Characterization of secreted proteins of 2-cell mouse embryos cultured in vitro to the blastocyst stage with and without protein supplementation

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Abstract

Purpose To identify the secreted proteins of murine embryos grown in vitro.

Methods Two-cell mouse embryos ($n=432$) were randomly allocated to culture to the blastocyst stage in protein-free and in protein-supplemented (3 % BSA) media. Proteins were separated by SDS-PAGE; bands were visualized by

coomassie staining, followed by in-gel trypsin digestion and liquid chromatography-tandem mass spectrometry. RT-PCR and confocal microscopy were used to confirm gene/protein expression in blastocysts.

Results Of all individually identified proteins, 34 and 23 were found in embryos cultured without and with BSA, respectively, and 20 were common. Identified proteins having an N-terminal secretory sequence or transmembrane domains located on the extracellular backbone were postulated as secreted proteins. Gene and protein expression for two selected molecules were confirmed. Functional analysis revealed over-represented processes related to lipid metabolism, cyclase activity, and cell adhesion/membrane functions.

Conclusions This study provided evidence to further characterize secreted proteins by mouse embryos grown from the 2-cell to the blastocyst stage in vitro. Because of homology between murine and human, these results may provide information to be translated to the clinical setting.

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Tanya Burch and Liang Yu have equal contribution to this manuscript.

Capsule Proteomic analysis was performed on conditioned media of murine blastocysts cultured to the blastocyst stage.

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Introduction

Assisted reproductive technologies (ART) provide infertile couples with a unique means to achieve a pregnancy. The discovery of a non-invasive method to identify biomarkers of embryo development during the in vitro culture period could significantly contribute to the optimization of ART by allowing (i) an improved selection of embryos with highest implantation competence for uterine transfer; and (ii) a concomitant reduction of the number of embryos transferred per attempt, which should significantly decrease the medical

complications and economic burden to the health system determined by the occurrence of multiple gestations.

It has been speculated that early embryo secretions analyzed from the culture medium can be detected and quantified by novel biochemical-proteomics methods with high sensitivity and specificity, providing information about embryo growth, developmental and implantation competences. Notwithstanding these expectations, so far, the proteomic analysis of “spent” (or “conditioned”) culture medium from early human embryos cultured *in vitro* has provided only limited clinical information [1–3]. This appears to be due in major part to the very small volumes in which embryos are cultured and the extremely low proteins/peptides concentrations in those volumes, and also to the confounding influence of serum proteins typically used in human IVF culture supplements.

On the other hand, the murine model can be highly informative as some of the available data indicate that key molecular determinants of early embryonic development in mice are likely to also be active in humans [4–7]. Moreover, this model can be subjected to a variety of experimental interventions not possible to perform in the human setting. Other advantages include the possibility to examine single or multiple embryos co-cultured in microdroplets, in order to augment concentration of putative secreted proteins, as well as other manipulations during *in vitro* development such as culturing embryos in serum-free conditions in order to eliminate interference of serum proteins [8, 9]. Importantly, knowledge gained from this research model may be thereafter translated to human biology in particular as many protein homologs exist among these species [7].

Here, we performed a prospectively designed, controlled investigational study using the murine model in order to identify secreted proteins/peptides by *in vitro*-cultured embryos up to the blastocyst stage using a comprehensive proteomics approach. Two-cell murine embryos were randomly allocated to culture conditions in protein-free and protein-supplemented media, and cultured up to the blastocyst stage *in vitro*. The objective of this discovery phase was to identify and compare the secreted proteins of these two groups of embryos, speculating that without the confounding influence of serum proteins in the culture medium, enhanced protein identification could be achieved.

