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Comparative gene expression profiling of early porcine embryos derived from different *in vivo* and *in vitro* sources

by

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Abstract

There are strong interests in the factors that affect the efficient production of viable porcine embryos using either *in vivo* or *in vitro* production methods based on assisted reproductive technologies (ART). During the pre-implantation period of development, the porcine embryo exhibits dramatic changes and many key events of embryonic development take place. In this research, a series of studies were carried out in order to identify the critical regulators during the pre-implantation period of porcine embryonic development, and to identify the gene networks that are responsible for the impaired development of embryos produced after different ART manipulations. The detailed transcriptome profile of *in vivo*-derived "normal" pre-implantation porcine embryos has been characterized by transcriptomic profiling analysis of porcine oocytes and embryos representing nine different developmental stages from GV stage oocytes to day 11 embryos. Results from this research also suggest that the molecular events associated with embryonic genomic activation (EGA) in porcine pig embryos are probably initiated at, or before, the 4-cell stage. The embryo-activated genes "take-over" the majority of the mRNA profile from 8-cell stage onward, and the second wave of EGA probably peaks around the early blastocyst stage. Further comparative transcriptomic analysis between the *in vivo* hatched blastocysts (HB) and HB produced after *in vitro* ART manipulations (parthenogenetic activation (PA) and somatic cell chromatin transfer (CT)) revealed 1492 and 103 genes that differentially expressed of in PA and CT HB, respectively, in comparison with *in vivo* HB. Several significantly altered critical gene networks and pathways were identified in the PA- and CT-derived HB. In addition, apoptotic process was predicted to be activated in both PA and CT HB, and the activation of this apoptotic process is likely to be greater in PA HB. Finally, the effect of porcine luteinizing hormone-induced ovulation on the transcriptome of early porcine embryos was also

investigated. Overall, result from this research provided useful information for the understanding of the molecular mechanism underlying early porcine embryonic development, and identified several critical genes / gene networks that are likely to contribute to the deficiencies of porcine embryos produced after different ART manipulations.

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Table of Contents

Chapter 1: General introduction 1

1.1 References 5

Chapter 2: Literature review 8

2.1 Embryonic development in early porcine embryos 8

2.1.1 Key developmental events during the pre-implantation period of the embryonic development 8

2.1.2 Key molecular regulators during the early embryonic development 11

2.2 Effect of assisted reproductive technologies (ART) on embryonic qualities 13

2.2.1 *In vitro* maturation/*in vitro* fertilization (IVM/IVF) 14

2.2.2 Somatic Cell Nuclear Transfer (SCNT) 15

2.2.3 Parthenogenetic activation (PA) 17

2.2.4 Criteria of embryo quality assessment 18

2.3 Platforms for gene expression profiling analysis 20

2.3.1 DNA microarray 20

2.3.3 Next generation sequencing (NGS) technologies 22

2.3.3 Applications of microarray and NGS technologies in studies with porcine embryos 27

2.4 Summary 27

2.5 References 32

Chapter 3: Verification of a porcine embryo-specific expression microarray platform 49

3.1 Introduction 49

3.2 Material and methods 51

3.2.1 Samples collection and RNA isolation 51
3.2.2 RNA amplification and labelling for microarray analysis 51
3.2.3 Microarray data acquisition and Spike-In quality control (QC) analysis..... 53
3.3 Results and Discussion..... 54
3.3.1 GO analysis of genes on EMPV1 microarray 54
3.3.2 Microarray quality assessment..... 55
3.3.3 Porcine COCs and embryo transcriptome profiling..... 57
3.3.4 GO analysis for biological processes in porcine COCs & embryos..... 58
3.4 Conclusion 59
3.5 References..... 69
Chapter 4: Comparative transcriptomic analysis between porcine early and hatched blastocysts 75
4.1 Introduction..... 75
4.2 Material and methods..... 77
4.2.1 Porcine embryo sample collection 77
4.2.3 Total RNA isolation..... 77
4.2.4 Microarray experimental design 78
4.2.5 RNA amplification for microarray analysis..... 79
4.2.6 Amplified antisense RNA (aRNA) labelling 79
4.2.7 Hybridization, washing and data acquisition for Microarray..... 79
4.2.8 Bioinformatics tools and analysis 81
4.2.9 Gene Ontology (GO) analysis..... 82
4.2.10 IPA biological functions, canonical pathways and upstream regulator analysis 83
4.2.11 Real-time quantitative PCR (QRT-PCR) verification of gene expression..... 83
4.3 Results..... 84

4.3.1 Re-annotation of microarray platforms 84
4.3.2 Global transcriptomic profiling of porcine blastocysts using different platforms 85
4.3.3 Comparative transcriptomic analysis between early and hatched blastocyst stages 87
4.3.4 QRT-PCR verification 90
4.4 Discussions 91
4.4.1 Global transcriptomic profiling of porcine blastocyst using different platforms 91
4.4.2 Gene expression profile differences between early and hatched blastocyst 93
4.5 Conclusion 97
4.5 References 113
Chapter 5: Characterization of the porcine embryo transcriptome during early embryonic development: from oocyte to hatched blastocyst 121
5.1 Introduction 121
5.2 Material and Method 122
5.2.1 Recovery of *in vivo* embryos 122
5.2.2Total RNA isolation 123
5.2.3 RNA amplification for microarray analysis 123
5.2.4 Reference amplified antisense RNA (aRNA) pool generation 124
5.2.5 Microarray experimental design 124
5.2.6 Microarray analysis and data acquisition 125
5.2.7 PANTHER Gene Ontology (GO) analysis 126
5.2.8 IPA (Ingenuity® Pathway Analysis) upstream regulator analysis and canonical pathways 126
5.2.9 Real-time quantitative PCR (QRT-PCR) verification of gene expression 127

5.3 Results 127
5.3.1 Global mRNA profile of porcine oocyte and pre-implantation embryo 127
5.3.2 Pre-miRNA profile from GV to D11stage 129
5.3.3 Patterns of gene expression profile changes from 4-cell to hatched blastocyst 130
5.3.4 Significantly altered gene networks and pathways from 4C to HB stage 132
5.3.5 QRT-PCR verification of gene expression 133
5.4 Discussion 134
5.4.1 Global mRNA profiling during early porcine embryonic development 134
5.4.2 Global pre-miRNA profile of porcine oocyte and pre-implantation embryo. 136
5.4.3 Detailed gene expression profile changes from 4-cell to hatched blastocyst. 137
5.4.4 Significantly altered gene networks and pathways from 4C to HB stage 138
5.5 Conclusion 141
5.6 References 159
Chapter 6: Characterization of the altered gene expression profile in early porcine embryos generated from parthenogenesis and somatic cell chromatin transfer..... 166
6.1 Introduction 166
6.2 Materials and Methods 167
6.2.1 Recovery of *in vivo* embryos 168
6.2.2 Production of *in vitro*-derived embryos 168
6.2.3 Total RNA isolation 169
6.2.4 Microarray experimental design 169
6.2.5 Microarray analysis and data acquisition 169
6.2.6 Gene expression data analysis 170
6.2.7 Real-time quantitative PCR (QRT-PCR) verification of gene expression 170
6.3 Results 170

6.3.1 Altered gene expression profile in PA- and CT-derived HB 170
6.3.2 Altered gene expression-regulation during blastocyst hatching of PA-derived embryos 174
6.4 Discussion 175
6.5 Conclusion 182
6.6 References 189
Chapter 7: Characterization of the gene expression profile of blastocysts generated from porcine luteinizing hormone (pLH)-induced superovulation in gilts 197
7.1 Introduction 197
7.2 Materials and Methods 198
7.2.1 Recovery of *in vivo* embryos 198
7.2.3 Total RNA isolation 199
7.2.4 Microarray experimental design 199
7.2.4 RNA amplification and labelling for microarray analysis 199
7.2.5 Real-time quantitative PCR (QRT-PCR) verification of gene expression result 200
7.3 Results and Discussion 200
7.3.1 Microarray analysis 200
7.3.2 QRT-PCR verification of gene expression 201
7.3.3 Altered gene expression in blastocysts produced from pLH-induced gilts 201
7.4 References 208
Chapter 8: General discussion **..... 213**
8.1 EMPV1 microarray as an effective platform for transcriptomic analysis of early porcine embryos 213
8.2 “Normal” gene expression profile of early porcine embryo 214

8.3 Effect of somatic cell chromatin transfer (CT) and parthenogenetic activation (PA) on early porcine embryo transcriptome 218
8.4 Effect of hormone (pLH)-induced superovulation on early porcine embryo transcriptome 222
8.5 Implications 223
8.6 Strengths and limitations of the present research 225
8.7 Future studies 227
8.8 Overall conclusions 229
8.9 References 230
Appendices A: Animal ethics statement **236**

List of Tables

Table 3-1 PANTHER pathway analysis of developmental processes-associated genes... 61

Table 4-1 Primer sequences used in QRT-PCR verification 99

Table 4-2 Probes and unique genes positively detected in porcine BL embryos using 4 different platforms. 100

Table 4-3 Detection of selected gene markers in BL embryos (4 platforms) 101

Table 4-4 SREBF1 targets that differentially expressed between EB and HB 102

Table 5-1 Primers used in the QRT-PCR analysis..... 143

Table 5-2 Differentially expressed genes among the 4C, 8C, MOR, EB, XB, and HB embryos..... 145

Table 5-3 Significantly activated or inhibited upstream regulators from 8C to HB embryos..... 146

Table 6-1 Primer sequences used in QRT-PCR verification 184

Table 7-1 Primers used in the QRT-PCR analysis..... 205

Table 7-2 Differentially expressed genes between blastocyst generated from pLH-induced and control gilts..... 206

List of Figures

Figure 2-1 Early porcine embryonic development (in vivo). 30

Figure 2-2 Layout of EMPV1 microarray 31

Figure 3-1 Biological process categories that significantly enriched in EMPV1 array probes with unique gene symbols. 62

Figure 3-2 A scatter plot of Cy3 and Cy5 normalized signal intensity. 63

Figure 3-3 QC plot showing the correlation coefficient across-arrays from the COCs dye-swapped direct comparison analysis (generated from the EmbryoGENE QC module). .. 64

Figure 3-4 MA plot for COC gene expression data. 65

Figure 3-5 PANTHER bar chart of gene count involved in biological pathways from early embryos. 66

Figure 4-1 Comparison of characterized unique genes in the EMPV1 and SOMV2 platforms. 103

Figure 4-2 PANTHER GO biological process analysis of common unique genes between EMPV1 and SOMV2 platforms. 104

Figure 4-3 - Comparison of unique genes positively detected from BL embryos using microarray and 454 sequencing. 105

Figure 4-4 IPA Biological Function Analysis of genes differentially expressed between early and hatched blastocysts. 107

Figure 4-5 IPA Canonical Pathway Analysis of genes differentially expressed between early and hatched blastocysts. 109

Figure 4-6 QRT-PCR Verification. 110

Figure 5-1 Microarray experimental design. 148

Figure 5-2 mRNA profiles from the GV to D11 stages. 149

Figure 5-3 mRNA profiles of genes with different molecular functions from GV to D11. 151
Figure 5-4 Pre-miRNA profile from GV to D11. 152
Figure 5-5 (A) The eight major patterns of gene expression changes from 4C to HB stage. (B) Number of genes belongs to these eight expression patterns 153
Figure 5-6 PANTHER GO molecular function (A) and biological process (B) overrepresentation analysis of genes displayed different expression patterns from 4C to HB. 154
Figure 5-7 Significantly activated or inhibited upstream regulators from 8C to HB embryos. 156
Figure 5-8 Significantly changed IPA canonical pathways from 4C to HB 157
Figure 5-9 QRT-PCR verification of gene expression 158
Figure 6-1 Altered biological function categories in PA and CT-derived HB 185
Figure 6-2 Altered canonical pathways in PA and CT-derived HB 186
Figure 6-3 QRT-PCR verification result 187
Figure 7-1 QRT-PCR verification results of seven selected genes (*ASNS*, *RGN*, *DPP4*, *GNPDA1*, *SLC41A1*, *ACTB*, and *GAPDH*) 207

List of Abbreviations

2C	2-Cell
4C	4-Cell
8C	8-Cell
aRNA	Amplified antisense RNA
AI	Artificial Insemination
ART	Assisted Reproductive Technologies
BH-FDR	Benjamini and Hochberg False Discovery Rate
BL	Blastocysts
BLAST	Blast Local Alignment Search Tool
cDNA	Complementary DNA
COC	Cumulus-Oocyte-Complexes
CT	Somatic Cell Chromatin Transfer
EB	Early Blastocyst
EGA	Embryonic Genome Activation
ELMA	EmbryoGENE LIMS and Microarray Analysis
EMPV1	EmbryoGENE Porcine Array Version 1
FC	Fold Change
GEO	Gene Expression Omnibus
GO	Gene Ontology
GPR	GenePix Results
GV	Germinal Vesicle
HB	Hatched Blastocyst
ICM	Inner Cell Mass
IPA	Ingenuity® Pathway Analysis

IVC	*In vitro* Culture
IVF	*In Vitro* Fertilization
IVM	*In vitro* Maturation
IVV	*In vivo*
miRNA	MicroRNA
MOR	Morula
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NT	Nuclear Transfer
PA	Parthenogenetic Activation
PANTHER	Protein Analysis Through Evolutionary Relationship database
pLH	Porcine Luteinizing Hormone
QRT-PCR	Real-time quantitative PCR
RIN	RNA Integrity Number
RT	Reverse Transcription
SAR-SAGE	Small amplified RNA-serial analysis of gene expression
SCNT	Somatic Cell Nuclear Transfer
SOMV2	Sus.Scrofa Oligo Microarray v2
TE	Trophectoderm
USDA	United States Department of Agriculture
XB	Expanded Blastocyst

Chapter 1: General introduction

During the pre-implantation period of embryonic development, the porcine embryo exhibits dramatic morphological changes and many key developmental events take place such as cleavage, morula compaction, embryonic genome activation (EGA), blastocyst formation, and hatching (Oestrup et al., 2009; Sirard, 2012). Although the morphological steps involved in these key developmental events are well documented, the molecular mechanisms underlying these events are not yet fully understood.

The domestic pig is not only an economically important livestock species (Dang-Nguyen et al., 2010), but also an increasingly recognized biomedical animal model (Rogers et al., 2008; Chorro et al., 2009; Vilahur et al., 2011) due to its physiological similarities with human (Betthauser et al., 2000; Abeydeera, 2002). It is known that embryos derived from different *in vitro* ART manipulations, such as *in vitro* fertilization, cloning, and parthenogenesis, are less competent than their *in vivo* counterparts are. In addition, the utilization of ART is much less efficient in the pig than in many other mammalian species such as cattle (Kikuchi et al., 2002; Gajda, 2009). As a result, there is strong interest in the factors that affect the efficient production of viable embryos and porcine offspring either *in vivo* or using *in vitro* production methods based on ART.

The success of ART applications is highly dependent on the quality or competence of the embryos used. The ultimate criterion for embryonic competence is the ability to produce viable offspring after embryo transfer into a recipient animal (Alexopoulos and French, 2009). To date, parameters such as timing of the first cleavage division, overall embryonic morphology and blastocyst formation rate have been commonly used to predict the developmental competence of embryos derived after *in vitro* manipulation

(Lonergan, 2007). However, embryos resulting from *in vitro* manipulation could exhibit molecular deviations without displaying significant changes in the embryos' pre-implantation morphological characteristics (Vejlsted et al., 2006; Nánássy et al., 2008; Rodriguez-Osorio et al., 2009). Hence, the morphological criteria and rate of blastocyst development are not sufficient to determine the viability of early porcine embryos (Whitworth et al., 2008). As an alternative, characterization of the altered gene expression profile in embryos generated after different ART manipulations could reveal pathways critical for embryonic development and potential gene markers of embryonic quality (Whitworth et al., 2005; Ka et al., 2008).

The ultimate goal of the present research was to better understand the molecular mechanisms behind the critical developmental events during early porcine embryonic development, as well as to improve the efficiency of ART in porcine, by identifying the gene networks and pathways affected by different ART manipulations and the potential gene markers for embryo competence. Specifically, this PhD thesis research focuses on: 1) characterization of the "normal" transcriptome profile of early porcine embryos; 2) identification of the significantly altered gene networks and pathways in porcine embryos produced after *in vitro* manipulations, such as parthenogenesis and cloning; 3) investigation of whether the hormone (pLH)-induced ovulation in gilts has effects on the transcriptome of early porcine embryos; and 4) identification of potential gene markers for early porcine embryo quality assessment.

The second chapter of this thesis provides a review of existing literature concerning the early embryonic development of porcine embryos, and the effects of ART manipulations on early embryonic quality. After that, the review provides an overview of the technologies and platforms that are currently available for gene expression profiling of early porcine embryos.

The EmbryoGENE Porcine Array Version1 (EMPV1, [GPL14925]) microarray platform is a recently developed gene expression microarray enriched with genes expressed during porcine early embryonic development based on the NGS sequencing data from pools of *in vivo* and *in vitro* derived porcine early embryos (Tsoi et al., 2012). Chapter 3 of this thesis describes the verification processes of the EMPV1 microarray platform.

In chapter 4, the re-annotation process of the EMPV1 microarray and a commercially available porcine microarray (Sus.Scrofa Oligo Microarray v2, SOMV2, Agilent [GPL15007]) are described. In addition, the EMPV1 platform's efficiency for transcriptomic profiling analysis of early porcine embryos is compared with two other porcine microarray platforms (SOMV2 microarray and Swine Protein-Annotated Oligonucleotide Microarray (PigOligoArray, [GPL7435])). Furthermore, the comparative transcriptomic analysis between early (before hatching) and hatched (after hatching) blastocysts using both the EMPV1 and SOMV2 platforms are presented in chapter 4.

Considering that the EMPV1 microarray has proven to be an efficient platform for the transcriptomic analysis of early porcine embryos (chapter 3 and 4), all of the transcriptomic analyses in chapter 5, 6, and 7 were performed using the EMPV1 microarray.

Chapter 5 describes the global gene expression profiling analysis of porcine oocytes and early embryos (before elongation) from nine different stages (GV, MII, 4-cell, 8-cell, morula, early blastocyst, expanded blastocyst (XB), hatched blastocyst (HB), and day 11 embryos), and the comparative transcriptomic analysis of early porcine embryos from 4-cell to hatched blastocyst using the EMPV1 microarray platform. This chapter attempts to characterize the global transcriptomic profile during the pre-implantation period of porcine embryonic development, and further to identify the critical gene networks and

pathways that underlying important embryonic developmental events, such as EGA and blastocyst formation.

Although it is known that the embryos derived after *in vitro* manipulations, including parthenogenetic activation (PA) and cloning using somatic cell chromatin transfer (CT), are less competent in comparison with *in vivo* embryos (Sullivan et al., 2004; Gupta et al., 2008; Mesquita et al., 2013), the molecular mechanisms underlying the deficiencies of PA and CT embryos are not entirely clear. Chapter 6 of this thesis focuses on the characterization of the transcriptomic profile differences among porcine HB embryos derived from *in vivo*, PA, and CT sources, and the identification of critical gene networks / pathways associated with the deficiencies observed in PA and CT embryos. Chapter 6 also attempts to identify critical genes that were not properly regulated during the blastocyst hatching process in embryos derived from PA.

Control and synchronization of ovarian follicular development and ovulation can provide practical advantages in livestock management and application of ART (Degenstein et al., 2008). However, it has been suggested that exogenous hormone treatment-induced oocyte maturation in pig could result in a poorer quality embryos (Wiesak et al., 1990). Chapter 7 of this thesis focuses on the effect of porcine luteinizing hormone (pLH)-induced ovulation on the transcriptome of porcine blastocyst stage embryos.

In the final chapter (chapter 8) of this thesis, the findings from chapters 5, 6, and 7 are summarized and are discussed in relation to existing literature. Final conclusions are drawn based on these combined findings. In addition, this chapter also discusses the questions raised from these findings and potential future research directions.

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Chapter 2: Literature review

2.1 Embryonic development in early porcine embryos

During the pre-implantation period of embryonic development, the porcine embryo exhibits dramatic morphological changes and many key developmental events take place, such as cleavage, morula compaction, EGA, blastocyst formation, and hatching (Oestrup et al., 2009; Sirard, 2012) (Figure 2-1).

2.1.1 Key developmental events during the pre-implantation period of the embryonic development

In pig, the first cleavage division takes place in the zygote about 14 to 16 hours after fertilization, which generates a two-celled embryo. Each cell of the two-celled embryo, which is referred to as blastomere, undergoes successive divisions and generates 4, 8 and then 16 daughter cells (Abeydeera, 2002). After the 8-cell stage, the embryo enters into the uterus from the oviduct during the 2-3 days after fertilization (Abeydeera, 2002; Gosden and Gibbons, 2003; Blomberg et al., 2005; Oestrup et al., 2009). After the 8-cell stage, the blastomeres begin to form a solid ball of cells, which is called a morula. In the pig, the compaction process initiates immediately after the morula has formed, when there are only 8 to 16 cells within the embryo (Oestrup et al., 2009). During compaction, the outer cells became more compacted than the cells in the center resulting in the separation of the inner cells and outer cells (Hyttel and Niemann, 1990). The outer cells connect with each other through tight junctions, while the inner cells remain as a tight cluster of lucent cells (Oestrup et al., 2009). This polarization of cells and morula compaction are considered the first morphological signs of differentiation leading to the separation of the inner cell mass (ICM) and the trophectoderm (TE) of the blastocyst (Reima et al., 1993). After the tight junctions in the outer cells are formed, the embryo

starts to accumulate fluid, and a fluid filled cavity inside of the embryo is formed. Once a recognizably distinct cavity is formed, the embryo is referred to as a blastocyst, and the cavity is called the "blastocoele" (Anderson, 1978). The porcine blastocyst forms at approximately day 5 after fertilization *in vivo* (Hyttel and Niemann, 1990). Similar to most other mammalian species, the porcine blastocyst stage embryo has a distinct morphological structure that consists of inner cell mass (ICM), internal cavity (blastocoele), and a single layer of epithelial trophectoderm (TE) with (before hatching) or without (after hatching) the protective zona pellucida (Watson and Barcroft, 2001; Duranthon et al., 2008).

Following blastocyst formation, the embryo expands in size and hatches from the zona pellucida to become a "free floating" hatched blastocyst in the uterus (Oestrup et al., 2009). This process is referred to as "hatching", which is a critical and tightly regulated event during early embryonic development of mammalian embryos (Seshagiri et al., 2009). In the pig, the blastocyst hatching process occurs approximately 5-6 days after fertilization *in vivo* (Oestrup et al., 2009). During the hatching process, the ICM separates into two distinct cell populations: the cell layer towards the blastocoele cavity flattens and further forms the hypoblast, while the dorsal cell population establishes the epiblast (Blomberg et al., 2008; Rielland et al., 2008). The hypoblast subsequently extends along the inside of the trophoblast. The hypoblast is sometimes also referred to as the primitive endoderm as opposed to the definitive endoderm formed as one of the germ layers during gastrulation (Oestrup et al., 2009).

Ungulate animals, such as the pig, sheep, and cattle, are characterized as having a long pre-implantation period and filamentous (or threadlike) embryonic structure prior to attachment (Spencer et al., 2004). Unlike human and mouse, the ungulate blastocyst remains detached in the uterus after hatching, and then the embryos experience a phase of

rapid trophoblast development that dramatically changes the embryonic morphology (from a sphere to ovoid to tubule to filament) prior to implantation, which is called elongation (Blomberg et al., 2005; Blomberg et al., 2008). In all ungulate species including the pig, the elongation process, which occurs during the peri-implantation period, is concomitant with gastrulation (Bazer et al., 1979; Geisert et al., 1982; Hue et al., 2001; Blomberg et al., 2008). Beginning at day 11 of gestation (D11), the porcine conceptus undergoes dramatic elongation from an 8-10 mm ovoid to a ~150 mm filament by day 12 after insemination (D12) (Anderson, 1978; Geisert et al., 1982; Blomberg et al., 2008). Trophoblast elongation initiates around D11 and D12 in the sheep and the cattle, respectively (Guillomot et al., 2004). During elongation in cattle, the conceptus displays more than a 1000-fold increase in size (Maddox-Hyttel et al., 2003). This morphological change is accompanied by a significant increase in cell number and protein synthesis (Thompson et al., 1998b; Degrelle et al., 2005; Blomberg et al., 2008). In the pig, the initial stages of trophoblast elongation take place through cellular reorganization and differentiation, while the proliferation occurs later (Enders et al., 1989; Blomberg et al., 2005). Expansion of the trophoblast provides an increased placental surface area, which enables the maternal-conceptus "cross-talk" and nutrient exchange that is necessary for the survival of the conceptus (Blomberg et al., 2008). Hence, the timing of elongation in porcine embryos may play an important role in conceptus survival: the blastocysts that differentiate earlier may have a competitive advantage over others in obtaining the necessary uterine surface for further development (Blomberg et al., 2005). Initial placentation in the pig occurs around day 13 to 14 of gestation, which is considerably later than the time of implantation in human (Oestrup et al., 2009).

Although the morphological steps of the embryonic developmental events during the pre-implantation period of porcine embryos have been well documented, the molecular mechanisms underlying these events are not yet fully understood.

2.1.2 Key molecular regulators during the early embryonic development

In comparison with the human and mouse, the molecular mechanisms underlying the pre-implantation period of embryonic development are less defined in domestic animals such as the pig.

In mouse embryos, CDX2 (caudal type homeobox 2) is the key regulator for the specification of the trophectoderm (Niwa et al., 2005; Strumpf et al., 2005; Ralston and Rossant, 2008), and CDX2 mutations result in implantation failure (Strumpf et al., 2005). During early developmental stages of the mouse embryo, CDX2 is co-expressed with POU5F1 (POU Class 5 Homeobox 1, also known as OCT4), but the CDX2 expression is restricted to the TE after blastocyst formation (Niwa et al., 2005; Ralston and Rossant, 2008; Suwińska et al., 2008). Expression of CDX2 mRNA has been reported in the human (Kimber et al., 2008), pig (Kuijk et al., 2008), and bovine (Hall et al., 2005). It is believed that the CDX2 has a conserved role in TE specification in mammals (Oestrup et al., 2009).

In mouse, GATA6 (GATA binding protein 6) is the key regulator for the specification of the primitive endoderm (PE) during early embryonic development (Kuijk et al., 2008), and embryonic stem (ES) cells with GATA6 over-expression develop into PE cells (Fujikura et al., 2002; Kuijk et al., 2008). GATA6 has been localized to a subset of the ICM cells, which are randomly distributed in the ICM during the blastocyst stage and direct PE development in the mouse (Chazaud et al., 2006). The same expression pattern of GATA6 was observed in porcine and bovine embryos (Kuijk et al., 2008), and GATA6

is believed to have a conserved role in PE formation during early embryonic development in mammals (Chazaud et al., 2006; Kuijk et al., 2008).

In the human and mouse, transcription factors OCT4, NANOG and SOX2 (sex determining region Y-box 2) are known to be key "pluripotency regulators" that are exclusively expressed within the ICM of the blastocyst (and later in the epiblast) but not in the TE (Boyer et al., 2005; Oestrup et al., 2009). However, in the pig blastocyst, expression of OCT4 is observed in both the ICM and TE (Keefer et al., 2007; du Puy et al., 2011). There is some controversy regarding NANOG, as its transcript was reported to be not detected in the ICM of porcine blastocysts in one study (Kuijk et al., 2008), while the presence of NANOG transcripts were reported in alternate studies (Brevini et al., 2007; du Puy et al., 2011). In addition, the expression of SOX2 mRNA is at very low levels in porcine blastocyst stage embryos (Magnani and Cabot, 2008) and is expressed exclusively in the epiblast of the D9.5 hatched blastocyst (du Puy et al., 2011). Findings from these studies suggest that several key regulators of embryonic development in early porcine embryos behave differently from other mammals, while other key embryonic developmental regulators have conserved roles among different mammalian species.

In mammals, oocyte-derived mRNAs degrade shortly after fertilization; hence, EGA and production of embryo-derived transcripts must occur during early embryonic development (Thompson et al., 1998a; Schultz, 2002). The precise timing of the onset of EGA in mammals varies among species, and the major embryonic genome activation (EGA) in the pig embryo is believed to be occurring at the 4-cell stage (Telford et al., 1990; Prather et al., 2009; Sirard, 2012). On the other hand, distinct differences in the gene expression profiles among the oocyte, 4-cell stage embryo and blastocyst has been reported in the pig (Whitworth et al., 2005). The distinct gene expression profile differences observed between oocytes and 4-cell stage embryos indicate the expression of

a large set of embryonic genes, which has raised questions regarding the precise timing of the molecular mechanisms underlying the initiation of EGA process in the pig.

The epigenetic remodeling of the specialized parental genomes into the totipotent genome of the zygote and initial blastomeres is necessary for the EGA and further development of mammalian embryos (Morgan et al., 2005; Oestrup et al., 2009). DNA methylation and histone modifications are the two most studied epigenetic modifications associated with early embryonic development. The regulation of DNA methylation and the histone deacetylation are synchronized processes that are associated with transcriptional repression and chromatin condensation (Oestrup et al., 2009). Mammalian embryos exhibit dynamic DNA methylation levels during the pre-implantation period of the embryonic development (Oestrup et al., 2009; Smith et al., 2012). The parental genomes are considered to be hypo-methylated at the time of fertilization, and the methylation levels of the embryonic genome decreases and reaches the lowest point at the blastocyst stage (Smith et al., 2012). These epigenetic regulation processes have important roles during embryonic development such as regulating gene expression, maintaining genomic integrity, and establishing parental-specific imprinting patterns. Hence, improper or incomplete epigenetic regulation processes during the early embryonic development could have perturbing effects on the survivability and further development of the embryo.

2.2 Effect of assisted reproductive technologies (ART) on embryonic qualities

As previously mentioned in chapter1, the domestic pig has become increasingly recognized not only as an economically important livestock species (Dang-Nguyen et al., 2010), but also as an biomedical animal model (Rogers et al., 2008; Chorro et al., 2009; Vilahur et al., 2011) due to its physiological similarities with humans (Betthauser et al., 2000; Abeydeera, 2002). Therefore, efficient reproduction of this species is of particular

interest to the scientific and livestock producer communities. Applications of ARTs have great potential to increase the efficiencies of swine reproduction. In comparison with other mammalian species, pig oocytes and embryos have higher lipid content stored mainly as lipid droplets in the cytoplasm, which has a negative influence on the efficiency of manipulations on them (Gajda, 2009). In general, the utilization of ART in the pig is much less efficient than many other mammalian species (Kikuchi et al., 2002; Gajda, 2009). As a result, there are strong interests in the factors that affect the efficient production of viable embryos and offspring in pig using either *in vivo* or *in vitro* production methods based on ART.

2.2.1 *In vitro* maturation/*in vitro* fertilization (IVM/IVF)

The *in vitro* production (IVP) of embryos mainly involves three steps: *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos (Lonergan, 2007). Surgical collection of oocytes from donor animals is time consuming and expensive, hence the ovaries from slaughterhouse animals are utilized for many *in vitro* ART techniques to generate mature oocytes and embryos (Abeydeera, 2002). In cattle, 30-40% of *in vitro* cultured embryos reach the blastocyst stage, at which point they often can be transferred to a female animal, with about 50% of the transferred embryos being able to produce viable offspring (Lonergan, 2007; Alexopoulos and French, 2009). IVP systems (including IVM, IVF, and IVC) in porcine have been modified by many researchers (Abeydeera, 2002; Viet Linh et al., 2009). However, the porcine IVP systems still have a relatively low efficacy in comparison with *in vivo* embryos and are more prone to having developmental failures (Bauer et al., 2010; Isom et al., 2013). Porcine embryos produced from IVP systems have poor embryonic qualities and low blastocyst developmental rates, which is believed to be the result of factors such as polyspermic oocyte penetration and the imperfect *in vitro* culture environment (Wheeler et al., 2004;

Bauer et al., 2010). The number and quality of oocytes that successfully enter the reproductive process is crucial in blastocyst developmental rate, while the post-fertilization culture environment has a major influence on the quality of the blastocyst (Abeydeera, 2002; Lonergan, 2007; Bauer et al., 2010).

2.2.2 Somatic Cell Nuclear Transfer (SCNT)

Somatic Cell Nuclear Transfer (SCNT) is a technique that replaces the nucleus of an unfertilized oocyte with the nucleus from a somatic cell (such as skin, heart, or nerve cell) and stimulates this reconstructed oocyte to begin dividing in the same manner as an embryo (De Sousa et al., 2002; Wilmut et al., 2002; Alexopoulos and French, 2009). SCNT, often referred to as cloning, has been performed with several different mammalian species including sheep, mice, cattle, and pigs (Betthauser et al., 2000; Forsberg et al., 2002). The SCNT procedure includes the *in vitro* maturation of source oocytes, culture of donor cells, nuclear transfer (NT), activation of the reconstructed oocytes following NT, and the *in vitro* culture of embryos and their transfer to recipient animals (Betthauser et al., 2000; Forsberg et al., 2002). SCNT has great potential applications in basic and biomedical research such as the production of genetically modified pigs for xenotransplantation (Ka et al., 2008), production of pharmaceutical proteins, and the enhancement of pig breeding programs (Betthauser et al., 2000; Byrne et al., 2007; Ezashi et al., 2009; Rodriguez-Osorio et al., 2009). However, the application of SCNT is limited by low embryo survival rates and the high incidence of abnormalities in individuals that develop to term, which are believed to be associated the incorrect or incomplete nuclear reprogramming (Wang et al., 2011; Mesquita et al., 2013). Although, the morula and blastocyst formation rates have been improved significantly in the SCNT systems (Farin et al., 2006), the quality of blastocysts produced by SCNT is still impaired in many ways: lower hatching ability (Gupta et al., 2008), less cells in the

inner cell mass (ICM), lower blastocyst formation rate (Hao et al., 2003), irregular-sized blastomeres (Gupta et al., 2008), and a high level of fragmentation and apoptosis (Farin et al., 2006; Alexopoulos and French, 2009). Although it is possible to generate adult animals through SCNT, the efficiency of SCNT remains very low (Ka et al., 2008). The problems associated with the development of SCNT derived embryos include large offspring syndrome (Young et al., 1998; Ka et al., 2008), high embryonic mortality (Hoffert et al., 2005; Ka et al., 2008) and abnormal placental morphology (Ono et al., 2001; Hoffert et al., 2005), poor developmental competence (Ka et al., 2008) and other aberrant phenotypes (Walker et al., 2002). In cattle, SCNT has similar blastocyst development rates to IVF (about 30-40%), but the rates of viable offspring production are much lower (around 10-15%) than those obtained through IVF or *in vivo* (Farin et al., 2006; Alexopoulos and French, 2009). Reconstructed porcine embryos have even lower blastocyst development rate (around 1%) and cloning efficiency and a higher embryonic death rate than in reconstructed bovine embryos (Ka et al., 2008; Alexopoulos and French, 2009).

Epigenetic changes are believed to be good indicators of the embryonic competence of early stage embryos produced after *in vitro* ART manipulations such as SCNT (Oestrup et al., 2009). Incorrect or incomplete nuclear reprogramming is believed to be associated with the low efficiency of SCNT (Wang et al., 2011; Mesquita et al., 2013). Somatic cell chromatin transfer (CT) is a cloning technology that was designed to facilitate the epigenetic reprogramming process (Sullivan et al., 2004; Rodriguez-Osorio et al., 2009), which involves *in vitro* remodeling of the donor nuclei prior to their transfer into enucleated oocytes by removing nuclear components that may interfere with nuclear remodeling (Sullivan et al., 2004; Collas et al., 2007). The *in vitro* donor nuclei remodeling process of the CT technology involves incubation of the donor nuclei under

"remodeling media" (e.g., supplemented mitotic cell extract) prior to their transfer, which could facilitate the proper remodeling of the donor nuclei (Collas et al., 2007). Although promising results (such as higher number of viable offspring) have been reported using CT, the embryos generated still exhibit abnormalities similar to those observed following conventional SCNT (Sullivan et al., 2004; Collas et al., 2007; Mesquita et al., 2013).

