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REVIEW PAPER

PROGRESS AND CHALLENGES IN THE PROTEOMICS OF DOMESTIC PIG IN RESEARCH ON THE FEMALE REPRODUCTIVE SYSTEM*

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Abstract

The proteomics of pigs is developing dynamically, attracting much attention among representatives of medical and health sciences, veterinary medicine, agricultural and natural sciences. The pig has been widely studied in genetics and genomics. However, proteomic applications are still awaiting more extensive implementation, both in the use of pigs as animal models in biomedicine sciences, and in the exploration of physiological tracts important for pig production. Underdeveloped databases for identification and analysis of porcine proteins as well as the scarcity of detailed information on similarities and differences between humans and pigs at the molecular level are hampering the pig proteomics. However, the use of pigs in proteomic studies in both physiological and biomedical sciences is prevalent comparing to other farm animals. The focus of the reported pig model proteomics studies is on exploring physiology and diseases, and on improving pig breading and productivity. This species has been used as a model in proteomics studies involved in ocular, brain, nutritional and reproduction research, etc. In the present paper we discuss technologies and bioinformatic tools used in studies of a proteome to verify the peptide- and protein-based content and we summarize the current status of proteomic studies of pigs. We focus on studies of the female reproductive system because the examination and understanding of the biology of oocytes, the oviduct and the uterus could facilitate the identification of mechanisms involved in the prenatal development, and it may help to develop new treatment for infertility of farm animals.

Keywords: domestic pig, proteomics, female reproductive system, uterus, oviduct, oocytes.

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DOMESTIC PIG AS A MODEL FOR PROTEOMIC STUDIES

Domestic pigs have been extensively studied for their economical value as one of the main sources of meat production, and as a model for physiological studies in large mammals (Hwang et al. 2005, Laville et al. 2005, Kwasiborski et al. 2008, Hollung et al. 2009, Xu et al. 2009, Mach et al. 2010). Domestic pig (*Sus scrofa domestica*) also holds promises for use as a well-suited model, which may be approximated to the humans, in biomedical and pharmaceutical studies. Pigs and humans show significant similarities in their physiological processes, and pigs' the size is comparable to that of humans. Pigs have also a great economical value in agriculture (Doherty et al. 2008). Thus far, however, pigs have played a limited role in studying human diseases and developing medical treatments.

Difficulties in using pigs as model organisms still arise from the lack of detailed data on similarities and differences between humans and pigs on the molecular level (Golovan et al. 2008). The information about the porcine proteome in databases is poor and fragmented. There is no porcine database which could be compared in completeness to human databases. There is an urgent need for a systematic development of proteome catalogues for different porcine organs in order to use the pig as a model organism more efficiently (Verma et al. 2011).

Sus scrofa domestica offers many advantages comparing to other model organisms, and these advantages drive the development of pig proteomics. Proteomics has been used to study ocular (Hauck et al. 2005, Azarian et al. 2006), cardiovascular (Fert-Bober et al. 2008, Sheikh et al. 2009), nutritional (Stephens et al. 2010, Herosimczyk et al. 2015, Lepczyński et al. 2015, Ożgo et al. 2015), nervous (Skalnikova et al. 2007), excretory (Havanapan, Thongboonkerd 2009, Tuma et al. 2015) and reproductive (e.g. Ellederova et al. 2004, Georgiou et al. 2005, Sostaric et al. 2006, Susor et al. 2007, Seytanoglu et al. 2008, Chae et al. 2011) systems. The publicly available PeptideAtlas project (www.peptideatlas.org.) so far has included data from 25 pig tissues and three types of pig body fluid mapped to 7139 canonical proteins. This repository provides information helpful to designe targeted proteome analyses needed for developing porcine biomedical models (Hessalger et al. 2015).

In this review, we discuss proteomics of the female reproductive system. The examination and understanding of the oviduct and uterine physiology will enable identification of mechanisms involved in fertilization and early embryonic development, and it may help to develop new treatment for infertility of farm animals (Verma et al. 2011).

PROTEOMIC PROFILES OF THE PIG REPRODUCTION SYSTEM

Many crucial events leading to the establishment of pregnancy occur within the female reproductive system, especially in the oviduct and uterus, such as final maturation of female and male gametes, fertilization, embryonic development, and transport of the embryo to the uterus (Menezo, Guerrin, 1997, Lee, DeMayo 2004, Demir et al. 2010). In this section we review the latest proteomics studies conducted on the female reproductive system in pigs.