Materials and methods

Murine embryos and culture conditions

For proteomics experiments frozen (propylene glycol/sucrose as cryoprotectants) two-cell mouse embryos obtained from B6D2F1 males × B6C3F1 females (Conception Technologies, San Diego, CA, USA) were thawed and thoroughly washed

three times for 5 min in Quinn’s Advantage Medium with HEPES (SAGE, Cooper Surgical, Trumbull, CT, USA) without protein at room temperature before being divided into two culture groups: Krebs without bovine serum albumin (BSA), and Krebs plus BSA (lyophilized 3 %, Sigma Chemical Company, St. Louis, MO). Extreme care was taken in media preparation to avoid any contamination; all Krebs media used in these experiments were prepared in-house and subjected to analysis by mass spectrometry to verify absence of any proteins/peptides.

Viable 2-cell embryos (post-thaw survival rate >99 %) were incubated at 37 °C in 5 % CO₂ (95 % humidity) in 50 µl aliquots for each culture group in duplicate using Falcon Microtest Primaria tissue culture plates (353872, Becton Dickinson, Franklin Lakes, NJ, USA) for 3 days until reaching the blastocyst stage. Typically, 25 embryos were cultured per 50 µl well, and for each duplicate culture media were pooled into 100 µl samples after extraction of embryos (blastocysts) at the end of the culture period of each experiment (late afternoon of day 3, or day 5 of embryo development).

We analyzed two embryo study groups: (i) embryos cultured in Krebs medium supplemented with BSA, and (ii) embryos cultured in serum-free conditions (Krebs only). Four culture groups were tested: (a) conditioned (spent) media of embryos grown in Krebs plus BSA; (b) conditioned (spent) media from embryos grown in Krebs without BSA; (c) Krebs medium plus BSA alone, as control (no embryos); and (d) Krebs medium alone (no BSA), as controls or blank (no embryos).

A total of nine independent experiments performed (embryo thawing and culture to blastocyst stage). Blastocyst formation rates were >98 % in all groups and in sibling wells and >70 % were at the hatching stage by the end of the culture period. Parallel cultures of culture media with and without BSA, and without embryos (controls) were performed under identical conditions. After the culture period, using a glass micropipette all blastocysts were removed from each 50 µl well with thorough elimination of any cellular debris (i.e., from broken or detached cells). Very few arrested embryos were also removed under direct microscopic view. The remaining media was withdrawn from each well for collection of conditioned and control media fluids, pooled within each category, and snap frozen at −20 °C until assayed. A total of 432 blastocysts were included in the study, 216 of them cultured in Krebs plus BSA and 216 in Krebs without BSA. Blastocysts were snap frozen at −196 °C for RNA extraction (see below).

Proteomic analysis

All components of the media were tissue culture grade (Sigma). All the reagents and chemicals used were of the highest possible purity and quality available. Ammonium

bicarbonate, iodoacetamide, DTT, trifluoroacetic acid and formic acid were obtained from Sigma. HPLC-grade acetonitrile and water were obtained from Fisher Scientific (Fairlawn, NJ, USA). Mass spectrometry-grade modified trypsin (porcine) was from Promega (Madison, WI, USA). The protein concentration of the complete culture media collected from embryos was determined using the BCA protein assay (Thermo Scientific Pierce, Rockford, IL, USA). Where appropriate 30 µg/ml of bovine serum albumin (BSA, Sigma) were added.

We used a proteomic approach to analyze the blank (control media) and conditioned media of embryos grown with and without BSA. Culture media collected from 432 embryos (half of them cultured in Krebs plus BSA and half in Krebs without BSA) were precipitated overnight in a 10-fold higher volume of acetone in order to enhance concentration to obtain a higher protein yield. The proteins were precipitated by centrifugation and solubilized in Laemmli buffer prior to SDS-PAGE. Bands were visualized on the gel by coomassie staining. Each band was excised, destained, and proteins reduced with 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 before alkylation with 55 mM iodoacetamide in 50 mM NH_4HCO_3 . Digestion with sequence grade trypsin was done overnight at a concentration of 20 ng/µl. The generated peptides were extracted with 50 % acetonitrile/0.1 % formic acid, and dried before reconstituting in 0.1 % formic acid. The peptides were separated on a C18 column (ResPrep C18 column, Bellefonte, PA, USA) and subjected to nano high performance liquid chromatography (HPLC, Accella, Thermo Fisher) and electro spray ionization (ESI) tandem mass spectrometry (LC-ESI-MS-MS) with a linear trap quadrupole instrument (LTQ, Thermo Fisher Scientific, Waltham, MA) [10–13]. Tryptic peptides extracted from identical sectors of the same media categories were combined to increase the concentration of the peptides 3-fold prior to mass spectrometry on the LTQ.

