  - easily detectable with minimum invasion
  - present at the earliest stages of disease
- Focus has been on the protein within blood/body fluids
- Proteomic methods useful in screening and identifying candidate biomarkers

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Proteome properties (problems?)

- ~25-30k genes encode for ??? proteins/peptides (millions?)
- Dynamic
  - A snapshot in time
- Diverse chemical/physical/structural properties
- Modified according to functions
  - PTMs
- Expression varies over 5-10 orders of magnitude
- Easily disrupted
  - Particularly during handling

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- Protein harvest is of key importance in good proteomic study

Ideally want to solubilize all proteins, including hydrophobic

Prevent protein aggregation

Avoid altering protein structure or chemistry

Completely remove all nucleic acids

Harvest sufficient protein for study

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Sample prep “rules”

- Minimise environmental contamination
- Minimise sample preparation to maintain sample size
- Keep cold
- Process as fast as possible
- Remove salts/contaminants
- Minimise/avoid unwanted processing
  - Protease activity/proteolysis
  - Chemical modification

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Step 1 - *LYSIS*

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Cell lysis

- Not required in all situations
- Often includes
  - Osmotic lysis
  - Rpt freeze/thaw
  - Enzymatic methods
  - Sonication

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Contaminants and how deal with them…

Contaminant	
Nucleic acids	Ppt protein DNase/RNase treatment Sonicate Extraction – Phenol/chloroform
Lipid	>2% detergent Ppt protein
Polysaccharides	Specific enzymatic action Ppt protein
Solid material	Centrifugation
Salts	Dialysis Ppt proteins Electrophoretic methods

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- Composition of lysis buffer varies between experiments and must be optimised
- Usually uses

Chaotropic agents – *e.g., Urea, thiourea. Disrupts hydrogen bonds usually used at 8M*

Detergents – *disrupt hydrophobic interactions and increases protein solubility. Often used for membrane proteins. Must be non ionic (eg octylglucoside) or zwitterionic (eg CHAPS)*

Reducing agents – *e.g., DTT. Disrupts disulphide bonds*

Rehydrates the IPG strip

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Step 2 – *PROTEIN SEPARATION*

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2D Gel electrophoresis

- Protein requires denaturing
  - Native protein exists in various conformations
  - Not always enter gel
  - Protein-protein interactions
  - Ease of matching pI/MW without tertiary/quaternary structure
- Function of urea
  - Thiourea used for v. hydrophobic proteins
  - Must avoid carbamylation (keep cool)

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- Separate the proteins systematically
- Most reliable method is 2-D polyacrylamide gel electrophoresis (PAGE)
- Electrophoresis in 2 dimensions
- Firstly, proteins are separated by isoelectric focussing, based on the pI value
  - pI is the pH at which a protein carries no charge and so does not migrate in an electrical field
- This is usually within the range of pH 3-12
  - Proteins beyond this range require separate processing

FIRST DIMENSION SEPARATION

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- Proteins have distinct chemical and physical properties relating to the amino acid structure
  - Amphoteric
- Move within an electrical field
- When net charge = 0, protein is at its isoelectic point
  - Stops migrating
- Use *Immoblised pH gradient strips*
  - Weakly acidic and basic buffers bonded to ploayacrylamide gel to give pH gradient

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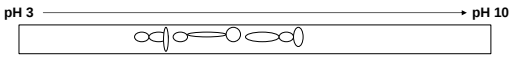
Two-Dimensional gel Electrophoresis (Iso-Electric Focusing)



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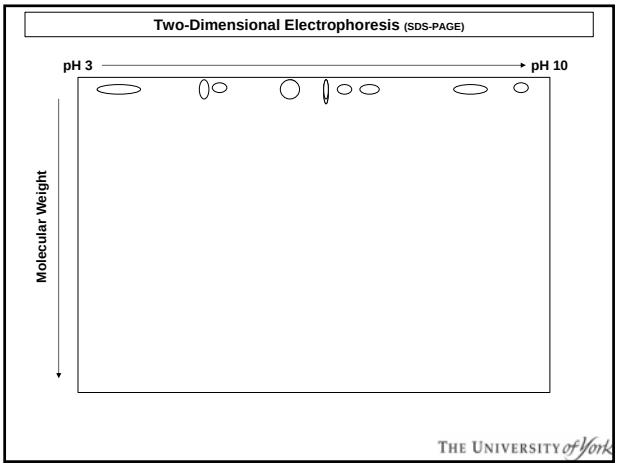
Second dimension