The research by Seytanoglu et al. (2008) was designed to identify the alterations in oviductal epithelial cells (OEC) proteome profiles during a reproductive cycle by using two-dimensional gel electrophoresis (2DE), mass spectrometry (MS) and Western Blotting. The authors described 51 differentially expressed proteins during the follicular phase of an estrous cycle, and 27 differentially expressed proteins during the luteal phase of an estrous cycle. The authors validated expression changes for five selected proteins: 70 kDa heat shock protein (Hsp70), calreticulin, ezrin, cytokeratins and gelsolinin. The first three proteins were found to be up-regulated in the oviduct harvested during the follicular phase of the estrous cycle, and the last two proteins were found to be up-regulated in the same tissue obtained during the luteal phase of the estrous cycle. The functional importance of these proteins was discussed, but no in-depth functional study was performed.

Sostaric et al. (2006) described a detailed profile of the surface plasma membrane proteome of the oviductal epithelium. The authors characterized molecules involved in gamete/embryo-oviduct interactions. In this study, two different techniques for the identification of biotinylated surface proteins were used: 1) a combination of two-dimensional gel electrophoresis with mass spectrometry and 2) 1D gel electrophoresis with mass spectrometry, a modified multidimensional protein identification technology (MudPIT) technique. This global profiling of the surface proteome of oviductal cells was validated by immunohistochemistry and Western Blot analysis. The number of proteins identified using MudPIT technology (276) was about 7-fold higher than the number of proteins identified using 2DE and MS (40). Some of the identified proteins had already been described in the oviduct (e.g. oviduct specific glycoprotein, elongation factor 1-beta, peroxiredoxin 2, heat-shock 70 kDa protein 1). The category mostly represented by identified proteins was the family of heat-shock proteins. Many of the identified proteins have cell surface associated functions, and others were classified as cytoplasmic or intracellular proteins. This was the first study to identify and characterize proteins at the surface of the epithelium of the mammalian oviduct in such a comprehensive manner.

Using a combination of 2DE and liquid chromatography-tandem mass spectrometry, Georgiou et al. (2005) demonstrated specific alteration of the

oviductal secretory proteomic profile in response to the presence of both gametes. The oviductal response to spermatozoa was different from its response to oocytes. The presence of spermatozoa or oocytes in the oviduct altered the secretion of specific proteins. The authors identified 19 proteins regulated only by sperm, 4 proteins regulated only by oocytes, and 1 protein regulated by both sperm and oocytes. The identified proteins were organized into following functional categories: 1) protein production, maintenance and repair (e.g. Hsp70 1A, ribonuclease UK114, cathepsin D), 2) antioxidant and free radical scavengers (e.g. thioredoxin, suoeroxide dismutase, peroxiredoxin 2), 3) metabolism (e.g. triose-phosphate isomerase, esterase, α enolase) and 4) miscellaneous (e.g. haptoglobin precursor, cytoskeleton-associated protein 1, lamin A/C). Most of the identified proteins were molecular chaperones and regulators of protein folding and stability and antioxidant and free radical scavenger proteins. The alteration of the oviductal secretory proteome profile in response to the gametes seems to provide a favourable microenvironment for gametes and prepare the oviduct's milieu for the arrival of an embryo. The summary of proteomics studies of the pig's oviduct is shown in Figure 1.

In the report by Ellederova et al. (2004), the proteomes of swine oocytes during *in vitro* maturation were studied. The authors found several proteins of high abundance which may play an important role in primary oocyte functions, such as fertilization and embryonic development. The identified proteins are peroxiredoxins, ubiquitin carboxyl-terminal hydrolase isozyme L1, spermine synthase. The other identified proteins were classified according to their function, as: 1) molecular chaperones (for example, heat shock protein 60 Hsp60 and endoplasmatic reticulum proteins calreticulin) which are implicated in correct folding of proteins and prevention of misfolding, 2) proteins involved in the energy metabolism (for example, alpha-enolase, triosephosphateisomerase, mitochondrial ATP synthase beta chain) whose presence may indicate that oocytes are preparing for modification of energy metabolism following fertilization, and 3) members of the reductase/dehydrogenase family. Among them, antiquitin (D7A1) was significantly increased in the first meiosis (MI) and the second meiosis (MII) stages compared to GV (germinal vesicle) oocytes.