The processed MS/MS data were searched with an in-house MASCOT Daemon server (Matrix Science, London, UK) using the latest version of the indexed combined *mammalian* non-redundant protein database from SwissProt (SP 2010.4). The following parameters were used for the searches: carbamidomethylation of cysteine as the fixed modification and oxidized methionine and deamidation of asparagine and glutamine as variable modifications. Precursor tolerance was set to 75 ppm and MS/MS fragment tolerance to 0.8 Dalton. Peptide scores >30 and expect score <0.05 were considered to be significant. In order to identify secreted proteins we further searched for the presence of an N-terminal signal sequence and of transmembrane domains (membrane-associated proteins). Secreted proteins have an N-terminal signal sequence that directs them to the secretory pathway. This was determined using SignalP at <http://www.cbs.dtu.dk/services/SignalP/>. Membrane proteins have

hydrophobic domains or transmembrane domains and these were identified with the program at TMPred <http://tmppred.sourceforge.net/>. Proteins with a transmembrane domain located to the extracellular site are also likely candidates for secretion as it is known that trans-membrane domains can undergo proteolytic processing prior to secretion [14]. Indirect biological connections among the identified secretory and membrane-associated secreted genes were established by String analysis (<http://string.embl.de/>) [15].

RNA extraction and analysis of gene expression by real time-polymerase chain reaction (RT-PCR)

Frozen/thawed mouse blastocysts were analyzed for mRNA detection, 27 blastocysts from cultures in Krebs's media with BSA, and 24 blastocysts from cultures in Krebs's media without BSA.

Total RNA was isolated using an RNeasy mini kit (Qiagen Sciences, MD) according to the manufacturer's instructions. The quality and quantification of total RNA was performed on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). A total of 10 µg of RNA was reverse-transcribed using a GeneAmp RNA PCR Core kit (Grand Island, NY). Relative quantitative real time PCR was performed using the Lightcycler Fastart DNA Master Plus SYBR Green I kit and the Lightcycler 2.0 instrument (Roche Applied Science, Indianapolis, IN). cDNA encoding prolipoprotein receptor-related protein 1 (*LRP1*) and integrin alpha-X (*ITAX* or *CD11c*) were amplified from 4 µl of the cDNA reaction mixture using specific gene primers. The polymerase chain reaction primers were designed based on published sequences of mouse *LRP1* and *ITAX*, as followings: *LRP1*, forward CCCTCCTACCACTTCCAACC and reverse CCCAGTCG ATAGCGATACC; *ITAX*, forward, CTTCTAAGAGCCAG AGTG, and reverse, GATAGCATTGGGTGAGTG. These primer sets specifically recognize only the interest genes as indicated by amplification of a single band of the expected size (104 and 313 bp for *LRP1* and *ITAX*, respectively).

The PCR products were analyzed on 3 % agarose gel in 1× Tris-acetate-EDTA (TAE buffer) containing ethidium bromide and were compared to DNA hyper ladder V (Bioline, Taunton, MA). 6X DNA loading dye was used for loading DNA markers and samples on agarose gel to allow better visual tracking of DNA migration during electrophoresis.