- Separates proteins according to molecular weight, using SDS-PAGE.
- Proteins from IEF applied to 2nd D gels
- Pores in the 2nd D gel 'sieve' proteins since dodecyl sulfate coats proteins according to mass
- The lighter the mass, the further the migration

•The second D gel can consist of homogenous single % gel or a gradient gel

•Single % gel give excellent resolution of proteins in a narrow Mr range

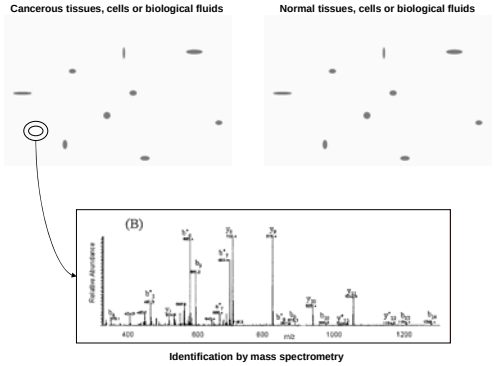
•Gradient gel allows larger range of proteins to be separated by decreasing the pore size



Detection of proteins

- Proteins are visualised by staining the gel
- Coomassie Blue
  - Requires 40ng total protein
- Fluorescent stains - more sensitive
- Silver staining is most sensitive
  - up to 100x more sensitive than coomassie blue
- Images collected by specialised equipment and spot profiles compared by image analysis software

Two-Dimensional Electrophoresis (Mass spectrometry)



Identification by mass spectrometry

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2D gel advantages/disadvantages

- Well established
- Visual
- MW and Isoelectric point
- Comparison is possible
- Databases exist
- Inexpensive

- Time and labour intensive
- Difficult
- Cannot display entire proteome
  - Limited dynamic range
  - Under-representation of low abundance proteins

REQUIRES LARGE AMOUNTS OF STARTING MATERIAL – LIMITED APPLICATION FOR EARLY EMBRYO SAMPLES

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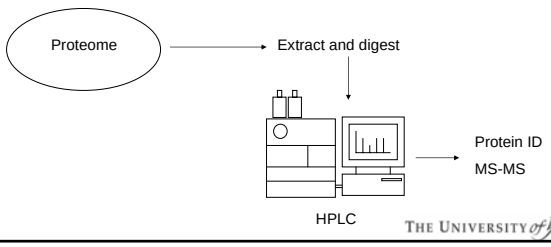
Shotgun proteomics

- Based on HPLC
- No prior protein purification/2D gel



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Shotgun advantages/disadvantages

- No 2-D gels
- Counteracts solubility issues
- Automated
- Dynamic range
- Sensitive
- Quantitative data possible

- No 2-D gels
- Only useful with sequenced genome
- LC
- Vast amounts of information
- Data analysis/ bioinformatics very time consuming

CAN USE SMALLER AMOUNTS OF STARTING MATERIAL – *MAY* BE APPLICABLE TO EMBRYO-BASED SAMPLES

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Next stage is protein identification

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Digestion

- Protein ID
  - Misnomer; peptides identified
- Uses 'controlled' proteolysis to produce mixture of peptides
  - Trypsin – *cleaves C-terminal of Arg and Lys when not followed by Pro*
  - Chymotrypsin – *cleaves C-terminal of Trp, Tyr and Phe when not followed by a Pro*
- Arg and Lys occur every ~10 residues

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ID of proteins

- Spots cut out of gel and placed into plates
- Spots de-stained and digested from gel by automated robotic systems
- Proteins identification
  - time of flight (TOF) of ionised for of protein
  - ion trapping

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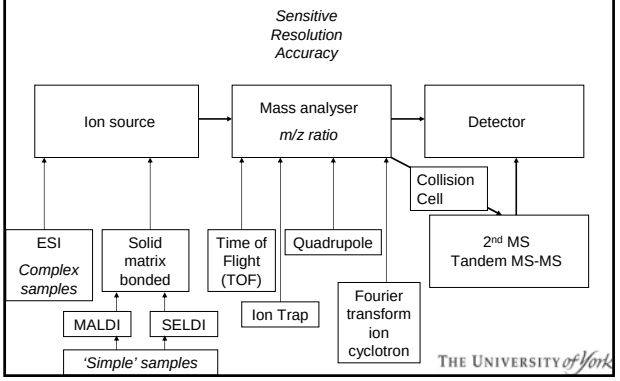
Mass Spec