The proteomic analysis of porcine oocytes during *in vitro* maturation was performed by Susor et al. (2007). Comparative analysis of oocytes at the initial and final meiotic division stages identified proteins that are differentially synthesized during *in vitro* maturation. Oocytes with compact cumuli, obtained by aspiration of antral follicles, were cultured and labeled with 50mCi [^{35}S]-methionine during *in vitro* culture to obtain cell populations of germinal vesicle, meiosis I and meiosis II stages. Four up-regulated proteins were found. Ubiquitin C-terminal hydrolase-L1 (UCH-L1) was identified by MS as an up-regulated protein. To elaborate the proteomics finding, a specific C30 inhibitor of this enzyme was used. The authors confirmed their hypothesis that UCH-L1 plays a role in the methaphase I-anaphase transition by regulating ubiquitin dependent proteasome mechanisms.

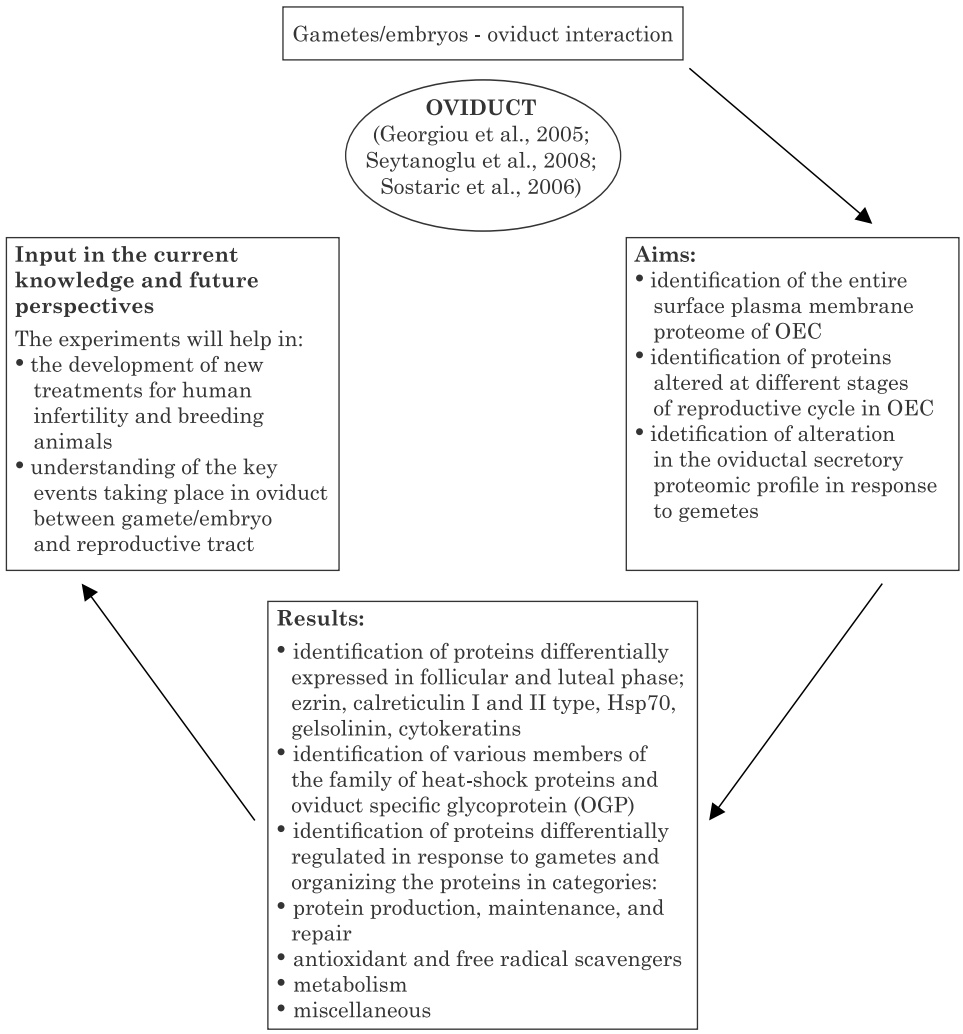

Fig. 1. The summary of proteomics studies of pig oviduct discussed in the review. The subjects, aims, results and future perspectives of the reported studies are indicated. For standardization of the presentation, the design of summaries is similar for different models presented in Figures 1 to 4

The purpose of the studies conducted by Powell et al. (2010) was to identify the biomarkers of oocyte quality and reprogramming and developmental potential. By comparing low and high quality oocytes and 110 proteins in *in vitro* maturation media (oocyte secretome), using a PerkinElmer ExacTag™ Kit, the authors identified 16 differentially expressed proteins in oocyte proteome. Candidate biomarkers, such as kelch-like ECH-associated protein 1, nuclear export factor CRM1 and ataxia-telangiectasia mutated protein kinase, were overexpressed in high-quality oocytes, while dystrophin

was more abundant in low-quality oocytes. In the oocytes' secretome, dystrophin and cystic fibrosis transmembrane conductance receptor were found to be overexpressed in high-quality oocytes, whereas monoubiquitin was more abundant in low-quality oocytes.