Protein detection by immunofluorescence and confocal microscopy

Animal use followed the protocol (#11-005) approved by the ODU institutional animal care and use committee (IACUC). Mice were housed and mated in the ODU vivarium, under a 14L:10D light cycle and had access to food and water ad libitum. Four to eight week old CBA/B₆ F1 (Jax Labs, Bar

Harbor, Maine) females were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG) followed 48 h later by 5 IU of human chorionic gonadotropin (hCG). Females were paired with CD1 (Harlan Labs, Frederick, Maryland) males of proven fertility and pregnancy were confirmed by the presence of a vaginal copulation plug 12 h post-hCG injection. All embryos were irrigated from excised oviducts into modified bicarbonate-buffered medium (mKBB) without protein and subsequently cultured in mKBB media with or without 0.3 mg/mL BSA. Embryos were cultured to the blastocyst stage at 37 °C, 5 % CO₂, in 100 µL volumes in 96-well plates overlaid by 100 µL of toxicity-tested heavy paraffin oil (Sigma). All components of the media were tissue culture grade (Sigma) and toxicity-tested prior to use in these experiments.

The blastocysts were fixed for 30 min in 4 % paraformaldehyde prepared from a 16 % stock solution (Electron Microscopy Sciences, Hatfield, PA) and refrigerated until immediately before use. The embryos were permeabilized with 2 % Triton X-100 in PBS for 10 min, followed by incubation overnight at 4 °C in blocking solution that contained 2 % normal goat serum (Abcam, Cambridge, MA) and 0.05 % Triton X-100. Primary antibodies were diluted in blocking solution and incubated with the embryos overnight at 4 °C. Then the embryos were washed several times in PBST (PBS-Tween 20 1 %) the next day and incubated with fluorescent secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200) for 1 h at room temperature. Antibodies used were as follows: mouse monoclonal to LRP1 (Abcam, 1:100) and mouse monoclonal to ITAX/CD11c (Abcam, 1:100). Isotype-matched negative controls were applied as appropriate (mouse IgG, Sigma). Nuclei were visualized with DAPI. Mounted embryos were analyzed and captured by a Zeiss 510 Meta confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). Argon (488 nm) and HeNe1 (543 nm) lasers were used for FITC and rhodamine, respectively. Image acquisition was done after optimizing LSM settings. Gain and offset in range indicator palette was optimized to enhance the visibility of image. A stack of images was collected along z-axis (Z-stacks) at an optimal thickness with 5 µm as interval in a 512×512 frame size.

Results

Proteomics

The in-gel trypsin digestion followed by mass spectra acquisition with LC-ESI-MS/MS on the LTQ demonstrated significant peptide hits for a variety of proteins in both categories of culture media of embryos grown with and without BSA. Figure 1, panel A, shows a Coomassie stained gel for in-gel trypsin digestion, and the representative excised sector areas.

Fig. 1 **a** Coomassie staining for in-gel trypsin digestion. *Left lane*: molecular marker; *A–J*: excised sector areas for individual digestion. **b** Venn diagram. Comparison of proteins identified in embryo conditioned media with and without BSA

Note absence of proteins in control media without BSA (blank, lanes 1 and 2). “Spent” media refers to conditioned media, with or without BSA. Conditioned media from embryos grown in Krebs without BSA showed a small amount of BSA, confirmed to be murine albumin by multiple sequence alignment (not shown). In-gel digestion and mass spectrometry results were followed by mammal and mouse taxonomy search results revealing a defined number of proteins present in each category.

In the BSA and common groups there were strong signals for bovine serum proteins (serum albumin, transthyretin, alpha 1 acid glycoprotein, alpha-1-microglobulin inter-alpha-trypsin inhibitor light chain *P*, and complement components C3 and C4) and also for porcine proteins (albumin, alpha fetoprotein, and trypsin) that were eliminated from the analysis. The subsequent analysis revealed 54 proteins identified in samples of embryos cultured without protein supplementation, and 43 proteins detected in samples of embryos cultured with BSA.