2.2.3 Parthenogenetic activation (PA)

Although parthenogenesis is not a form of natural reproduction in mammals (Paffoni et al., 2008), it has been artificially induced in many mammalian species such as human (Paffoni et al., 2007), bovine (Méo et al., 2004), rabbit (Liu et al., 2002), cat (Grabiec et al., 2007) and mice (Kono et al., 2002). Given that the development of parthenogenetically activated oocytes (parthenotes) share identical morphological characters with embryos generated from IVF and SCNT systems in several animal models (Gupta et al., 2007; Gupta et al., 2008; Hosseini et al., 2008; Paffoni et al., 2008), parthenogenesis may be used as a possible embryological developmental model (Paffoni et al., 2008). In humans, the embryological model based on parthenogenetic activation (PA) of oocytes could overcome many of the ethical limitations to human embryos research in the timing and embryonic development stages (Paffoni et al., 2008). Human parthenotes are able to maintain early embryological development until the blastocyst stage, hence, they could serve as a potential experimental model for embryo development, stem cell, and regenerative medicine researches (Brevini and Gandolfi, 2008). In addition, parthenogenesis systems do not involve the male/sperm factors, hence, they can be considered as a good model system for the analysis of the maternal influence on embryonic development including gene imprinting (Naturil-Alfonso et al., 2012) (Gupta et al., 2008; Naturil-Alfonso et al., 2012). In addition, the PA systems are often used as

control systems for IVF or SCNT research (Katayama et al., 2006; Gupta et al., 2008; Paffoni et al., 2008).

Mammalian parthenotes obtained *in vitro* using oocytes from different stages along oocyte meiosis result in parthenotes with different chromosome complements (haploid or diploid) (Paffoni et al., 2008). Parthenogenetically activated oocytes (diploid or haploid) are able to resume meiosis and proceed through early development (Nánássy et al., 2008; Petr et al., 2008), and the development of haploid PA embryos tends to be more delayed than diploid parthenogenetic embryos (Hao et al., 2004). In comparison with fertilized embryos, PA embryos always exhibit delayed development, reduced total cell number, and fewer cells in the inner cell mass of blastocysts, (Paffoni et al., 2008; Petr et al., 2008). Apoptotic cell death plays an important role in pre-implantation mammalian embryonic development (Hao et al., 2004). For instance, apoptotic cell death in the human blastocyst seems to correlate with cell number and embryo quality (Levy et al., 2001). In general, parthenogenetic embryos exhibit a higher level of apoptotic cell death during culture compared to embryos generated by *in vitro* fertilization systems (Hao et al., 2003; Hao et al., 2004; Jin et al., 2007). In addition, porcine embryos produced from PA systems displayed altered epigenetic regulation mechanisms (Oestrup et al., 2009).

2.2.4 Criteria of embryo quality assessment

Many efforts has been made to characterize the factors affecting embryo quality and the key molecular mechanisms responsible for impaired development in porcine embryos generated after different *in vitro* ART manipulations (Whitworth et al., 2004; Whitworth et al., 2008; Prather et al., 2009; Bauer et al., 2010; Isom et al., 2013). However, the molecular mechanisms underlying the impaired development of embryos derived from *in vitro* ART manipulation systems such as IVM/IVF, SCNT, and PA have not been well established.

The ultimate criterion for embryonic competence is the ability to produce viable offspring (Alexopoulos and French, 2009). To date, the morphology characteristics and blastocyst formation rate are still two of the major parameters commonly used in embryonic developmental competence assessment (Lonergan, 2007; Lonergan, 2007). These criteria along with other non-invasive assessment criteria such as the timing of the first cleavage division, which has been linked to developmental ability (Lonergan, 2007), have been utilized for the selection of viable embryos prior to embryo transfer (Van Soom et al., 2003).

However, it is known that different culture and induced environmental conditions can significantly change the fetal developmental pathway without obvious changes in pre-implantation morphology of *in vitro* produced and cloned embryos (Edwards et al., 2003; Chavatte-Palmer et al., 2004; Wells, 2005; Farin et al., 2006). At least in cattle and pigs (Vejlsted et al., 2006), embryos produced from IVM/IVF (Giritharan et al., 2007), SCNT (Rodriguez-Osorio et al., 2009), and PA systems (Hao et al., 2004; Nánássy et al., 2008; Bebbere et al., 2010) display molecular deviations without significant changes in morphological characteristics (Vejlsted et al., 2006). Since different *in vitro* ART manipulations can have disruptive effects on the pattern of gene expression in the embryo with potential long-term consequences, morphological criteria and blastocyst development rate alone are not sufficient for the embryo quality assessment (Shiraki et al., 2003; Whitworth et al., 2004; McHughes et al., 2007; Whitworth et al., 2008; Rodriguez-Osorio et al., 2009). Hence, reliable gene markers for embryo quality assessment are needed. Characterization of the gene expression profile of the early porcine embryo could identify the pathways critical for embryo development and gene markers for embryo quality determination (Whitworth et al., 2005; Ka et al., 2008). A

quantitative examination of gene expression could be a valuable tool for embryo quality assessment.

2.3 Platforms for gene expression profiling analysis

Recent advances in molecular biology tools for studying gene expression have resulted in the availability of a variety of options for gene expression profiling such as DNA microarrays, and next generation sequencing (NGS) technologies (Pariset et al., 2009).

2.3.1 DNA microarray

The DNA microarray is a very powerful high throughput method for gene expression profiling, which can analysis the expression of thousands of genes at the same time (HornshÃ j et al., 2009). After being first introduced in 1995 (Schena et al., 1995), the DNA microarray has been widely utilized in many research fields such as gene expression profiling (Hue et al., 2007), genotyping (Sachse et al., 2009), pathogen detection (Suo et al., 2010), and Genome-wide detection of single feature polymorphisms (SFP) including single nucleotide polymorphisms (SNPs) and indels (Bischoff et al., 2008).

A DNA microarray consists of an arrayed series of thousands of specific DNA sequences that are used to hybridize with targets, hence the microarray can only analyses the expression of a predefined set of genes (Li et al., 2006; Ka et al., 2008). The probes present on DNA microarrays may be either cDNA (cDNA microarray) or oligonucleotides (Oligonucleotide microarrays) (Pariset et al., 2009). In cDNA microarrays, the cDNA probes, usually amplified cDNA fragments, are spotted onto a solid substrate such as a glass slide (Kothapalli et al., 2002). In Oligonucleotide microarrays, the probes can be either pre-synthesized and then spotted on the array surface (such as the PigOligoArray (Steibel et al., 2009)), or synthesized in situ on the

array (such as Affymetrix (Pease et al., 1994) and Nimblegen (Nuwaysir et al., 2002) microarrays). In addition, the lengths of the probes in different microarray platforms can vary. For example, the Agilent microarray utilizes an ink-jet technology to print 60-mers probes on to the slides (Hughes et al., 2001), PigOligoArray uses 70-mer probes (Steibel et al., 2009), while Affymetrix utilizes a set of 11 short probes (25-mers) for each target gene (Pease et al., 1994; Kothapalli et al., 2002).

The CombiMatrix oligonucleotide microarray platform is a relatively new technology, which is based on a silicon microchip containing arrays of thousands of platinum microelectrodes, which can simultaneously synthesize different oligonucleotides in response to digital control (Ghindilis et al., 2007). Since each microelectrode can synthesize a different oligonucleotide, this technology enables one to design a microarray of any desired configuration (Pariset et al., 2009).

The gene expression observed with microarrays is determined by the hybridization between the probes on the microarray and the fluorescent-labeled targets (Shalon et al., 1996). Based on differences in fluorescent signal detection channels, DNA microarray platforms can also be classified into one-coloured microarrays such as Affimetrix (Pease et al., 1994) and two coloured microarrays (Ghindilis et al., 2007; Pariset et al., 2009) such as PigOligoArray (Steibel et al., 2009)). For the one-coloured microarray, only one fluorescent-labeled target sample is hybridized with each slide, and the gene expression differences are determined by comparison among different slides (Pease et al., 1994). In the two-coloured microarray (Shalon et al., 1996; Kothapalli et al., 2002), two different target samples, which were labeled with two different fluorescent dyes, are hybridized with one slide, and the gene expression differences are determined by the ratio of the two fluorescent signals (Shalon et al., 1996).

Microarrays have become a fundamental tool for gene expression profiling and have been used as a clinical diagnostic tool (Pariset et al., 2009). However, the application of microarrays is limited by many factors. For instance, due to nonspecific hybridization or cross hybridization to non-specific sequences (Kothapalli et al., 2002), microarrays shows lower sensitivity and dynamic signal range in comparison with other gene expression analyses, such as the real-time quantitative PCR (Yuen et al., 2002). There are concerns about the reliability of microarray results because of the low repeatability between different microarray platforms (Tan et al., 2003; Pariset et al., 2009). In addition, because previous gene and sequence knowledge are pre-required for the microarray development and data interpretation, the utilization of microarray is somewhat limited in species with incomplete genome sequences or annotations (Pariset et al., 2009).

2.3.3 Next generation sequencing (NGS) technologies

In recent years, growing interests in genome resequencing and high demand for low-cost sequencing have driven the development of a new generation of sequencing technologies that parallelize the sequencing process, producing thousands or millions of sequences at once (Hall, 2007). Advances in sequencing technologies are driving down sequencing costs and increasing sequence capacity dramatically, and making whole-genome resequencing by individual laboratories possible (Mardis, 2008; Varshney et al., 2009). NGS are revolutionizing genomic studies by allowing novel applications in biology and medicine such as genome-wide transcription factor binding-site profiling, transcriptome sequencing, whole-genome resequencing (Turner et al., 2009), and non-coding RNA expression profiling (Morozova and Marra, 2008) to take place. The NGS technologies have provided unprecedented opportunities for high-throughput genomic researches. The NGS technologies are relatively new, and they generate new types of very large datasets, which make the sequencing data assembling, annotation, and interpretation very

challenging (Mardis, 2008). Although it is currently difficult to generate complete *de novo* assemblies of higher-vertebrate genomes solely using next-generation sequencing, improvements in sequence read lengths and throughput, coupled with new assembly algorithms for large data sets, will soon make this a reality (Turner et al., 2009). Currently, there are many different NGS technologies available for genomic research applications such as the 454 sequencing/Roche, Illumina/Solexa, SOLiD/ABI (Applied Biosystems), Ion torrent (life technologies), and Pacific Biosciences (PacBio) (Nyren, 2007; Morozova and Marra, 2008; Quail et al., 2012).

Although these NGS technologies are based on different principles, they share several features in comparison with the Sanger sequencing method. First, NGS platforms have the ability to process millions of sequence reads in parallel rather than 96 or 384 at a time (Meyer et al., 2009; Reis-Filho, 2009; Walter et al., 2009; Quail et al., 2012). This massively parallel throughput may require only one or two instrument runs to complete an entire experiment. Secondly, the reads of NGS are produced from fragmented 'libraries' that have not been subjected to the conventional vector-based cloning and *Escherichia coli*-based amplification stages used in capillary sequencing. As such, some of the cloning bias issues that affect genome representation in sequencing projects may be avoided, although each sequencing platform may have its own associated biases (Mardis, 2008; Quail et al., 2012). Third, relatively little input DNA (several micrograms at most) is needed to produce a library, and with modification in the library production process, most of the NGS platforms can sequence the paired ends of fragments (Quinn et al., 2008; Varshney et al., 2009). Finally, most of the NGS platforms produce much shorter read lengths (100~400 bp, depending on the platform) than Sanger sequencing (except the 454 sequencing and the PacBio technology), which can impact the utility of the data for various applications such as *de novo* assembly and genome resequencing (Mardis,

2008; Pop and Salzberg, 2008; Varshney et al., 2009). The 454 sequencing and the PacBio technology are superior to the other NGS platforms in terms of obtaining longer sequence reads (Gupta, 2008; Ansorge, 2010; Quail et al., 2012).

Although the PacBio provides outstanding reads length (mean reads length > 1500 bp) that are actually longer than the Sanger sequencing, the large sample-input requirements and the amplification-free library preparation workflow of PacBio limits its utilization in applications involving significant prior enrichment such as ChIP-seq and exome sequencing (Quail et al., 2012). Alternatively, the long read length of the PacBio technology might have applications in *de novo* sequencing and transcription variant discovery (Quail et al., 2012).

454 sequencing was the first commercially available NGS technology, which was introduced by 454 Life Science/Roche in 2005 (Hall, 2007). 454 sequencing is an ultra-high-throughput amplification-dependent DNA sequencing technology based on the principle of 'pyrosequencing' (Nyren, 2007). Currently, the 454 Sequencer has the ability to sequence 400-600 million bp in a single run with a Sanger sequencing-like read length of 500-700 bp (http://454.com/products/gs-flx-system/index.asp). The read length of 454 sequencing is longer than many other NGS technologies such as the Illumina and SOLiD technologies. In addition, 454 sequencing has significant advantages over Sanger sequencing because of its ultra-high-throughput, real time detection of base incorporation, and lower cost (Varshney et al., 2009). However, the precision of Roche/454 sequencing technology in handling homopolymers (short stretches of the same contiguous nucleotides) is less reliable than many other commercially available NGS technologies (Varshney et al., 2009). In terms of costs per bp of sequence data, 454 sequencing is more expensive than either the Illumina or SOLiD technologies (Varshney et al., 2009).

Illumina/Solexa sequencing was introduced to the market in 2006 (Varshney et al., 2009). The principle of the Illumina/Solexa sequencing system is based on an ingenious sequencing-by-synthesis chemistry, with novel reversible terminator nucleotides for the four bases each labelled with a different fluorescent dye and a special DNA polymerase enzyme that can incorporate those dyes (Mardis, 2008). The Illumina/Solexa system has the ability to generate 60 - 600 Gb of data in a run, and the sequence read length is about 100 nucleotides (http://www.illumina.com/). Unlike Roche/454 sequencing, Illumina/Solexa has no problems in sequencing homopolymeric regions, and the accuracy of Illumina/Solexa is comparable to or better than that of Roche/454 sequencing. For each base position sequenced, the Illumina/Solexa platform requires incorporation, imaging and cleavage of the reversible terminators, thus limiting the read length of sequences. Owing to the short reads, *de novo* genome sequencing for large genomes is problematic because of the difficulty of accurately assembling shorter reads. However, if a nearly identical genome or reference genome sequence is available, this can be used to assemble and/or align individual sequence reads (Varshney et al., 2009). The Illumina/Solexa technology was widely utilized in the quantitative analysis of gene expression (Morozova and Marra, 2008; van Iterson et al., 2009), detection of methylated regions (Zhang et al., 2008; DiGuistini et al., 2009), and determination of protein binding sites on DNA or RNA (Morozova and Marra, 2008; Ansorge, 2010).

SOLiD (Sequencing by Oligo Ligation and Detection) /ABI system was introduced to the market in 2007 (Mardis, 2008). The SOLiD/ABI technology utilized a unique sequencing chemistry based upon the ligation catalysed by DNA ligase and octamer labelling (Ansorge, 2010). The unique attribute of this ligation-based approach and the octamer labeling is an extra quality check of read accuracy, which reduced the error rate during sequencing (Ansorge, 2010). Each SOLiD run has the ability to produce 80 - 320 Gb of

sequence data with an average read length of 50 - 75 bp. Because of the high data quality and the huge amount of data can be produced from each run, the SOLiD sequencing is utilized in researches including mutation discovery, metagenomic characterization, non-coding RNA and DNA–protein interaction discovery (Hall, 2007; Mardis, 2008; Ansorge, 2010).

The ultra-high-throughput and the lower cost of the NGS technologies made large-scale sequencing within the reach of many scientists (Pop and Salzberg, 2008). However, the short reads generated by most of the NGS platforms (except the 454 sequencing and the PacBio technology) provides potential problems for sequence assembly particularly in areas associated with sequence repeats (Morozova and Marra, 2008; Quail et al., 2012). Although the accuracies of NGS technologies are improving, users generally rely on relatively high redundancy of sequence coverage to determine reliability of the sequence for a region, particularly those containing a polymorphism (Thomas et al., 2006). Hence, the improvement in the reaction chemistry has the potential to further decreasing the cost associated with the NGS technologies. Although the costs of NGS technologies have been decreasing dramatically, it is still prohibitively expensive for many researchers, especially for research projects requiring transcriptomic level analysis of many samples. Alternatively, DNA microarrays continue to be widely used as an efficient tool to analyse the expression levels of tens of thousands of different predetermined transcripts in many different samples (Ka et al., 2008; HornshÃ j et al., 2009).

Real-Time Quantitative PCR (QRT-PCR) is widely utilized in quantifying mRNA and cDNA due to its sensitivity and repeatability (Jamnikar Ciglenečki et al., 2008). Although microarray and NGS can both roughly determine gene expression differences (Pariset et al., 2009), QRT-PCR confirmation is still needed to accurately determine gene expression level differences. At present, QRT-PCR is the most sensitive and reliable method for the

detection of gene expression levels, hence it is often utilized in the verification of microarray data and NGS expression data (Robinson et al., 2007; Ka et al., 2008).

2.3.3 Applications of microarray and NGS technologies in studies with porcine embryos

There have been efforts to characterize the gene expression profile of *in vivo* developing porcine embryos and early embryos produced after *in vitro* manipulations using NGS and microarray platforms (Smith et al., 2001; Whitworth et al., 2004; Blomberg et al., 2005; Blomberg et al., 2008; Isom et al., 2013). However, full description of novel genes expressed during early embryonic development, and the altered gene expression profile in porcine embryos produced after different *in vitro* ART manipulations are still needed. Although there are gene expression microarray platforms available for various species (including pig), most of these platforms are designed based on somatic cell gene expression profiles. It has been shown that the embryonic transcriptome differs significantly from that of somatic cells (Vallee et al., 2009). EmbryoGENE Porcine Array Version1 (EMPV1, [GPL14925]) is a porcine pre-implantation embryo-specific microarray platform that has been recently designed based on a 454-pyrosequencing analysis of nine different stages from oocytes to blastocysts (Tsoi et al., 2012). The EMPV1 microarray is a custom Agilent 4X44K gene expression array with the only porcine-early embryo specific gene expression microarray, which allows for parallel analysis of many samples (Figure 2-2).

2.4 Summary

The pre-implantation period of mammalian embryonic development is critical for the future development of the individual. Although the morphological steps associated with

the key embryonic developmental events during the pre-implantation period of porcine embryos have been well documented, the molecular mechanisms underlying these events are not yet fully understood. Although some of the key regulators of embryonic development have conserved functions among different mammalian species, several key regulators of embryonic development in early porcine embryos behave differently from other mammals.

After artificial insemination, almost 100% of all the fertilized embryos in pig can develop into blastocysts *in vivo* (Isom et al., 2013). However, porcine embryos derived from *in vitro* ART manipulation systems, exhibit slower development, lower cleavage and blastocyst formation rate, and are less competent than *in vivo* embryos. Although many previous studies have been performed (Whitworth et al., 2004; Blomberg et al., 2005; Blomberg et al., 2008; Isom et al., 2013), the molecular mechanisms responsible for the impaired development of porcine embryos derived from *in vitro* ART manipulation systems are not fully established.

The overall hypothesis of the present research is that altered, or improperly regulated, gene expressions of critical genes / gene networks in the early porcine embryos are responsible for the deficiencies observed in porcine embryos produced after ART manipulation. The overall objectives of the present research are to better understand the molecular mechanisms underlying the key pre-implantation developmental events; to better understand the molecular mechanisms underlying the impaired development of embryos produced after *in vitro* ART manipulation.

Chapter 3 and 4 of this thesis describes the verification and re-annotation process of the EMPV1 microarray. The studies described in the following chapters will be focused on characterization of the "normal" transcriptome profile for the pre-implantation period of porcine embryos, and characterization of the altered gene expression profiles in porcine

embryos produced from after *in vitro* ART manipulation (CT and PA) systems. In addition, the effect of porcine luteinizing hormone (pLH)-induced ovulation on the gene expression profile of early porcine embryos will be investigated.

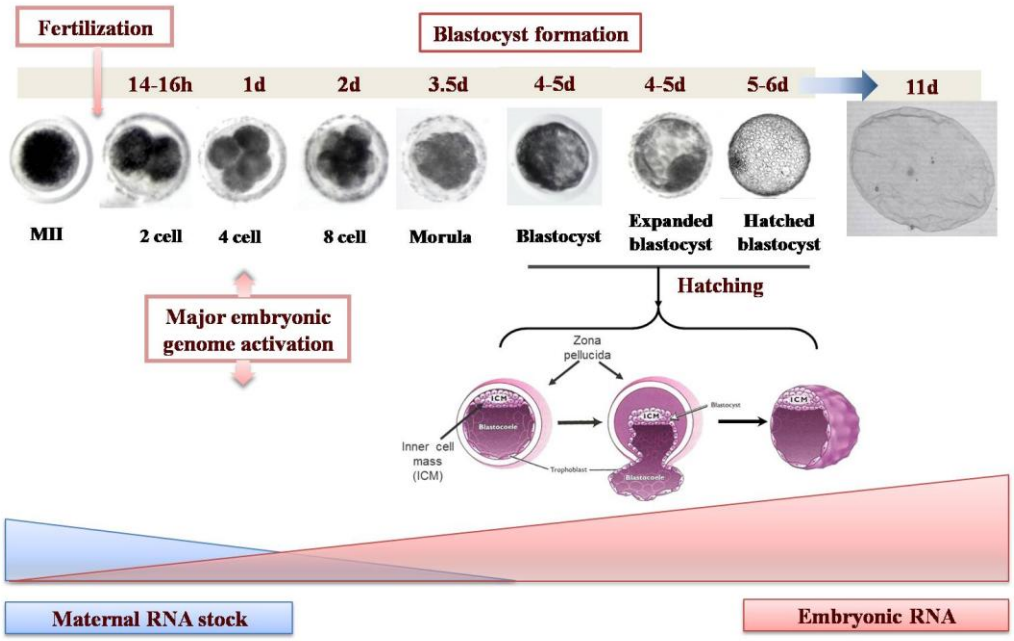

Figure 2-1 Early porcine embryonic development (in vivo).

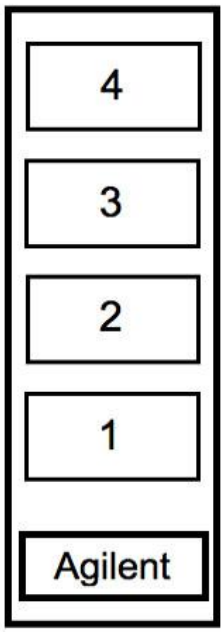

Figure 2-2 Layout of EMPV1 microarray

Each EMPV1 microarray slide contains four microarrays, which allow for the parallel analysis of four different samples.

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Chapter 3: Verification of a porcine embryo-specific expression microarray platform[1]

3.1 Introduction

The domestic pig is an economically important livestock species, with pork constituting 40% of the world's meat consumption, making it one of the most important meat source globally (Dang-Nguyen et al., 2010). However, swine are also a well-recognized biomedical animal model for improving human health. Recent research has focused on using the pig as a medical model for renal transplantation (Giraud et al., 2011), cardiovascular-related diseases (Chorro et al., 2009), atherosclerosis (Vilahur et al., 2011) and cystic fibrosis (Rogers et al., 2008). As well, advances in induced pluripotent stem cell (iPSCs) technologies (Ezashi et al., 2009; Roberts et al., 2009) make the pig an attractive model for regenerative medicine and stem cell research. As a result, there is a strong interest in the factors that affect the efficient production of viable embryos and offspring in this species using either *in vivo* or *in vitro* production methods.

As described in chapter 2, during the pre-implantation period of embryonic development, the mammalian embryo exhibits dramatic morphological changes and many key developmental events take place. Until recently, studies to determine the effects of various factors on embryonic development and competence have been limited to morphological and phenotypic evaluations (Hazeleger et al., 2000; Crosier et al., 2001; Fujino et al., 2006). Current understanding of the molecular events taking place during

[1] A version of the information presented in this chapter has been included as a major part of the manuscript published in BMC Genomics 13: 370. Available: http://www.biomedcentral.com/1471-2164/13/370 via the Internet.

the development of porcine pre-implantation embryos is limited. Increased knowledge in this area will contribute to our understanding of basic reproductive biology. It will also allow us to identify molecular markers indicative of embryonic quality, and facilitate improved maternal management as well as *in vitro* production and manipulation of embryos.

Powerful high-throughput genomic tools, such as microarray technologies and deep sequencing have been developed to study gene expression at the whole genome level of domestic animals during development (Niemann et al., 2007). Deep sequencing allows for a detailed analysis of transcript levels, as well as data mining and identification of transcript isoforms. Alternatively, gene expression microarrays allow for efficient analysis of a large number of different predetermined transcripts in biological samples, but are limited by prior knowledge and gene discovery. As previously described in chapter2, there are gene expression microarray platforms available for various species, but most of these platforms are based on somatic cell expression. It has been shown that the embryonic transcriptome differs significantly from that of somatic cells (Vallee et al., 2009). To date, the development of embryo-specific gene-expression microarrays has only been reported for cattle (Robert et al., 2011). Although there have been efforts to characterize the gene expression profile of the developing porcine embryo (Smith et al., 2001; Whitworth et al., 2004a; Blomberg et al., 2005; Blomberg et al., 2008), a full description of novel genes expressed during pre-implantation development in the pig is still needed. With the on-going effort in porcine genome mapping and sequencing (Archibald et al., 2010), the capacity to achieve this endeavor is now available. EmbryoGENE Porcine Array Version1 (EMPV1, [GPL14925]), which contains 43,795 probes, is a porcine pre-implantation embryo-specific microarray platform that has been

recently designed based on a 454-pyrosequencing analysis of nine specific stages from oocytes to early blastocysts.

To verify the reliability of the EMPV1 platform for transcriptomic studies of pre-implantation porcine embryos, gene expression profiling analysis of porcine cumulus-oocyte-complexes (COC) and pooled embryos representing 2-cell, 4-cell and 8-cell stages were performed using EMPV1.

3.2 Material and methods

3.2.1 Samples collection and RNA isolation

Ovaries from gilts were collected at a local slaughterhouse and cumulus–oocyte complexes (COC) were collected by aspiration from mature follicles and washed in saline solution. Individual COC samples were stored at -80 ℃ until RNA extraction.

In vivo collection of 2-cell, 4-cell and 8-cell stage embryos was carried out as previously described (Degenstein et al., 2008). For each of these developmental stages, five morphologically identical embryos from each stage were pooled for RNA extraction. PicoPure RNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA) was used for both single COC and pooled-embryo extraction. Total RNA quality of each sample was evaluated using Agilent 2100 Bioanalyzer using RNA 6000 Pico chip (Agilent Technologies, Mississauga, ON, Canada). The RIN (RNA integrity number) value of the two COC samples were greater than 8. The RIN value of 2-cell, 4-cell and 8-cell embryos was 5.9, 6, and 6.8, respectively. It is known that there are consistently low levels of ribosomal 28S RNA present in 2-cell, 4-cell and 8-cell embryos which result in lower RIN values (Gilbert et al., 2009), so these 2-cell, 4-cell and 8-cell embryos samples were still considered suitable for microarray analysis.

3.2.2 RNA amplification and labelling for microarray analysis

RiboAmp HSPlus kit (Applied Biosystems, Carlsbad, CA, USA) was used to amplify the low quantities of total RNA isolated from the samples. Five ng of total RNA from each of the two COC samples was used for amplification of adequate antisense RNA (aRNA) for labelling. A total of 1.5 ng of total RNA from pooled embryos were utilized in amplification. Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the aRNA quantity. The Agilent two-color RNA Spike-In kit (Agilent Technologies, Mississauga, ON, Canada) is a mixture of 10 different viral poly-adenylated RNAs. Five ng of Spike-In RNA was also used for amplification to serve as controls in the microarray analysis.

Amplified RNA of the two COC samples were labeled with Cy3 and Cy5 dye, and were further compared with each other in a dye-swap manner. Aliquots of amplified RNA of the pooled embryos sample was labeled with either Cy3 or Cy5 dye and hybridized on the same array.

Two μg of aRNA from each sample were labeled with either Cy3 or Cy5 dye using the ULS Fluorescent Labelling Kit (Kreatech Diagnostics, Amsterdam, Netherlands). The same kit was used for the Spike-In aRNA except the amount for labelling was 5 μg of each, using Cy3 for spike A and Cy5 for spike B. All the labeled probes were purified using picopure RNA extraction kit (Applied Biosystems, Carlsbad, CA, USA). Concentration and labelling efficiencies were determined using a Nanodrop ND-1000.

A total of 110 μl of hybridization mixture was prepared according to the manufacturer's instructions (Agilent Gene Expression Hybridization Kit 60-mer oligo microarray protocol version 4.0). In brief, 825 ng of each Cy3 and Cy5 labeled aRNA plus 2.75 μl of labeled Agilent Spike-In (0.01X, spike A and B) was prepared with 25X fragmentation and 10X blocking buffers. After incubating the mixture at 60 ℃ for 15 minutes, it was immediately cooled on ice for one minute before adding an equal volume of 2X GEx

hybridization buffer HI-RPM (Agilent Technologies, Mississauga, ON, Canada). Array was hybridized for 17 hours at 65 ℃ rotating at 10 rpm. The washing, stabilization and drying steps were performed strictly following the established Agilent protocols.

3.2.3 Microarray data acquisition and Spike-In quality control (QC) analysis

After the washing, stabilization and drying steps, the microarrays were immediately scanned using an Axon 4200AL scanner. The images obtained were analyzed with GenePix Pro 6.0 software (Molecular Device, Sunnyvale, CA 94089 USA), and saved as GenePix Results (GPR) format for further analysis. There are 120 Spike-In probe sequences printed on the EMPV1 array corresponding to Agilent Spike-In external RNA controls for the assessment of microarray performance developed by Agilent (Zahurak et al., 2007).

Details related to the microarray experiments were deposited into the EmbryoGENE LIMS and Microarray Analysis (ELMA) web platform (Robert et al., 2011). The EmbryoGENE microarray QC module within ELMA was used to generate QC graphs for the microarray data. Hybridization quality of each array was evaluated by the graphical distribution of signals that generated by both Cy3 and Cy5, and the negative and Agilent Spike-In controls (Robert et al., 2011).

Simple background subtraction and within array lowess global normalization was performed on raw data from each array through FlexArray 1.6.1 software (http://genomequebec.mcgill.ca/FlexArray) (Robert et al., 2011). The normalized microarray data of direct comparison of two COC samples was analyzed using the "Limma" package (Smyth, 2005) of Bio-conductor through FlexArray (Robert et al., 2011). The threshold for positive spots selection for the COC and pooled embryo microarray data was calculated as the mean value of all the dark corner spots plus 2 standard deviations (Vallée et al., 2005).

3.2.4 PANTHER gene ontology (GO) analysis

The unique gene symbol lists from the EMPV1 array and a widely utilized commercial Affymetrix porcine genome array (http://www.affymetrix.com/estore/index.jsp) were uploaded into PANTHER analysis tools (http://www.pantherdb.org/) (Thomas et al., 2003; Mi et al., 2010) in order to identify the Gene Ontology (GO) terms associated with the genes, transcripts, and proteins. These GO term lists were then uploaded into PANTHER list analysis tools in order to identify biological processes that were statistically over and under-represented in comparison with a reference list (*Homo sapiens* genome) using the PANTHER "Statistical overrepresentation test" under Bonferroni multiple testing correction condition (Thomas et al., 2003). Only biological processes and pathways with a Bonferroni corrected P-value (Bon P-value) < 0.05 were considered significantly over- or under-represented. More details related to the expected value and P-value calculation algorithm (Cho and Campbell, 2000) are available on the PANTHER help website.

3.3 Results and Discussion

3.3.1 GO analysis of genes on EMPV1 microarray

To verify if the EMPV1 platform is enriched with genes related to developmental processes, we selected a commercially available Affymetrix porcine array for comparison using PANTHER tools as described in the Methods. The Affymetrix GeneChip® Porcine Genome Array is widely used in pig functional genomics researches (Tuggle et al., 2007). After removing the common gene symbols from both arrays, 5221 and 9425 unique gene symbols were identified from the Affymetrix Porcine Genome array and the EMPV1 array, respectively (Additional file 3-S1). Further PANTHER over-representation analysis (Thomas et al., 2003; Mi et al., 2010) of these unique gene symbols from

Affymetrix Porcine Genome array and the EMPV1 array was performed as described in methods section.

PANTHER over-representation analysis revealed 37 (highlighted with yellow in Additional file 3-S2) and 23 (highlighted with green in Additional file 3-S2) statistically significantly enriched biological processes (Bon P-value < 0.05) in EMPV1 and Affymetrix Porcine Genome arrays, respectively. None of the enriched biological processes in Affymetrix Porcine Genome array categories was associated with development processes. However, approximately 1/3 of the 37 significantly enriched biological processes in EMPV1 were associated with development (Figure 3-1). This indicates that the EMPV1 array is more efficient in detection of early pre-implantation embryonic development-associated genes. Further pathway analysis of these development-associated genes revealed six major molecular pathways (Table 1) including Wnt signaling pathway, TGF-beta signaling pathway, cadherin signaling pathway, interleukin signaling pathway, PI3 kinase pathway and insulin/IGF pathway- protein kinase B signaling cascade (Table 1). The important role of these six pathways and other extrinsic regulators has been reviewed in mouse and human pre-implantation embryonic development and stem cell related studies (Liu et al., 2007; Pera and Tam, 2010). The roles of these pathways in self-renewal, pluripotency and differentiation of pre-implantation embryonic development and embryonic stem cells is under active investigation (Abu-Remaileh et al., 2010; Tanaka et al., 2011), but is not well understood in pigs. Representation of these development-associated genes on the EMPV1 microarray is expected to facilitate cost effective and fruitful functional genomics research related to early porcine embryo development in the future.

3.3.2 Microarray quality assessment

With the current incompleteness of the porcine genome map and the limited ESTs resources, next generation RNA-sequencing is not a cost effective tool to study the effect of *in vivo* and *in vitro* factors on the development of early porcine embryos. After 2005, high background cDNA microarrays were generally replaced by oligo-based microarrays generated by companies such as Affymetrix and Agilent. Recently, the commercially available porcine oligo arrays from Agilent and Affymetrix have been widely used to study gene expression related to meat quality (Li et al., 2010), nutrition (Jun et al., 2010), female reproduction (Paczkowski et al., 2011; Sun et al., 2011), and peri-implantation embryos (Blomberg le et al., 2010). The EMPV1 microarray platform is a custom Agilent Array enriched with porcine transcripts from different early developmental stages.

In the present study, self-hybridization analysis of pooled early porcine embryos and dye-swapped direct comparison analysis of two porcine COC samples were performed to assess the intra- and inter-array variability of EMPV1 microarray platform.

The aRNA from the pooled early porcine embryo sample was labeled with Cy3 and Cy5 to test the fluorescent dyes' effect due to labelling and hybridization. High degree of correlation between the Cy3 and Cy5 signals ($r^2 = 0.97$) was obtained in the self-hybridization analysis with very few spots over the two-fold change threshold intensity line (Figure 3-2).

Dye-swapped direct comparison analysis of the two COCs samples was performed to assess the inter-array variability of the EMPV1 array. The inter-array variability was assessed by the correlation coefficient generated from the EmbryoGENE QC module based on the data of Spike-In controls within and across the arrays. In the present study, high degrees of correlation coefficient ($r^2 \geq 0.97$) was observed across the arrays from the COCs dye-swapped direct comparison analysis (Figure 3-3). A MA plot of contrast between two COCs samples was generated after the Limma analysis through FlexArray.