Jiang et al. (2011) explored protein profiles during porcine oocyte aging and the effects of caffeine on protein changes. The authors compared MII stage oocytes, 24 hours aged oocytes and 24 hours aged and caffeine-treated oocytes. Using Two-Dimensional Difference Gel Electrophoresis (2D DIGE) combined with matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS), the authors identified 38 proteins and classified them into 5 regulation patterns. Proteins involved in metabolism, stress response, chaperones and antioxidants were found to be involved in the aging processes. The authors found also several proteins which were modified during oocyte aging. Physiological aging can be prolonged by caffeine and the expression of most proteins restores the normal level after caffeine treatment, what was also shown in the above study. However, numerous proteins became changed when caffeine was added. These proteins might effectively participate in anti-aging mechanisms on molecular levels. The summary of proteomics studies of pig oocytes is shown in Figure 2.

Thus far, there has been limited research in which the authors would aim at establishing a detailed profile of the proteome of the porcine endometrium harvested during pregnancy in comparison to the non-gravid state. Chae et al. (2011) investigated changes in expression patterns during pregnancy days (40, 70 and 93 days) using 2DE, MALDI TOF, MALDI TOF/TOF MS and Western Blotting. They detected 98 proteins regulated differentially between tissues from non-pregnant and pregnant animals, and identified 63 proteins which were up- or down-regulated. Among these proteins, 10 are related to development, cytoskeleton and chaperones (for example, septin 2, cofilin 1, galectin-1, Hsp 27). The reported study expands the list of regulators of the endometrium, in both physiological and abnormal conditions, including infertility, endometriosis or endometrial cancer. It is important to note that variations in timing and conditions of the animal models will help to explain the function of the proteins crucial for maintaining the pregnancy. This may facilitate the identification of factors affecting even human implantation.

Jalali et al. (2015) compared proteome profiles of porcine endometrium from days 9 and 12 of the estrous cycle and pregnancy using 2D-DIGE. The authors observed that abundance of several proteins was altered depending on the pregnancy status. MALDI-TOF/TOF was used to identify some of these proteins. Examples of proteins that increased from day 9 to 12 of cycle in the endometrium are annexin A4, beta-actin, apolipoprotein, ceruloplasmin and afamin. The authors observed also changes in protein abundances associated with conceptus secreted factors, e.g. haptoglobin, prolyl-4-hydroxylase, aldose-reductase and transthyretin. Functional analysis showed that endometrial proteins with altered abundance on day 12 in both reproductive sta-