A Venn diagram was constructed based on the protein profiles detected by MS in order to distinguish proteins present in the conditioned media of embryos grown without BSA, those found in embryos cultured with BSA, and those that were common proteins seen in both groups (Fig. 1, panel B). Of all 77 individually identified proteins, 34 proteins were only found under protein-free conditions, and 23 proteins only

found in BSA-grown conditions. There were 20 proteins common to both culture conditions (found in samples of embryos cultured with and without BSA).

Table 1 presents a list of putative secreted mouse proteins with an N-terminal secretory sequence and/or transmembrane domains (membrane-associated) found in conditioned media from embryos cultured without BSA, conditioned media from embryos cultured with BSA, and those proteins common to both groups. A total of ten proteins were identified. Six proteins have an N-terminal secretory sequence. One of these proteins, arylsulfatase J (ARSJ) does not have a transmembrane domain, and is therefore a clear candidate as a secreted soluble protein. Five other proteins have an N-terminal secretory sequence making them candidates for secretion; all of them also possess a transmembrane domain, and in 2 of these proteins the domains are located to the extracellular site, integrin alpha-X or CD11c (ITAX) and phospholipase B1 (PLB1) also making those likely candidates for secretion as it is known that transmembrane domains can undergo proteolytic processing prior to secretion [14]. The three other proteins, i.e., extracellular matrix protein FRAS1 (FRAS1), integrin alpha-6 (ITA6) and prolow-density lipoprotein receptor-related protein 1 or Apolipoprotein E receptor (LRP1), have an N-terminal secretory sequence and also a transmembrane domain but that is not located to the extracellular site.

In addition, Table 1 shows 4 other proteins without an N-terminal secretory sequence, and all of them having a transmembrane domain. Two of these proteins have an extracellular site, making them potential candidates for proteolysis and perhaps secretion, including armadillo repeat containing, X-

linked 1 (ARMX1) and potassium channel subfamily U member 1 (KCNU1), whereas guanylyl cyclase-activating protein 2 (GUC1B) and insulin receptor substrate 4 (IRS4) did not.

Functional analysis was performed with Pathway Studio (Elsevier Inc, NY), a computer software used for visualization, analysis and integration of cell signaling and biochemical pathways, protein interaction maps and gene regulation networks. Analysis revealed the over-representation of different molecular functions, cellular components and biological processes (presented in Table 2), with remarkable representation of molecules and processes involved in lipid metabolism and cell adhesion, among others, particularly for LRP1, GUC1B and ITA6 genes. Plasma membrane related terms were the most common cellular components, as expected, and finally, the most important molecular functions terms belong to lipid metabolism (Table 2).

LRP1 and ITAX mRNA expression levels

RT-PCR resulted in the detection of mRNA transcripts encoding *LRP1* and *ITAX* producing the expected size amplicons of 104 bp and 313 bp, respectively (Fig. 2). The identity of both RT-PCR products was confirmed by direct sequencing and BLAST analysis. RT-PCR products amplified using *LRP1* and *ITAX* primers possessed 100 % sequence identity with their respective Gene Bank mouse nucleotide sequences. These results suggested that LRP1 and ITAX genes expressed at transcriptional level in both the embryos cultured in Krebs media with and without BSA.

Table 1 List of putative secreted mouse proteins with an N-terminal secretory sequence and/or transmembrane domains (membrane-associated) found in conditioned media from embryos cultured without BSA,

conditioned media from embryos cultured with BSA, and those proteins common to both groups

Protein accession no.	Protein description	# Identified peptides	N-terminal secretory sequence	Transmembrane domains
A. Proteins detected only in conditioned media without BSA				
ARMX1	Armadillo repeat-containing X-linked protein 1	4	No	Yes ^a
ARSJ	Arylsulfatase J	1	Yes	None
FRAS1	Extracellular matrix protein FRAS1	1	Yes	Yes
GUC1B	Guanylyl cyclase-activating protein 2	1	No	Yes
IRS4	Insulin receptor substrate 4	7	No	Yes
ITAX	Integrin alpha-X	11	Yes	Yes
LRP1	Prolow-density lipoprotein receptor-related protein 1	9	Yes	Yes
B. Proteins detected only in conditioned media with BSA				
KCNU1	Potassium channel subfamily U member 1	11	No	Yes ^a
C. Proteins conditioned media of embryos cultured with and without BSA				
ITA6	Integrin alpha-6	27	Yes	Yes ^a
PLB1	Phospholipase B1, membrane-associated	12	Yes	Yes ^a