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Ionisation

- Two main types of ionisation
  - Electrospray Ionisation (ESI)
    - Ionises analytes out of a solution
    - Coupled to LC
    - Suitable for complex mixtures
  - Matrix-Assisted Laser Desorption/Ionisation (MALDI)
    - Laser sublimates dry sample out of a crystalline matrix
    - Appropriate for relatively simple peptide mixtures
    - Can use Surface Enhanced matrices (SELDI)
- MS of whole proteins less sensitive than for peptides

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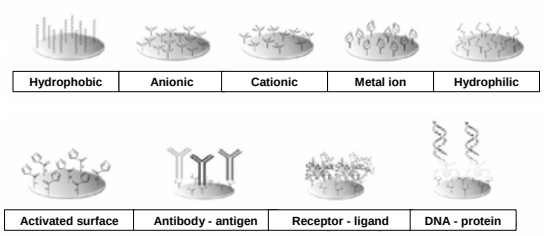
SELDI (Surface Enhanced Laser Desorption / Ionization) [1]



Issaq *et al.*, Biochemical and Biophysical Research Communications, 2002

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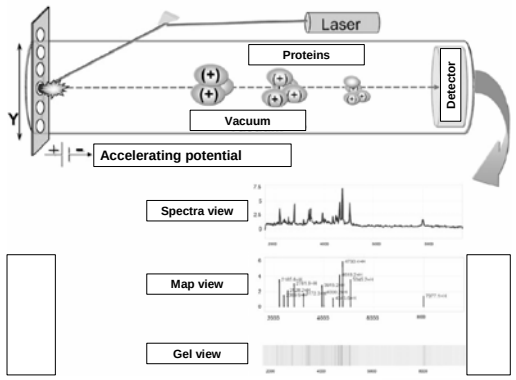
SELDI (Surface Enhanced Laser Desorption / Ionization) [2]



Issaq *et al.*, Biochemical and Biophysical Research Communications, 2002

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Mass Analysers

- Detect and measure mass:charge (*m/z*) ratio
- Four main types
  - TOF
    - Ions accelerated to high energy and separate according to different velocities
  - Quadrupole
    - Select by varying electric fields giving a stable trajectory for ions of desired *m/z*
  - Ion Trap
    - Captures ions via electrical excitation, which are then released according to specific *m/z* to produce a mass spectrum
  - Fourier Transform
    - Captures ions similar to above, using strong magnetic fields

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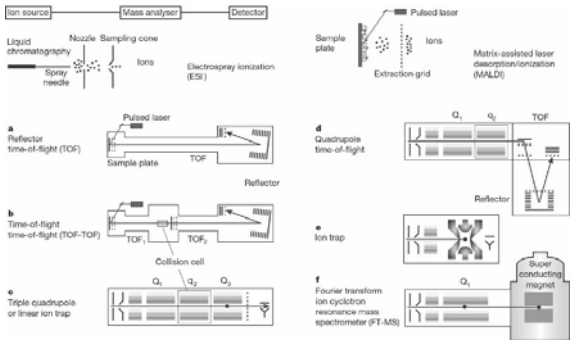

Aebersold & Mann, Nature, 2003

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Word about databases

- *De novo* peptide sequencing can be carried out for small data sets
  - *Requires operator expertise*
- Vast data needs to be matched to database
  - MS only gives *m/z* ratios – not protein or peptide identification
- Amino acid sequence can be obtained and matched to expected and pre-identified sequences
  - Only applicable to organisms whose entire genome is sequenced.

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Proteomics Vs Genomics

- Proteomic experiments more complex and technically demanding than genomics
  - PCR can amplify DNA – *no proteomic equivalent*
  - Genomic methods and technology more established
  - Gene = gene – *splicing or translational variation of same gene can lead to different protein isoforms*
- Genomic material is fixed; *protein levels can range more than 10 o.m.*
- *The relatively low number of human genes generates an enormous and highly complex proteome!*

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General challenges

- No method capable of identifying and quantifying complex protein mixtures in a simple, single step
- 2D approaches have consistently identified same proteins
  - Limited dynamic range – *getting better!*
- Relationship between analyte abundance and measured signal is complex
  - Quantification difficult

No complete 'proteome' yet analysed – without a suitable reference point this may never be achieved

How would you know????