There were 72 probes that showed differential expression (fold change ≥ 2 or ≤ 0.5, P-value<0.05) between the two COCs samples, which were considered to be the result of variation between the two biological samples.

3.3.3 Porcine COCs and embryo transcriptome profiling

The COCs are composed of both reproductive (oocyte) and somatic (cumulus) cells, which were optimal samples for these validation purposes. A global mRNA gene expression analysis of COC was carried out by selecting positive signals as described in the Methods. Approximately 74% of the probe sets representing 28,715 transcripts were detected in porcine COC (Additional file 3-S3). This number is in accordance with the 16,066 transcripts (67.16% of all probe sets) detected using the Affymetrix GeneChip. Porcine Genome Array in hormonally stimulated pre-ovulatory ovary follicles from Large White sows (Sun et al., 2011). The greater number of expressed genes in the present study is probably due to different physiological conditions of the female and additional cumulus cells with the oocytes. In addition, the different array platforms used for analysis may also have contributed to these differences.

To confirm the accuracy of EMPV1 microarray data in biological relevance, six transcripts of conserved oocyte markers were identified from the COCs gene expression profiling in mammals (Figure 3-4). Zona pellucida glycoprotein 2 & 3 (ZP2, ZP3) (Vallée et al., 2005; Wassarman and Litscher, 2008), B-cell translocation gene 4 (BTG4) (Vallée et al., 2005), myeloid leukemia factor 1 interacting protein (MLF1IP) (Vallée et al., 2005) and growth differentiation factor 9 (GDF9) (McGrath et al., 1995; Vallée et al., 2005; Adjaye et al., 2007), and bone morphogenetic protein 15 (BMP15) (Juengel et al., 2002; Vallée et al., 2005) were highly expressed in COCs. On the other hand, several cumulus cells markers were also identified from the positively detected genes in COCs (Figure 3-4). Studies from human indicated that hormone receptors and secretary proteins such as

progesterone receptor membrane component 1 (PGRMC1) and bone morphogenetic protein 1 (BMP1) were significantly over-expressed in cumulus oophorous cells when compared to oocytes (Assou et al., 2006). Peroxiredoxin 4 (PRDX4) and ADAM metallopeptidase with thrombospondin type 1 (ADAMTS1) have been reported as a cumulus cells marker in human (Assou et al., 2006; Yung et al., 2010). Secreted protein acidic, cysteine-rich (SPARC) was found to be exclusively expressed in bovine cumulus cells (Regassa et al., 2011). As expected, transcripts related with these cumulus cells markers were positively detected in COCs using EMPV1 array.

Positively detected probes were selected from the self-hybridization microarray analysis of the pooled early embryos as described in method section. A total of 28,597 transcripts were positively detected from pooled porcine embryos of 2-cell, 4-cell and 8-cell stages (Additional File 3-4). It should be noted that in this study our intent was not to quantify gene expression between different developmental stages, but to simply identify genes, from the literature that may be present in the 2- to 8-cell stages. Little is known regarding global gene expression during these early cleavage stages in the pig. Sequencing from the porcine EST project on early developmental stages has been generated from *in vitro*- and *in vivo*-derived 4-cell embryos (Whitworth et al., 2004b). Most of the ESTs were poorly annotated at that time and only few highly expressed genes, such as porcine casein kinase II beta subunit (CSNK2B), cyclin-dependent kinase-2 alpha (CDK2), ribosomal protein S10 (RPS10) and eukaryotic translation initiation factor 3 (EIF3), were identified in 4-cell embryos. These genes were positively detected in both the COCs and pooled early embryo expression data (Additional file 3-S3 and 3-4).

3.3.4 GO analysis for biological processes in porcine COCs & embryos

To highlight differences in the biological processes between the COC and embryos, we removed the common gene symbols from the positively detected transcripts in COCs

(28,715) and pooled early embryos (28,597) (Additional file 3-S3 and 3-4). Genes only positively detected in the COCs or pooled early embryos were obtained after removing redundancies from both data sets. A total of 793 and 4,388 unique gene symbols were positively detected in the COCs and the pooled early embryos, respectively, while 7,822 appeared in both (Additional file 3-S5). The unique gene symbols from COC and pooled early embryos were analyzed using the PANTHER tools, and only one pathway related to apoptosis was found to be statistical significant (Additional file 3-S6) in COC, but not in embryos.

Studies have shown that apoptosis is important during *in vitro* culture of bovine (Rubio Pomar et al., 2004; Hussein et al., 2005) and porcine embryos (Tatemoto et al., 2000). However, the gene count revealed additional unique pathways, which were statistically significant in the porcine embryos. The three pathways with the highest gene counts were primarily related with inflammation signaling (mediated by chemokine and cytokine), interleukin signaling and TGF-beta signaling (Figure 3-5). Specifically, the interleukin-signaling and TGF-beta signaling pathways may play an important role during porcine pre-implantation embryonic development as we have discussed in the previous section. In addition, the inflammation-signaling pathway likely plays a role in the establishment of pregnancy, including cellular proliferation, attachment and development of the conceptus (Dyck and Ruvinsky, 2011).

3.4 Conclusion

The EMPV1 microarray platform was confirmed with a high level of reproducibility that is provided by the current Agilent microarray technology. PANTHER GO analysis confirmed that the EMPV1 microarray is enriched with transcripts related with developmental processes-associated genes. With more than 20 thousand unique

transcripts that represented on the EMPV1 microarray, this platform will provide the foundation for future research into the *in vivo* and *in vitro* factors that affect the viability of the porcine embryos, as well as the effects of these factors on the live offspring that result from these embryos.

Table 3-1 PANTHER pathway analysis of developmental processes-associated genes

Category name (Accession)	**# genes**	**Percent of gene hit against total # genes**	**Percent of gene hit against total # Pathway hits**
Wnt signaling pathway (P00057)	109	5.20%	8.60%
TGF-beta signaling pathway (P00052)	80	3.80%	6.30%
Interleukin signaling pathway (00012)	71	3.40%	5.60%
Insulin/IGF pathway-protein kinase B signaling cascade (P00033)	56	2.70%	4.40%
Pl3 kinase pathway (P00048)	56	2.70%	4.40%

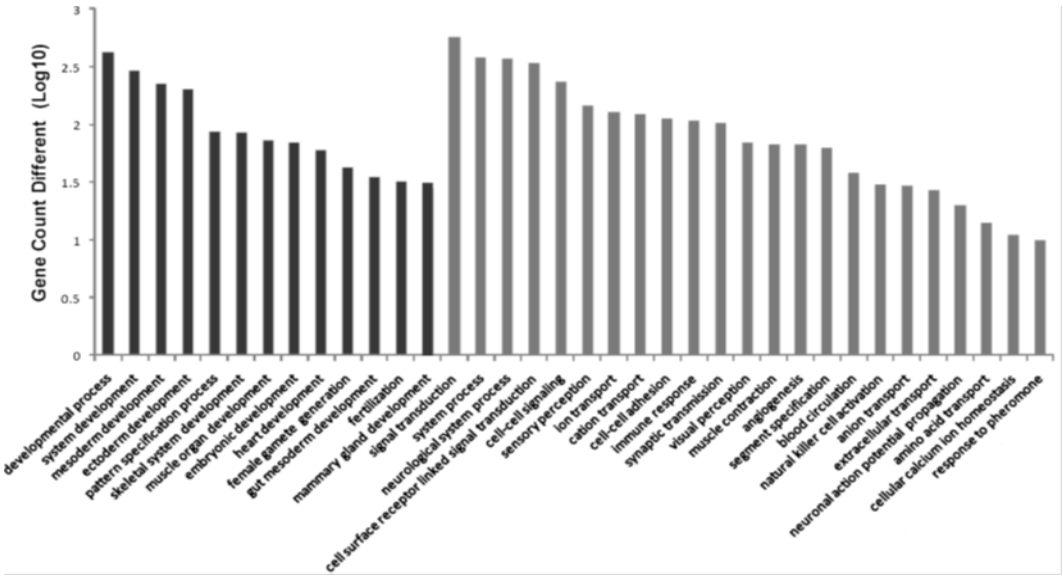

Figure 3-1 Biological process categories that significantly enriched in EMPV1 array probes with unique gene symbols.

Y-axis showing log10 of the number of genes associated with each biological process. Black bars showing the biological process categories significantly enriched in EMPV1 array that related to development processes. Large format version of this figure can be access through the following link:

https://docs.google.com/file/d/0B0QxwqYwWLkfWnNveUF5aG9NRVU/edit?usp=sharing

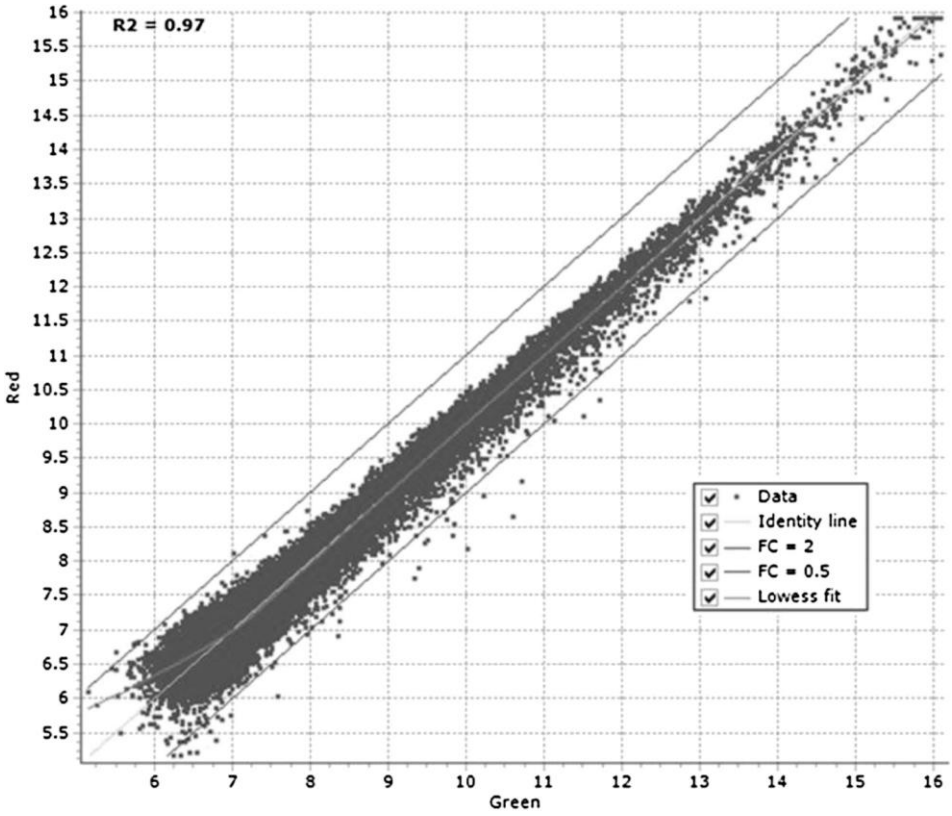

Figure 3-2 A scatter plot of Cy3 and Cy5 normalized signal intensity.

X and Y-axis showing the signal intensity after the same aRNA from pooled embryos were labeled with Cy3 and Cy5 respectively. FC = fold change.

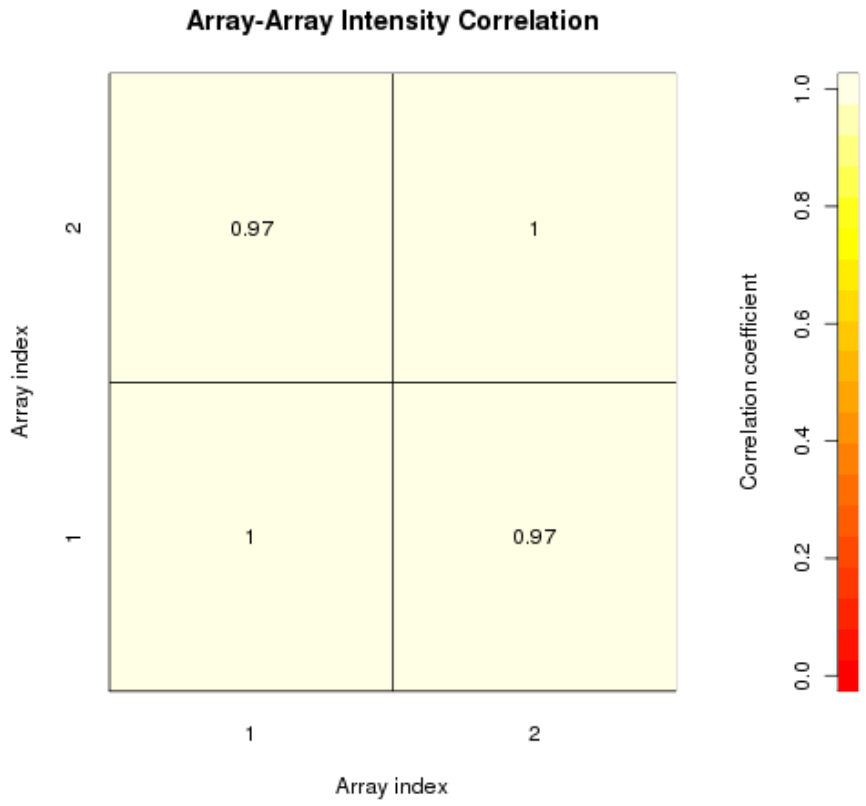

Figure 3-3 QC plot showing the correlation coefficient across-arrays from the COCs dye-swapped direct comparison analysis (generated from the EmbryoGENE QC module).

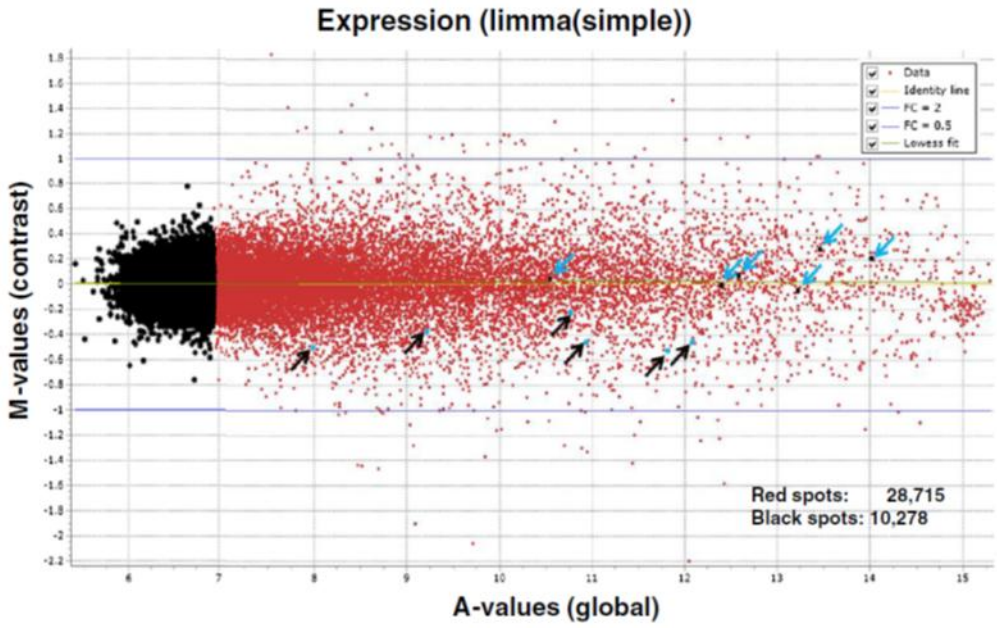

Figure 3-4 MA plot for COC gene expression data.

Six blue arrows pointed downward represented oocyte specific markers and six black arrows pointed upward represented cumulus cells markers. 28,715 red spots represented positive signals above background signals (10,278 black spots).

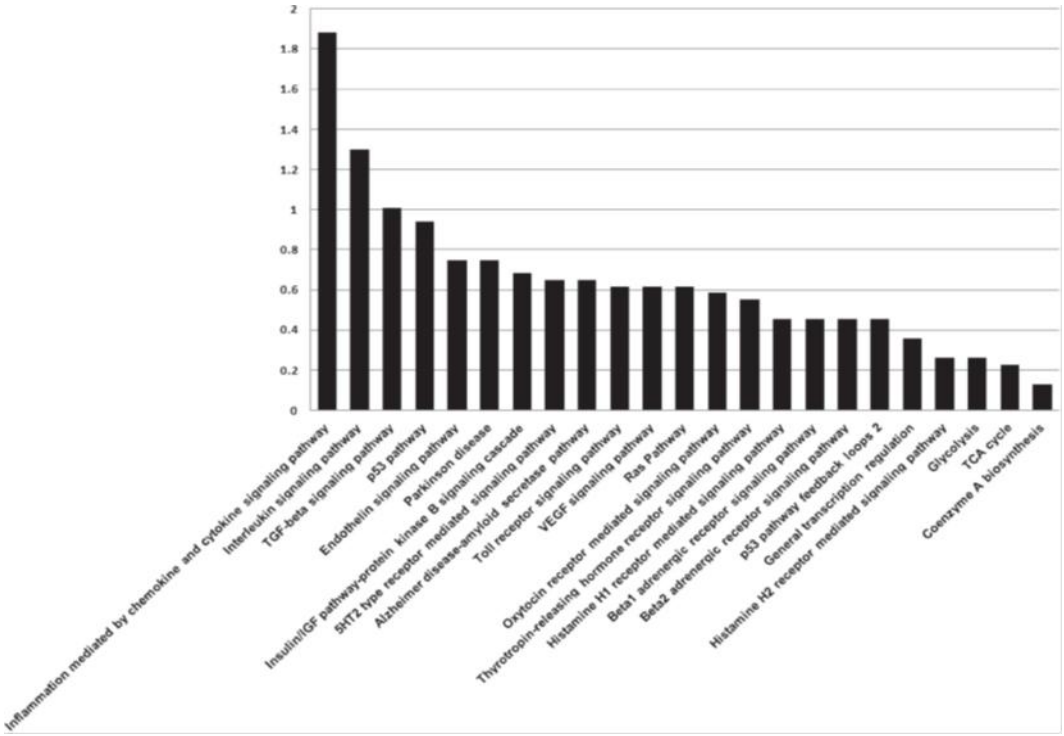

Figure 3-5 PANTHER bar chart of gene count involved in biological pathways from early embryos.

The % of gene list (Y axis) in the category is calculated for each testing list as: (# genes for the category/ # total genes in the list)* 100. Large format version of this figure can be access through the following link:

https://docs.google.com/file/d/0B0QxwqYwWLkfRlRzNDRtb0JOZDQ/edit?usp=sharing

Additional file 3-S1: gene symbols list from Affymetrix and EMPV1 microarray. Excel file containing column of gene symbols only found in Affymetrix and EMPV1 microarray after removing all the common gene symbols.

http://www.biomedcentral.com/content/supplementary/1471-2164-13-370-s9.xls

Additional file 3-S2: PANTHER GO biological process difference in Affymetrix and EMPV1 porcine microarray. Excel file containing statistical significance of the gene count differences between unique gene symbols from EMPV1 array and Affymetrix porcine array over different categories of biological processes using PANTHER expression tool. Yellow indicates genes over-represented in EMPV1 (p-value <0.05) and Green indicates genes over-represented in Affymetrix (p-value <0.05).

http://www.biomedcentral.com/content/supplementary/1471-2164-13-370-s10.xls

Additional file 3-S3: Positive spots selection in COCs microarray analysis data. An excel file contains all positive signals higher than the threshold for positive spots selection (log intensity signal > 6.69) calculated according to Methods.

http://www.biomedcentral.com/content/supplementary/1471-2164-13-370-s12.xls

Additional file 3-S4: Positive spots selection in embryos microarray analysis data. An excel file contains all positive signals higher than the threshold for positive spots selection (log intensity signal > 6.69) calculated according to Methods.

http://www.biomedcentral.com/content/supplementary/1471-2164-13-370-s13.xls

Additional file 3-S5: Unique gene symbols list from COC and embryos array data. Excel file containing unique gene symbols from microarray data with genes only found in COC, embryos and both.

http://www.biomedcentral.com/content/supplementary/1471-2164-13-370-s14.xls

Additional file 3-S6: An excel file containing the result of pathway analysis from PANTHER. Yellow indicates the p-value is significant.)

http://www.biomedcentral.com/content/supplementary/1471-2164-13-370-s15.xls

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Chapter 4: Comparative transcriptomic analysis between porcine early and hatched blastocysts[2]

4.1 Introduction

As described in chapter 1, the formation and hatching of the blastocyst are important developmental events that take place during the pre-implantation period of mammalian embryonic development (Oestrup et al., 2009). The mammalian blastocyst stage embryo has a distinct morphological structure that consists of inner cell mass (ICM), internal cavity (blastocoele), and a single layer of epithelial trophectoderm (TE) with (before hatching) or without (after hatching) the protective zona pellucida (Watson and Barcroft, 2001; Duranthon et al., 2008). The morphology of porcine blastocysts is similar to most other mammalian species.

Blastocyst is the embryonic stage that is most frequently transferred into female recipients after *in vitro* ART manipulation (Glujovsky et al., 2012; Yoshioka et al., 2012) in pig and is therefore of particular scientific and economic interest. The porcine blastocyst forms approximately 5 days after fertilization *in vivo* (Hyttel and Niemann, 1990; Oestrup et al., 2009). Following blastocyst formation, the embryo expands in size and hatches from the zona pellucida to become a "free floating" hatched blastocyst in the uterus approximately 5-6 days after fertilization (Oestrup et al., 2009). This process is referred to as "hatching", which is a critical and tightly regulated event during the early embryonic development, and any dysregulation of the hatching process leads to early

[2] A version of this chapter is under submission with Molecular Reproduction and Development.

embryonic loss (Seshagiri et al., 2009). However, the molecular mechanisms underlying the hatching process are not yet fully understood.

Characterization of the gene expression profile of early porcine embryos could identify pathways critical for embryonic development and potential gene markers of embryonic quality (Whitworth et al., 2005; Ka et al., 2008). Technologies, such as microarray and next generation sequencing (NGS) allow for the study of gene expression at the transcriptomic level in domestic animals during embryonic development (Niemann et al., 2007). NGS allows for gene expression profiling analysis without the requirement of prior gene sequence knowledge, and thus can be used to discover new genes. Alternatively, gene expression microarrays continue to be widely used as an efficient tool to analyse the expression levels of tens of thousands of different predetermined transcripts in many different samples. Detailed and accurate annotation of genes on the microarray platform is critical for microarray data analysis: complete annotation facilitates the identification of critical pathways and regulatory elements. The power of microarray technology is dependent on, and limited by, the probe sequences spotted on the microarray surface.

As shown in chapter 3, the EmbryoGENE Porcine Array Version1 (EMPV1, NCBI Gene Expression Omnibus (GEO) platform: GPL14925) (Tsoi et al., 2012) is a microarray platform with high reproducibility and is enriched with genes expressed during early porcine embryonic development. To date, the EMPV1 microarray is the only porcine embryo-specific gene expression microarray. To evaluate the EMPV1 microarray's capability in transcriptomic analysis of early porcine embryos, global transcriptomic profiling analysis of *in vivo* porcine blastocyst stage embryos was performed using EMPV1 and two other commonly used somatic cell-based porcine microarray platforms (Swine Protein-Annotated Oligonucleotide Microarray (PigOligoArray, [GPL7435])

(Steibel et al., 2009) and Sus.Scrofa Oligo Microarray v2 (SOMV2, [GPL15007])). To better interpret microarray expression data in the present study, re-annotations using the Sus scrofa10.2 genome were performed on both the EMPV1 and the SOMV2 microarrays. In addition, comparative transcriptomic profiling analyses between early and hatched blastocysts were performed using both the EMPV1 and SOMV2 microarray platforms in order to identify the key genes / gene networks associated with the blastocyst hatching process in the porcine embryo.

4.2 Material and methods

4.2.1 Porcine embryo sample collection

An initial set of *in vivo* derived porcine blastocyst (BL) stage embryos (including embryos before and after hatching), which were staged based on their morphological characteristics, were collected 5-6 days after artificial insemination and stored in pools of 10.

Another set of *in vivo* derived porcine embryo samples, which were also collected 5-6 days after artificial insemination, were classified into early blastocyst (EB; unexpanded, zona intact) and hatched blastocyst (HB; zona shed) stages based on their morphological characteristics and stored in pools of 5.

All embryo samples were collected from gilts as previously described (Degenstein et al., 2008). In brief, the porcine embryos was flushed from the reproductive tract of gilts an washed twice with 37 ℃ BPS solution, and all embryo samples were collected and stored individually. All samples were placed on dry ice immediately after collection and stored at -80 ℃ until RNA extraction.

4.2.3 Total RNA isolation

Total RNA was extracted from each pool of *in vivo* derived porcine embryo samples using Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA). The quality and integrity of each total RNA sample was evaluated using the Bioanalyzer RNA 6000 Pico LabChip (Agilent Technologies, Mississauga, ON, Canada), and only the RNA samples with RIN (RNA integrity number) value higher than 7.5 were used for downstream applications.

4.2.4 Microarray experimental design

Three different microarray platforms used in this study were: the Swine Protein-Annotated Oligonucleotide Microarray (PigOligoArray, USDA, NCBI GEO platform GPL7435) (Steibel et al., 2009), the EmbryoGENE Porcine Array Version1 (EMPV1, Agilent custom array, NCBI GEO platform GPL14925) (Tsoi et al., 2012) and the Sus.Scrofa Oligo Microarray v2 (SOMV2, a commercially available Agilent microarray, NCBI GEO platform GPL15007).

To characterize the global transcriptomic profile of *in vivo* derived porcine BL, microarray analysis was initially performed on porcine BL embryos using the Swine Protein-Annotated Oligonucleotide Microarray (PigOligoArray) platform. Total RNA samples extracted from pools of 8-10 embryos were used in the microarray assessment. A dye-swapped loop design was used, and 3 biological replicates were amplified, labeled and hybridized with each other in a dye-swapped manner.

In order to further determine the detailed gene expression profile changes before and after hatching of porcine blastocysts, comparative transcriptomic analysis was performed on morphologically-staged *in vivo*-derived EB and HB porcine embryos using both the EMPV1 and SOMV2 microarray platforms. Total RNA samples extracted from pools of 5 embryos were used in the microarray assessment. A dye-swapped direct comparison

design was used, and 3 biological replicates from each embryonic stage of interest (EB or HB) were used in the comparative microarray analyses.

4.2.5 RNA amplification for microarray analysis

Due to the low quantities of total RNA obtained from each sample, all RNA samples were amplified using RiboAmp HS^{Plus} kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions to generate amplified antisense RNA (aRNA) targets for microarray reactions. A total of 2.5 ng total RNA was utilized in each amplification reaction, and the quantity and quality of aRNA products from RNA amplifications were evaluated by the Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Agilent two-colour RNA Spike-In® kit (Agilent Technologies, Mississauga, ON, Canada) was amplified, labeled and utilized as positive control in hybridizations with both the EMPV1 and SOMV2 platform as described previously (Tsoi et al., 2012).

4.2.6 Amplified antisense RNA (aRNA) labelling

Two µg of aRNA from each aRNA sample was labeled with either Cy3 or Cy5 using the ULS Fluorescent Labelling Kit (Kreatech Diagnostics, Amsterdam, Netherlands) following the manufacturer's instructions. The labelling of aRNA targets was carried out in an ozone-free environment, to minimize degradation of the fluorescent dyes. All the labeled probes were purified using the Picopure RNA extraction kit. Probe concentration and labelling efficiency of each labeled sample was evaluated using Nanodrop ND-1000.

4.2.7 Hybridization, washing and data acquisition for Microarray

Swine Protein-Annotated Oligonucleotide Microarray (PigOligoArray, USDA)

The aRNA samples labeled with Cy3 and Cy5 dyes were hybridized together on one Swine Protein-Annotated Oligonucleotide Microarray (PigOligoArray). A total of 80 µl of hybridization mixture containing 8 µl 20X SSC, 3.2 µl 2% SDS, 4.8 µl Liquid

Blocking reagent (Amersham, USA), and 120 pmol of samples labeled with each dye (Cy5 and Cy3) was prepared. The hybridization reaction mixture was then incubated at 65 ℃ for 5 minutes and then applied directly to the PigOligoArray slide covered with a lifter slip (Erie Scientific, Portsmouth, USA). The microarray slides were then hybridized and washed following the procedure previously described by Steibel et al (Steibel et al., 2009).

After the washing and drying steps, the microarray slides were immediately scanned at 10μm resolution using the Axon 4200AL microarray scanner (Molecular Device, Sunnyvale, CA 94089 USA). The optimum scanning intensity of each dye was determined by the auto-PMT function of the scanner. The scanned image of each array was analyzed with GenePixPro 7.0 software to extract signal intensities for each feature on the array (Molecular Device, Sunnyvale, CA 94089 USA). The analysis result from each microarray was saved as GenePix Results (GPR) format for further array analysis. FlexArray is a software package, which uses R and Bio-Conductor (Gentleman et al., 2004) and provides a user-friendly interface that facilitates data processing, visualization, and statistical analysis (http://genomequebec.mcgill.ca/FlexArray). Simple background subtraction and within array print-tip lowess normalization was performed on raw data from each array using the FlexArray software package. The threshold for positive spots selection from BL embryos microarray data was determined by the mean value of all the negative control spots plus 2 standard deviations (Tsoi et al., 2012).

EMPV1 and SOMV2 microarray assessment

The hybridization, washing and drying steps of EMPV1 and SOMV2 were conducted following the procedure previously described in chapter 3. In short, aRNA samples were labeled with either Cy5 or Cy3 dyes and hybridized on one array, which were then incubated at 65 ℃ with rotation at 10 rpm for 17 hours in a hybridization oven. After

washing, stabilization and drying steps that strictly followed the manufacturer's instructions, microarrays were immediately scanned at 5μm resolutions using an Axon 4200AL scanner. The scanned image of each array was analyzed with GenePixPro 7.0 software. The analysis result from each microarray was saved as GenePix Results (GPR) format for further array analysis. The hybridization quality of each was evaluated by the graphical distributions and signals generated by both channels from the negative and spiked in controls (Robert et al., 2011).

Simple background subtraction and within array lowess global normalization was performed on raw data from each array through FlexArray (Robert et al., 2011). To identify the differentially expressed genes between EB and HB, the normalized microarray data was analyzed using "limma" package (Smyth, 2005) of Bio-conductor through FlexArray under the Benjamini and Hochberg false discovery rate (BH-FDR) (Benjamini and Hochberg, 1995) multiple comparison correction condition through FlexArray (Robert et al., 2011). For any particular comparison, only genes with a BH-FDR adjusted P value (B-H P-value) ≤ 0.05 and a fold change (FC) ≥ 2 (or ≤ 0.5) were considered to be significantly up- or down-regulated.

4.2.8 Bioinformatics tools and analysis

A sequence assembly program "SeqMan NGen" within LaserGene 9.0 package (DNASTAR, Madison, WI, USA) was used to compare the SOMV2 and EMPV1 probe sequences with the pig RefSeq RNA database (25752 sequences) from the Sus scrofa10.2 genome downloaded from NCBI (ftp://ftp.ncbi.nih.gov/genomes/Sus_scrofa/RNA/). The program parameters were adjusted slightly from their default settings (Additional file 4-S1). Further microarray probes without annotations were then compared against the pig RefSeq RNA (25651 sequences) and human RefSeq RNA (44477 sequences) database using NCBI Blast with an E-value cut off of 1.0E-6. Due to the incompleteness of the Pig

genome annotation, many of the positive pig RefSeq matches of microarray probes did not have official gene symbols. These pig RefSeq sequences were then further characterized through identification of their human homologs, which were identified by a cross-species BLASTN analysis against human RefSeq RNA (44477 sequences) database with an E-value cut off of 1.0E-10.

The EMPV1 is a custom Agilent microarray platform designed based on 454 sequencing data (Tsoi et al., 2012). The source 454 contig sequences of the remaining uncharacterized probes in EMPV1 were identified and further compared against the human RefSeq RNA (44477 sequences) database using NCBI Blast with an E-value cut off of 1.0E-10.

Probe sequences of all the uncharacterized positively expressed probes from the porcine BL PigOligoArray data were compared with the pig RefSeq RNA database (26217 sequences) from the Sus scrofa10.2 genome downloaded from NCBI using "SeqMan NGen" program. The program parameters were adjusted slightly from their default settings (Additional file 4-S2). Annotation of a previously published porcine BL 454 sequencing dataset [SRA: SRX039506] (Tsoi et al., 2012) was also performed with the pig RefSeq RNA database from the Sus scrofa10.2 genome using the same "SeqMan NGen" program parameters. Positive pig RefSeq matches that did not have official gene symbols were then further characterized through identification of their human homologs by a cross-species BLASTN analysis against the human RefSeq RNA database with an E-value cut off of 1.0E-10.

4.2.9 Gene Ontology (GO) analysis

The unique gene symbol lists of genes that were positively detected by the 454 sequencing, PigOligoArray, EMPV1 and SOMV2 microarray analyses of the BL were uploaded into PANTHER analysis tools (http://www.pantherdb.org/) (Thomas et al.,

2003; Mi et al., 2010) in order to identify the Gene Ontology (GO) terms associated with the genes, transcripts, and proteins.

4.2.10 IPA biological functions, canonical pathways and upstream regulator analysis

Expression data obtained from the comparative transcriptomic analysis between EB and HB using the EMPV1and SOMV2 microarray platforms were analyzed using the IPA (Ingenuity® Pathway analysis, Ingenuity® Systems, www.ingenuity.com) Biological Functions Analysis, Canonical Pathway Analysis, and Upstream Regulator Analysis tools. The biological functions and canonical pathways analyses were performed under HB-FDR multiple testing correction conditions. Only the biological functions and canonical pathways with an HB-FDR corrected P-value (HB-FDR P-value) <0.05 were considered significant. IPA Upstream Regulator Analysis predicts the activation status of the upstream regulator by calculating a regulation Z-score and an overlap p-value, which are based on the number of known regulation target genes from the dataset of interest, expression changes of these target genes, and their agreement with literature findings. Upstream regulators with an overlap p-value of overlap ≤ 0.05 and an IPA activation Z-score ≥ 2.0 (or ≤ -2.0) were considered significantly activated (or inhibited). Description of the calculation of the IPA regulation Z-score and overlap p-value is available in IPA white paper "A Novel Approach to Predicting Upstream Regulators". Description of IPA analysis is available on IPA website (http://www.ingenuity.com) under "Upstream Regulator Analysis", "Biological Functions Analysis", and "Ingenuity Canonical Pathways Analysis".

4.2.11 Real-time quantitative PCR (QRT-PCR) verification of gene expression

Eleven genes (*APOA2*, *ACADL*, *RGN*, *JARID2*, *KRT18*, *SRSF11*, *ITGB1*, *PDHB*, *LDHB*, *CCPG1*, and *ACTB*) were selected from the comparative gene expression data, and were evaluated using SYBR Green I-based QRT-PCR. The primer sequences for all target

genes are listed in Table 4-1, and the QRT-PCR efficiencies of all the target genes are within the range of 90% to 110%. Four biological replicates from each embryonic stage were utilized. A total of 1 ng total RNA isolated from each pool of 5 embryos was reverse transcribed into cDNA using a high capacity reverse transcriptase (SuperScript® VILO™ cDNA Synthesis Kit, Invitrogen™) following the manufacturer's instructions using a 20 µl reverse transcription (RT) reaction volume. An equal amount (10000 copies) of a synthetic RNA transcript (Xeno™ RNA Control, SYBR® Green Cells-to-CT™ Control Kit, Ambion®) was added to each reverse transcription reaction to serve as an external reference for SYBR Green I-based QRT-PCR analysis and as a positive control for reverse transcription in order to assess variability resulting from any RT or PCR inhibitors. The cDNA products were then diluted 5 times, and 2.5 µl of the diluted cDNA was used as template in each of the QRT-PCR reactions performed with StepOnePlus™ QRT-PCR System (Life technologies) and Fast SYBR® Green Master Mix (Applied Biosystems®). No template control reactions (water used as template) and negative QRT-PCR reactions were performed on each QRT-PCR runs with 96 wells plates in order to rule out contaminations. The QRT-PCR data was adjusted with the external control gene (Xeno™ RNA Control, Ambion®) using the qbase^{PLUS} software (Biogazelle) (Hellemans et al., 2007). The adjusted QRT-PCR data was then further analyzed using $2^{-\Delta\Delta CT}$ method (Yuan et al., 2006; Hellemans et al., 2007) to determine the relative differential expression (fold changes) of the target genes between EB and HB embryos.