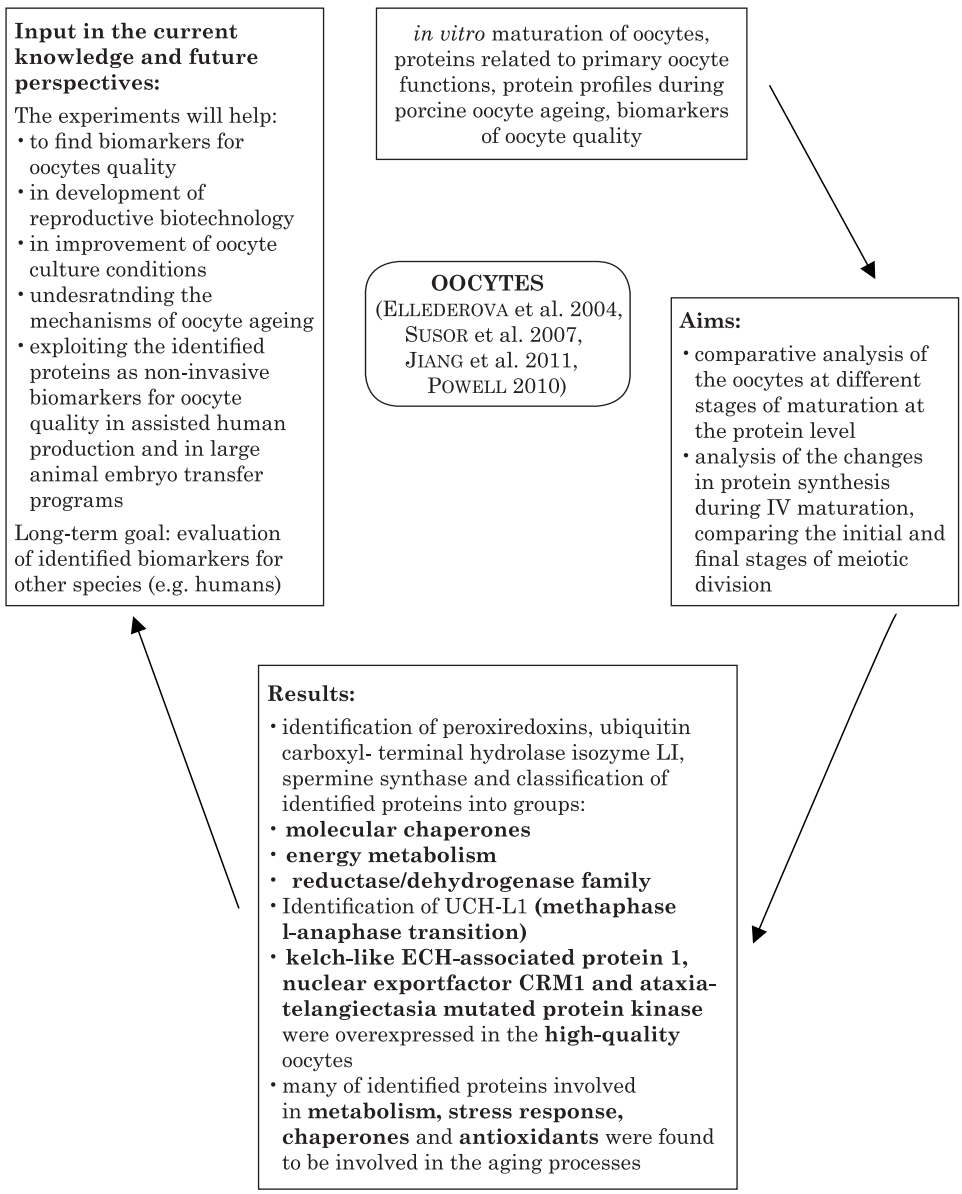

Fig. 2. The summary of the reviewied proteomics studies of pig oocytes.
The subjects, aims, results and future perspectives of the reported studies are indicated.
For standardization of the presentation, design of summaries is similar for different models presented in Figures 1 to 4

tuses, i.e. during estrous and pregnancy, were related to growth and remodeling, acute phase response and free radical scavenging. Transport and small molecule biochemistry were activated in the pregnant endometrium when

compared to the cyclic endometrium. These results may assist in predicting the expression of which proteins is important for successful pregnancy and which proteins are potentially involved in abnormal endometrial receptivity, placentation and embryo loss. The summary of proteomics studies of the porcine endometrium is shown in Figure 3.