^a Some of the identified peptides are located in the extracellular domain of these proteins

Table 2 Functional analysis performed with the genes presented in Table 1

	Functional analysis	Genes	<i>p</i> -value	GO terms
Gene ontology (GO) terms and names of the identified biological processes (BP), cellular components (CC) and molecular functions (MF) are indicated in the last column. Names of genes and <i>p</i> -values are also indicated	Integrin-mediated signaling pathway	ITA6,ITAX	0.00039	BP
	Leukocyte migration	ITA6,ITAX	0.00054	BP
	Integrin complex	ITA6,ITAX	0.00010	CC
	Membrane	KCNU1,LRP1,ITA6,ITAX,FRAS1,IRS4,GUC1B,PLB1,ARMX1	0.00011	CC
	Plasma membrane	LRP1,ITA6,ITAX,FRAS1,IRS4,GUC1B,PLB1	0.00032	CC
	Basement membrane	ITA6,FRAS1	0.00063	CC
	External side of plasma membrane	ITA6,ITAX	0.0042	CC
	Integral to membrane	KCNU1,LRP1,ITA6,ITAX,FRAS1,PLB1,ARMX1	0.014	CC
	Protein complex binding	LRP1,ITA6	0.0033	MF

Immunofluorescence and confocal microscopy for expression and localization of LRP1 and ITAX protein

To further explore the presence of LRP1 and ITAX proteins in the blastocysts, we performed immunofluorescence with antibodies against both proteins (Fig. 3). Immunofluorescence coupled with confocal microscopy allows for the assessment of the cellular location of these proteins. Results for LRP1 showed staining predominantly associated with the cell membrane, punctuated in some cells, and more diffuse in others. Compared to embryos cultured in Krebs' media with BSA, embryos grown without BSA showed increased staining. Similar findings were observed for ITAX. Negative staining demonstrated no detection of non-specific IgG (shown in Supplemental Material 1).

Discussion

It remains unclear what proteins function during the preimplantation stages of embryo development. Embryos might consume and release proteins into the growth media while in

culture. To illustrate the potential of using specific proteins indicating successful embryo development, we combined MS-based proteomics approach and gene/protein detection methods in biomarker discovery.

We speculated that the characterization of the secreted proteins could represent a first step toward the identification of biomarkers predictive of in vitro developmental capacity (from the 2-cell through the expanded-hatching blastocyst stage). The results of these studies could also lead to prediction of implantation potential following blastocyst transfer to the uterus.

Nieder et al. [16] studied protein synthesis and secretion of blastocysts recovered from intact mice. The array of proteins synthesized and secreted by late stage blastocysts was found to be qualitatively and quantitatively different from those released by embryos at earlier stages of development. Furthermore, there was a temporal correlation between the appearance of certain proteins secreted by the embryos and changes in specific proteins synthesized and released by the uterus. It is known that the development of mouse embryos in vitro is affected by strain and culture medium [17]. Development of two-cell mouse embryos to the blastocyst stage can be

Fig. 2 Detection of mRNA transcripts encoding LRP1 and ITAX during mouse preimplantation development. RT-PCR products encoding *LRP1* and *ITAX* were detected in cDNA from blastocysts cultured in Krebs's media with and without BSA. Representative image of three independent replicates is shown