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…and the embryo?

- To date only a very few reports where full proteomic approaches have been applied to the early embryo

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BIOLOGY OF REPRODUCTION 71, 1533–1539 (2004)
Published online before print 30 June 2004.
DOI 10.1095/biolreprod.104.030304

Protein Patterns of Pig Oocytes During In Vitro Maturation[1]

Zdenka Ellederova,[3] Petr Haladа,[4] Petr Man,[4] Michal Kubelka,[3] Jan Motlik,[3] and Hana Kovarova[2,3]
Institute of Animal Physiology and Genetics,[3] Academy of Sciences of the Czech Republic, 277 21 Libechov, Czech Republic
Institute of Microbiology,[4] Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic

- Proteomic analysis of groups of 200-500 pig oocytes
  - 2D minigels, MALDI AND MS-MS approaches
- Only able to ID 35 protein spots
  - Pig genome incomplete
- Of these, 18 spots represented single protein; some proteins found in numerous spots
- Comparison of "proteome" of oocytes at different stages
  - 6 proteins appear to alter abundance during maturation

- Encouraging – however, too large a sample required
- Probable underestimation of low-abundance protein

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A proteomic analysis of mammalian preimplantation embryonic development

Mandy G Katz-Jaffe, Donald W Linck, William B Schoolcraft and David K Gardner
Colorado Center for Reproductive Medicine, 799 E Hampden Ave, Suite 520, Englewood, Colorado 80113, USA
Correspondence should be addressed to M Katz-Jaffe; Email: mkatz-jaffe@ccrcdocs.com

- Groups of 5 murine embryos bound to protein chips
  - SELDI approach, anionic and cationic chips *m/z* range of 1-20kDa
- Obtained spectra
  - Identities purely on *m/z* ratios of proteins; no sequencing
- Reported differences between embryo stages and embryos grown under 5% Vs. 20% oxygen
  - ~40 potential biomarkers, based on different expression
- Not truly quantified
- Again encouraging
  - Data suggests potential biomarkers which could be investigated further by "conventional" methods

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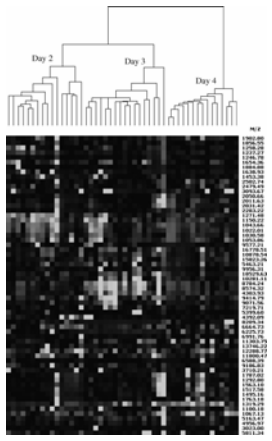

Katz-Jaffe *et al* 2005

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Volume 85, Issue 1, Pages 101-107 (January 2006)

Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability

Mandy G. Katz-Jaffe, Ph.D., David K. Gardner, Ph.D., William B. Schoolcraft, M.D.

- SELDI-TOF approach for single HUMAN freeze/thawed blastocysts
- Proteome analysed based on molecular weight using single MS approach
  - Candidate protein identities based on *m/z* ratios, with no sequencing
- Observed differences in profiles between early, expanded and degenerated blastocysts
  - Used "quasi"-quantitative approach, based on signal normalisation
- Suggested identities for 6 proteins observed
  - Caution as based purely on *m/z*!
- Interesting approach again for the discovery of potential biomarkers, but requires follow up work

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CLONING AND STEM CELLS
Volume 8, Number 4, 2006

Molecular Analysis of Maturation Processes by Protein and Phosphoprotein Profiling during *In Vitro* Maturation of Bovine Oocytes: A Proteomic Approach

MONIKA BHOJWANI,[1] EBERHARD RUDOLPH,[2] WILHELM KANITZ,[1] HARTMUT ZUEHLKE,[1] FALK SCHNEIDER,[1] and WOLFGANG TOMEK[1]

- Performed 2D gels on groups of 400 bovine oocytes
  - Stained for total and phosphorylated protein
- Spots cut and tryptically digested for identification by MALDI-TOF
  - ID based on peptide mass fingerprinting (theoritcal mass, isoelectric point, sequence coverage and cross species matching
- Obtained protein maps