4.3 Results

4.3.1 Re-annotation of microarray platforms

Currently, the porcine genome annotation is not complete and is an on-going process. To increase the number of unique genes detected and to maximize the gene expression and

pathway analyses using the three microarray platforms, re-annotation with the most updated genome database (Sus scrofa10.2 genome) was necessary. Re-annotation was performed with the previously uncharacterized probe sequences from EMPV1 and SOMV2 microarray platforms, as well as the PigOligoArray probes positively detected in BL embryos as described in the methods.

A total of 16672 probe sequences (38% of all the probes on EMPV1) were newly characterized in EMPV1 (Additional file 4-S3), and a total of 8119 probes (18% of all the probes on SOMV2) were newly characterized in SOMV2 (Additional file 4-S4) after re-annotation. Combining the pre-characterized probes and newly characterized probes after re-annotation, a total of 32797 (75% of the whole probe set) probes representing a total of 15555 unique genes were characterized in EMPV1, and a total of 22480 probes (51% of the whole probe set) representing a total of 11396 unique genes were characterized in SOMV2. A total of 7530 unique genes were in common between EMPV1 and SOMV2 platforms (Figure 4-1) (Additional file 4-S5), and 7216 of these common unique genes were mapped with human GO terms in the PANTHER database. A major portion of the GO biological processes were related to the following terms: **"**metabolic process" (GO: 0008152, 3210 genes, 44%), "cellular process" (GO: 0009987, 2298 genes, 31%), "cell communication" (GO: 0007154, 1573 genes, 21%), "transport" (GO: 0006810, 1042 genes, 14 %), and "developmental process" (GO: 0032502, 902 genes, 12%) (Figure 4-2).

4.3.2 Global transcriptomic profiling of porcine blastocysts using different platforms

Unique genes positively detected in porcine BL embryos using four different ultra-high-throughput platforms (3 different microarray platforms and 454 sequencing) were determined as described in the methods section. There were 10352 and 7825 unique genes positively detected from the porcine BL embryos using EMPV1 microarray [GEO: GSE38882] and SOMV2 microarray [GEO: GSE38882], respectively. A total of 2486

probes (12% of the whole probe set) were positively detected in BL embryos by the PigOligoArray analysis [GEO: GSE38923], which represented a total of 1789 unique genes after the re-annotation. Annotation of the 454 sequencing data [SRA: SRX039506] revealed 2263 unique genes that expressed in porcine BL embryos.

Further effort was made to identify genes with human GO term matches in the PANTHER-classified genes, transcripts, and proteins database from the positively detected unique gene lists generated from the 4 ultra-high-throughput platforms. The total number of sequences, annotated probes and unique genes, as well as the number of positively detected probes and related unique genes in BL embryos are summarized in Table 4-2.

The unique gene symbol list identified from the EMPV1 microarray analysis had more overlap (1858 genes, Figure 4-3(A)) with the 454 sequencing data than the SOMV2 microarray analysis (1158 genes, Figure 4-3(B)) and PigOligoArray analysis (423 genes, Figure 4-3(C)), and the unique gene list generated from PigOligoArray analysis had the least overlap with the 454 sequencing data. The overlap between the unique genes positively detected from PigOligoArray and 454 sequencing data accounts for 18% of all the unique genes identified from the 454 sequencing data.

We further examined the expression of several pluripotency (*NANOG, POU5F1, SOX2*) (du Puy et al., 2011) and TE (*CDX2* (Strumpf et al., 2005; El-Hashash et al., 2010), *DLX3* (Ealy and Yang, 2009; El-Hashash et al., 2010), *BMP4* (Zernicka-Goetz et al., 2009), *KRT*8 (Ralston and Rossant, 2008)) marker genes among the porcine BL embryos global gene expression profiles obtained from the 3 microarray platforms and 454 sequencing analyses.

Expression of all of these seven genes was detected in porcine BL embryos with most of them detected by the EMPV1 and SOMV2 microarray platforms (Table 4-3). The

expression of the three TE markers (*CDX2*, *DLX3* and *KRT8*) and *NANOG* genes have not been detected in porcine 2- to 8-cell stage porcine embryos (Tsoi et al., 2012).

In order to elucidate the global transcriptomic profile of the *in vivo* porcine BL embryos, the transcriptomic profiles obtained from the 3 microarray platforms and 454 sequencing analysis were combined. A total of 19694 unique genes (Additional file 4-S6) were positively detected from *in vivo* derived porcine BL embryos.

4.3.3 Comparative transcriptomic analysis between early and hatched blastocyst stages

Once the efficiency of the four different platforms to profile the porcine early embryonic transcriptome was evaluated, the primary objective of this study was to understand how the global transcriptomic profile changes before and after the hatching process of porcine blastocysts.

As shown in 4.3.2, the EMPV1 and SOMV2 platforms both have better coverage of the BL transcriptome than PigOligoArray. In order to characterize the transcriptomic profile changes between EB and HB efficiently, both the EMPV1 and the SOMV2 platforms were utilized in the comparative transcriptomic analysis. A dye-swapped direct comparison design with 3 biological replicates from each embryonic stage (EB or HB) was utilized in the microarray analysis with the EMPV1 and SOMV2 platforms. The microarray results were submitted to NCBI Gene Expression Omnibus (GEO) Database and can be accessed through accession number of GSE38882.

A total of 10352 and 7825 unique genes were positively detected from porcine BL embryos using the EMPV1 and the SOMV2 platforms, respectively. Among the 7530 unique genes that were common in both the EMPV1 and the SOMV2 platforms (Additional file 4-S5), around half of these genes (3938 genes) were positively detected in BL embryos.

Comparative transcriptomic analysis using the EMPV1 and the SOMV2 microarray platforms identified 492 and 452 unique significant differentially expressed genes between EB and HB, respectively (Additional file 4-S8). A total of 76 genes were identified as significant differentially expressed between EB and HB in both of the EMPV1 and the SOMV2 analysis, and 75 of these genes showed the same direction (up-regulation or down-regulation) of gene expression changes from EB to HB in both analyses.

Significant differential expression of several critical genes that are associated with early embryonic development (*GATA2* and JARID2) and Ca^{2+} homeostasis (RGN) were observed after hatching. In addition, the TE development-associated genes *KRT18* and *KRT8* displayed trends of up-regulation (2> FC >1.5) after hatching. The expression data of these 5 genes were consistent between the comparative transcriptomic profiling analyses using the SOMV2 and the EMPV1 platforms. The down-regulation of *JARID2* and up-regulation of *KRT18* genes were further confirmed by QRT-PCR. Significant up-regulation of epigenetic modification regulators of DNA methylation (*DNMT1* and *DNMT3B*) and histone de-acetylation (*HDAC3*) after hatching were also observed from the microarray analysis.

IPA (Ingenuity® Pathway Analysis) Biological Function, Canonical Pathway, and Upstream Regulator Analysis

The expression data obtained from the comparative transcriptomic analysis using the EMPV1 and the SOMV2 platforms were analyzed using the Ingenuity® Pathway Analysis (IPA) tools as described in the methods section.

IPA Biological Function Analysis revealed 13 biological function categories that were significantly changed (B-H P-value <0.05, with more than 5 genes in the analysis) between EB and HB in both of the SOMV2 and the EMPV1 analyses including “lipid

metabolism", "cell death and survival", "tissue morphology", and "energy production" (Figure 4-4A). "Lipid metabolism" was the most significantly changed biological function category, with 67 and 94 "lipid metabolism" functions-associated genes showing significantly differential expression between EB and HB in the EMPV1 and the SOMV2 analysis, respectively. These "lipid metabolism"-associated genes accounted for approximately 14% and 21% of all the significant differentially expressed genes identified by the EMPV1 and SOMV2 platforms, respectively. Specifically, 26 significantly changed (B-H P-value <0.05, with more than 5 genes in the analysis) "lipid metabolism"-associated biological functions were identified from the SOMV2 microarray analysis (Figure 4-4B). The "synthesis of lipid", " fatty acid metabolism, "quantity of steroid" and "synthesis of fatty acid" functions are the largest groups among these "lipid metabolism"-associated biological functions. Significant differential expression of genes associated with these 26 biological functions were also observed in the EMPV1 analysis, but only the "oxidation of fatty acid" function was statistically significantly changed (B-H P-value <0.05, with more than 5 genes in the analysis) from EB to HB.

IPA Upstream Regulator Analysis revealed 17 upstream regulators that were predicted to be significantly activated (or inhibited) from EB to HB (Additional file 4-S7). Specifically, 8 and 20 known regulation target genes of the transcription factor SREBF1 exhibit significantly differential expression between EB and HB from the EMPV1 and SOMV2 analyses, respectively (Table 4-4). Seven (*IL6*, *CYP51A1*, *FN1*, *FASN*, *APOA2*, *NPC1*, and *LGALS3*) regulation target genes of SREBF1 exhibited significant differential expression in both the EMPV1 and the SOMV2 analyses. The direction of expression changes (up- or down- regulation) for these seven genes were consistent between the EMPV1 and the SOMV2 analyses. The transcription factor SREBF1 is predicted to be

significantly activated (overlap P-value ≤ 0.05, Z-score ≥ 2) in HB in comparison with EB in both the EMPV1 and the SOMV2 analyses (Table 4-4).

Neither SREBF1 nor SREBF2 were included in the EMPV1 platform. Positive expression of the transcription factors SREBF1 and SREBF2 were observed in SOMV2, but no significant differential expression was observed between EB and HB stage embryos.

IPA Canonical Pathway Analysis revealed 10 canonical pathways that were significantly changed (B-H P-value <0.05, with more than 5 genes in the analysis) between EB and HB in the SOMV2 analysis (Figure 4-5). Five of these 10 canonical pathways are associated with lipid, fatty acid and cholesterol homeostasis, including "FXR/RXR activation", "LXR/RXR activation", "PPARα/RXRα activation", "atherosclerosis signalling" and "PXR/RXR activation" pathways.

Although significant differential expression of genes associated with these 10 canonical pathways were also observed in the EMPV1 analysis, none of these canonical pathways showed statistically significant changes between EB and HB (Figure 4-5).

4.3.4 QRT-PCR verification

To confirm the results of microarray transcriptomic analyses, 11 genes were selected for QRT-PCR verification from genes that were positively detected in both EMPV1 and SOMV2 analysis. These 11 genes were selected from genes that showed different levels of expression changes (significant differential expression, trend of differential expression, and similar level of expression) from EB to HB in the microarray analyses. In general, the expression data obtained from QRT-PCR analyses are consistent with data obtained from the comparative transcriptomic analysis using the EMPV1 and SOMV2 platforms (Figure 4-6). The *APOA2*, *ACADL*, *RGN*, and *JARID2* genes exhibited significant differential expression (FC > 2 or < 0.5, B-H P-value < 0.05) between EB and HB in both EMPV1

and SOMV2 analysis, and the QRT-PCR analyses data showed the same gene expression changes. A trend toward differential expression (FC >1.5 or <0.7, P-value <0.05) of the *KRT18* (trend of up-regulated in HB) and *SRSF11* (trend of down-regulation in HB) genes were observed between EB and HB in both EMPV1 and SOMV2 analyses, and their B-H P-values were larger than, but close to, the cut off for significance (B-H P-value < 0.05). The QRT-PCR analysis revealed significant up-regulation of KRT18 and significant down-regulation of *SRSF11* from EB to HB. The *ITGB1*, *PDHB*, *LDHB*, and *ACTB* genes showed similar level of expression between EB and HB in both of the EMPV1 and SOMV2 analyses, and QRT-PCR analyses results were consistent with the microarray data. Significant down-regulation of *CCPG1* in HB was observed in SOMV2, but the down-regulation was not significant in EMPV1. *CCPG1* expression data obtained from QRT-PCR analysis was consistent with the EMPV1 analysis.

4.4 Discussions

The power of microarray technology is dependent on, and limited by, the probe sequences spotted on the microarray surface. The re-annotation performed in the present study has improved the number of annotated probes on the EMPV1 and the SOMV2 platforms by 38% and 18%, respectively. After the microarray platform re-annotation, the total number of annotated unique genes included in EMPV1 and SOMV2 microarray platforms increased to 15555 and 11396, respectively.

4.4.1 Global transcriptomic profiling of porcine blastocyst using different platforms

In the present study, global transcriptomic profiling of *in vivo* porcine BL embryos was performed using PigOligoArray, EMPV1, SOMV2, and 454 sequencing platforms. Results from the global transcriptomic profiling analyses using PigOligoArray, EMPV1, and SOMV2 platforms showed that the number of positively detected genes in the BL

embryos are in the following order: EMPV1 > SOMV2 > PigOligoArray. The transcriptomic profiling data obtained by the 3 microarray platforms (PigOligoArray, EMPV1, and SOMV2) were further compared with the 454 sequencing data respectively (Figure 4-3). The 454 sequencing of porcine BL embryos was performed with 1/8 plate (Tsoi et al., 2012), which indicates a comparatively limited coverage of the whole transcriptome. It was not surprising that the genes positively detected in 454 sequencing data did not cover all of the genes detected by microarray analysis. However, this 454 sequencing data still represent a significant part of the BL transcriptome. The overlap between the gene expression profiles observed by the microarray platforms and the 454 sequencing data are still indicative for their capabilities in detecting the genes expressed in BL embryos. The global gene expression data obtained from EMPV1 microarray analysis has the highest coverage among the 3 microarray platforms in comparison with the 454 sequencing data, and data obtained from PigOligoArray analysis has the lowest coverage. These result indicated that the EMPV1 data had the highest and the PigOligoArray data had the lowest coverage of the transcriptome for porcine BL embryos among the three microarray platforms.

After combining the gene expression data from the 3 different microarray platforms with data from a 454 sequencing analysis, positive expression of 19694 unique genes were detected from *in vivo* derived BL embryos (Additional file 4-S6). Our approach in this study has allowed us to find more genes that are expressed in BL embryos when compared to previous microarray studies performed in mouse (10357 unique genes) (Hamatani et al., 2004) and cattle (12956 transcripts) (Kues et al., 2008).

The total number of positively detected genes in BL embryos (19694) is very close to a previous transcriptomic profiling study performed on Day 6 *in vivo* porcine blastocysts (20029 transcripts) using a SAR-SAGE (small amplified RNA-serial analysis of gene

expression) platform (Miles et al., 2008). This indicates that the combined global transcriptomic profile obtained from the present study provides good coverage of the whole transcriptome of porcine blastocysts.

4.4.2 Gene expression profile differences between early and hatched blastocyst

Although, the transcriptomic profiling of *in vivo*-derived porcine blastocysts has been conducted (Whitworth et al., 2005; Miles et al., 2008; Bauer et al., 2010), the present study focused on a critical early embryonic developmental event - blastocyst "hatching", which occurs approximately 5-6 days after fertilization (Oestrup et al., 2009). Blastocyst hatching is a tightly regulated event, and the blastocyst needs to hatch from the zona pellucida prior to implantation. Any dysregulation in the hatching process leads to implantation failure and results in early embryonic loss (Seshagiri et al., 2009). The genes contained on the EMPV1 and the SOMV2 platforms are complementary with each other. Using both the EMPV1 and SOMV2 microarray platforms, we have characterized the transcriptomic changes of porcine blastocyst from EB to HB.

The number of significant differentially expressed genes that were identified by both the EMPV1 and SOMV2 platforms is limited. This difference is probably due to 3 major factors: (1) The differences in the unique genes that are included in the EMPV1 and the SOMV2 platforms. (2) Different positive detection intensity cut-offs during the microarray data analysis of the two platforms due to their different background signals. (3) The limited reproducibility between microarray platforms may also account for part of the differences.

GATA binding protein 2 (GATA2) belongs to the GATA transcription factor family which is necessary for embryonic development and differentiation (Tsai et al., 1994; Fujikura et al., 2002). GATA2 is an important regulator of multipotent hematopoietic precursor cells (Lugus et al., 2007) and plays important roles in migration and

differentiation of neurons during early embryonic development (Willett and Greene, 2011). Jumonji, AT rich interactive domain 2 (JARID2) is an ortholog of the mouse jumonji gene (Jarid2), which encodes a nuclear protein essential for organogenesis including that of the liver, thymus and spleen development (Motoyama et al., 1997), as well as neural tube formation (Bergé-Lefranc et al., 1996). Keratins 18 and keratins 8 (KRT18 and KRT8) are predominantly expressed in simple epithelial tissues and are associated with the regulation of cell cycle, cell growth and apoptosis (Alam et al., 2011). Keratins are essential for the integrity of a specialized embryonic epithelium (trophoblast giant cells) layer (Hesse et al., 2000). In the present study, significant up-regulation of GATA2 and down-regulation of JARID2 expression were observed from EB to HB. The KRT18 and KRT8 genes showed trends of up-regulation from EB to HB in the microarray analyses. Regucalcin (RGN) is a Ca^{2+} binding protein which activates (Ca^{2+} - Mg^{2+})-ATPase and is involved in the maintenance of intracellular Ca^{2+} homeostasis (Takahashi and Yamaguchi, 1997; Wu et al., 2008). In the present study, up-regulation of RGN gene was observed from EB to HB stage. The down-regulation of JARID2 and up-regulation of KRT18 and RGN genes from EB to HB were further confirmed by QRT-PCR results (Figure 4-6). The regulation of the JARID2, GATA2, KRT18, KRT8, and RGN genes may be necessary for the blastocysts hatching process in pig.

DNA methylation and histone deacetylation are two major epigenetic modification regulation events during early embryonic development (Chen et al., 2005). DNMT1 and DNMT3B are members of DNA methyltransferase family, which is necessary for the de novo methylation of unmodified DNA (Bird, 2002). DNMT1 and DNMT3B are shown to be epigenetic reprogramming regulators during the blastocyst hatching process in mouse (Chen et al., 2005). In the present study, up-regulation of DNMT1 and DNMT3B genes was observed after blastocyst hatching, which is consistent with a previous report in

mouse embryos (Chen et al., 2005). HDAC3 (histone deacetylase 3) is a member of histone deacetylase family (HDACs), which is necessary for histone modification (Shi and Wu, 2009). In the present study, up-regulation of HDAC3 was observed after hatching. These results indicate that DNMT1, DNMT3B, and HDAC3 may be involved in the regulation of epigenetic reprogramming in during early porcine embryonic development.

In comparison with other mammals, the porcine embryo has a relatively high lipid content in the form of intracellular lipid droplets (McEvoy et al., 2000; Romek et al., 2009). This high lipid content is believed to be responsible for the compromised efficiency of various biotechnological manipulations with pig oocytes and embryos (McEvoy et al., 2001; Romek et al., 2011). Lipid droplets in porcine embryos contain many types of lipids such as triglycerides, free fatty acids, phospholipids and cholesterol (Romek et al., 2009). Although the amounts of triglycerides are believed to remain unchanged in pig embryos until the late blastocyst stage (Sturmey and Leese, 2003), a decrease in the total lipid content has been reported at blastocyst and late blastocyst stages in comparison with earlier stages (Romek et al., 2009). In the present study, "lipid metabolism" is the most significantly changed biological function category observed between EB and HB. Specifically, the biological function of "oxidation of fatty acid" was significantly changed from EB to HB in both the EMPV1 and the SOMV2 analyses. Pig embryos may utilize intracellular lipids as an energy source through β-oxidation, which requires oxygen (Romek et al., 2009). Oxygen consumption in pig embryos stays low throughout the cleavage stages and reaches a peak at the hatching blastocyst stage, after which the oxygen consumption falls back to low levels in the hatched blastocyst stage (Sturmey and Leese, 2003). Down-regulation of the ACADL gene, which is involved in the β-oxidation pathway, from EB to HB was observed in the present study. These results

suggest that regulation of lipid metabolism-associated functions, specifically the "oxidation of fatty acid" function, took place and probably was necessary during the blastocyst hatching process in the pig.

In humans and other mammals, three members of the Sterol regulatory element binding proteins (SREBPs) family have been described: SREBP-1a, SREBP -1c, and SREBP-2 (Im et al., 2009). SREBP-1a and SREBP-1c are isoforms encoded by the SREBF1 gene, and SREBP-2 is encoded by SREBF2 gene (Weber et al., 2004). SREBPs are essential for embryonic viability as deletion of both isoforms of SREBP-1 in mice results in significant embryonic lethality at day 10 (Shimano et al., 1997; Im et al., 2009) and SREBP-2 inactivation in mice results in 100% lethality during early embryonic development (Horton et al., 2002; Im et al., 2009). In vivo studies performed with mice suggested that SREBP-1 is selectively involved in fatty acid synthesis, lipogenesis and insulin-induced glucose metabolism (Eberle et al., 2004). On the other hand, SREBP-2 is activated in a cholesterol-dependent manner (Sato, 2010) and selectively regulates genes directly involved in cholesterol homeostasis (Horton et al., 1998; Eberle et al., 2004). In the present study, the transcription factor SREBF1 was predicted to be significantly activated from EB to HB by the IPA Upstream Regulator Analysis. Although no differential expression was observed in SREBF1 and SREBF2, significant differential expression of 8 and 20 regulation target genes of SREBF1 were observed between EB and HB from the EMPV1 and the SOMV2 analyses, respectively. These results suggest that the transcription factor SREBF1 was activated from EB to HB.

Most nuclear receptors, including peroxisome proliferator-activated receptors (PPARs), the cholesterol-sensing liver X receptor (LXRα/β, NR1H3/2), the bile-acid-activated farnesoid X receptor (FXR, NR1H4), and the pregnane X receptor (PXR, NR1I2) are active as dimers with the retinoid X receptor (RXR, NR2B1/2/3) and may be activated

through binding to their ligands (Francis et al., 2003). These nuclear receptors play critical roles in the control of lipid, cholesterol, and bile acid homeostasis in a very complex cross-regulatory manner (Francis et al., 2003). In the present study, significant expression level changes of genes associated with the lipid, fatty acid and cholesterol homeostasis associated pathways including the "FXR/RXR activation", "LXR/RXR activation", "PPARα/RXRα activation", "atherosclerosis signalling" and "PXR/RXR activation" pathways were observed from EB to HB. LXRα/β and the FXR are lipid-activated nuclear receptors that are activated by oxysterols and bile acids, respectively (Chawla et al., 2001; Handschin and Meyer, 2005). LXR is an activator of SREBP-1c, which triggers triglyceride biosynthesis (DeBose-Boyd et al., 2001; Handschin and Meyer, 2005). Activated FXR inhibits the LXR-mediated induction of SREBP-1c; activated PPARα and LXR antagonize each other's functions (Watanabe et al., 2004; Handschin and Meyer, 2005). PPARα (NR1C1) is involved in fatty acid, triglyceride, and lipoprotein metabolism, and activated PPARα induces the expression of genes encoding enzymes in the β-oxidation pathway (e.g. ACADL) and stimulates fatty acid oxidation (Barger and Kelly, 2000; Francis et al., 2003). PPARα also induces the synthesis of apolipoprotein A-I (APOA1) and apolipoprotein A-II (APOA2), the two major proteins of high-density lipoprotein (HDL) in human (Vu-Dac et al., 1995; Vu-Dac et al., 1998). Significant down-regulation of ACADL and up-regulation of the APOA2 gene after hatching were observed in the present study. These results suggest that nuclear receptors FXR, LXR, PPARα, PXR, and RXR-involved regulation of lipid, cholesterol, and bile acid homeostasis probably occurred during the blastocyst hatching process in the pig, and the transcription factor SREBF1 may be involved in this complex regulation.

4.5 Conclusion

The re-annotation performed in the present study has improved the annotation of the EMPV1 and SOMV2 microarray platforms: 38% and 18% of the probes on the EMPV1 and SOMV2 microarray have been newly characterized, respectively. The EMPV1 microarray has the highest coverage of the porcine blastocyst stage transcriptome among the 3 microarray platforms analyzed in the present study. Comparative transcriptomic analysis using both the EMPV1 and SOMV2 microarray platforms showed that the "lipid metabolism" is the most significantly changed biological function category from EB to HB stage. Both the EMPV1 and the SOMV2 analyses revealed statistically significant changes in the "oxidation of fatty acid" function between EB and HB. In addition, significant changes in the lipid fatty acid, and cholesterol homeostasis-associated pathways were also observed between EB and HB. Furthermore, results from the IPA Upstream Regulator Analysis suggest an activation of the transcription factor SREBP-1 from EB to HB. These results suggest that the regulation of a battery of "lipid metabolism" and "lipid, fatty acid, and cholesterol homeostasis"-associated genes took place during the "hatching" process from EB to HB, and the transcription factor SREBF1 is involved in this regulation process.

Results of the present study increased our understanding of the developmental biology of this pivotal developmental period in pig, and should facilitate the identification of marker genes for embryo quality assessment and hence increase our ability to define embryonic competence in pig embryos.

Table 4-1 Primer sequences used in QRT-PCR verification

Official Gene Symbol	Gene name	Associated Porcine RefSeq Accession No.	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	PCR product size
ACADL	Acyl-CoA dehydrogenase, long chain	NM_213897.1	GAGTTACAAAACAGCGTGGC	AACTCGGGCATCCACATAAG	102 bp
APOA2	Apolipoprotein A-II	NM_001186294.1	CTTTGGCAAGGACCTGATAGAG	GGCCAATGAAACTGCTCAAG	149 bp
CCPG1	Cell cycle progression 1	NM_001243301.1	CTGAACCCTCTAAGGATCTGTG	TCTGACTAACTGTTGACGCTTC	146 bp
ITGB1	Integrin beta-1 subunit (CD29)	NM_213968.1	TGAAGACATGGACGCTTACTG	ATTATCACACTCGCAGAATTTGC	145 bp
JARID2	Jumonji, AT rich interactive domain 2	XM_003128194.1	GTGGAGAAGCAGAAGTCCTG	GTGGGTTTACCGAGACAGTTC	140 bp
KRT18	Keratin 18	XM_003126180.3	TTGACCGTGGAGTTGGATG	ACCACTGAGGTGCTCTCC	149 bp
LDHB	Lactate dehydrogenase B	NM_001113287.1	AACAATGGTGCAGGGCATGTATGG	TCTTGAGCTGGGCAACCTCATCAT	131 bp
PDHB	Pyruvate dehydrogenase (lipoamide) beta	NM_001244398.1	GATGGGCTTTGCTGGAATTG	CCCCGACATGTAATAGGTCTTG	139 bp
RGN	Regucalcin (senescence marker protein-30)	NM_001077220.1	TGCTTTGGAGGGAAGGATTAC	CCCCAGGCCAGTTATCTT	120 bp
SRSF11	Serine/arginine-rich splicing factor 11	NM_001044587.1	AGACGTTCCAGAAGTGCAAG	TGGTGAGCGAGACAACTTTC	108 bp
ACTB	Actin, beta	XM_003124280.2	Control primer from SYBR® Green Cells-to-CT™ Control Kit(Ambion)		135 bp
Xeno™	Xeno™ artifical synthetic RNA Control		Control primer from SYBR® Green Cells-to-CT™ Control Kit(Ambion)		105 bp

Table 4-2 Probes and unique genes positively detected in porcine BL embryos using 4 different platforms.

Platform	Total number of sequences on platform	Total number of annotated probes with GS on platform	Total number of Unique GS on platform	Total number of probes positively detected in BL embryos	Unique Genes positively detected in BL embryos	Positively detected Unique genes mapped with human GO term in PANTHER
PigOligoArray	20360	13536	10963	2486	1789	1593
EMPV1	43542	32797	15555	23263	10352	9183
SOMV2	43803	22480	11396	22090	7825	5596
454 sequencing	18149				2263	2001

Table 4-3 Detection of selected gene markers in BL embryos (4 platforms)

	454 Sequencing	PigOligoArray	EMPV1	SOMV2
KRT8	PD	PD	PD	PD
NANOG	ND	ND	PD	PD
POU5F1	ND	ND	PD	PD
BMP4	ND	ND	PD	PD
SOX2	ND	ND	ND	PD
CDX2	PD	ND	PD	ND
DLX3	ND	ND	ND	PD

PD: Positively detected; ND: Not detected.

Table 4-4 SREBF1 targets that differentially expressed between EB and HB

SREBF1 regulation targets			Transcription factor SREBF1 (IPA Upstream Regulator Analysis Z-score=2.399, overlap P-value < 0.05) EMPV1 analysis			Transcription factor SREBF1 (IPA Upstream Regulator Analysis Z-score=2.112, overlap P-value < 0.05) SOMV2 analysis		
Offical Gene Symbol	Gene name	GO biological process	Genes ID in dataset SOMV2	Log2 Fold Change	IPA Prediction (based on expression direction)**	Genes in dataset EMPV1	Log2 Fold Change	IPA Prediction (based on expression direction)**
APOA2	Apolipoprotein A-II	lipid transport;lipid metabolic process	APOA2	1.711	Activated	APOA2	1.514	Activated
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1	respiratory electron transport chain;cholesterol metabolic process;proteolysis	CYP51A1	2.698	Activated	CYP51A1	2.748	Activated
FASN	Fatty acid synthase	cellular amino acid metabolic process;fatty acid biosynthetic process	FASN	1.893	Activated	FASN	1.949	Activated
FN1	Fibronectin 1	intracellular protein transport;phagocytosis;cell-matrix adhesion;signal transduction;cell-cell adhesion;cellular component morphogenesis;	FN1	2.611	Activated	FN1	1.355	Activated
G6PD	Glucose-6-phosphate dehydrogenase	monosaccharide metabolic process				G6PD	1.171	Activated
IL6	Interleukin-6	immune system process;negative regulation of apoptosis;cytokine-mediated signaling pathway;JAK-STAT cascade;negative regulation of apoptosis;JNK cascade;cell-cell signaling;	IL6	-2.691	Affected	IL6	-2.629	Affected
LGALS3	Lectin, galactoside-binding, soluble, 3	immune system process;induction of apoptosis;cell adhesion;	LGALS3	1.277	Affected	LGALS3	1.088	Affected
NPC1	Niemann Pick type C1	lipid transport;intracellular protein transport;exocytosis;cell surface receptor linked signal transduction;lipid metabolic process;	NPC1	1.096	Activated	NPC1	1.967	Activated
APOC3	Apolipoprotein C-III	lipid transport;lipid metabolic process	APOC3	1.582	Affected			
CDK4	Cyclin-dependent kinase 4	mitosis;protein amino acid phosphorylation;	CDK4	1.003	Affected			
DBI	Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)	lipid transport;lipid metabolic process	DBI	1.168	Activated			
FABP5	Fatty acid binding protein 5 (psoriasis-associated)	lipid transport;vitamin transport;signal transduction;lipid metabolic process;ectoderm development	FABP5	-1.337	Inhibited			
HMOX1	Heme oxygenase (decycling) 1	porphyrin metabolic process	HMOX1	-1.236	Affected			
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble	tricarboxylic acid cycle;carbohydrate metabolic process	IDH1	-1.037	Inhibited			
IL1B	Interleukin-1 beta	immune response;macrophage activation;cytokine-mediated signaling pathway;cell-cell signaling;response to stimulus	IL1B	-1.408	Affected			
LDLR	Low-density lipoprotein receptor	female gamete generation;cell adhesion;	LDLR	1.226	Activated			
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	gluconeogenesis	PCK2	1.963	Affected			
PIK3R3	Phosphatidylinositol 3-kinase regulatory subunit gamma	intracellular signaling cascade;	PIK3R3	-1.163	Affected			
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	proteolysis	SERPINE1	-1.248	Inhibited			
SQLE	Squalene epoxidase		SQLE	1.041	Activated			
STAR	Steroidogenic acute regulatory protein	female gamete generation;lipid metabolic process	STAR	1.072	Activated			

*: Log2 Fold Change=log2 (HB/EB); **: Activated=Expression direction consistent with activation of SREBF1). Inhibited=Expression direction consistent with inhibition of SREBF1.

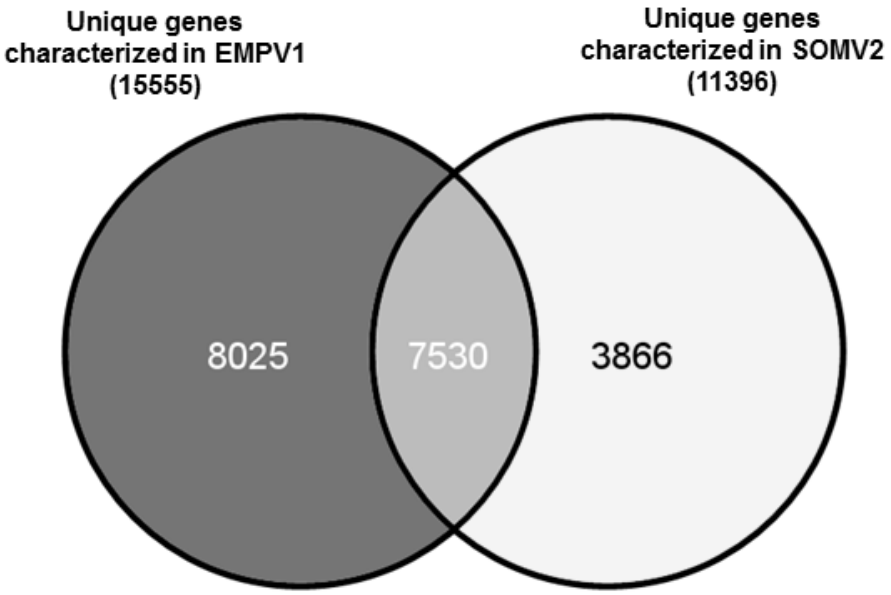

Figure 4-1 Comparison of characterized unique genes in the EMPV1 and SOMV2 platforms.

Venn diagram showing the number of unique genes that were characterized in both the EMPV1 and SOMV2 platforms after the re-annotation analysis. Note that 7530 unique genes were observed in common between EMPV1 and SOMV2 microarray platforms.

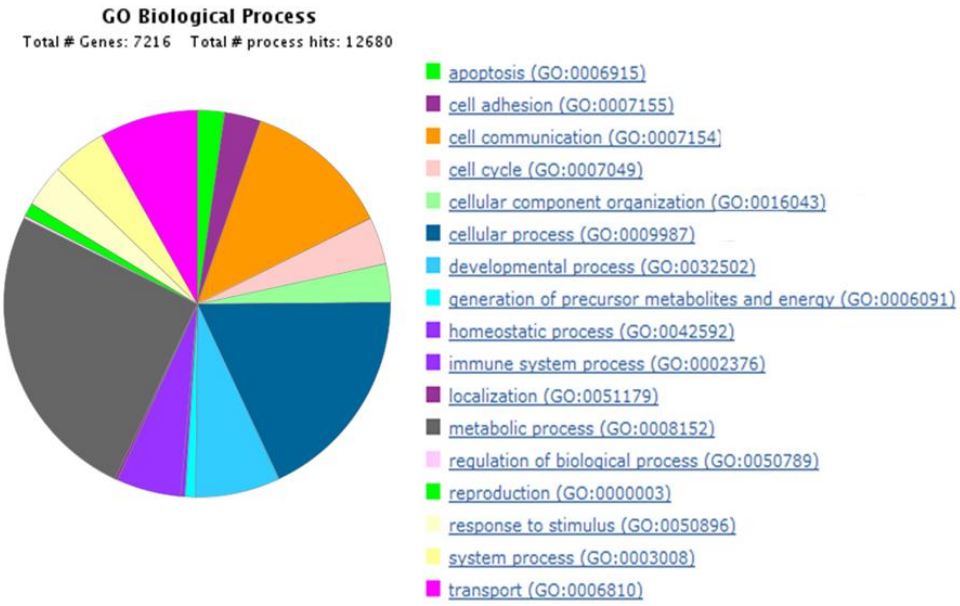

Figure 4-2 PANTHER GO biological process analysis of common unique genes between EMPV1 and SOMV2 platforms.