Fig. 3 Immunofluorescent localization of LRP1 and ITAX in the mouse blastocyst using confocal microscopy. LRP1 and ITAX proteins were detected in embryos cultured in Krebs' media with and without BSA. Green indicates positive staining for LRP1 or ITAX protein. In addition, blastocysts were stained for F-actin detection (red, rhodamine-

phalloidin), and merged images are presented (orange/yellow in co-localization areas). Both LRP1 and ITAX immunofluorescence appeared to be predominantly associated to the membrane (arrows), either in punctate (granular) or more diffuse form, and with stronger staining in blastocyst without BSA, compared to the ones with BSA

achieved in protein-free conditions [8]. In mice, an in vitro phenomenon known as the “2-cell block” takes place in some of the strains, which ceases embryo development at that stage. Jang et al. [9] studied development of mouse two-cell embryos of block (ICR) or non-block (F1 of C57BL × DBA) strains in media supplemented with bovine serum albumin or polyvinyl alcohol. Presence or absence of protein supplementation did not influence blastocyst formation, inner cell mass and trophoctodermal cells in the blastocysts, rates of pregnancy and delivery, mean litter size, or the expression of several genes related to pluripotency, organogenesis, and implantation. In addition, it has been reported that human embryonic stem cells derived from the embryo at the blastocyst stage can grow in a chemically defined medium devoid of serum and feeders [18].

Here we cultured 2-cell embryos to the blastocyst stage; the observed development and proportions of blastocysts reaching expanded and hatching stages were similar under culture conditions with and without protein supplementation. We have used this system for embryo toxicity and quality control in our IVF program for several years [19]. We pooled samples and used concentration methods in order to increase protein abundances, and embryos were cultured without change in media for the 3-day period in an effort to accumulate secretory products. We found that in samples from embryos cultured under protein-free conditions we could detect only a few more total proteins than in those ones grown in BSA as identified by in-gel digestion followed by LC-ESI-MS/MS on a LTQ. It has been shown that molecular weight determination and structural information can be accurately provided by biomolecular mass spectrometry and in particular by ESI- and MALDI-MS [13]. Further analysis was focused

on those detected mouse proteins that were membrane-associated or secreted (having an N-terminal signal sequence). As initially expected, a number of proteins identified in the BSA-grown embryos, and those common to both culture conditions, were related to various bovine and porcine “contaminants” (bovine as the source of serum albumin, porcine as the source of trypsin). The confounding effects of albumin and other serum proteins in the BSA medium are partly derived from adsorption or binding of other proteins to albumin, as has been described for example for paf [20]. These results strengthened our speculation that the detection and identification of secreted embryo proteins could be higher in protein-free medium. Importantly, we identified unique murine proteins, particularly in the culture media of embryos grown without protein supplementation, but also in the other two groups (with BSA and common) which might represent part of the embryonic secretome.

Ten putative secreted mouse proteins were identified having an N-terminal secretory sequence and/or transmembrane domains (membrane-associated). These included ARSJ, ITA6, PLB1, FRAS1, ITA6, LRP1, KCNU1, ITA6, GUC1B, and IRS4. These proteins have extensive human homology. Putative functional roles of these proteins as related to embryogenesis and implantation are discussed in Supplemental Material 2. The results of RT-PCR and immunofluorescence/confocal microscopy confirmed blastocyst gene and protein expression of two validated genes/proteins, LRP1 and ITAX, with more robust staining in embryos grown in BSA-free media thus confirming MS results.

Using surface enhanced laser desorption and ionization time of flight (SELDI-TOF) mass spectrometry, Katz-Jaffe et al. [21] described in detail only one protein from analysis

of mouse and human embryos. The putative protein had a molecular weight of 8.5 kDa and was identified as ubiquitin. It is agreed that SELDI-TOF does not provide for correct identification of protein peaks [22], and questions have been raised about this primary identification [1]. Use of HPLC coupled with ESI has been shown to allow for flow rates in the low nL/min range increasing the resolution and sensitivity for detection of proteins derived from in-gel-digested silver-stained bands from 1-D and 2-D gels [23]. McReynolds et al. [24] identified the protein lipocalin-1 in the secretome of human blastocysts in association with chromosome aneuploidy. Samples were depleted of human serum albumin; proteins were separated by SDS-PAGE, followed by in-gel digestion and analysis on an LTQ-FT Ultra Hybrid Mass Spectrometer (and results corroborated by ELISA). We did not detect any of these proteins in our murine study using HPLC and ESI tandem mass spectrometry (LC-ESI-MS-MS).