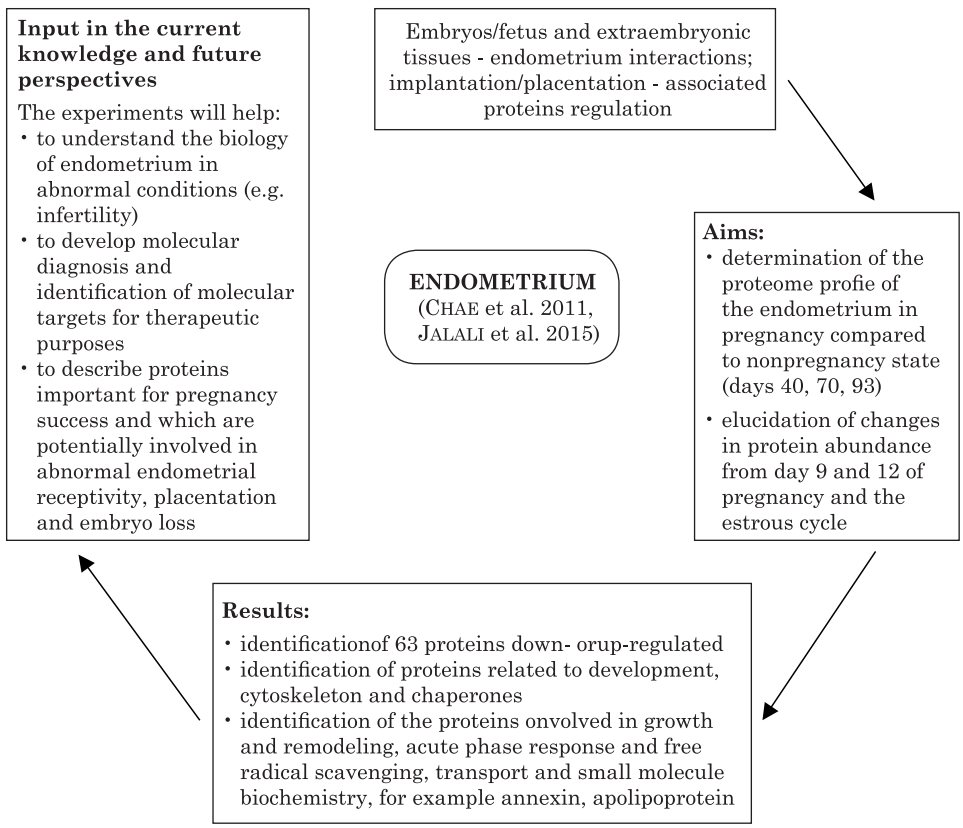

Fig. 3. The summary of the reviewed proteomics studies of pig endometrium. The subjects, aims, results and future perspectives of the reported studies are indicated. For standardization of the presentation, design of summaries is similar for different models presented in Figures 1 to 4

There are several papers describing embryonic development in detail (for example OESTRUP et al. 2009), but there are only a few proteomics studies focusing on embryonic development. DEGRELLE et al. (2009) aimed to establish a comprehensive profile of abundant proteins of the pig conceptus prior to implantation in order to understand the regulatory network involved in the elongation of the conceptus. The authors compared the abundant proteins of a homogenous population of gestational day-11 ovoid (0,7-1 cm) and gestational day-12 filamentous (15-20 cm) porcine conceptus by extracting proteins from three independent conceptus pools and separating the proteins in 2DE gels. Then, proteins in 305 spots were analyzed using MALDI-TOF or liquid

chromatography – tandem mass spectrometry (LC-MS/MS), from which 275 spots were identified representing 174 proteins. The identified proteins were divided into different categories e.g. cell proliferation/differentiation, cytoskeleton, metabolism and stress response. A total of 35 proteins, associated with cell proliferation, differentiation, apoptosis and embryo/maternal signaling were found to be differentially expressed between the two types of embryos, including elongation factor G, palmitoyl-protein thioesterase 1, regucalcin, villin 2 (over-expressed in ovoid embryos) and interleukin-1 beta, legumain, translational elongation factor 1 delta, transaldolase 1 (over -expressed in filamentous embryos). The role of these proteins relates to embryonic differentiation and embryo loss. The embryo loss may approach 20% in pigs following artificial insemination or natural mating, and the reported studies may help to decrease this number.

In another study, Gupta et al. (2009) used reverse phase LC-MS/MS combined with 1D sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), and identified 1625 proteins by homology to different mammalian species and 735 *Sus scrofa* proteins from porcine zygotes. These proteins included both cytosolic and membrane proteins. The big difference in the numbers implicates some shortcomings of pig databases. A different pattern between parthenogenetically activated (PA) and *in vitro* fertilized (IVF) embryos with several differentially expressed proteins was also detected. These data gave rise to a global protein profiling of PA and IVF zygotes, which may be useful as a reference map for future studies. This study may help to understand the mechanism underlying embryonic development and to identify an embryo's quality markers.

In order to understand the cause of early porcine embryo losses, and to address the question why the birth rate in cloned pigs is low, somatic cell nuclear transfer (SCNT)-derived from 26-day-old extraembryonic tissues was analyzed (Chae et al. 2008). The results are crucial for understanding the involvement in embryonic genesis of signaling pathways such as Notch, hedgehog (Hh), receptor tyrosine kinase (RTK), Janus kinase/signal transducer and activator of transcription (JAK/STAT), wingless related (Wnt) and transforming growth factor-β (TGF-β). Using the 1DE and Western Blotting methods, the authors showed down-regulation of the expression of key molecules involved in the Notch, Hh, RTK and JAK/STAT signaling pathways, while most Wnt and TGF-β signaling pathway regulators were up-regulated in cloned extraembryonic tissues, in comparison to non-manipulated tissues. The results indicate that unbalanced regulation of signaling pathways may impair the early development of transplanted cloned porcine embryos and may be associated with embryonic losses during early pregnancy. The summary of proteomics studies of pigs conceptus, zygotes and extraembryonic tissues is shown in Figure 4.