Pie chart showing the distribution of common unique genes between EMPV1 and SOMV2 platforms associated with GO biological process. Total # genes: Total number of genes with human GO term matches from the input gene list. Total # process hits: the total number of GO biological processes associated with the input genes.

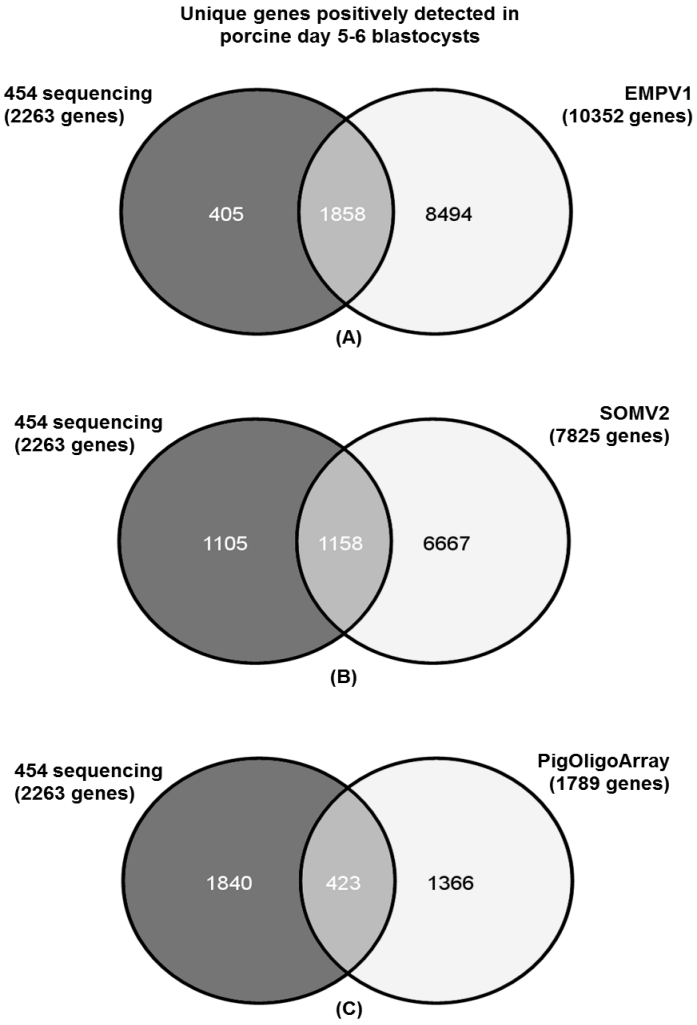

Figure 4-3 - Comparison of unique genes positively detected from BL embryos using microarray and 454 sequencing.

Venn diagram showing comparison between gene lists positively detected in BL embryo using microarray platforms and 454 sequencing. (A) 1858 unique genes were in common between the unique gene lists positively detected in BL embryo using 454 sequencing and EMPV1 platform. (B) 1158 unique genes were in common between the unique gene lists positively detected in BL embryo using 454 sequencing and SOMV2 platform. (C) 423 unique genes were in common between the unique gene lists positively detected in BL embryo using 454 sequencing and PigOligoArray platform.

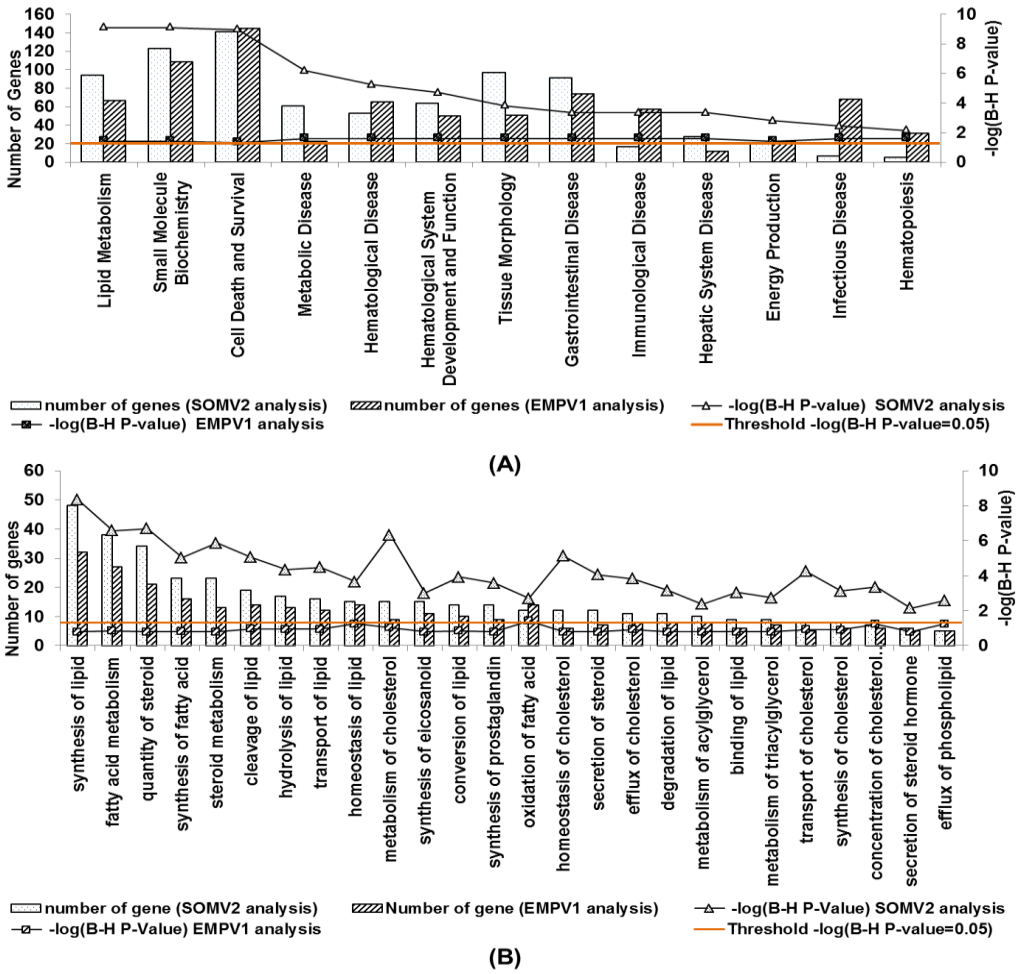

Figure 4-4 IPA Biological Function Analysis of genes differentially expressed between early and hatched blastocysts.

Bar chart showing the significantly enriched (B-H P-value <0.05, more than 5 genes included in the analysis) biological function categories (A) and lipid metabolism-associated biological function (B) in the differentially expressed gene from the comparative transcriptomic analyses between early and hatched blastocysts using EMPV1 and SOMV2 platforms. Primary Y-axis (left): number of genes associated with the biological function; Secondary Y-axis (right) [-log (B-H P-value)]: the -log of B-H P-value of the biological function. Large format version of this figure can be access through the following link:

https://docs.google.com/a/ualberta.ca/file/d/0B0QxwqYwWLkfNkZGaEY0X3hSVWs/edit?usp=sharing

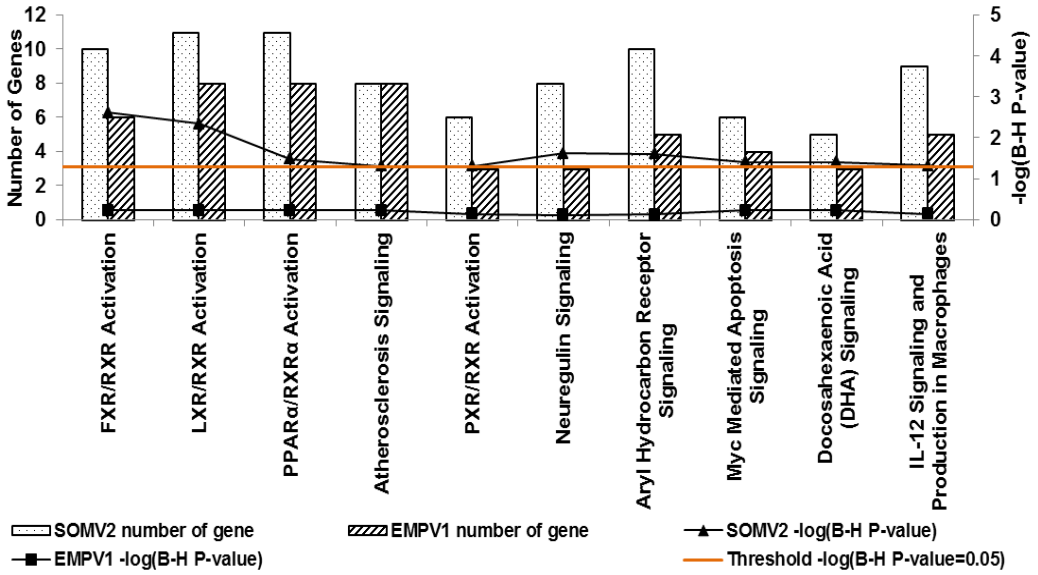

Figure 4-5 IPA Canonical Pathway Analysis of genes differentially expressed between early and hatched blastocysts.

Bar chart showing the 10 canonical pathways that significantly enriched (B-H P-value <0.05, more than 5 genes included in the analysis) in the genes differentially expressed between early and hatched blastocysts. Primary Y-axis (left): number of genes associated with the canonical pathway; Secondary Y-axis (right) [-log (B-H P-value)]: displays the -log of B-H P-value of the canonical pathway. EMPV1: EmbryoGENE Porcine Array Version 1; SOMV2: Sus.Scrofa Oligo Microarray v2.

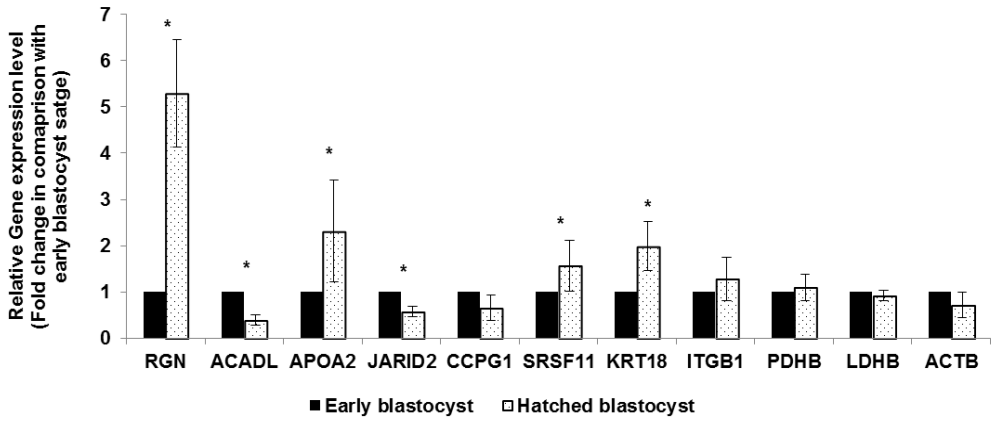

Figure 4-6 QRT-PCR Verification.

QRT-PCR verification of 11 genes (APOA2, ACADL, RGN, ITGB1, CCPG1, KRT18, JARID2, SRSF11, PDHB, LDHB and ACTB) that were positively detected in both EMPV1 and SOMV2 microarray analyses. Bar chart showing the relative gene expression level of the 11 selected genes in hatched blastocyst (fold change in comparison with early blastocyst stage.) *: Significant differentially expressed between early and hatched blastocysts (P-value<0.05).

Additional file 4-[S1-S2]:

http://www.biomedcentral.com/imedia/8518904869990146/supp2.docx

Additional file 4-S1 Assembly parameter for gene annotation using SeqMan NGen for probe sequences from EMPV1 and SOMV2 microarray

Additional file 4-S2 Assembly parameter for gene annotation using SeqMan NGen for 454 sequencing and PigOligoArray data

Additional file 4-[S3-S7]:

https://drive.google.com/file/d/0B0QxwqYwWLkfRmQ4b0s4SmE2dVU/edit?usp=sharing

Additional file 4-S3 EMPV1 newly characterized probes. An excel sheet contains the re-annotation result of newly characterized probes in EMPV1 with GEO probe reference ID, probe Sequence, probe ID, accession number, gene description, gene symbol and description.

Additional file 4-S4 SOMV2 newly characterized probes. An excel sheet contains the re-annotation result of newly characterized probes in SOMV2 with GEO probe reference ID, probe Sequence, probe ID, accession number, gene description, gene symbol and description.

Additional file 4-S5 Unique gene symbols and common gene symbols in the EMPV1 and SOMV2 platforms. An excel sheet contains the list of gene symbols of genes only characterized in EMPV1, genes only characterized in SOMV2, and genes that characterized in both EMPV1 and SOMV2 platforms.

Additional file 4-S6 Combined unique genes positively detected in porcine BL embryos

An excel sheet contains combined unique gene symbol of all positively detected genes in porcine BL embryos in the present study.

Additional file 4-S7 Upstream regulators predicted to be significantly activated (or inhibited) from EB to HB. An excel sheet contains the IPA Upstream Regulator Analysis results of upstream regulators that predicted to be significantly activated (or inhibited) from EB to HB.

Additional file 4-S8 Unique genes differentially expressed between early and hatched blastocyst identified by EMPV1 and SOMV2

An excel file contains the unique gene symbol of differentially expressed genes that only identified in EMPV1 (4-S8.1), only identified in SOMV2 (4-S8.2), and the differentially expressed genes that identified by both EMPV1 and SOMV2 platforms (4-S8.3).

https://docs.google.com/file/d/0B0QxwqYwWLkfa3NRZDdfbHJtTDA/edit?usp=sharing

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Chapter 5: Characterization of the porcine embryo transcriptome during early embryonic development: from oocyte to hatched blastocyst[3]

5.1 Introduction

As described in chapter 2, the embryonic genome activation (EGA), morula compaction, blastocyst formation and hatching are important developmental events that take place during the pre-implantation period of mammalian embryonic development (Oestrup et al., 2009; Sirard, 2012). Although the morphological steps of these developmental events have been well documented, the molecular mechanisms underlying these events are not yet fully understood.

In mammals, oocyte-derived mRNAs are degraded shortly after fertilization. Hence, EGA and production of embryo-derived transcripts must occur during early embryonic development (Thompson et al., 1998; Schultz, 2002). EGA is a gradual process, with a small portion of the embryonic genome activating early and the major embryonic genome activation of abundant transcription occurring later (Oestrup et al., 2009). The precise timing of the onset of EGA in mammals varies among species (Telford et al., 1990; Oestrup et al., 2009; Sirard, 2012). As discussed in chapter 2, the major embryonic genome activation (EGA) in pig embryo is believed to be initiated at 4C stage (Telford et al., 1990; Prather et al., 2009; Sirard, 2012), which is different from many other mammalian species including the mouse (second cell cycle) and the rabbit (the 5th cell cycle) (Oestrup et al., 2009). On the other hand, distinct variation in gene expression profile between the oocyte and 4C and blastocyst has been reported in pig, where

[3] A manuscript based on this chapter is under preparation.

differential expression of 3214 transcripts were observed between oocyte and 4C (Whitworth et al., 2005). However, the precise timing and key regulators of the molecular mechanism that associated with the initiation of EGA process in porcine embryos remains to be further elucidated.

Characterization of the gene expression profile of early porcine embryos could identify pathways critical for embryonic development and potential gene markers of embryonic quality (Whitworth et al., 2005; Ka et al., 2008). Although there have been efforts to characterize the transcriptomic profile of *in vivo*-derived early porcine embryos (Whitworth et al., 2005; Miles et al., 2008; Bauer et al., 2010), most of these studies were focused on few selected embryonic stages. In the present study, global gene expression profiling analysis and comparative transcriptomic analysis were performed with a relative complete set of *in vivo*-derived porcine oocytes and early pre-implantation embryos in order to characterize the detailed gene expression profile change during the pre-implantation period of porcine embryonic development.

The EMPV1 (EmbryoGENE Porcine Array Version1) microarray (Tsoi et al., 2012) was shown to be an efficient microarray platform for transcriptomic profiling analysis in early porcine embryos (chapter 3 and 4). Drawing on data from this unique microarray platform we report on 1) The global gene expression profile of 9 porcine oocyte and embryonic stages during early embryonic development from germinal vesicle stage oocytes to day 11 embryos (in terms of the number of positively expressed genes in different stages); 2) Critical genes / gene networks that displayed significant gene expression changes from 4-cell to hatched blastocyst stage.

5.2 Material and Method

5.2.1 Recovery of *in vivo* embryos

In-vivo derived porcine Germinal vesicle (GV), MII, 2-cell (2C), 4-cell (4C), 8-cell (8C), morula(MOR), early blastocyst (EB), expanded blastocyst (XB), hatched blastocyst (HB) and embryonic day 11 (D11) HB (hatched blastocyst before elongation) stage embryos were collected from gilts as described previously (Degenstein et al., 2008) and stored individually. The day of artificial insemination is considered to be day 0 (D0). All embryo samples were placed on dry ice immediately after collection and stored at -80 ℃ until RNA extraction.

5.2.2Total RNA isolation

Total RNAs were extracted from pools of five *in vivo* derived porcine embryos using Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA). The RNA quality and integrity of each total RNA sample was evaluated by Bioanalyzer RNA 6000 Pico LabChip (Agilent Technologies, Mississauga, On, Canada). Only high quality RNA samples (RNA integrity number (RIN) $\geq$7.5) were used in RNA amplifications except samples from MII, 2C and 4C and 8C stages. It has been demonstrated that embryos from pre-embryonic genome activation (pre-EGA) stages contains very low amounts of 28S rRNA which results in lower total RNA RIN value (Gilbert et al., 2009). Therefore, total RNA samples from MII, 2C and 4C and 8C stages with lower RIN values (range from 5.8 to 6.8) were utilized in this study.

5.2.3 RNA amplification for microarray analysis

Due to the low quantities of each total RNA samples, all RNA samples were amplified using RiboAmp HS^{Plus} kit (Applied Biosystems, Carlsbad, CA, USA). Amplified antisense RNA (aRNA) products were used as targets for microarray reactions. One ng of total RNA was utilized in each amplification reaction. The quantity and quality of aRNA products were evaluated using Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

5.2.4 Reference amplified antisense RNA (aRNA) pool generation

A reference aRNA pool was generated from 10 different embryonic stages (GV, MII, 2C, 4C, 8C, MOR, EB, XB, HB, and D11 HB). Total RNA samples were amplified individually using RiboAmp HS^{Plus} kit (Applied Biosystems, Carlsbad, CA, USA); 1 ng of total RNA was used in each amplification and the quality and quantity of each aRNA sample was assessed using Bioanalyzer RNA 6000 Nano LabChip (Agilent Technologies, Mississauga, On, Canada) and Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, USA). A total of 360 μg of reference aRNA was generated by pooling 36 μg of aRNA from each of the 10 embryonic stages. The reference aRNA pool was stored in aliquots at -80 ℃ until use.

When applied to the EMPV1 platform, the reference aRNA pool produced reference signals (signals that were higher than the average signal of negative controls) for 95% of all the genes spotted on the microarray.

5.2.5 Microarray experimental design

To characterize the "normal" transcriptome profile of early porcine embryos, global gene expression profiling analysis of *in vivo*-derived porcine oocytes (GV and MII) and embryos from seven different developmental stages (4C, 8C, MOR, EB, XB, HB, and D11) were performed with the EMPV1 microarray (Figure 5-1 A). The aRNA samples from each embryonic stage were pooled (n=15), and the resulting pooled aRNA samples of each embryonic stage was labeled with both Cy5and Cy3 dye and hybridized on the EMPV1 array in a dye-swapped manner. Two technical replicates of each embryonic stage were analysed. The data of genes that positively detected from each oocyte or embryonic stages were determined as described in 4.2. 8.

To further characterize the detailed gene expression profile changes from the 4C to HB stages, comparative transcriptomic analysis of *in vivo*-derived porcine embryos from 5

different embryonic developmental stages (4C, 8C, MOR, EB, XB, and HB) was performed with the EMPV1 microarray platform following a reference design (Figure 5-1 B). Samples of aRNA from each embryonic stage were labeled with Cy5 dye and hybridized with Cy3 dye labeled "reference aRNA pool" (5.2.5) on EMPV1 array. Three biological replicates (each biological replicate sample was generated from 5 morphologically identical embryos) from the six embryonic developmental stages were analysed.

Agilent two-colour RNA Spike-In® (Agilent Technologies, Mississauga, ON, Canada) were amplified, labeled and utilized as positive controls in each hybridization reaction as previously described (Tsoi et al., 2012).

5.2.6 Microarray analysis and data acquisition

The labelling, hybridization, washing and drying steps of EMPV1 microarray were conducted following the procedure described in chapter 4. In short, aRNA samples were labeled with different dyes (Cy5 or Cy3), and hybridized on one microarray. Arrays were incubated at 65 ℃ with rotation at 10 rpm for 17 hours. After washing and drying steps that strictly followed the manual from Agilent, microarrays were immediately scanned using an Axon 4200AL scanner (Molecular Device, Sunnyvale, USA).

Microarray data were analysed using the FlexArray software package, which uses R and Bio-Conductor (Gentleman et al., 2004) and provides a user-friendly interface that facilitates data processing, visualization, and statistical analysis (Michal Blazejczyk, Mathieu Miron, Robert Nadon (2007). FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL http://genomequebec.mcgill.ca/FlexArray). Simple background subtraction and within array global lowess normalization was performed on raw data from each array using the FlexArray software package. The threshold for positive spots selection from microarray

data was determined by the mean value of all the negative control spots plus two standard deviations (Tsoi et al., 2012). To identify the differentially expressed genes, the normalized microarray data was analyzed using "limma" package (Smyth, 2005) of Bio-conductor through FlexArray under the Benjamini and Hochberg false discovery rate (BH-FDR) (Benjamini and Hochberg, 1995) multiple comparison correction condition through FlexArray (Robert et al., 2011). For any particular comparison, only genes with a BH-FDR adjusted P value (B-H P-value) ≤ 0.05 and a fold change (FC) ≥ 2 (or ≤ 0.5) were considered to be significantly up- or down-regulated.

5.2.7 PANTHER Gene Ontology (GO) analysis

The gene expression data obtained from the global transcriptomic profiling analysis were uploaded into PANTHER analysis tools (http://www.pantherdb.org/) (Thomas et al., 2003; Mi et al., 2010) in order to identify the Gene Ontology (GO) terms associated with the genes, transcripts, and proteins. These GO term lists were later uploaded into PANTHER expression analysis tools in order to identify biological processes that were statistically over- and under-represented in comparison with a reference list using the PANTHER "Statistical overrepresentation test" under Bonferroni multiple testing correction condition (Thomas et al., 2003). Only biological processes and pathways with a Bonferroni corrected P-value (Bon P-value) < 0.05 were considered to be significantly over- or under-represented. More details related to the expected value and P-value calculation algorithm (Cho and Campbell, 2000) are available on the PANTHER help website.

5.2.8 IPA (Ingenuity® Pathway Analysis) upstream regulator analysis and canonical pathways

Expression data obtained from the comparative microarray analysis were analysed using the IPA (Ingenuity® Pathway Analysis, Ingenuity® Systems, www.ingenuity.com)

Biological Functions Analysis, Canonical Pathway Analysis, and Upstream Regulator Analysis tools as described in chapter 4 (4.2.10).

5.2.9 Real-time quantitative PCR (QRT-PCR) verification of gene expression

Ten genes were selected from the gene expression data and verified using SYBR Green I-based QRT-PCR. The QRT-PCR analysis was performed as previously described in chapter 4 (4.2.11). In brief, total RNA samples were extracted from pools of five morphological identical embryos (or oocytes), and three biological replicates from each developmental stage were analysed. One ng of total RNA from each biological sample was reverse transcribed into cDNA using a high capacity reverse transcriptase (SuperScript® VILO™ cDNA Synthesis Kit, Invitrogen™). Equal amount of Xeno™ RNA Control (SYBR® Green Cells-to-CT™ Control Kit, Ambion®) was added to each reverse transcription reaction to serve as an external reference for the QRT-PCR analysis and a positive control for reverse transcription. The QRT-PCR reactions performed with StepOnePlus™ Real-Time PCR System (Life technologies) and Fast SYBR® Green Master Mix (Applied Biosystems®). The primer sequences for all target genes were listed in Table 5-1. The QRT-PCR data were normalized with the external control gene (Xeno™ RNA Control) using the qbase^{PLUS} software (Biogazelle) (Hellemans et al., 2007). The adjusted QRT-PCR data was then further analysed using $2^{-\Delta\Delta CT}$ method (Yuan et al., 2006; Hellemans et al., 2007) to determine the relative differential expression (fold changes) of the target genes.

5.3 Results

5.3.1 Global mRNA profile of porcine oocyte and pre-implantation embryo

To characterize the "normal" transcriptome profile of pre-implantation porcine embryos, global gene expression profiling analysis of *in vivo*-derived porcine oocytes (GV and

MII) and embryos from seven different developmental stages (4C, 8C, MOR, EB, XB, HB, and D11) was performed with EMPV1 microarray.

The global gene expression profiling analysis positively detected 12649, 10133, 11127, 11015, 11206, 12385, 11620, 10820, and 11421 genes in GV, MII, 4C, 8C, MOR, EB, XB, HB, and D11 stage embryos (or oocytes), respectively (Figure 5-2 A, B). Genes detected in each embryonic stage (4C to D11 stage) were characterized into two groups (Figure 5-2 A): (1) "Embryo-activated genes": genes that were detected in the present stage but were not detectable in at least one of the preceding stages prior to the present stage (genes that were "activated" in the embryos) (Figure 5-2 C). (2) "Maternal genes": genes that were positively detected in the present stage and all preceding stages examined in this study (Figure 5-2 D). The "maternal genes" detected in each embryonic stage include both the maternally stocked genes and the embryonic genes that were activated before the maternal mRNA stocks were completely degraded.

The number of "embryo-activated genes" and the number of total positively detected genes in each stage showed similar trends from 8C to D11 stages, whereas they displayed different trends from GV to 4C stages (Figure 5-2 B, C). The number of "maternal genes" displayed a gradual decrease from GV to D11 stages (Figure 5-2 D).

In addition, the "embryo-specific genes", which refers to genes that were detected in the embryonic stages examined in this study (4C to D11) but were not detected in the oocytes (GV and MII), were further identified from each embryonic stage (Figure 5-2 E). The number of the "embryonic specific genes" increases gradually from the 4C to MOR stages, and then increases dramatically from the MOR to EB stages followed by a significant decrease from the EB to HB stages, after that, the number of genes increased again from the HB to D11 stages.

Genes with “transcription regulator activity”, “ion channel activity”, and “RNA splicing factor activity” were identified from the lists of total “positively detected genes” (Figure 5-3 A-C) and the lists of “embryo-activated genes” (Figure 5-3 D-F) that were obtained from the GV to D11 stages using the PANTHER function classification tool. The results showed that the number of genes with “transcription regulator activity” displayed a similar trend as for the total number of positively detected genes from GV to D11 stage, while the number of genes with “ion channel activity” (Figure 5-3 B, E) and “RNA splicing factor activity” (Figure 5-3 C, F) showed different trends. The number of “embryo-activated genes” with “ion channel activity” stays the same from 4C to 8C, and then significantly increased from 8C to EB followed by a significant decrease from EB to HB.

5.3.2 Pre-miRNA profile from GV to D11stage

The EMPV1 array included annotated probes for 218 precursor-microRNAs (pre-miRNA), and the pre-miRNA positively detected in all stages examined were identified (Figure 5-4 A, B). The positively detected pre-miRNAs in each embryonic stages (4C to D11) examined were classified into two major categories (Figure 5-4 A). 1) “Embryo-activated pre-miRNAs”: pre-miRNAs that were detected in the present stage but were not detected in at least one of all the preceding stages prior to the present embryonic stage (pre-miRNAs that were transcribed and processed into pre-miRNA in the embryos). 2) “Maternal pre-miRNA”: pre-miRNAs that were detected in the present stage and all preceding stages examined in this study. “Maternal pre-miRNAs” detected in each stage include both the maternally stocked pre-miRNA and the pre-miRNAs that were transcribed and processed in embryos before their maternal stock were completely processed into mature miRNA (or degraded).

The total number of positively detected pre-miRNA from the GV to D11 stages (Figure 5-4 A) showed the same trend as the total positively detected genes (Figure 5-2 B). The number of "embryo-activated pre-miRNAs" and the number of all positively detected pre-miRNAs displayed a similar trend from the 8C to D11 stages (Figure 5-2 B, D). In addition, the "embryonic specific pre-miRNAs", which refers to the pre-miRNAs that were only detected in the embryonic stages examined in this study (4C to D11) but not detected in oocytes (GV and MII), showed the same trend as the total embryo-activated genes from GV to D11 stage (Figure 5-4 C).

5.3.3 Patterns of gene expression profile changes from 4-cell to hatched blastocyst

Comparative transcriptomic analysis was performed following a reference design, which allows for reliable comparison among the six groups in the analysis (Konig et al., 2004), as described in the methods. The number of significant differentially expressed genes between groups that were identified by the comparative transcriptomic analysis among the six developmental stages (4C, 8C, MOR, EB, XB, and HB) has been summarized in Table 5-2.

In the stage wise transcriptomic comparisons, 5492 genes showed differential expression in at least one comparison from 4C to HB. The genes differentially expressed from 4C to HB were classified based on the patterns of their gene expression changes from 4C to HB, and the majority of the differentially expressed genes (92% of all the differentially expressed genes) belong to 8 major expression patterns (Figure 5-5A-B). Genes with these 8 expression patterns were further analysed with the PANTHER over-representation test (using all the positively detected genes as a reference list). Specifically, the pattern 1 genes account for 29% of the total number of differentially expressed genes from 4C to HB. Pattern 1 genes showed significant up-regulation from 4C to 8C but decreased significantly to a lower level from 8C to MOR, and the expression level of the pattern 1

genes remained lower from MOR to HB stage. The pattern 2 genes showed a significant down-regulation from 4C to 8C, but expression of these genes increased from 8C to MOR, and then remained at that level for the HB stage. The pattern 3, pattern 6, and pattern 7 genes displayed different levels of down-regulation from 4C to MOR stage, and the expression of these genes remains the same after the down-regulation. On the other hand, the pattern 4, pattern 5, and pattern 8 genes all showed different levels of up-regulation from 4C to MOR, and the expression levels of these genes stays in the same range after up-regulation.

Further PANTHER GO over-representation analysis was performed for genes belonging to the 8 gene expression patterns using all the positively detected genes from 4C to HB as a reference list. The PANTHER GO molecular function over-representation analysis (Figure 5-6A) showed that genes with molecular functions associated with "nuclear acid binding", "RNA binding", and "translation regulator activity" were significantly over-represented in genes with expression pattern 1. The genes with molecular functions associated with "translation regulator", "translation factor", and "translation initiation factor" activities were significantly over-represented in genes with expression pattern 4. In addition, genes with molecular functions associated with "structural constituent of ribosome" were significantly over-represented in genes with expression patterns 4, 5 and 8.

The PANTHER GO biological process overrepresentation analysis (Figure 5-6B) showed that genes associated with "mRNA processing" and "RNA metabolic" was enriched in pattern 1 genes. Genes associated with primary metabolic process were enriched in pattern 1 and pattern 4 genes. Genes associated with translation, metabolic and protein metabolic processes were enriched in pattern 4 genes. While genes exhibiting expression patterns 4, 5 and 8 were enriched for "translation" and "oxidative phosphorylation"

processes and genes associated with “generation of precursor metabolites and energy” processes were enriched in both pattern 5 and pattern 8 genes. In addition, genes associated with “protein complex assembly” and “respiratory electron transport chain” were enriched in pattern 8 genes.

5.3.4 Significantly altered gene networks and pathways from 4C to HB stage

IPA upstream regulator analysis revealed 24 upstream regulators (Table 5-3) that were predicted to be significantly activated or inhibited from the 4C to HB stages. Transcription factors MYCN and MYC are predicted to be the most significantly activated factors across these stages. In comparison with 4C, the transcription factor *MYCN*, *MYC*, *NRF1*, *HSF2*, and *PPARGC1A* were predicted to be activated in all the stages from 8C to HB. Additionally, when compared to 4C stage, activation of ESSRA was not significant in 8C stage, but it was predicted to be significantly activated in MOR, EB, XB, and HB (Figure 5-7).

IPA canonical pathway analysis revealed 49 pathways that were significantly changed from the 4C to HB stages. “EIF2 signalling”, “Regulation of eIF4 and p70S6K signalling”, “Mitochondrial Dysfunction”, “mTOR signalling” pathways are the most significantly altered across these stages. All of these pathways were significantly altered in all stages from 8C to HB and were most dramatically changed at the MOR stage, in comparison with 4C (Figure 5-8). ”. The “Protein Ubiquitination Pathway”, and “NRF2-mediated Oxidative Stress Response” pathways were also significantly altered in all stages from 8C to HB, in comparison with 4C stage. In addition, the “Estrogen Receptor signalling” pathway was significantly altered from 8C to XB, in comparison to the 4C stage. In comparison to the 4C stage, both the “Protein Ubiquitination Pathway” and the “Estrogen Receptor signalling” pathways were most dramatically changed at the 8C stage.

5.3.5 QRT-PCR verification of gene expression

To confirm the gene expression data obtained from the comparative microarray analysis, QRT-PCR verification of five transcription regulators (*EIF4E2, MYC, MYCN, NRF1*, and *PPARG*), several pluripotency, embryonic development, and trophectoderm development-associated genes (*GATA2*, *KRT8*, and *KRT18*), and epigenetic regulators (*DNMT1*, *HDAC1*) with oocytes (or embryos) samples from GV, 4C, 8C, MOR, EB, XB, HB, and D11 stages (Figure 5-9). The mRNA level of *EIF4E2* decreased from GV to 4C stage followed by a dramatic increase from 4C to MOR, and the mRNA abundance of *EIF4E2* decreased from MOR to HB stage and increased again from HB to D11 stage. The expression of *MYCN* was not detectable until MOR stage, and the abundance of *MYCN* mRNA decreased from MOR to EB stage and remained at the lower level from EB to D11 stage. The mRNA level of *MYC* gene was not detectable in all the samples examined in this study. The mRNA level of *NRF1* gene was increased from GV to 4C to 8C stage followed by a gradual down-regulation from 8C to EB stage, and the mRNA abundance of *NRF1* remained at the lower level from MOR to D11 stage. The mRNA level of *GATA2* was not detectable until MOR stage, and displayed constant up-regulation from MOR to D11 stage. The *PPARG* gene displayed constant down-regulation from GV to EB stage, and the mRNA level of *PPARG* was not detectable in XB and HB stage. A significant up-regulation of the PPARG mRNA expression was observed from HB to D11 stage. The expression level of *KRT18* was not detectable from GV to 8C stage, and *KRT8* gene was not detectable in GV stage oocytes. The mRNA levels of both *KRT8* and *KRT18* genes displayed gradual up-regulation until EB stage, and the mRNA level of these two genes remained at the higher levels from EB to HB followed by a dramatic increase from HB to D11 stage. The *DNMT1* gene displayed dramatic and constant down-regulation from 4C to EB stage, and was remained at the low

expression level from EB to D11 stage. *HDAC1* displayed up-regulation from GV to 4C stage followed by a down-regulation from 4C to 8C stage, the mRNA abundance of *HDAC1* significantly increased from 8C to MOR stage and remained at the higher level from MOR to D11 stage.