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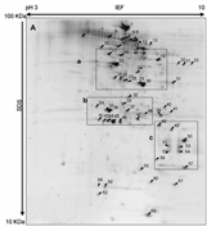
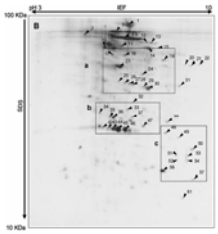


'Total' protein



'Phosphorylated' protein

- 550 protein spots found in total, of which 60 were identified
- Majority = cytoplasmic protein
  - May indicate loss of membrane protein during handling?
- Looked at PTMs – increased by ~20% during IVM
- Quantification based on protein abundance (from gel) not MS signal
- Suggested 3 potential markers of maturation status – one matched Ellederova study (PDX2)

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Available online at www.sciencedirect.com
ScienceDirect
Toxicology in Vitro 21 (2007) 521–526

Two-dimensional electrophoresis of protein from cultured postimplantation rat embryos for developmental toxicity studies

Makoto Usami [a,*], Katsuyoshi Mitsunaga [b], Ken Nakazawa [a]

[a] Division of Pharmacology, National Institute of Health Sciences, 1-23-1, Kamiyoga, Setagaya, Tokyo 158-8501, Japan
[b] School of Pharmaceutical Sciences, Toho University, Japan
Received 6 January 2006; accepted 10 November 2006
Available online 17 November 2006

- Produced a protein list based on 2D gels from day 10.5 and 11.5 rat embryos
- 81 proteins identified
- Protein resource

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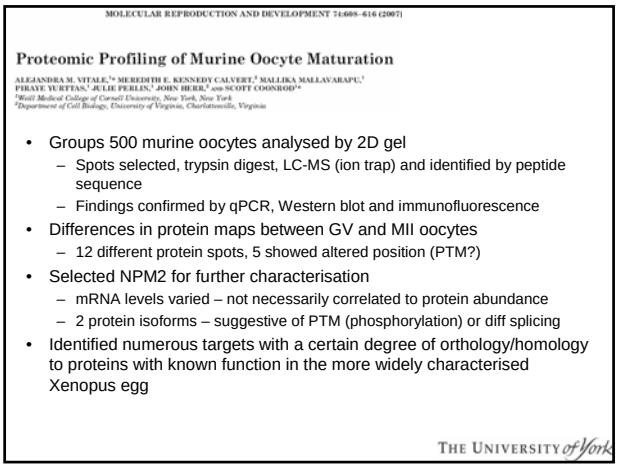

MOLECULAR REPRODUCTION AND DEVELOPMENT 74:600–616 (2007)

Proteomic Profiling of Murine Oocyte Maturation

ALEJANDRA M. VITALE,[1]* MEREDITH E. KENNEDY CALVERT,[2] MALLIKA MALLAVARAPU,[1] PIRAYE YURTTAS,[1] JULIE PERLIN,[1] JOHN HERR,[2] AND SCOTT COONROD[1]*
[1]Weill Medical College of Cornell University, New York, New York
[2]Department of Cell Biology, University of Virginia, Charlottesville, Virginia

- Groups 500 murine oocytes analysed by 2D gel
  - Spots selected, trypsin digest, LC-MS (ion trap) and identified by peptide sequence
  - Findings confirmed by qPCR, Western blot and immunofluorescence
- Differences in protein maps between GV and MII oocytes
  - 12 different protein spots, 5 showed altered position (PTM?)
- Selected NPM2 for further characterisation
  - mRNA levels varied – not necessarily correlated to protein abundance
  - 2 protein isoforms – suggestive of PTM (phosphorylation) or diff splicing
- Identified numerous targets with a certain degree of orthology/homology to proteins with known function in the more widely characterised Xenopus egg

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ScienceDirect

BBA

The protein profile of mouse mature cumulus–oocyte complex

Yan Meng[a,b], Xiao-hui Liu[a,b], Xiang Ma[b], Ya Shen[b], Lu Fan[b], Jing Leng[a], Jia-Yin Liu[a,b,*], Jia-Hao Sha[a]

- 2000 mouse oocytes used for protein extraction
  - 2DE, proteins stained with silver stain and coomassie blue
  - Spots cut, trypsin digest and analysed by MALDI-TOF
  - Proteins Id'ed on peptide fragment mass, p*I* and theoretical MW
- >2000 spots showed silver stain
- >700 spots stained with CB
  - Most abundant proteins
- 259 identified – many showed evidence of PTM
- Generated "protein profile" to be used as a resource.