The works described above focus on exploring fundamental physiological mechanisms. They cover only small part of our knowledge of swine reproduction, which is economically relevant for pig production. Proteomic studies of

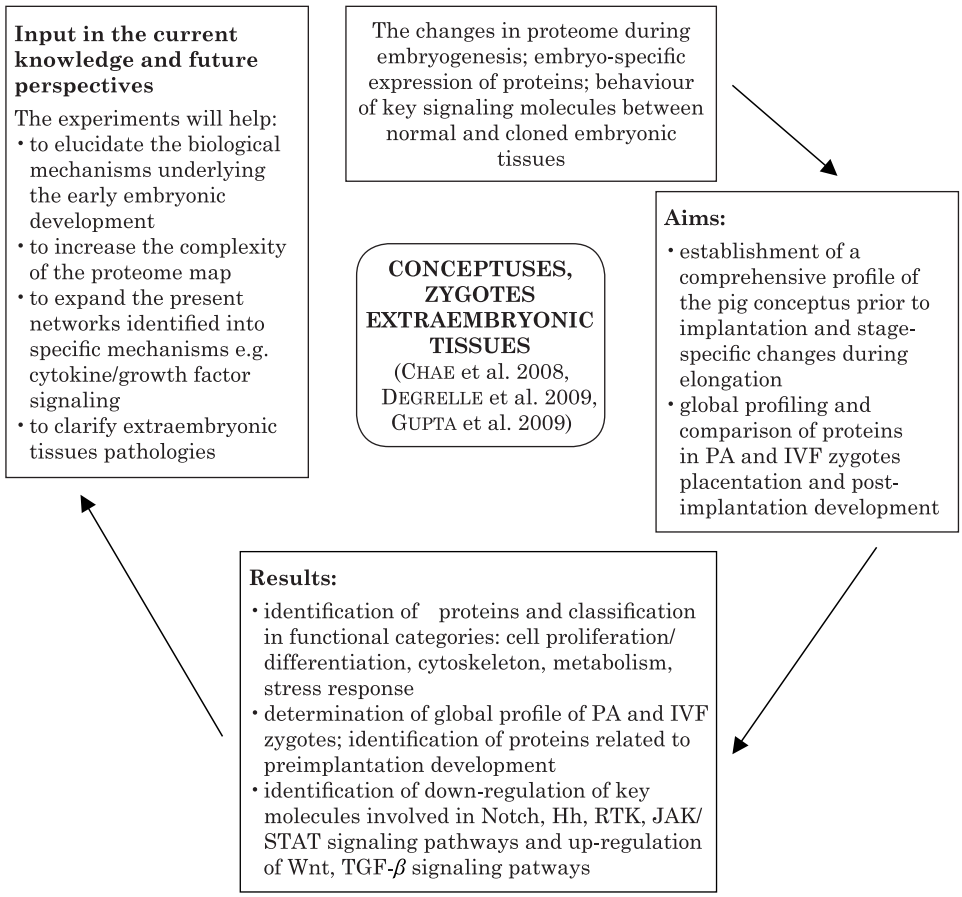

Fig. 4. The summary of the reviewed proteomics studies of pig conceptus, zygotes, and extraembryonic tissues. The subjects, aims, results and future perspectives of the reported studies are indicated. For standardization of the presentation, design of summaries is similar for different models presented in Figures 1 to 4

pigs' gestation in the context of pig production, especially at the level of embryo losses as a consequence of rearing, environmental or nutritional factors, might help to define biomarkers used for pig selection (de Almeida, Bendixen 2012). Proteomics has had great impact on the discovery of markers which are now used in clinical studies, and has been also helped to explore the pathogenesis of porcine reproductive and respiratory syndrome (Ding et al. 2012), classical swine fever (Li et al. 2010) and post-weaning multisystemic wasting syndrome (Cheng et al. 2012). These diseases are most harmful for pig production, as they cause high mortality rates of piglets (Kennedy et al. 2000, Opriessning et al. 2008). The methods and databases used to study proteome profiles of pig female reproductive tract are shown in Figure 5.