In general, the QRT-PCR analysis results were consistent with the microarray data (Figure 5-9).

5.4 Discussion

5.4.1 Global mRNA profiling during early porcine embryonic development

The major embryonic genome activation (EGA) in the pig embryo is considered to start at the 4C stage (Telford et al., 1990; Prather et al., 2009; Sirard, 2012). On the other hand, distinct differences in mRNA abundance between the oocyte and 4C and blastocyst have been reported in pig (Whitworth et al., 2005). In the present study, we have successfully characterized the global mRNA profiles of porcine oocytes (GV and MII stages) and pre-implantation stage embryos from 4C to D11 stages.

The number of embryo-activated genes showed a similar trend as the number of total positively detected genes from 8C to D11 stage, while they showed different trend from the oocyte to 4C stage embryos. This result indicates that the "embryonic-activated genes" probably "take-over" the majority of the mRNA profile from the 8C stage in pig embryos. In addition, the dramatic increase in the number of "embryonic-activated genes" from MII to 4C indicates that the molecular mechanism of EGA in pig embryos probably is initiated at the early 4C stage or even before the early 4C stage. However, without data from 2-cell (2C) stage embryos in this study, it is difficult to determine the exact timing of the initiation of the molecular events associated with EGA in swine. The decrease in the number of positively detected "embryonic-activated genes" from EB to

HB stage indicates that the expression of these genes might be necessary for the blastocyst formation process, and only very low expression levels (or no expression) of these genes were needed after the HB stage. In addition, the size of the porcine embryo increased significantly from the HB to D11 stage, hence, the "embryonic-activated genes" that activated from HB to D11 stage probably were associated with the fast developmental process during this period.

A gradual decrease in the number of "maternal genes" from MII to D11 was observed in the present study. However, since the genes classified as "maternal genes" in this study contain both maternally stocked RNA as well as the genes activated in embryos before the complete degradation of their maternal stocks, there is no guarantee that the number of maternal stocked genes showed the same decreasing trend.

Results from the present study showed that the number of total positively detected genes and the "embryo-activated genes" with different molecular functions exhibited different trends in gene expression from GV to D11 stage.

Transcription factors regulate the transcription of their target genes, and it is not unexpected that the number of "embryo-activated genes" with "transcription factor" activity displayed a similar trend as the total number of positively detected genes and "embryo activated genes" from 8C to D11 stage.

The ion transport system plays important roles during blastocyst formation and blastocoel cavitation in mammalian embryos (Watson et al., 2004). In the trophectoderm, the Na^+-K^+ ATPase pump is involved in the formation and maintenance of the blastocoel cavity (Watson et al., 2004; Krisher and Prather, 2012). In the present study, the number of "embryo-activated genes" with "ion channel activity" dramatically increased during blastocyst formation, and then decreased during blastocyst hatching. This result supports

the proposed role of these genes in the formation and maintenance of the blastocoel cavity.

5.4.2 Global pre-miRNA profile of porcine oocyte and pre-implantation embryo

MicroRNAs (miRNA) are involved with cell fate and are required for cell lineage destinations during embryogenesis (Prather et al., 2009), and miRNAs are temporally associated with zygotic and early embryonic development (Tang et al., 2007; Prather et al., 2009).

In animals, miRNA genes are initially transcribed as primary miRNAs (pri-miRNA), and then processed into precursor miRNAs (pre-miRNA) within the nucleus, which are further processed to become mature miRNAs in the cytoplasm (Wahid et al., 2010). The EMPV1 platform contains annotated probes for 222 pre-miRNAs, and aproximately 37% of these pre-miRNAs were positively detected in GV stage oocytes.

Global gene expression profiling analysis revealed that the number of positively detected "maternal pre-miRNAs" decreased from the GV to MII to 8C stages and remained at similar levels from 8C to D11 stages. However, this group of "maternal pre-miRNAs" detected in all stages from 4C to D11 could contain both the maternally stocked pre-miRNAs and the pre-miRNAs that were transcribed in embryos before the complete processing (or degradation) of their maternal pre-miRNA stock. Hence, further study is necessary to determine the trend of maturation and degradation of maternal stocked pre-miRNA and miRNA during embryonic development.

To date, in humans, there are 2578 mature miRNA and 1872 pre-miRNA recorded in the miRBase, which is a searchable database of published miRNA sequences and annotation (http://www.mirbase.org/). On the other hand, there are only 326 mature miRNA and 280 pre-miRNA records for the pig in miRBase, which is likely an incomplete list of all the porcine miRNA and pre-miRNA. In the present study, using the EMPV1 platform, we

were only able to examine expression of 222 pre-miRNAs during early embryonic development in pig. Therefore, the complete profile of miRNA and pre-miRNA, and their functions during the pig embryonic development remains to be elucidated.

5.4.3 Detailed gene expression profile changes from 4-cell to hatched blastocyst

To characterize the key gene networks and pathways involved in pre-implantation embryonic development, comparative transcriptomic analysis was performed among embryos from six (4C, 8C, MOR, EB, XB, and HB) different embryonic stages. Among the eight major patterns of differential expression patterns across the 6 embryonic stages examined, expression pattern 1 has the greatest number of genes represented. The expression pattern 1 genes only displayed significant up-regulation from 4C to 8C stages and were then down-regulated and remained at the lower expression levels from MOR to HB, similar to their expression levels observed at the 4C stage. This would suggest that these genes are necessary for the embryonic development at 8C stage. PANTHER over-representation analysis showed that the pattern 1 expression profile was enriched with genes associated with nucleic acid (specifically RNA) binding, metabolism and splicing. Our results from the global mRNA profiling analysis suggest that the embryonic-activated genes probably "take-over" the majority of the mRNA profile from the 8C stage. Hence, the expression pattern1 genes that are associated with nucleic acid (specifically RNA) binding, metabolism and splicing probably are involved in the degradation of maternal mRNA stock and the splicing of the embryonic mRNA produced from the EGA.

Genes with expression patterns 4, 5, and 8 were all enriched with genes associated with the "structural constituent of ribosome", "translation", and "oxidative phosphorylation". Porcine embryos experience rapid growth from 4C to HB stage, and many important developmental events, including morula compaction, blastocyst formation, and blastocyst

hatching, take place during this period. ATP production via oxidative phosphorylation is essential for embryonic development during morula compaction and blastocyst formation (Thompson et al., 2000). Oxygen consumption related to mitochondrial oxidative phosphorylation displayed a dramatic increase from cleavage stage to blastocysts stage, and this change in oxygen consumption is believed to be indicative of developmental competence (Trimarchi et al., 2000). Hence, it is not unexpected that the genes associated with translation and oxidative phosphorylation showed increased expression from the 4C to MOR stages and remained at this higher expression level until HB.

The pattern 4 genes displayed a significant increase in mRNA abundance from the 4C to 8C stages, and the mRNA levels for these genes remained high from the 8C to HB stages. Among the 8 major expression patterns observed in the present study, only genes with expression pattern 4 were enriched with translation initiation factors and translation factors. This would suggest that major transcription of mRNAs for translation regulators occurs during the 4C to 8C period in porcine embryos. Further investigation with embryos prior to 4C stage is needed to determine the exact timing and detailed trend of the transcriptional activation of these translation regulators.

Genes with expression pattern 2 were down-regulated from 4C to 8C, and then were up regulated from the 4C to MOR stages. However, without the expression data from 2-cell stage embryos, it is not guaranteed that genes with expression pattern 2 are maternal stocked mRNAs that were degraded from 4C to 8C and then activated in embryos at the 8C stage.

Genes with expression patterns 3, 6, and 7 were down-regulated or degraded as the embryo develops from the 4C to MOR stage, and further study is necessary to determine if these genes are maternal mRNA stocks that were degraded as the embryos develop.

5.4.4 Significantly altered gene networks and pathways from 4C to HB stage

Transcription factors MYC and MYCN play important roles in maintenance of pluripotency, self-renewal, and cell cycle in embryonic stem (ES) cells (Rahl et al., 2010; Varlakhanova et al., 2010; Chappell et al., 2013). In mice, disruption of MYC and MYCN causes embryonic lethality by D10.5 (Davis et al., 1993) and D11.5 (Stanton et al., 1992), respectively. In addition, MYC plays a key role in regulating transcriptional elongation by RNA polymerase II (Pol II) in ES cells (Rahl et al., 2010). In the present study, MYC and MYCN were predicted to be the most significantly activated transcription regulator in all embryonic stages from 8C to HB, compared to the 4C stage. This result suggests that the MYC and MYCN may also play important roles during early porcine embryonic development.

Heat shock transcription factor 2 (HSF2) belongs to the heat shock transcription factors family, which are major transactivators of heat shock protein genes in response to stress (Wang et al., 2003). HSF2 is involved in embryonic development, brain development, and gametogenesis (Kallio et al., 2002; Wang et al., 2003). HSF2 has been previously reported to be activated at the 8C stage in mouse embryos (Mezger et al., 1994). In the present study, HSF2 was also predicted to be activated at 8C stage in the pig and remain activated during the period from 8C to HB stages, which is consistent with previous finding in mice.

NRF2 has an important role in the regulation of antioxidant gene expression during early embryonic development (Leung et al., 2003). The degradation and *de novo* synthesis mechanism of NRF2 is part of the cellular protection system that protects the cells against oxidative and electrophilic stresses, prevents apoptosis, and promotes cell survival (Kobayashi et al., 2006; Kaspar et al., 2009). NRF1 has overlapping functions with NRF2, mostly in the regulation of antioxidant genes, during early embryonic development (Leung et al., 2003; Motohashi and Yamamoto, 2004). In the present study, the "NRF2-

mediated oxidative stress response" pathway was significantly altered from the 8C to HB stages, in comparison with 4C stage. In addition, NRF1 is predicted to be significantly activated from the 8C to HB period. These results suggest that the NRF1 and NRF2 mediated cellular protection system against oxidative stress is activated from 8C stage in pig embryos.

Eukaryotic translation initiation factor 2 (eIF2) plays a key role in recognition of the correct start codon during translation initiation process (Schmitt et al., 2010). Phosphorylation of eIF2 reduces global translation and activates the transcription of "stress recovery" genes in response to environmental stresses such as amino acid deficiency and heavy metal toxicity (Schmitt et al., 2010; Shrestha et al., 2012). The "mTOR signalling pathway" plays a critical role in the regulation of cell growth, proliferation, translation, protein synthesis, and survival (Ruvinsky and Meyuhas, 2006; Laplante and Sabatini, 2009; Dowling et al., 2010).The eIF4 initiation factors are responsible for recruiting mRNA to ribosomes during translation (Gingras et al., 1999). The translation eIF4 initiation factors and p70 S6 kinase (p70S6k) are both regulation targets of mTOR, which play critical roles in translation and protein synthesis regulation (Gingras et al., 1999; Ruvinsky and Meyuhas, 2006). Many stimuli including growth factors, hormones, oxygen level, and nutrient availability can regulate the eIF4 and p70S6K through "mTOR signalling pathway" (Laplante and Sabatini, 2009). In comparison with 4C stage, significant expression changes in genes associated with the "EIF2 signalling", "mTOR signalling", and "Regulation of eIF4 and p70S6K signalling" pathways were observed from 8C to HB stages in the present study. Regulation of genes associated with these pathways from the 8C stage indicate that they probably are involved with the critical developmental events, such as morula compaction and blastocyst formation, during early embryonic development in pig embryos.

Ubiquitination of cellular protein is essential for the ubiquitin-proteasome pathway-dependent cellular protein degradation (Dudek et al., 2010). Degradation of maternal proteins through the "ubiquitin–proteasome pathway" is believed to be important for the oocyte-to-embryo transition (DeRenzo and Seydoux, 2004). In comparison with the 4C stage, the number of differentially expressed "protein ubiquitination pathway"-associated genes peaks at the 8C stage, and then decreased from the MOR to HB stages. Further investigation is needed to determine if the degradation of maternal proteins followed the same trend of decrease from 8C to HB.

Estrogens play an important role in differentiation, development and maintenance of female reproductive organs, and are also risk factors for breast and endometrial cancer (Couse and Korach, 1999; Matsuda et al., 2001). Estrogen receptors (ER) are ligand-activated transcriptional factors, which interact with estrogen response elements in the target gene promoters, directly regulate their transcription, and have important roles in the regulation of estrogen action (Mangelsdorf et al., 1995; Mosselman et al., 1996; Paech et al., 1997; Matsuda et al., 2001). In comparison with 4C stage, the number of differentially expressed "Estrogen Receptor signalling" pathway-associated genes decreased from the 8C to HB stages. This result suggests that these genes were only differentially expressed in 8C stage and are likely to be necessary for the embryonic development during this specific period.

5.5 Conclusion

We have successfully characterized the mRNA and miRNA profile changes during early porcine embryonic development. Results from the present study suggest that the number of embryo-activated genes "take-over" the majority of the mRNA profile from 8C stage, and that the molecular events associated with EGA in porcine embryos are probably

initiated at or before the 4C stage. In addition, genes with different molecular activities may display distinct trends of gene expression during the early embryonic development. Related to this, we further identified eight major patterns of gene expression changes and several significantly altered gene networks from the 4C to HB stages. The *MYC* and *MYCN* were predicted to be the most significant transcriptional regulators that were activated at the 8C stage, and probably play central roles during porcine embryonic development from 8C to HB stage. The findings of the present study have increased our understanding of the molecular mechanisms involved in early porcine embryonic development.

Table 5-1 Primers used in the QRT-PCR analysis

Gene Symbol	Associated Porcine Sequence Accession No.	Primer	Primer Sequence (5'-3')	Product size (bp)
DNMT1	NM_001032355	Forward	AAG AAC GCA TCC AGT ATC GAG	154
		Reverse	GTA GTC AGA GTA TTT CCG GTA GTG	
GATA2	NM_213879	Forward	CTC CAG CTT CAC CCC TAA G	157
		Reverse	CCC GTT CAT CTT GTG GTA CAG	
KRT18	XM_003126180	Forward	TTGACCGTGGAGTTGGATG	149
		Reverse	ACCACTGAGGTGCTCTCC	
KRT8	NM_001159615	Forward	AGA TCC AAA AGC GTA CCG AC	136
		Reverse	AGC TGC CTG TAG AAG TTG ATC	
EIF4E2	NM_001100191	Forward	TCACAGAGCTACGAACAGAATATC	125
		Reverse	GAAGAGATGGAAGTCACTGTGG	
HSF2	XM_003121229	Forward	CTTTGGAAGGAGGTGTCAGAA	150
		Reverse	TCCATTAGTGTTTAGAAGAAGAGGC	
MYC	NM_001005154	Forward	CCTTCGGATAGTGGAAAACCC	104
		Reverse	AGTAGAAATAAGGCTGCACCG	
MYCN	XM_003125346	Forward	GACTCAGATGACGAAGATGACG	119
		Reverse	ACAGTGATGGTGAAGGTGG	
NRF1	XM_003134690	Forward	GTCGGAGCACTTACTGGAG	147
		Reverse	GCTGTCCGATATCCTGGTG	

Xeno™	Control primer for Xeno™ from SYBR® Green Cells-to-CT™ Control Kit (Ambion)	105

Table 5-2 Differentially expressed genes among the 4C, 8C, MOR, EB, XB, and HB embryos

	8C vs. 4C	MOR vs. 4C	EB vs. 4C	XB vs. 4C	HB vs. 4C	MOR vs. 8C	EB vs. MOR	XB vs. B	HB vs. XB
Up regulated genes	2413	1263	1270	1041	1017	1590	107	130	94
Down regulated genes	2005	1332	1521	1322	1440	2268	86	20	141
Total differentially expressed genes	4418	2595	2791	2363	2457	3858	193	150	235

Table 5-3 Significantly activated or inhibited upstream regulators from 8C to HB embryos

Upstream Regulator	Molecule Type	Analysis	IPA Predicted Activation State	IPA Activation Z-score	IPA P-value of overlap
MYCN	transcription regulator	8C vs. 4C	Activated	4.496	9.63E-20
MYC	transcription regulator	8C vs. 4C	Activated	4.116	2.22E-13
TP53	transcription regulator	8C vs. 4C	Activated	2.916	3.48E-11
NRF1	transcription regulator	8C vs. 4C	Activated	2.547	9.25E-05
TBX2	transcription regulator	8C vs. 4C	Inhibited	-2.33	7.45E-04
ATF4	transcription regulator	8C vs. 4C	Activated	2.156	8.74E-04
KDM5B	transcription regulator	8C vs. 4C	Activated	2.104	1.49E-03
MAX	transcription regulator	8C vs. 4C	Inhibited	-2.619	1.50E-03
NOBOX	transcription regulator	8C vs. 4C	Inhibited	-2.433	3.80E-03
FOS	transcription regulator	8C vs. 4C	Activated	2.108	6.91E-03
NFE2L2	transcription regulator	8C vs. 4C	Activated	3.036	9.30E-03
FLI1	transcription regulator	8C vs. 4C	Activated	2.305	1.38E-02
MYCN	transcription regulator	EB vs. 4C	Activated	5.369	1.30E-28
MYC	transcription regulator	EB vs. 4C	Activated	4.63	1.28E-23
ESRRA	ligand-dependent nuclear receptor	EB vs. 4C	Activated	2.718	1.30E-06
HOXA9	transcription regulator	EB vs. 4C	Inhibited	-3	3.86E-05
NOBOX	transcription regulator	EB vs. 4C	Inhibited	-2.414	3.29E-04
NRF1	transcription regulator	EB vs. 4C	Activated	3.136	4.86E-04
PGR	ligand-dependent nuclear receptor	EB vs. 4C	Inhibited	-2.526	6.89E-04
PPARGC1A	transcription regulator	EB vs. 4C	Activated	2.21	1.47E-03
MITF	transcription regulator	EB vs. 4C	Inhibited	-2.867	4.02E-03
NR1I2	ligand-dependent nuclear receptor	EB vs. 4C	Inhibited	-3.06	3.53E-02
MYCN	transcription regulator	HB vs. 4C	Activated	5.667	1.67E-24
MYC	transcription regulator	HB vs. 4C	Activated	3.962	6.01E-21
HOXA9	transcription regulator	HB vs. 4C	Inhibited	-2.646	6.00E-05
ESRRA	ligand-dependent nuclear receptor	HB vs. 4C	Activated	3.066	1.21E-04

NRF1	transcription regulator	HB vs. 4C	Activated	2.977	1.22E-04
NOBOX	transcription regulator	HB vs. 4C	Inhibited	-2.414	1.63E-04
CDKN2A	transcription regulator	HB vs. 4C	Activated	2.343	1.86E-04
PPARGC1A	transcription regulator	HB vs. 4C	Activated	2.752	3.24E-03
NUPR1	transcription regulator	HB vs. 4C	Activated	2.029	9.88E-03
MITF	transcription regulator	HB vs. 4C	Inhibited	-2.581	1.50E-02
PPARGC1B	transcription regulator	HB vs. 4C	Activated	2.621	1.84E-02
MXI1	transcription regulator	HB vs. 4C	Inhibited	-2.2	2.46E-02
HSF2	transcription regulator	HB vs. 4C	Activated	2.449	2.73E-02
RARG	ligand-dependent nuclear receptor	HB vs. 4C	Inhibited	-2.49	2.89E-02
MYCN	transcription regulator	MOR vs. 4C	Activated	6.188	8.35E-30
MYC	transcription regulator	MOR vs. 4C	Activated	4.677	5.49E-20
HOXA9	transcription regulator	MOR vs. 4C	Inhibited	-2.646	1.01E-06
ESRRA	ligand-dependent nuclear receptor	MOR vs. 4C	Activated	3.151	2.46E-05
NRF1	transcription regulator	MOR vs. 4C	Activated	3.136	2.15E-04
NOBOX	transcription regulator	MOR vs. 4C	Inhibited	-2.414	2.16E-04
PPARGC1A	transcription regulator	MOR vs. 4C	Activated	3.682	1.76E-03
PPARGC1B	transcription regulator	MOR vs. 4C	Activated	2.373	9.45E-03
MITF	transcription regulator	MOR vs. 4C	Inhibited	-2.687	1.56E-02
HSF2	transcription regulator	MOR vs. 4C	Activated	2.449	3.48E-02
RARG	ligand-dependent nuclear receptor	MOR vs. 4C	Inhibited	-2.49	3.76E-02
MYCN	transcription regulator	XB vs. 4C	Activated	6.212	2.15E-24
MYC	transcription regulator	XB vs. 4C	Activated	3.976	5.13E-21
ESRRA	ligand-dependent nuclear receptor	XB vs. 4C	Activated	3.189	3.23E-06
NOBOX	transcription regulator	XB vs. 4C	Inhibited	-2.414	1.29E-04
NRF1	transcription regulator	XB vs. 4C	Activated	2.991	3.01E-04
PPARGC1B	transcription regulator	XB vs. 4C	Activated	2.562	3.99E-04
PPARGC1A	transcription regulator	XB vs. 4C	Activated	2.771	8.46E-04

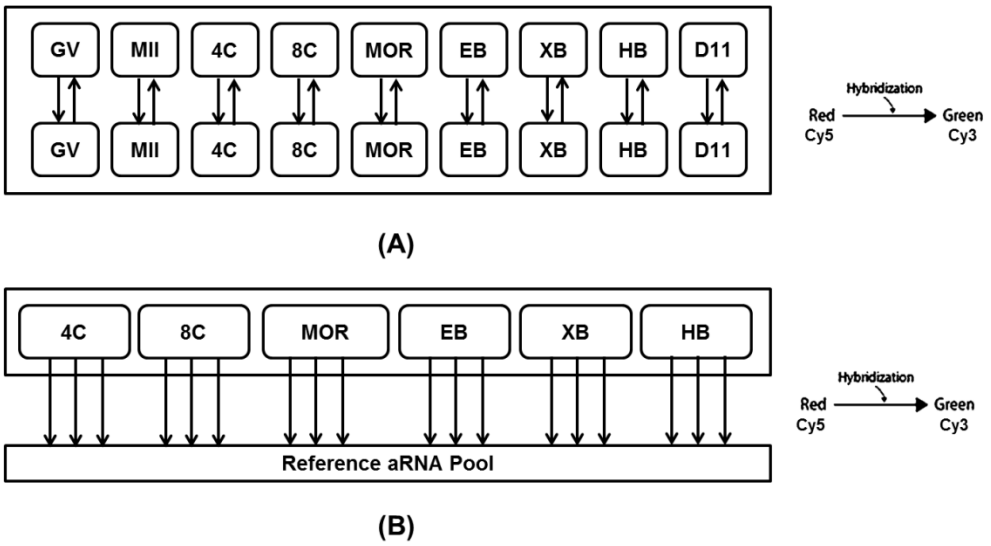

Figure 5-1 Microarray experimental design. (A) Microarray experimental design of the global transcriptomic profiling analysis of 9 different developmental stages from GV to D11 stages. (B) Microarray experimental design of the comparative transcriptomic analysis of 5 embryonic stages from 4C to D11 stages.

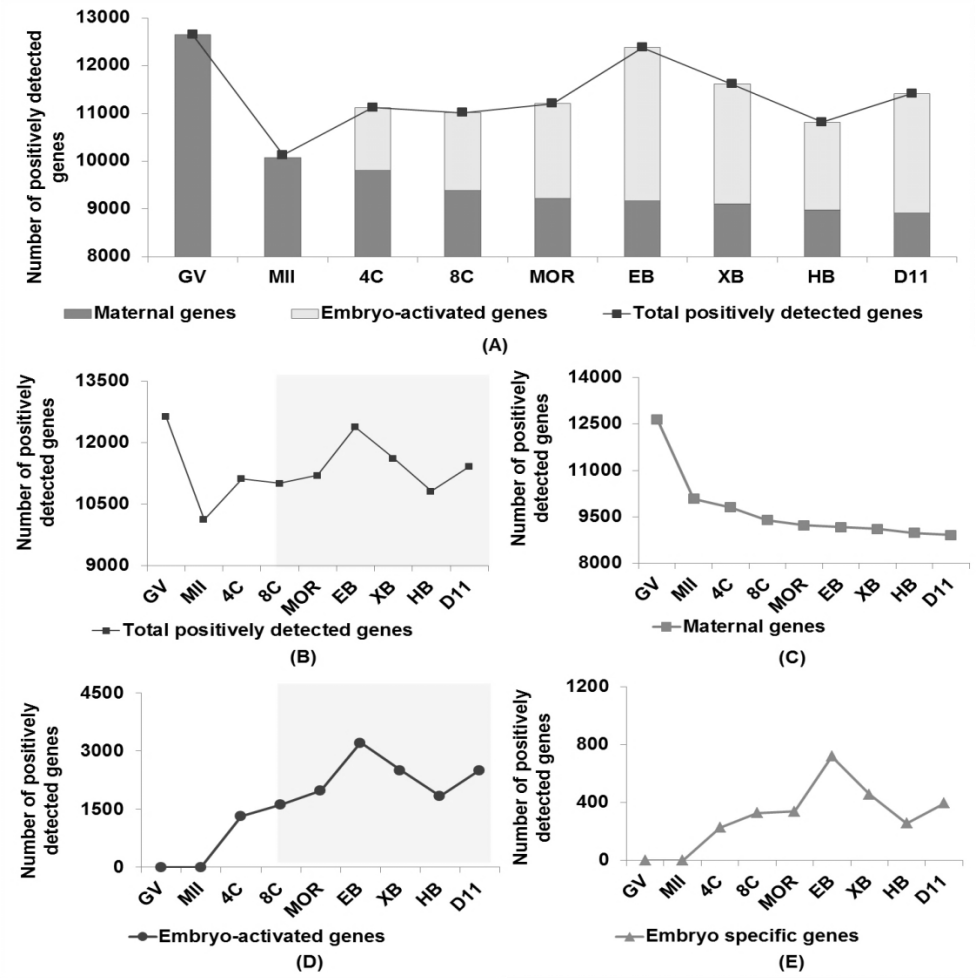

Figure 5-2 mRNA profiles from the GV to D11 stages.

(A)Stacked bar chart shows the number of positively detected “embryo-activated genes” and “maternal genes” from the GV to D11 stages. The line chart on top of the bar chart shows the total number of positively detected genes from the GV to D11stages. (B) Re-plot of the total number of positively detected genes from GV to D11stages in line chart. (C) Re-plot of the total number of “maternal genes” detected from GV to D11 stages in line chart. (D) Re-plot of the number of “embryo-activated genes” detected from GV to D11 stages in line chart. (E) Re-plot of the total number of “embryo-specific genes” detected from GV to D11 stages in line chart.

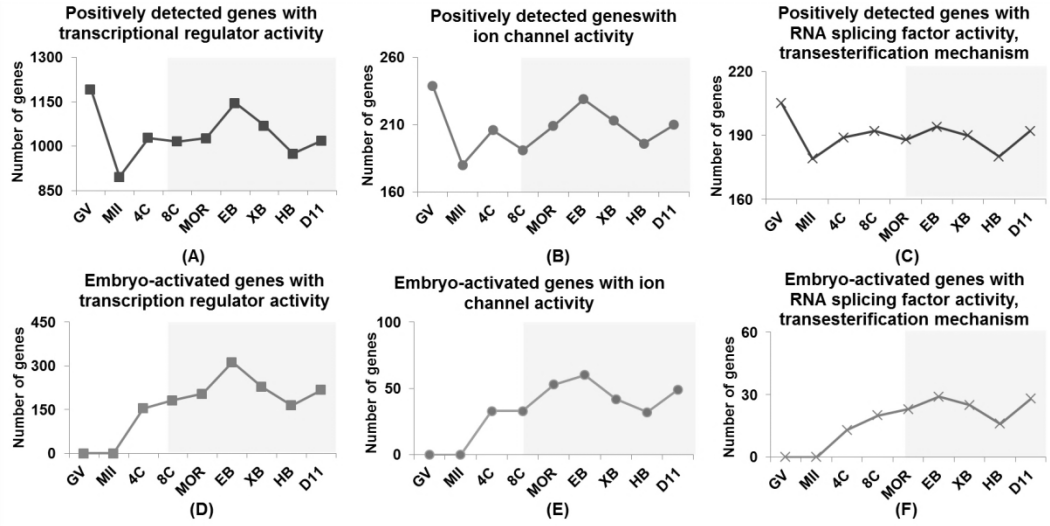

Figure 5-3 mRNA profiles of genes with different molecular functions from GV to D11.

Line charts A-C shows the total number of positively detected genes with “transcriptional regulator” (A), “ion channel” (B), and “RNA splicing factor” (C) activities from the GV to D11 stages. Line charts D-F shows the number of embryo-activated genes with “transcriptional regulator” (D), “ion channel” (E), and “RNA splicing factor” (F) activities from the GV to D11 stages.

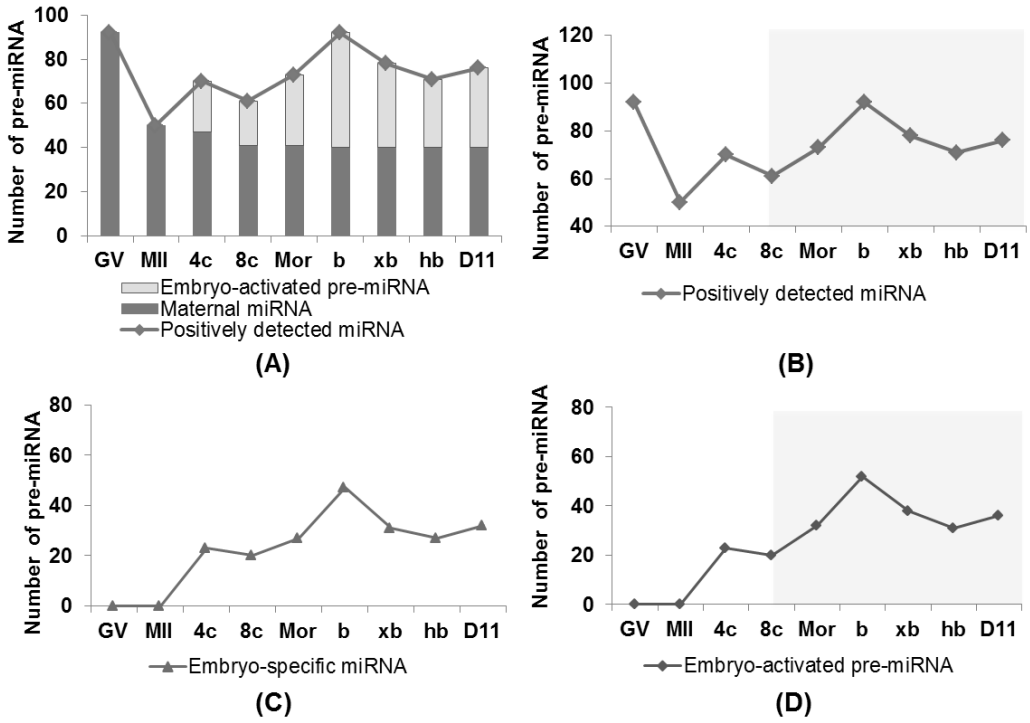

Figure 5-4 Pre-miRNA profile from GV to D11.

(A) Stacked bar chart shows the number of positively detected "embryo-activated pre-miRNA" and "maternal pre-miRNA" from GV to D11. The line chart on top of the bar chart shows the total number of positively detected pre-miRNA from the GV to D11stages. (B) Re-plot of the number of "embryo-activated pre-miRNA" from the GV to D11stages in line chart. (C) Line chart shows the number of "embryo-specific pre-miRNA" the GV to D11stages. (D) Re-plot of "embryo-activated maternal pre-miRNA" number in line chart.

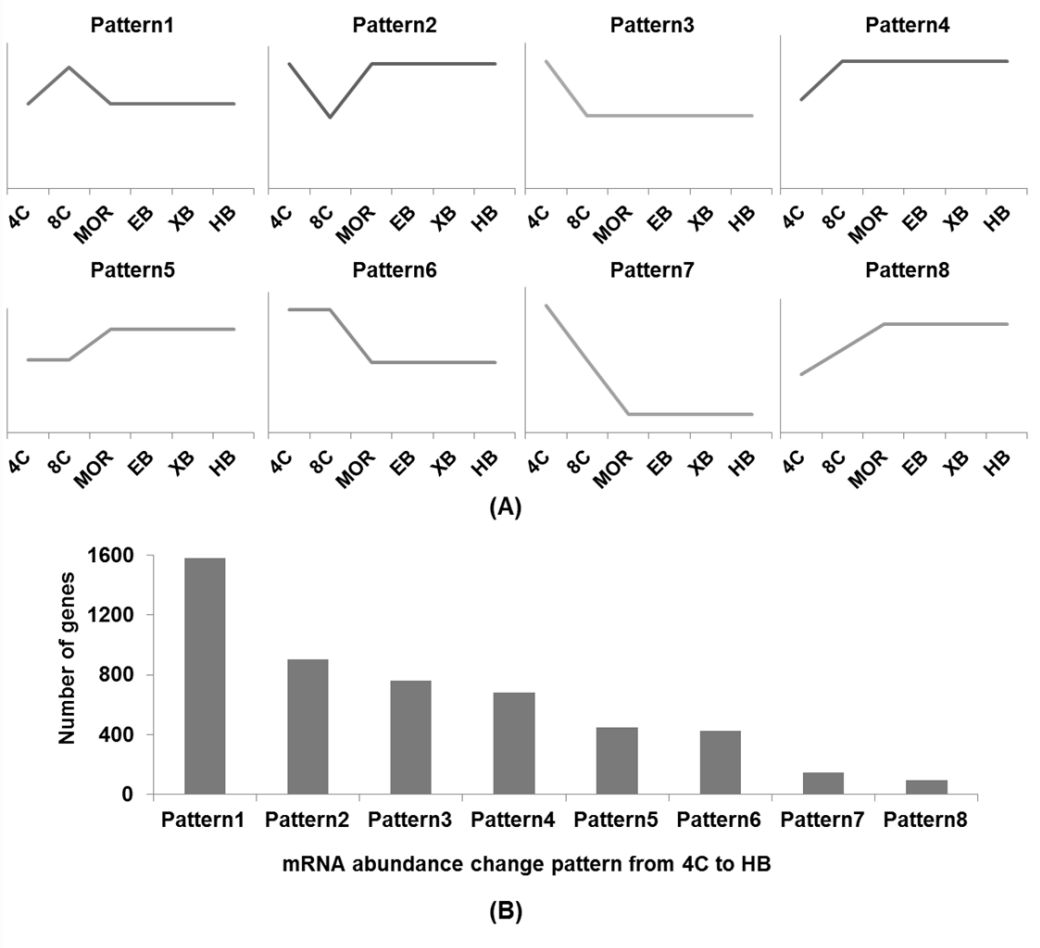

Figure 5-5 (A) The eight major patterns of gene expression changes from 4C to HB stage. (B) Number of genes belongs to these eight expression patterns.