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- Invasive proteomic profiling can only ever be a research tool – aiding in the discovery of potential biomarkers
  - Require additional validation
- Challenges – can't amplify
  - Embryos are small – any criteria aimed as selecting embryos developmental potential *must* be applicable to individual embryos!
- No set sequence
- Dynamic range – 8-10 orders of magnitude
- 'Status' of protein – Free/bound/modified?

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So where for proteomics and embryology?

- Focussed approach
  - Specific aspects of proteome – *less ambitious (glycosylome/phosphorylome)*
- Use for discovery science
  - Biomarker discovery
  - Additional, independent validation
- More basic "proteomics"
  - Less ambitious – investigate specific proteins of interest, using hypothesis-driven approaches based on existing and newly acquired knowledge
- Protein(s) may form yet the basis of marker of viability
- Protein(s) added to culture medium may help improve developmental potential

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- The comprehensive study of proteins and their functions.

plan2005.cancer.gov/glossary.html

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Name	Symbol	Embryo culture	Target effect	Reference
Platelet-activating factor	PAF	Human	↑ pregnancy rate	O'Neill et al., 1980
Transforming growth factor α	TGFα	Mouse	Blastocyst development	Paria and Dey, 1990
Epidermal growth factor	EGF	Mouse	Blastocyst development	Paria and Dey, 1990
	EGF	Bovine	↑ blastocyst rates	Lonergan et al., 1996
Platelet-activating factor	PAF	Mouse	Higher mitotic index in blastocyst	Roberts et al., 1993
Leukaemia inhibitory factor	LIF	Mouse	↑ hatching rate and trophoblast outgrowth	Lavranos et al., 1995
Insulin-like growth factor-binding protein 3	IGFBP-3	Mouse	↑ hatching	Lai et al., 1996
Insulin-like growth factor 1	IGF-1	Human	↑ inner cell mass number	Lighten et al., 1998
Granulocyte-macrophage colony-stimulating factor	GM-CSF	Mouse	Blastocyst development	Sjoblom et al., 1999; 2005
Leptin	Ob	Mouse	Blastocyst development	Kawamura et al., 2002
Oviduct-specific glycoprotein	OVGP1	Porcine	Enhanced cleavage and blastocyst formation	McCauley et al., 2003
Complement component 3	C3	Mouse	↑ hatching rate	Lee et al., 2004
Hyaluronic acid or heparin	HA/HP	Porcine	↑ blastocyst rates	Kim et al., 2005
Demilune cell and parotid protein	DCPP	Mouse	Blastocyst development	Lee et al., 2006

Lee & Yeung, 2006, Hum Fert.

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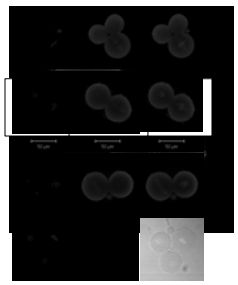
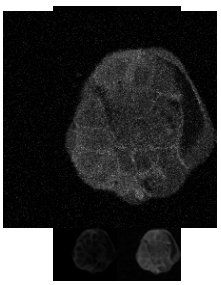
“Proteo-cytomics”

Expression, activity and localisation of protein





- Interaction between Creatine Kinase and spindles
- Interaction between CK and Na^+/K^+ATPase

KE Forsey et al, in preparation

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Secretome

- Search for markers secreted into the culture environment
- Attractive idea
- sHLA-G one example

Caution

- Need to ensure that protein/factor is produced in measurable quantities
- Needs to be easily measured
- Needs to relate to developmental potential!

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Functional genomics

- Unlike genomics and proteomics, functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein-protein interactions, as opposed to the static aspects of the genomic information such as DNA sequence or structures.

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Conclusions

- Need for selection
- Proteomic methodology
- “Full” proteomic studies
- Challenges
- FUTURE????

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Prof Henry Leese
Dr Katherine Forsey

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Embryology in the era of proteomics

Roger Sturmey
Department of Biology
THE UNIVERSITY of York
rgs102@york.ac.uk