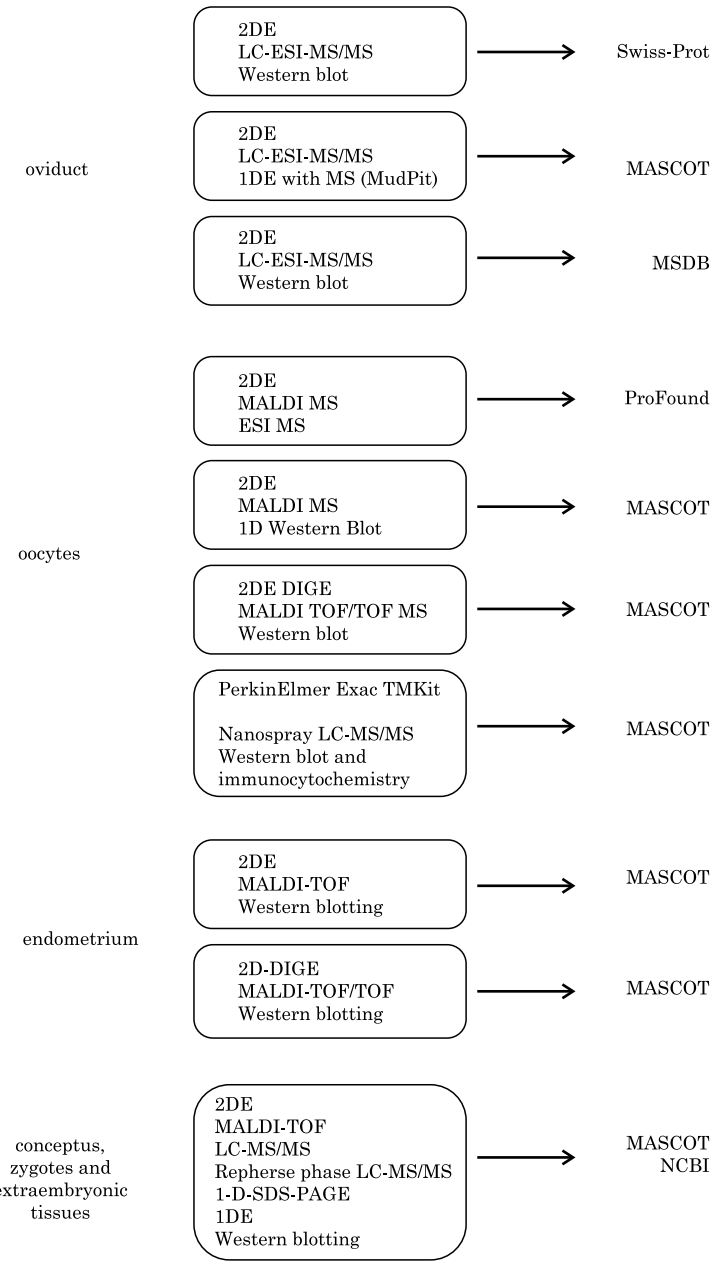

Fig. 5. Methods and databases used to study proteome profiles of the pig female reproductive system and embryos. The left column indicates types of tissues studied, the middle column shows proteomics technologies which were used, and the right part refers to databases used for identification of proteins

CONCLUSIONS AND FUTURE PERSPECTIVES

Proteomics is of growing interest to representatives of agricultural, veterinary and biomedical sciences (de Almeida, Bendixen 2012). Despite many advances in proteomics methods and technologies, there are still many issues to address. The most important goals of future studies are: 1) to improve sensitivity of analysis; 2) to develop automation of proteome analysis, which would allow fast proteome profiling; 3) to develop novel tools for complete proteome profiling (Chait 2011).

Farm animal proteomics, including pig proteomics, has been successfully applied to optimize animal welfare and productivity in the farming and food industry (Bendixen et al. 2011). The progressive advance of proteomics tools is of great importance for developing and describing pig models used in biomedical research (de Almeida, Bendixen 2012). As mentioned previously, that development of pig databases would strongly promote pig proteomics.

Model organisms play a critical role in understanding and studying human diseases. The human-like physiology of domestic pig ensures high accuracy of data obtained in pigs for human-related therapeutic research (Verma et al. 2011). Proteome profiling of pig reproductive system is under development. The reports reviewed here indicate that proteomics can help us gain insight into regulatory processes involved in reproductive functions of pigs. However, the extent of proteome profiling of the pig reproductive system is still relatively small and the cited reports have to be followed by more research.

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