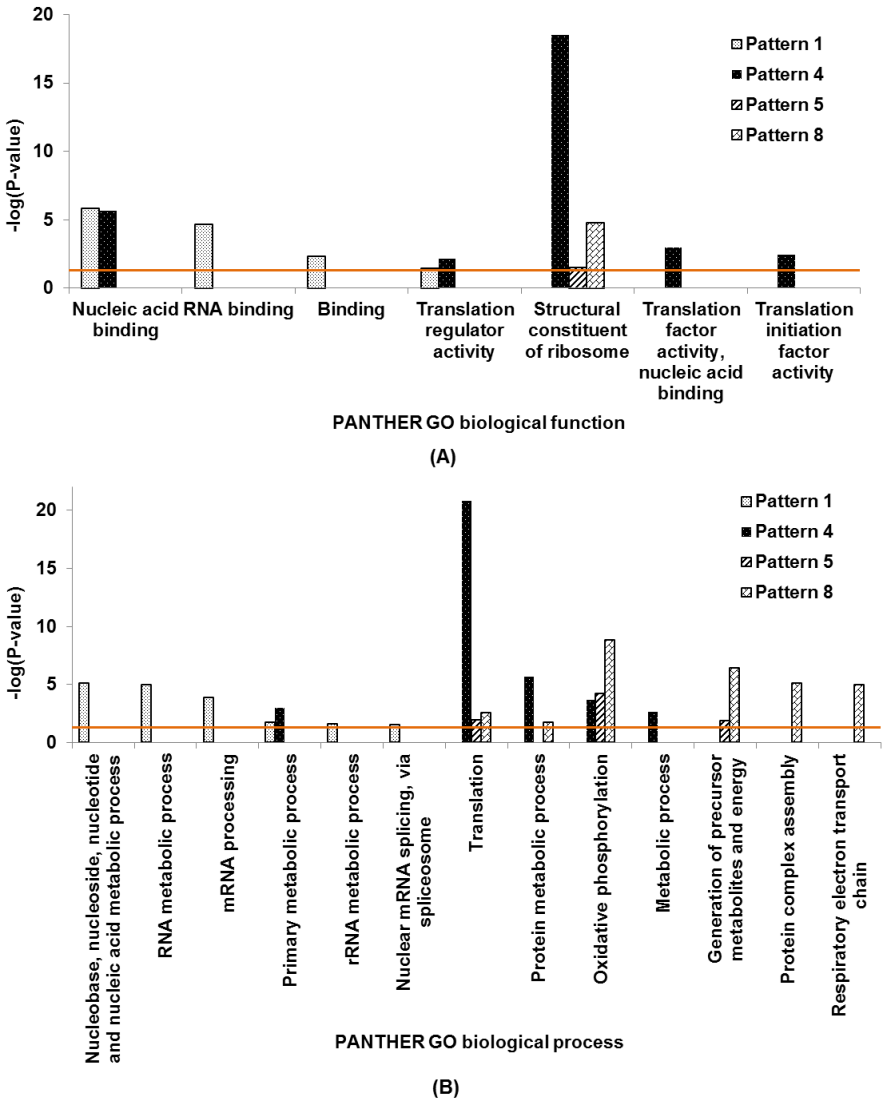

Figure 5-6 PANTHER GO molecular function (A) and biological process (B) overrepresentation analysis of genes displayed different expression patterns from 4C to HB.

Y-axis (left) displays the -log of Bonferroni multiple testing corrected P-value (Bon P-value) of the biological function. Orange line shows the significance threshold of -log (Bon P-value=0.05).

(No significant molecular functions and biological process was identified in genes with expression patterns 2, 3, 6, and 7)

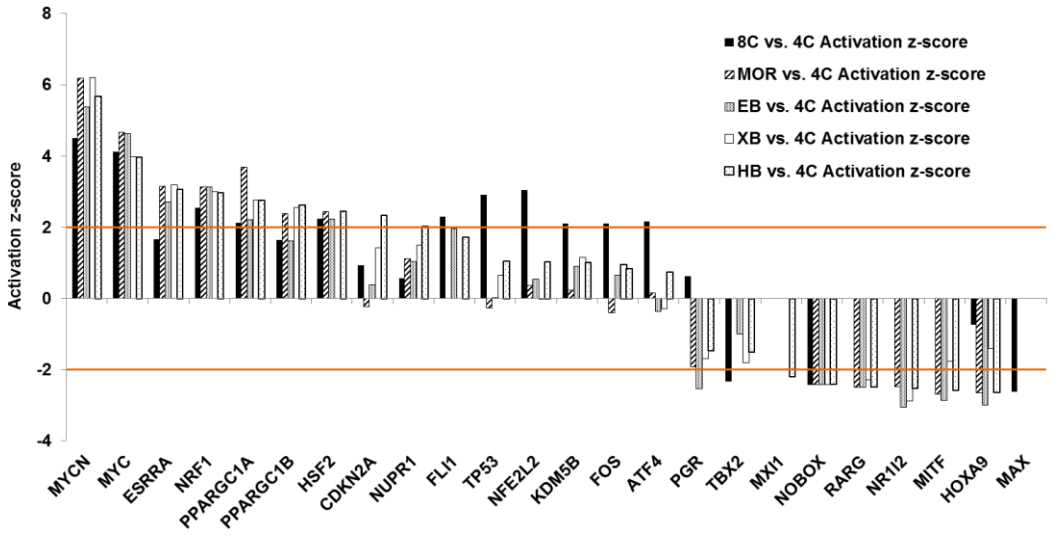

Figure 5-7 Significantly activated or inhibited upstream regulators from 8C to HB embryos.

Y-axis displays the IPA activation Z-score, which was calculated based on the number of known regulation target genes from the dataset of interest, expression changes of these target genes, and their agreement with literature findings in the IPA knowledge base. Orange line shows the significance threshold of Z-score > 2 (or < -2).

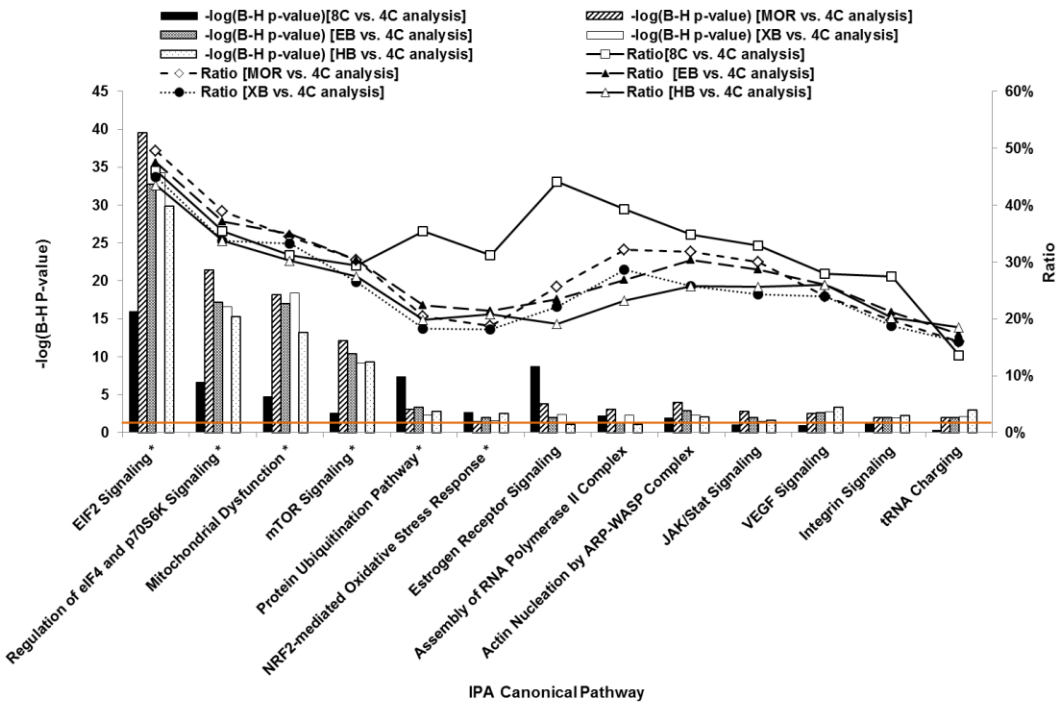

Figure 5-8 Significantly changed IPA canonical pathways from 4C to HB

Bar chart shows the significantly altered canonical pathways in 8C, MOR, EB, XB, and HB stages in comparison with 4C stage. Primary Y-axis (left) [-log (B-H P-value)]: displays the -log of B-H P-value of the canonical pathway. Orange line shows the significance threshold of –log (P-value=0.05).Secondary Y-axis (right) [ratio]: ratio = 100 % * (number of differential expressed genes in this pathway) / (number of all genes in this pathway in IPA knowledge base).

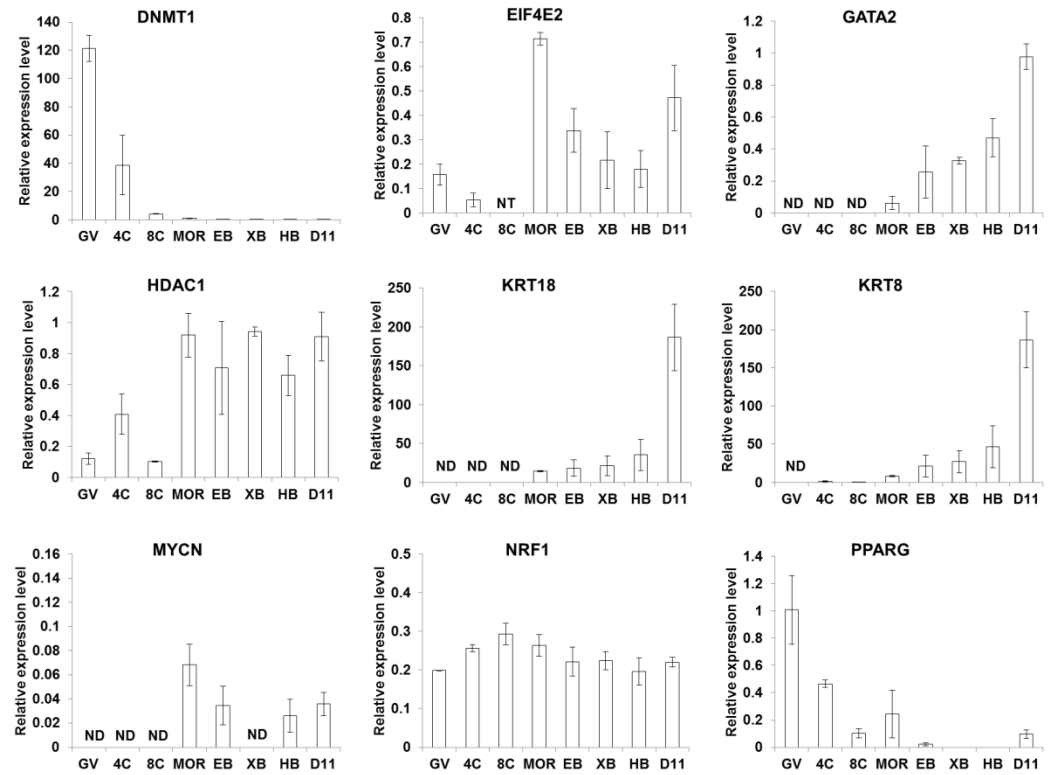

Figure 5-9 QRT-PCR verification of gene expression

QRT-PCR verification results of nine selected genes. The mRNA expression levels of these genes were normalized with the external control gene (Xeno™), and were calculated with $2^{-\Delta\Delta Ct}$ relative quantification. Bar charts showing the relative expression levels of DNMT1, EIF4E2, GATA2, HDAC1, KRT18, KRT8, MYCN, NRF1, and PPARG genes in embryos from *in vivo* GV, 4C, 8C, MOR, EB, XB, HB, and D11 stages. The relative expression levels of these genes in each sample were standardized with their mRNA levels of Xeno. Error bar shows the standard error. ND: not detected; NT: not tested.

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Chapter 6: Characterization of the altered gene expression profile in early porcine embryos generated from parthenogenesis and somatic cell chromatin transfer[4]

6.1 Introduction

The *in vitro* production of early porcine embryos is of particular scientific and economic interest. As discussed in chapter 2, embryos produced from *in vitro* based systems using Assisted Reproductive Technologies (ART) are generally less developmentally competent in comparison with *in vivo* embryos. In swine, the *in vitro* production of pre-implantation embryos is much less efficient than in many other mammalian species (such as cattle) (Kikuchi et al., 2002). *In vitro* ART manipulations could have perturbing effects on embryonic gene expression, which potentially results in important negative long-term consequences (Lonergan, 2007), without displaying significant changes in the embryos' pre-implantation morphological characteristics (Vejlsted et al., 2006; Nánássy et al., 2008; Rodriguez-Osorio et al., 2009).

Somatic cell nuclear transfer (SCNT) is a technology with great potential applications in basic and biomedical researches. However, the application of SCNT is limited by its low embryonic survival rate and the high incidence of abnormalities in individuals that develop to term, and are believed to be associated with the incorrect or incomplete nuclear reprogramming (Wang et al., 2011; Mesquita et al., 2013). Somatic cell chromatin transfer (CT) is a cloning technology that was designed to facilitate the reprogramming process (Sullivan et al., 2004b; Rodriguez-Osorio et al., 2009), which involves *in vitro* remodelling of the donor nuclei prior to their transfer into enucleated

[4] A version of this chapter is under submission with PlosOne (http://www.plosone.org/).

oocytes to remove nuclear components that may interfere with nuclear remodelling (Sullivan et al., 2004b). Although promising results have been reported using chromatin transfer (CT), the CT-derived embryos still exhibit abnormalities similar to those observed following conventional SCNT (Sullivan et al., 2004b; Mesquita et al., 2013). Embryos derived from parthenogenetic activation (PA) are valuable for studies on gene imprinting (Naturil-Alfonso et al., 2012) and are a potential alternative source of embryonic stem cells (Brevini and Gandolfi, 2008; Naturil-Alfonso et al., 2012). However, embryos generated from PA experience severe development failure (Hao et al., 2004). The mechanisms behind the deficiencies of embryos generated from PA and CT are not completely understood.

The blastocyst is an embryonic stage that is frequently transferred into female recipients after ART manipulation (Glujovsky et al., 2012; Yoshioka et al., 2012) in pig, and is, therefore, of particular scientific and economic interest. As discussed in chapter 4, the hatching process of blastocyst is a critical and tightly regulated event during early mammalian embryonic development and any dysregulation of the hatching process leads to implantation failure and results in early embryonic loss (Seshagiri et al., 2009).

In the present study, comparative transcriptomic analyses of *in vivo* (IVV) expanded blastocysts (XB), IVV hatched blastocyst (HB), PA XB, PA HB, and somatic cell chromatin transfer (CT) HB were performed using the EMPV1 microarray platform. The objectives of the present study were (1) to characterize the effect of somatic cell chromatin transfer (CT) and parthenogenetic activation (PA) on the gene expression patterns of hatched blastocyst (HB) stage porcine embryos; (2) to identify genes and gene networks dysregulated in PA embryos during blastocyst hatching.

6.2 Materials and Methods

6.2.1 Recovery of *in vivo* embryos

In vivo (IVV) derived porcine XB, and HB stage embryos were collected from gilts as described previously and stored individually (Degenstein et al., 2008). The day of artificially insemination is considered day 0 (D0). All embryo samples were placed on dry ice immediately after collection and stored at -80 ℃ until RNA extraction.

6.2.2 Production of *in vitro*-derived embryos

All of the *in vitro* (somatic cell nuclear transfer (CT) and parthenogenetic activation (PA)) embryos used in the present study were produced by the International Center of Biotechnology, Minitube of America®, MT Horeb, Wisconsin, USA (http://www.minitube.com/). In brief, the CT reconstructed embryos were produced by using the Chromatin Transfer technology (Sullivan et al., 2004a; Collas et al., 2007) under license from Hematech to Minitube (Verona, WI, USA). Oocyte collection, maturation, and micromanipulation were performed following established standard operating procedures (Collas et al., 2007; Carlson et al., 2011). The CT reconstructed embryos (for CT embryo production) and mature oocytes (for PA embryo production) were activated with incubation in 15 μM calcium ionomycin (Calbiochem, CA, USA) supplemented mNCSU23 medium (Minitube, WI, USA) and subsequently an incubation of 1.9 mM 6-dimethylaminopurine (DMAP) supplemented mNCSU23 medium following previously established procedures (Carlson et al., 2011). The *in vitro* activated CT reconstructed embryos and the parthenogenetically activated oocytes were both cultured in the PorcPRO mNCSU-23 (Minitube, WI, USA) pig embryo culture medium system in 38.7 ℃, 5% CO_2, and 95-98% humidity for up to 8 days for expanded blastocyst and hatched blastocyst development.

All of the *in vitro* (CT and PA)-derived embryo samples were placed on dry ice immediately after collection and stored at -80 ℃ until RNA extraction.

6.2.3 Total RNA isolation

Total RNA was extracted from pools of 5 embryos using Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, CA, USA). The RNA quality and integrity of each total RNA sample was evaluated by Bioanalyzer RNA 6000 Pico LabChip (Agilent Technologies, ON, Canada). Only high quality RNA samples (RNA integrity number (RIN) ≥7.5) were used for subsequent RNA amplification.

6.2.4 Microarray experimental design

The comparative transcriptomic analyses were performed using a custom designed porcine embryo-specific microarray platform (EMPV1: EmbryoGENE Porcine Array Version1 [GPL14925]) (Tsoi et al., 2012).

To characterize the effects of *in vitro* manipulations (PA and CT) on the porcine blastocyst transcriptome, comparative transcriptomic analyses among *in vivo* XB, *in vivo* HB, PA XB, PA HB, and CT HB were performed. Total RNA samples extracted from pools of 5 embryos from the same stage were amplified, labeled with Cy5 dye, and hybridized with a Cy3 dye-labeled reference amplified antisense RNA (aRNA) pool (5.2.4) on EMPV1 microarray following a reference design (Kerr and Churchill, 2001; Konig et al., 2004; Novoradovskaya et al., 2004) using three biological replicates from each group.

Agilent two-colour RNA Spike-In® (Agilent Technologies, ON, Canada) were amplified, labeled and utilized as positive controls in each hybridization reaction as previously described (Tsoi et al., 2012).

6.2.5 Microarray analysis and data acquisition

Due to the low quantities of each total RNA sample, all RNA samples were amplified following the procedures described in chapter 5 (5.2.3) using RiboAmp HS^{Plus} kit (Applied Biosystems, CA, USA). The labelling, hybridization, washing and drying steps

of EMPV1 microarray analysis were conducted following the procedure described in chapter 4. The microarray data acquisition was performed following the procedure described in chapter 5 (5.2.5).

6.2.6 Gene expression data analysis

Expression data obtained from the comparative transcriptomic analysis were analysed using the IPA (Ingenuity® Pathway Analysis, Ingenuity® Systems, www.ingenuity.com) Biological Functions Analysis, Canonical Pathway Analysis, and Upstream Regulator Analysis tools as described in chapter 4 (4.2.10).

6.2.7 Real-time quantitative PCR (QRT-PCR) verification of gene expression

Fourteen genes selected from the comparative gene expression data were evaluated using SYBR Green I-based QRT-PCR. The primer sequences for all target genes are listed in Table 6-1. The QRT-PCR analysis was performed as described in chapter 4 and 5 (4.2.10). Three biological replicates from each embryonic stage were utilized.The QRT-PCR data was normalized with the external control gene (Xeno™ RNA Control, Ambion®) using the qbase^{PLUS} software (Biogazelle) (Hellemans et al., 2007). The normalized QRT-PCR data was then further analysed using the $2^{-\Delta\Delta CT}$ method (Yuan et al., 2006; Hellemans et al., 2007) to determine the relative differential expression (fold changes) of each target gene.

6.3 Results

6.3.1 Altered gene expression profile in PA- and CT-derived HB

The reference design, which was used in the microarray comparative transcriptomic analysis among embryos derived from the PA and CT, allows for reliable comparisons among different groups in the analysis (Konig et al., 2004), as described in the methods.

Comparative microarray analysis revealed 1492 and 103 significant differentially expressed (FC > 2 or < 0.5, B-H P-value<0.05) genes in PA- and CT-derived HB, respectively, in comparison with IVV HB (Additional file 6-S1). In comparison with IVV HB, 55 genes showed significant differential expression in both PA and CT HB, and 54 out of these 55 genes showed the same direction of expression changes (up- or down-regulation) in PA and CT HB.

IPA biological function (bio-function) analysis revealed 19 and 48 biological function categories that were significantly altered (B-H P-value<0.05, and have more than 8 molecules included in the analysis) in PA- and CT-derived HB, respectively (Additional file 6-S2). The most significantly altered (B-H P-value<0.01, and have more than 8 molecules included in the analysis) bio-function categories in PA HB and CT HB were further identified (Figure 6-1). The four most significantly altered bio-functions in PA HB were associated with "cellular growth and proliferation", "cellular development", "cell cycle", and "neurological disease"; and the four most significantly altered bio-functions in CT HB were associated with "cell cycle", "neurological disease", "skeletal and muscular disorders", and "nucleic acid metabolism". The "cell cycle", and "neurological disease"-associated bio-functions were significantly altered in both PA and CT-derived HB stages embryos.

IPA canonical pathway analysis revealed eight canonical pathways that were significantly altered (B-H P-value<0.05, and have more than six molecules included in the analysis) in PA HB in comparison with IVV HB (Figure 6-2). The "eIF2 signalling", "mitochondrial dysfunction", "regulation of eIF4 and p70S6K signalling", "protein ubiquitination", and "mTOR signalling" pathways were the five most significantly changed canonical pathways between PA HB and IVV HB. Specifically, most of the differentially expressed genes associated with these pathways were down-regulated in PA HB (Figure 6-2).

IPA Upstream Regulator Analysis revealed five (MYC, MYCN, NOBOX, PPARGC1A, and TP53) and one (TP53) transcription factors that predicted to be significantly activated (or inhibited) in PA and CT-derived HB, respectively, in comparison with IVV HB (Additional file 6-S3). Transcription factors PPARGC1A, MYC, and MYCN were predicted to be inhibited in PA HB; and transcription factors NOBOX and TP53 were predicted to be activated in PA HB.

Transcription factor TP53 was predicted to be significantly activated in both PA and CT HB. Although no significant differential expression of the TP53 gene was observed, 136 and 23 regulation targets of TP53 showed significant differential expression in PA and CT-derived HB, respectively, in comparison with IVV embryos (Additional file 6-S4). In addition, 11 regulation targets (*ANXA8, CTSH, CTSK, GSTP1, HSP90AA1, IL6, MYO6, PERP, PHLDA3, PRDX3*, and *PSEN2*) of TP53 showed differential expression in both PA and CT HB compared with IVV HB. The down-regulation of *PSEN2* (Figure 6-3A) and the up-regulation of *ANXA8* (Figure 6-3B) in PA and CT HB were confirmed by QRT-PCR. *ANXA8* displayed detectable expression levels in both PA and CT HB, and *ANXA8* displayed significantly higher expression in PA HB than CT HB. No detectable expression of *ANXA8* was observed in IVV HB by QRT-PCR analysis.

Significant differential expression of four "notch signalling"-associated genes (*PSEN2*, *HEY2*, *HES1*, and *JAG1*) were observed in PA HB in comparison with IVV HB embryos. In comparison with IVV HB, the microarray analysis revealed significant down-regulation of *HEY2*, *HES1*, and *JAG1* genes, and significant up-regulation of *PSEN2* showed in PA HB. Significant down-regulation of the *PSEN2* genes was also observed in CT-derived HB in comparison with IVV HB. Another three (*NCSTN*, *HES1*, and *JAG1*) "notch signalling"-associated genes showed altered expression in CT HB in comparison

with IVV HB, but with less statistical significance (FC>2 or <0.5, P-value<0.05 but B-H P-value >0.05).

Five "notch signalling"-associated genes (*PSEN2*, *HEY2*, *HES1*, *NCSTN*, and *JAG1*) were selected for QRT-PCR verification. *HES1* (Figure 6-3C) was up-regulated in both PA and CT HB in comparison with IVV HB. *PSEN2* (Figure 6-3A) was down-regulated in CT HB in comparison with IVV HB, and did not display detectable expression in PA HB. *JAG1* (Figure 6-3D) did not display detectable expression in IVV HB, but was expressed in both PA HB and CT HB. *HEY2* expression (Figure 6-3E) was not detectable in PA, CT, and IVV HB embryos in the QRT-PCR analysis. No significant expression change of NCSTN (Figure 6-3F) was observed among PA, CT and IVV HB in the QRT-PCR analysis.

In comparison with IVV HB, significant down-regulation (FC=0.3, B-H P-value<0.05) of *KRT18* (Figure 6-3G) was observed in PA HB, and a less significant down-regulation (FC=0.66, P-value<0.05 but B-H P-value>0.05) of *KRT18* was observed in CT HB. QRT-PCR analysis of *KRT18* (Figure 6-3G) and *KRT8* (Figure 6-3H) expression showed that the *KRT18* and *KRT8* genes were down-regulated in PA HB, and the *KRT18* was down-regulated in CT HB, in comparison with IVV HB.

In addition, microarray analysis revealed significant down-regulation (FC<0.5, B-H P-value<0.05) of *GATA2* and *NANOG* in PA HB in comparison with IVV HB. QRT-PCR analysis results confirmed this down-regulation of *GATA2* in *NANOG* in PA HB (Figure 6-3I and 3J). However, the down-regulation of *GATA2* in CT HB was not statistically significant. In the QRT-PCR analysis, *NANOG* expression was only detectable in IVV HB, and no detectable expression of *NANOG* was observed in PA and CT HB.

Microarray analysis also revealed significant up-regulation (FC > 2, B-H P-value<0.05) of four precursor-microRNAs (pre-miRNA) (*MIR1343*, *MIR149*, *MIR505*, and *MIR192*)

in PA HB in comparison with IVV HB. Only trends (P-value<0.05 but B-H P-value>0.05) of differential expression of the pre-miRNA of MIR505 (FC=0.57) and MIR192 (FC=1.57) were observed in the CT HB in comparison with IVV HB.

6.3.2 Altered gene expression-regulation during blastocyst hatching of PA-derived embryos

Comparative transcriptomic analysis among IVV XB, IVV HB, PA XB, and PA HB revealed that during the transition from XB to HB, differential expression (FC > 2 or <0.5, B-H P-value<0.05) of 3 and 31 genes were observed in PA and IVV-derived embryos, respectively (Additional file 6-S5).

The comparative microarray analysis revealed three genes (*KCTD3*, *ANXA8*, and *SLC36A2*) that showed statistically significant up-regulation from XB to HB in PA embryos. However, no significant differential expression of these three genes was observed in between IVV XB and IVV HB.

QRT-PCR analysis confirmed the up-regulation of *SLC36A2* (Figure 6-3K) and *ANXA8* (Figure 6-3B) from the XB to HB stage in PA embryos. *SLC36A2* showed no significant differential expression between IVV XB and IVV HB, and *ANXA8* expression was not detectable in IVV XB and IVV HB. No significant differential expression of *KCTD3* was observed between PA XB and PA HB in the QRT-PCR analysis (Figure 6-3L).

Significant up-regulation (FC > 2, B-H P-value<0.05) of *DPP4* and *LGMN* from XB to HB in *in vivo*-derived embryos were observed in the microarray analysis. Trends toward up-regulation of the *DPP4* and *LGMN* were also observed in the PA embryos from XB to HB. In addition, a trend (P-value<0.05 but B-H P-value >0.05) of up-regulation of the trophectoderm development-associated gene *KRT8* (FC=1.9) and *GATA2* (FC=2.4) from XB to HB in IVV embryo was observed in the microarray analysis. However, no differential expression of *KRT8* was observed between PA XB and PA HB embryos.

Results from QRT-PCR analysis confirmed the up-regulation of *DPP4*, *LGMN*, *GATA2* and *KRT8* from XB to HB *in vivo* (Figure 6-3M, 3N, 3H, and 3G). In comparison with IVV embryos, *DPP4* and *LGMN* displayed a smaller up-regulation from XB to HB in PA embryos. No differential expression of *GATA2* and *KRT8* was observed between PA XB and PA HB in the QRT-PCR analysis.

Three (*HEY2*, *HES1*, and *JAG1*) "Notch signalling"-associated genes showed down-regulation (FC<0.5), but with reduced statistical significance (P-value<0.05 but B-H P-value >0.05), from XB to HB in IVV embryos in the microarray analysis. *HES1* showed more than 2.5 fold down-regulation from XB to HB in both IVV and PA embryos. *HEY2* and *JAG1* showed more than 2.4 fold down-regulation from XB to HB in IVV embryos, but no significant differential expression of these two genes was observed in PA embryos.

Results from QRT-PCR analysis confirmed the up-regulation of *HES1* and the down-regulation of *HEY2* and *JAG1* from XB to HB in IVV embryos (Figure 6-3C-E). Although up-regulation of *HES1* and down-regulation of *JAG1* from XB to HB in PA embryos were observed in the QRT-PCR analysis, the expression changes of these two genes were less significant than IVV embryos (Figure 6-3C-D). HEY2 displayed a higher expression in IVV XB than PA XB, and *HEY2* expression was not detectable in both PA and IVV HB in the QRT-PCR analysis (Figure 6-3E).

6.4 Discussion

The embryos generated after *in vitro* manipulations such as parthenogenetic activation and nuclear transfer displayed slower and less effective development (Whitworth et al., 2005; Liu et al., 2010; Whitworth et al., 2011; Isom et al., 2013), and dysregulation of critical gene networks is probably associated with these deficiencies.

The first objective of the present study was to characterize the effects of somatic cell chromatin transfer (CT) and parthenogenetic activation (PA) on the gene expression patterns of hatched blastocyst stage porcine embryos.

Comparative microarray analysis revealed 1492 and 103 significantly differentially expressed genes in PA and CT-derived HB, respectively, in comparison with IVV HB. The large gene expression profile differences between PA HB and IVV HB observed in the present study is consistent with previous studies in different species (Whitworth et al., 2005; Liu et al., 2010; Naturil-Alfonso et al., 2012; Isom et al., 2013). The gene expression profile differences between CT and IVV HB observed in the present study was less pronounced than the differences previously reported between SCNT and IVV-derive porcine blastocyst stage embryos (Whitworth et al., 2011).

In comparison with IVV HB, the"eIF2 signalling", "mTOR signalling", "regulation of eIF4 and p70S6K signalling", "mitochondrial dysfunction", and "protein ubiquitination pathway" pathways were the 5 most significantly altered pathways in PA HB, and of the differentially expressed genes associated with these 5 pathways were down-regulated in PA HB.

Eukaryotic translation initiation factor 2 (eIF2) plays a key role in the recognition of the correct start codon during translation initiation process (Schmitt et al., 2010). Phosphorylation of eIF2 reduces global translation and activates the transcription of "stress recovery" genes in response to environmental stresses such as amino acid deficiency, heavy metal toxicity, and bacterial infection (Schmitt et al., 2010; Shrestha et al., 2012). It has been reported that cells with defective eIF2 signalling were more susceptible to bacterial invasion (Shrestha et al., 2012). The "mTOR signalling pathway" plays a critical role in the regulating of cell growth, proliferation, translation, protein synthesis and survival (Ruvinsky and Meyuhas, 2006; Laplante and Sabatini, 2009;

Dowling et al., 2010). The eIF4 initiation factors are responsible for recruiting mRNA to a ribosome during translation process (Gingras et al., 1999). The translation eIF4 initiation factors and p70 S6 kinase (p70S6k) both play critical roles in the translation and protein synthesis regulation, and both eIF4 and p70S6k are regulation targets of mTOR (Gingras et al., 1999; Ruvinsky and Meyuhas, 2006). Many environmental stimuli including growth factors, hormones, and nutrient availability can regulate the eIF4 and p70S6K through "mTOR signalling pathway" (Laplante and Sabatini, 2009). The down-regulation of genes associated with the "eIF2 signalling", "mTOR signalling", "Regulation of eIF4 and p70S6K signalling" pathways suggest that the general translation and protein synthesis are affected in PA HB; and many "mTOR signalling"-associated critical biological processes are also significantly affected in PA HB.

Mitochondria, especially as an ATP generation source, are critical for the development of early embryos, and perturbation in their functions is associated with compromised embryonic competence (Mitchell et al., 2009). Mitochondrial dysfunction in oocytes is directly responsible for the high levels of developmental retardation and early arrest of pre-implantation embryos produced *in vitro* (Thouas et al., 2004). In the present study, the down-regulation of "mitochondrial dysfunction"-associated genes in PA HB suggests compromised mitochondria function in PA HB.

The "Ubiquitin–proteasome pathway" is responsible for the selective degradation of soluble cellular proteins in most cases (Hochstrasser, 1996). Ubiquitination of cellular protein is essential for the ubiquitin-proteasome pathway-dependent cellular protein degradation (Dudek et al., 2010). Degradation of maternal proteins through the "ubiquitin–proteasome pathway" is believed to be important for the oocyte-to-embryo transition (DeRenzo and Seydoux, 2004). In this study, significant differential

expressions in genes associated with "protein ubiquitination pathway" were observed, suggesting an altered protein degradation process in PA embryos.

TP53 (tumor protein p53) is a well- known cell-cycle regulator and apoptosis mediator (Molchadsky et al., 2010), and it has been previously reported that the embryos derived from parthenogenetic activation experience a higher apoptotic cell death rate (Hao et al., 2004). Results from the present study showed that the transcription factor *TP53* is predicted to be activated in both PA and CT HB in comparison with the IVV HB, where the number of differentially expressed *TP53* regulation targets in PA HB was more than four times higher than the number of differentially expressed *TP53* regulation targets in CT HB. In addition, *ANXA8* (annexin A8) is a member of the annexins (ANXs) family, which is a group of Ca^{2+}-dependent phospholipid-binding proteins. ANXs are involved in many important biological processes including vesicle trafficking, calcium signalling, cell growth, cell cycle, and apoptosis (Hata et al., 2012). Over expression of *ANXA8* has been reported to be associated with cancer and apoptosis (Hammond et al., 2006). In the present study, *ANXA8* displayed significantly higher expression in PA HB than CT HB, and no detectable expression of *ANXA8* was observed in IVV HB. These results suggest that an activated apoptotic process might be induced in both PA and CT derived HB, and that the activation of this apoptotic process appears to be greater in PA HB than in CT HB.

NOTCH is an important regulator of development in many animals (Shepherd et al., 2009), which participate in many critical biological processes including cell fate specification, differentiation, proliferation, apoptosis, migration, and angiogenesis (Bolos et al., 2007). Small perturbations in Notch activity could lead to numerous developmental defects and diseases (Shepherd et al., 2009). Notch signalling is initiated through ligand-receptor interactions between neighbouring cells (Bolos et al., 2007). The NOTCH-

mediated HES1 expression plays an important role in the regulation of cell fate decision (Bolos et al., 2007). In mammals, the two highly homologous presenilin genes (*PSEN1* and *PSEN2*) play important roles during early embryonic development and both of the presenilin genes are positive regulators of the "notch signalling" pathway (Donoviel et al., 1999; Ye et al., 1999). Results from the present study showed that one of the mammalian Notch ligands Jagged1 (encoded by *JAG1* gene) (Lindsell et al., 1995; Bolos et al., 2007) and two other members (*HES1* (Ohtsuka et al., 1999) and *PSEN2* (Ye et al., 1999; Ferjentsik et al., 2009)) of "Notch signalling" pathway were significantly differentially expressed between PA and IVV HB. Less dramatic differential expression of these three "notch signalling"-associated genes were also observed in the CT HB. These results suggest that the "notch signalling" pathway is dysregulated in both PA and CT HB, and this dysregulation is more significant in PA HB than in CT HB. The altered regulation in Notch signaling probably contributes to the impaired development of PA and CT-derived embryos.

As one of the key regulators of pluripotency, the transcription factor *NANOG* functions as a repressor of the extra-embryonic endoderm (ExE) or primitive endoderm (PE) cell fate (Strumpf et al., 2005). In comparison with IVV HB, significant down-regulation of *NANOG* in both PA and CT HB was observed in the present study, which suggests a compromised regulation of cell fate specification and TE differentiation in PA HB and CT HB.