Beardsley et al. [22] aimed to characterize the secretome generated by the mouse pre-implantation embryo in vitro using SELDI-TOF and also ESI-MS. The strains of mice used in the experiments were C57BL/6 (B6) and F1 (C57BL/6 × CBA/He and CBA/He × C57BL/6; B6CBF1). Zygotes were collected, exposed to hyaluronidase, and cultured in groups of 10 to the blastocyst stage in modified HTF plus BSA. The two use methods generated different outcomes. SELDI-TOF analysis with CM10 or IMAC30 protein chips was able to detect only a single protein peak at *m/z* approximately 8570. On the other hand, ESI-MS of tryptic digests of embryo-conditioned media identified a total of 20 proteins released during development from the zygote to blastocyst stage. Using this approach, the 8.5 kDa protein could not be detected as ubiquitin. No clear differences were seen between the studied mouse strains. Although there was variability of expression of the proteins in the various cultures tested, this analysis identified a range of potential targets. Some of the identified proteins are membrane- or cell surface-associated, but no information about secretion (sequence signals) was provided.

Previous published studies have detected the expression of proteins in conditioned (spent) culture media from early embryos grown in vitro in a variety of species, including the human. Some of these molecules were identified by immunological methods, including among others, paf [20, 25], IGF-II [26], HLA-G [27], hCG [28], Apolipoprotein A1 [29] and superoxide dismutase-1 [30]. However, there has been considerable debate as to the reliability of some of these measurements. While it appears that the embryos may indeed release some of these signals, the sensitivity of the assays, the impact of IVF culture conditions (various media and protein supplements used clinically), and the true levels of the secreted molecules (orders of magnitude of their concentrations) have been questioned [31, 32]. It is noteworthy that neither in Beardsley et al. [22] nor in our study, this highly sensitive proteomic method was able to detect any of the expected

targets such as the HLA-G homologue Qa-2, IGF-II, ubiquitin, or the range of other protein growth factors thought to have potential paracrine/autocrine roles. This could be due to a limitation of proteomics due to the mixture of serum and secreted proteins (in case of embryos grown in BSA) or it may be due to the fact that these factors are released at levels too low to be detected by these methods. Alternatively, it may mean that these molecules act as trophic factors for the embryo, but do not do so in the soluble phase [22].

Because the current studies were conducted on pooled samples (in order to enhance detection), we will now move on to explore individual embryo samples, both with ESI and other derived immunoaffinity assays for those identified murine secretory proteins having potential functional roles related to embryo development and implantation, and also having extensive human homology (i.e., ARSJ, ITAX, LPR1, and PLB1). In addition, the functional analysis confirmed over-representation of molecules and processes involved in lipid metabolism, cyclase activity, and cell adhesion, remarkably for LRP1, GUC1B and ITA6 genes. Although there were more biological processes over-represented in the gene ontology analysis, they were obtained for only one candidate gene and are not presented herein. In conclusion, the present study has presented data to further characterize secreted proteins by the murine blastocyst during in vitro development from the 2-cell stage.

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Authors' roles Tanya Burch, PhD, performed proteomics experiments including in gel digestion and MS.

Liang Yu, PhD, performed PCR and immunofluorescence-confocal microscopy studies.

Julius Nyalwidhe, PhD, participated in study design, data analysis and manuscript writing.

Jose Horcajadas, PhD, performed String-gene analysis and participated in study design.

Silvina Bocca, MD, PhD, participated in study design and manuscript writing.

R. James Swanson, PhD, participated in study design and mouse embryo collection.

Sergio Oehninger, MD, PhD, participated in study design, data analysis and manuscript writing.

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