Transcription factor GATA binding protein 2 (*GATA2*) is expressed in trophoblast giant cells and acts as important regulator for trophoblast-specific gene expression and placental function (Ma et al., 1997; Assou et al., 2012). Expression of *GATA2* genes is essential for normal embryonic development (Ma et al., 1997). Keratins 8 (*KRT8*), keratin 18 (*KRT18*) and keratins 19 (*KRT19*) are predominantly expressed in epithelial

components of glandular tissues (Alam et al., 2011). Expression of keratin 8 and keratin 18/19 are expressed in TE and are essential for the integrity of a specialized embryonic epithelium (trophoblast giant cells) layer and the survival of embryos (Baribault et al., 1993; Hesse et al., 2000; Assou et al., 2012). In the present study, *GATA2*, *KRT18*, and *KRT18* showed significant down-regulation in PA HB, but only *KRT18* showed significant down-regulation in CT HB embryos in the QRT-PCR analysis. These results suggest impaired trophoblast development in both PA HB and CT HB, and trophoblast development in CT HB is less affected than PA HB.

Although the DPP4 (dipeptidyl peptidase 4) was reported to be differentially regulated in the CT-derived bovine day 45 placenta (Mesquita et al., 2013), no significant differential expression of *DPP4* was observed in PA and CT HB in the present study.

MicroRNAs (miRNA) are believed to be key regulators in pre-implantation embryonic development and differentiation (Yang et al., 2008; Viswanathan et al., 2009). Recent reports suggest that the microRNA reprogramming is incomplete and inconsistent in cloned embryos (Castro et al., 2010; Isom et al., 2013). In the present study, microarray analysis revealed significant differential expression of four pre-miRNAs in PA HB in comparison with IVV HB. Two of these 4 pre-miRNAs showed trends of differential expression, and no statistically significant differentially expressed pre-miRNA was observed between CT and IVV HB. During pre-implantation development of embryos, dynamic synthesis and degradation of miRNAs coexists (Yang et al., 2008). Hence the differential expression of pre-miRNA does not guarantee the differential expression of mature miRNA.

The second objective of the present study was to identify dysregulated genes and gene networks in PA embryos during blastocyst hatching. Hatching is a critical and necessary process during the early development of mammalian embryos. Blastocyst hatching is a

well programmed and tightly regulated event, and dysregulation of this critical process leads to implantation failure and results in early embryonic loss (Seshagiri et al., 2009). Dysregulation of critical genes and gene networks during blastocyst hatching process are probably contributed to the deficiencies in embryos generated from PA.

In the present study, significant differential expression of 31 genes were observed during the blastocyst hatching process (from XB to HB) in IVV embryos, but these 31 genes were not properly regulated in PA embryos during blastocyst hatching. On the other hand, *SLC36A2* and *ANXA8* showed significant up-regulation during the blastocyst hatching process in PA embryos, but no up-regulation of these two genes were observed in IVV embryos. SLC36A2 (Solute carrier family 36 (proton/amino acid symporter), member 2) mediates the transport of amino and fatty acids, which are critical to early embryonic development (Foltz et al., 2004; Klein et al., 2010). Further work is necessary to determine if this up-regulation of *SLC36A2* is compensating for the function of other dysregulated genes in PA embryos and reflecting the increased need for nutrients in the rapidly developing embryos.

LGMN (legumain), also known as cysteine protease 1, is involved in protein processing and is highly expressed in the placenta (Degrelle et al., 2009). Legumain has been reported to be expressed in bovine trophoblast and associated with the regulation of trophoblast invasiveness and endometrial remodelling during implantation (Ledgard et al., 2009). DPP4 (dipeptidyl peptidase 4) is a membrane-bound aminopeptidase, which is associated with placental development and the establishment of proper fetal-maternal interactions (Fujiwara et al., 2005; Mesquita et al., 2013). In the present study, marked up-regulation of *LGMN* and *DPP4* were observed during hatching process in IVV embryos, but the expression changes of *LGMN* and *DPP4* observed during hatching process in PA embryos are much less dramatic. Results from the present study showed

that the expression of several critical pluripotency, trophoblast development, and implantation-associated genes (*NANOG*, *GATA2*, *KRT8*, *LGMN*, and *DPP4*) were not properly regulated during the blastocyst hatching process in PA embryos. In addition, altered regulation of "notch signalling"-associated genes was also observed during the blastocyst hatching process in PA embryos. Failing to regulate the expression of these critical genes during the hatching process is probably contributed to the delayed and less efficient development of PA embryos.

6.5 Conclusion

In the present study, we have successfully characterized the altered gene expression profiles in porcine HB embryos derived from parthenogenetic activation and somatic cell chromatin transfer, in comparison with *in vivo*-derived HB. Specifically, we have identified several signalling pathways, critical genes, and critical gene networks that were significantly altered in the PA- and CT-derived HB stage embryos. In addition, we have also identified several critical genes that were not properly regulated during the blastocyst hatching process in embryos derived from PA.

To date, morphological characteristics and blastocyst formation rate are still two of the major parameters commonly used in embryonic developmental competence assessment (Lonergan, 2007). Results from the present study showed that embryos produced from PA and CT could develop into expanded blastocyst and hatched blastocyst stage, even with dysregulations of critical pathways and gene networks. Hence, the morphological criteria and blastocyst development ratio are insufficient to determine the ultimate competence of embryos generated after *in vitro* ART manipulations (such as PA and CT). The critical genes that exhibited altered expression in CT and PA embryos are indicative

of underlying developmental deficiencies and could serve as potential candidate genes for the embryonic competence gene markers selection and validation.

Table 6-1 Primer sequences used in QRT-PCR verification

Gene symbol	Associated Porcine RefSeq Accession No.	Primer	Primer sequence (5'-3')	Product size (bp)
LGMN	XM_001927082.4	Forward	AGA CGC TCC ACA AAC AGT AC	95
		Reverse	CAA CTT CAT GGC AGA GAT GGA	
GATA2	NM_213879.1	Forward	CTC CAG CTT CAC CCC TAA G	157
		Reverse	CCC GTT CAT CTT GTG GTA CAG	
KRT8	NM_001159615.1	Forward	AGA TCC AAA AGC GTA CCG AC	136
		Reverse	AGC TGC CTG TAG AAG TTG ATC	
PSEN2	NM_001078666.1	Forward	CTC AAC TCC GTG CTC AAC A	148
		Reverse	GAT GTA GGT GAA GAG GAA GAG C	
NCSTN	XM_001928786.1	Forward	CCC CGC AAT GTC ATG TTT G	92
		Reverse	AAC TTG CCC TTC TCC ATA TCG	
HES1	NM_001195231.1	Forward	CTG GAG AAG GCG GAC ATT C	92
		Reverse	GCT CGG GTC TGT GCT TAG	
HEY2	NM_001243329.1	Forward	CTG CAA AGT TAG AAA AGG CCG	145
		Reverse	TCT GTT AAG CAC TCT CGG AAT C	
ANXA8	NM_001243599.1	Forward	AGA CAT ACA AGC AGA TAC CAG TG	142
		Reverse	CTT CTC ACC CGC TGC ATA C	
SLC36A2	XM_003134141.2	Forward	CAT CAC CCA GTA CAT CAT CCA G	127
		Reverse	CAG AAC CAC ACC AAT GCT TTC	
KCTD3	XM_003357619.2	Forward	AGA AGT TCC CTC TGC GAA TG	149
		Reverse	CGT ACC ATA GGC GAT CTC AAT C	
NANOG	NM_001129971.1	Forward	GGACTTTTCCTACAATCCAGC	153
		Reverse	CCCATAAACCTCAGGCATTG	
JAG1	XM_001926559.2	Forward	ACA TAG CCC GAA ACA GTA GC	158
		Reverse	GTT GTA GCA GGG ATG AGG AC	
DPP4	NM_214257.1	Forward	TGCGGATTCCATACCCAAAG	137
		Reverse	ATCCCCTATTAACACAGACGC	
KRT18	XM_003126180.3	Forward	TTGACCGTGGAGTTGGATG	149
		Reverse	ACCACTGAGGTGCTCTCC	
Xeno™	Control primer Xeno™ from SYBR® Green Cells-to-CT™ Control Kit (Ambion)			105

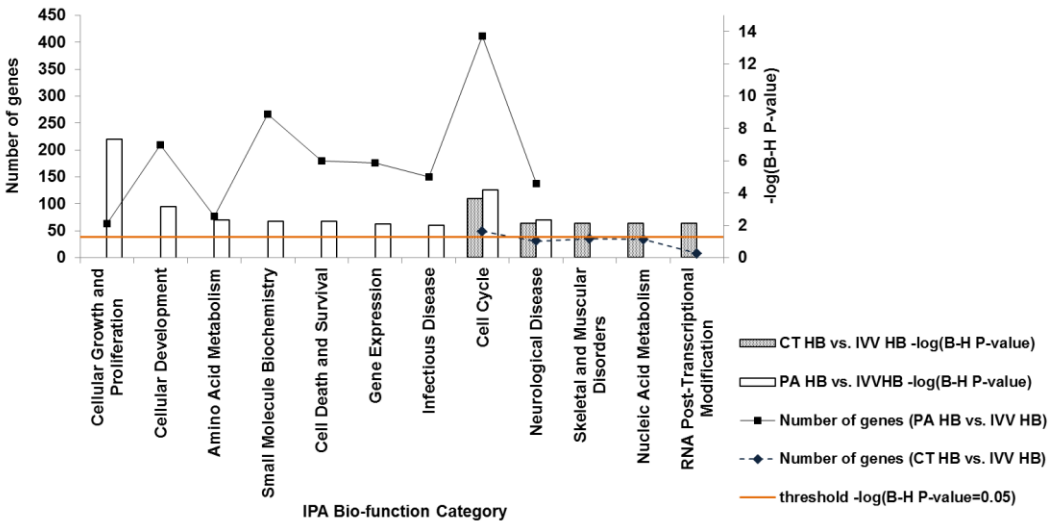

Figure 6-1 Altered biological function categories in PA and CT-derived HB

Bar chart shows the significantly altered biological function categories in IPA biological function (bio-function) analysis. Major Y axis on the left shows the number of differentially expressed genes that involved in the biological function category. Secondary Y axis on the right shows the significance (-log (B-H P-value)) of the altered biological function category. The orange line shows the significance threshold of cut off of -log (B-H P-value=0.05).

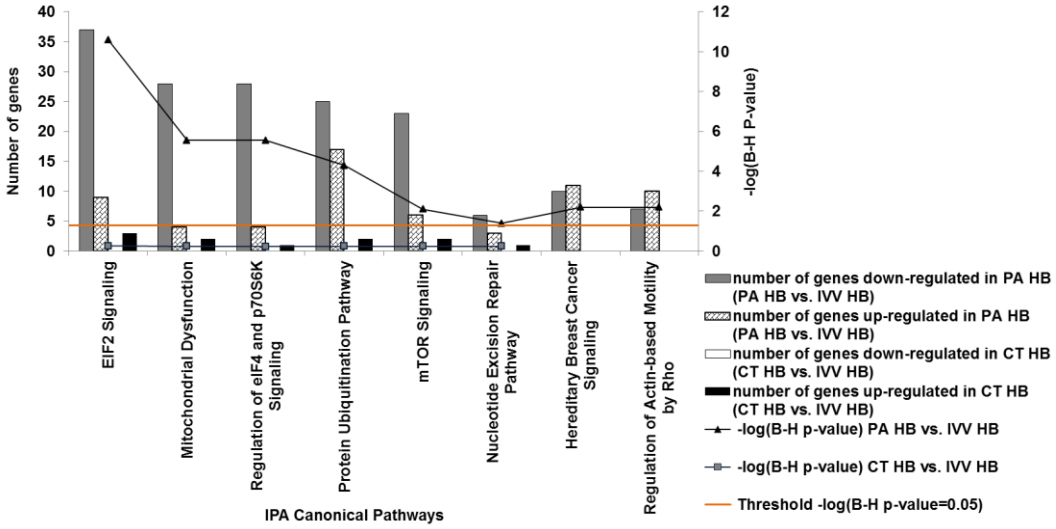

Figure 6-2 Altered canonical pathways in PA and CT-derived HB

Bar chart shows the altered canonical pathways in IPA canonical pathways analysis. Major Y axis on the left shows the number of differentially expressed genes that involved in the canonical pathway. Secondary Y axis on the right shows the significance (-log (B-H P-value)) of the canonical pathway. The orange line shows the significance threshold of cut off of -log (B-H P-value=0.05).

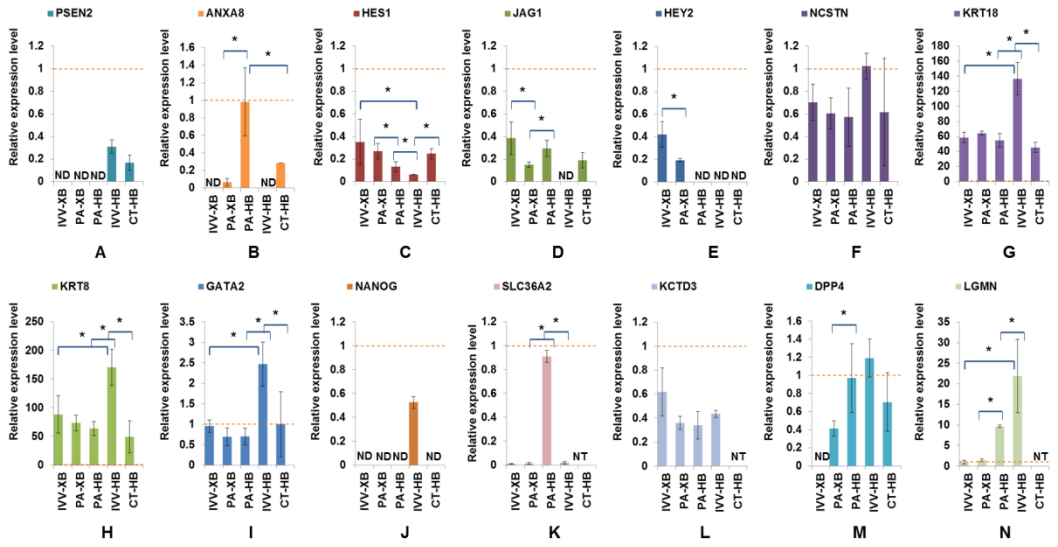

Figure 6-3 QRT-PCR verification result

QRT-PCR verification result of 14 selected genes. The mRNA expression levels of these genes were normalized with the external control gene (Xeno™), and were calculated with $2^{-\Delta\Delta Ct}$ relative quantification. Bar charts showing the relative expression levels of HES1, NCSTN, PSEN2, JAG1, HEY2, ANXA8 NANOG, GATA2, KRT18, KRT8, DPP4 KCTD3, SLC36A2, and LGMN genes in IVV XB, IVV HB, PA XB, PA HB, and CT HB (KCTD3, SLC36A2, and LGMN genes were not tested in CT HB). The relative expression levels of these genes in each sample were standardized with their mRNA levels of Xeno. Error bar shows the standard error. Dashed lines indicate 1.0 expression level. ND: not detected. NT: not tested. Bars marked with "*"are with significant differential expression. The large format version of this figure can be access through following link:

https://docs.google.com/file/d/0B0QxwqYwWLkfRk9XOENqbnl1Uk0/edit?usp=sharing

Additional file 6-[S1-S5]

https://docs.google.com/file/d/0B0QxwqYwWLkfSUotM2I3MVZ6Xzg/edit?usp=sharing

Additional file 6-S1 Expression data of significant differentially expressed (B-H P-value<0.05, FC>2 or <0.5) genes in PA HB vs. IVV HB and CT HB vs. IVV HB analyses.

Additional file 6-S2 IPA bio-function analysis result of significantly altered (B-H P-value<0.05, molecules involved in the analysis ≥ 8) biological function categories in PA HB vs. IVV HB and CT HB vs. IVV HB analyses.

Additional file 6-S3 IPA upstream regulator analysis result of transcription factors that predicted to be significantly activated or inhibited (overlap P-value<0.01, IPA Activation z-score >2 or <-2) in PA HB vs. IVV HB and CT HB vs. IVV HB analyses.

Additional file 6-S4 Expression data and IPA upstream regulator analysis prediction of differentially expressed (B-H P-value<0.05, FC>2 or <0.5) regulation targets of transcription factor TP53 in PA HB vs. IVV HB and CT HB vs. IVV HB analyses.

Additional file 6-S5 Expression data of genes that differentially expressed (FC > 2 or <0.5, B-H P-value<0.05) during the blastocyst hatching process in PA and IVV-derived embryos.

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Chapter 7: Characterization of the gene expression profile of blastocysts generated from porcine luteinizing hormone (pLH)-induced superovulation in gilts[5]

7.1 Introduction

Control and synchronization of ovarian follicular development and ovulation can provide practical advantages in livestock management and application of assisted reproductive technology (ART) (Degenstein et al., 2008).

Although a relationship between estrus duration and the time of ovulation after the onset of estrus has been established in sows (Nissen et al., 1997) and gilts (Almeida et al., 2000), substantial variation in the actual time of ovulation within the estrous period has been reported (Soede et al., 1995; Soede and Kemp, 1997). The frequency of estrus detection (Almeida et al., 2000) and the observational skills of the stockperson (Soede et al., 1995) can also affect the recorded onset and duration of estrus, which have large influences on the appropriate timing of insemination and subsequent fertility (Soede et al., 1995). Controlling the timing of ovulation using exogenous hormones can eliminate the need for heat detection and facilitates the use of fixed-time artificial insemination (AI) procedures in swine (Cameron et al., 2010).

Previous studies with gilts have shown that porcine luteinizing hormone (pLH) treatment can reliably synchronize the ovulation and shorten the period between artificial insemination (AI) and ovulation without detrimentally influence pregnancy rate or litter

[5] Data presented in this chapter is part of a manuscript currently under preparation that will also include procedures and results described in Cameron et al. 2010

size in cyclic gilts (Degenstein et al., 2008; Cameron et al., 2010). In addition, it was reported that the pLH treatment in gilts does not have significant effect on the embryo morphology and assumed quality (total cell counts and embryo diameter) of blastocyst stage embryos (Degenstein et al., 2008). However, Degenstein et al. (2008) also found that the diameter of the largest follicles prior to ovulation was smaller in pLH-induced animals than control animals (Degenstein et al., 2008). This has raised questions regarding the implications of this ovulation induction protocols on factors such as embryonic quality and survival. Furthermore, embryos with molecular deviations may display similar morphological characteristics as "normal" embryos in pre-implantation stages (Nánássy et al., 2008; Rodriguez-Osorio et al., 2009). Whether pLH treatment in gilts has an effect on the gene expression profile of porcine embryos remains to be elucidated.

The objective of the present study was to determine the effect of porcine luteinizing hormone (pLH)-induced ovulation on the transcriptome of porcine blastocyst stage embryos. Comparative transcriptomic analysis between blastocyst stage embryos produced from control and pLH- induced gilts was performed using a custom designed porcine embryo-specific microarray platform: EmbryoGENE Porcine Array Version1 (EMPV1, [GPL14925]) (Tsoi et al. 2012).

7.2 Materials and Methods

7.2.1 Recovery of *in vivo* embryos

In-vivo (IVV) derived porcine blastocyst (BL) stage embryos were collected from control and pLH-induced gilts at 5 days (D5) after artificial insemination, as described previously

(Degenstein et al., 2008; Cameron et al., 2010). The day of artificial insemination was considered to be day 0 (D0).

7.2.3 Total RNA isolation

Total RNA was extracted from pools of 5 blastocyst stage embryos using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA) as described chapter 4. The RNA quality and integrity of each total RNA sample was evaluated by Bioanalyzer RNA 6000 Pico LabChip (Agilent Technologies, Mississauga, On, Canada). Only high quality RNA samples (RNA integrity number (RIN) ≥7.5) proceeded to RNA amplification.

7.2.4 Microarray experimental design

In the present study, all the comparative transcriptomic analyses were performed using a custom designed porcine embryo-specific microarray platform: EmbryoGENE Porcine Array Version 1 (EMPV1, [GPL14925]) (Tsoi et al., 2012).

To characterize the gene expression profile changes in blastocyst stage embryos generated from pLH-induced ovulation, comparative transcriptomic analysis was performed between blastocyst stage embryos from control and pLH-induced gilts. Total RNA samples extracted from pools of 5 embryos were amplified, labeled with Cy5 and Cy3 dye and hybridized on the EMPV1 Microarray following a direct comparison design with dye-swap. Three biological replicates from each group were included in the comparative microarray analysis.

Agilent two-colour RNA Spike-In® (Agilent Technologies, Mississauga, ON, Canada) were amplified, labeled and utilized as positive controls in each hybridization reaction as previously described (Tsoi et al., 2012).

7.2.4 RNA amplification and labelling for microarray analysis

All RNA samples were amplified following the procedures described in chapter 5 (5.2.3) using RiboAmp HS^{Plus} kit (Applied Biosystems, CA, USA). The labelling, hybridization, washing and drying steps of EMPV1 microarray analysis were conducted following the procedure described in chapter 4. The microarray data acquisition was performed following the procedure described in chapter 5 (5.2.5).

7.2.5 Real-time quantitative PCR (QRT-PCR) verification of gene expression result

Seven genes of interest genes (*ASNS*, *RGN*, *DPP4*, *GNPDA1*, *SLC41A1*, *ACTB*, and *GAPDH*) were selected from the comparative gene expression data, and were evaluated using SYBR Green I-based QRT-PCR. The primer sequences for all target genes are listed in Table 7-1. The QRT-PCR analysis was performed as described in chapter 4 and 5 (4.2.10). Four biological replicates from each embryonic stage were utilized.The QRT-PCR data was normalized with the external control gene (Xeno™ RNA Control, Ambion®) using the qbase^{PLUS} software (Biogazelle) (Hellemans et al., 2007). The normalized QRT-PCR data was then further analysed using the $2^{-\Delta\Delta CT}$ method (Yuan et al., 2006; Hellemans et al., 2007) to determine the relative differential expression (fold changes) of each target gene.

7.3 Results and Discussion

7.3.1 Microarray analysis

Results from the comparative microarray analysis revealed 11 genes that showed significant differential expression (P-value<0.05, and FC ≥ 2 (or ≤ 0.5)) between blastocysts (BL) produced from control and pLH-induced gilts. Of these 11 genes, 10 were up-regulated and 1 gene was down-regulated in pLH BL, respectively (Table 7-2). However, after the BH-FDR multiple testing adjustment, these 11 genes displayed less

significant BH-FDR P-values (B-H P-value>0.05). To confirm the differential expression of these genes, half of these genes were selected for QRT-PCR verification.

This limited number of differentially expressed genes (11 genes) accounts for only 0.08% of all the genes detected in control and pLH BL embryos (13373 genes). This result suggests that the pLH-induced ovulation in gilts has limited influence on the general gene expression profile of early stage embryos. Previous studies in gilts showed that the pLH treatment does not have detectable effect on the quality of porcine BL based on evaluation of morphological characteristics (Degenstein et al., 2008). Hence, the small differences in gene expression profile between control and pLH BL that observed in the present study is not unexpected.

7.3.2 QRT-PCR verification of gene expression

To confirm the gene expression data obtained from the microarray analysis, QRT-PCR analysis was performed on control and pLH BL stage embryos. Among the 11 differentially expressed genes, only 5 genes (*ASNS, RGN, DPP4, GNPDA1*, and *SLC41A1*) have porcine RefSeq mRNA records containing sequences spanning intron-exon junctions, and these 5 genes were selected for QRT-PCR analysis. In addition to these 5 differentially expressed genes, 2 common housekeeping genes (*ACTB* and *GAPDH*) were also selected for QRT-PCR verification. Results obtained from the QRT-PCR analysis in the present study are consistent with microarray data (Figure 7-1). These results suggest that the gene expression data obtained from microarray analysis in the present study is reliable.

7.3.3 Altered gene expression in blastocysts produced from pLH-induced gilts

Dipeptidyl-peptidase 4 (*DPP4*) is a membrane-bound aminopeptidase, which is a marker for non-invasive trophoblast cells in humans and cattle (Fujiwara et al., 2005; Mesquita et

al., 2013). DPP4 is associated with placental development and the establishment of proper fetal-maternal interaction (Fujiwara et al., 2005; Mesquita et al., 2013). The down-regulation of DPP4 is positively linked to cell migration and invasion in humans (Sato et al., 2002; Fujiwara et al., 2005), and the up-regulation of DPP4 is believed to be associated with the abnormal placental development in cloned cattle (Mesquita et al., 2013). In the present study, up-regulation of DPP4 was observed in pLH BL in comparison with control BL. This result indicates that the embryos produced from the pLH-induced gilts may have potential effects on trophoblast development, which could lead to abnormal placenta development.

Asparagine synthetase (glutamine-hydrolyzing) (*ASNS*) catalyzes the ATP-dependent formation of asparagine and glutamate from aspartate, ATP, and glutamine (Richards and Schuster, 1998; Chen et al., 2004). The transcription of *ASNS* is highly regulated by nutritional deprivation and other forms of cellular stress (Kilberg and Barbosa-Tessmann, 2002; Chen et al., 2004; Balasubramanian et al., 2013). *ASNS* can be transcriptionally activated by amino acid and glucose deprivation (Kilberg and Barbosa-Tessmann, 2002), and is involved in maintaining the homeostasis of asparagine, glutamate, aspartate, and glutamine (Balasubramanian et al., 2013). In the present study, significant up-regulation of *ASNS* was observed in the pLH BL embryos. This result suggest a more activated ASNS transcription in pLH BL, which indicating that the pLH embryos probably are experiencing nutritional limitation or other forms of cellular stress.

Stratifin (*SFN*), also known as 14-3-3 Protein Sigma, is a trophoblast protein associated with cell cycle, growth, and migration (Fu et al., 2000; Laronga et al., 2000). Up-regulation of *SFN* has been reported in less competent porcine embryos during elongation (Blomberg et al., 2005; Blomberg et al., 2008). In the present study, *SFN* showed

significant up-regulation in pLH BL, indicating the pLH BL maybe less competent than the control BL.

In the present study, significant up-regulation of *RGN*, *GNPDA1*, and *MPEG1* genes were observed in pLH BL in comparison with control BL. Regucalcin (*RGN*), also known as senescence marker protein-30 (SMP-30), is a Ca^{2+} binding protein which activates (Ca^{2+}-Mg^{2+})-ATPase and is involved in the maintenance of intracellular Ca^{2+} homeostasis (Takahashi and Yamaguchi, 1997; Wu et al., 2008) and aging (Jung et al., 2004). Previous studies in rats and chicken suggested that the down-regulation of RGN is associated with increased oxidative stress (Jung et al., 2004). Glucosamine-6-phosphate deaminase 1 (*GNPDA1*) is an allosteric enzyme that catalyzes the reversible conversion of glucosamine-6-phosphate into fructose-6-phosphate and ammonium (COMB and ROSEMAN, 1958; Wolosker et al., 1998; Alvarez-Anorve et al., 2011). The glucosamine 6-phosphate deaminase (GNPDA) may have a role in the acrosome reaction (Montag et al., 1999). Macrophage-expressed gene 1 protein-like (*MPEG1*) is expressed almost exclusively in differentiated myelomonocytic cells in human and murine (Spilsbury et al., 1995). *MPEG1* has been used as a macrophage-specific marker gene in mammalian systems (Karlsson et al., 2008) and in *in vivo* early zebra fish embryos (Zakrzewska et al., 2010). Further investigation is required to determine if the up-regulation of these genes in pLH embryos indicating any potential developmental deficiencies.

In addition, significant down-regulation of *SLC41A1*, and significant up-regulation of *CHAC1*, *MSMO1*, and *IDI1* were observed in pLH BL embryos. These genes are important in maintaining the ion and lipid homeostasis in porcine embryos. Solute carrier family 41, member 1 (*SLC41A1*) is a relatively nonselective divalent cation transporter, which transports a variety of divalent metal cations (Goytain and Quamme, 2005). SLC41A1 proteins are central components the plasma membrane Mg^{2+}-uptake system in

vertebrate (Mandt et al., 2011), and the transcription of *SLC41A1* is up-regulated in response to low magnesium concentration (Goytain and Quamme, 2005). ChaC, cation transport regulator homolog 1 (*CHAC1*) is a cation transport regulator that promotes apoptosis (Mungrue et al., 2009; Joo et al., 2012). Methylsterol monooxygenase 1 (*MSMO1*, also known as sterol-C4-methyl oxidase) and Isopentenyl-diphosphate delta isomerase 1 (*IDI1*) are both involved in the sterol/cholesterol biosynthetic and metabolic processes (Fukushima et al., 2010). Further investigation is required to determine if the differential expressions of these genes are associated with any deficiencies in pLH embryos.

It should be noted that the 18S ribosomal RNA (*RN18S*) gene is differentially expressed between pLH and control BL embryos. This result suggests that the *RN18S*, a commonly used "housekeeping" gene (Martinez-Beamonte et al., 2011; Gendelman and Roth, 2012), probably is not suitable to be used as "housekeeping gene" in studies of early porcine embryos.

In summary, pLH-induced superovulation in gilts has limited influence on the gene expression profile of blastocyst stage porcine embryos, where only 11 genes showed differential expression between pLH and control blastocysts. Several of the 11 differentially expressed genes in pLH BL are considered to play important roles during early embryonic development. However, a review of the literature would indicate that the induction of ovulation in pigs does not generally affect key fertility parameters such as pregnancy rate and litter size (Draincourt, 2013). More specifically, induction of ovulation with pLH followed by fixed-time insemination has been shown not to impact fertility by our research group (unpublished) and others (Cassar et al., 2005). Further study is required to determine if indeed pLH treatment in gilts has perturbing effects on embryonic quality.

Table 7-1 Primers used in the QRT-PCR analysis

Gene Symbol	Description	Associated Porcine Sequence Accession No.	Primer	Primer Sequence (5'-3')	PCR product size
ASNS	Asparagine synthetase (glutamine-hydrolyzing)	NM_001167640	Forward	GCCTTTATTTTACTGGATACTGCC	150 bp
			Reverse	CATGGAGTGCTTCAAGTTAACG	
DPP4	Dipeptidyl-peptidase 4	NM_214257	Forward	TGCGGATTCCATACCCAAAG	137 bp
			Reverse	ATCCCCTATTAACACAGACGC	
GNPDA1	Glucosamine-6-phosphate deaminase 1	NM_001244093	Forward	CCTGGAGCTGAAAGTGAAAAC	141 bp
			Reverse	GGCTAGTCGCTGTATGGTTTC	
RGN	Regucalcin (senescence marker protein-30)	NM_001077220	Forward	TGCTTTGGAGGGAAGGATTAC	120 bp
			Reverse	CCCCAGGCCAGTTATCTT	
SLC41A1	Solute carrier family 41, member 1	NM_001243667	Forward	TCA GAC CCC AAC TTT GCA G	141 bp
			Reverse	GAC TTG CTC AGA GTT CTC CC	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AF017079	Forward	GTGAAGGTCGGAGTGAACGGATTT	191 bp
			Reverse	AGCTTCCCATTCTCAGCCTTGACT	
ACTB	Actin, beta	Control primer Xeno™ from SYBR® Green Cells-to-CT™ Control Kit (Ambion)			135 bp
Xeno™	Xeno™ artifical synthetic RNA Control	Control primer Xeno™ from SYBR® Green Cells-to-CT™ Control Kit (Ambion)			105 bp

Table 7-2 Differentially expressed genes between blastocyst generated from pLH-induced and control gilts

Probe Name on EMPV1	Gene Symbol	Description	Differential Expression (pLH Vs. Con)*	P-value
EMPV1_20059	CHAC1	ChaC, cation transport regulator homolog 1	2.35	3.07E-02
EMPV1_17330	ASNS	Asparagine synthetase (glutamine-hydrolyzing)	1.58	1.67E-02
EMPV1_38049	MPEG1	Macrophage-expressed gene 1 protein-like	1.22	3.71E-03
EMPV1_08235	MSMO1	Methylsterol monooxygenase 1	1.16	3.12E-03
EMPV1_17154	RGN	Regucalcin-like, transcript variant 1	1.12	3.40E-05
EMPV1_24958	DPP4	Dipeptidyl-peptidase 4	1.10	5.36E-03
EMPV1_22948	GNPDA1	Glucosamine-6-phosphate deaminase 1	1.08	8.63E-05
EMPV1_18633	SFN	Stratifin	1.06	1.87E-04
EMPV1_03685	IDI1	Isopentenyl-diphosphate delta isomerase 1	1.06	5.92E-04
EMPV1_36757	RN18S	18S ribosomal RNA	1.05	3.42E-03
EMPV1_17384	SLC41A1	Solute carrier family 41, member 1	-1.09	2.92E-04

*: Differential Expression (pLH Vs. Con)=log2(pLH/Con) , where pLH and Con are the expression levels of the particular gene in blastocysts generated from pLH-induced and Control gilts, respectively.

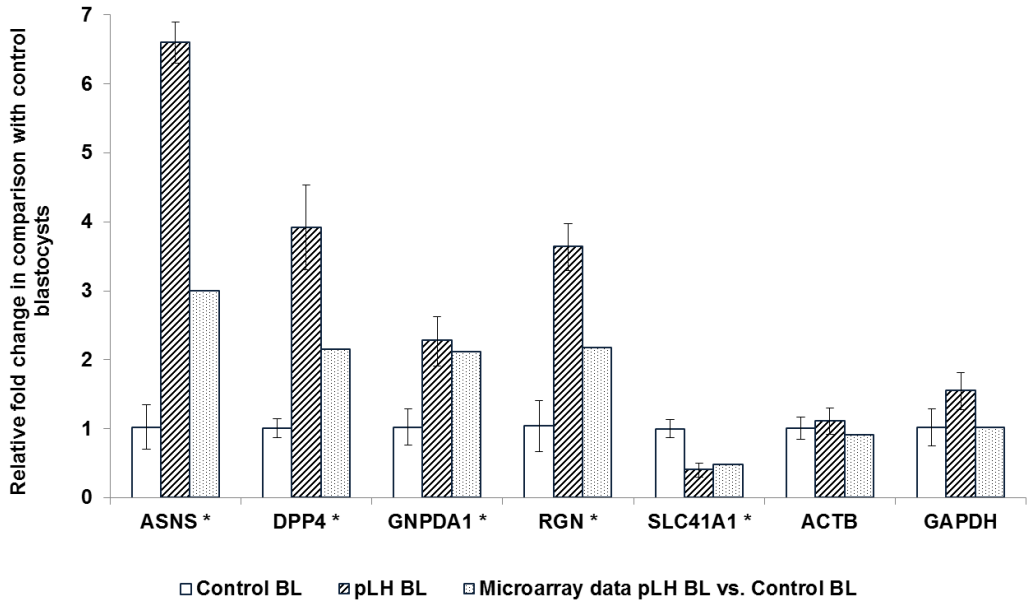

Figure 7-1 QRT-PCR verification results of seven selected genes (*ASNS*, *RGN*, *DPP4*, *GNPDA1*, *SLC41A1*, *ACTB*, and *GAPDH*).

Bar chart showing the relative gene expression level of the seven selected genes in pLH BL embryos (fold change in comparison with control BL). Genes marked with "*" are significantly differentially expressed (P-value<0.05) between BL generated from control and pLH treated gilts.

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Chapter 8: General discussion

In this chapter, the findings from chapter 3 to 7 are summarized and discussed in the context of existing literature. Final conclusions are drawn based on these combined findings. In addition, this chapter discusses the questions raised from the combined findings and potential future research.

The main drive behind the present research is the need to address factors that affecting the efficiencies of different ARTs in pig. Specifically, the studies described in the present research focused on: 1) Characterization of the "normal" transcriptome profile of early porcine embryos, thus providing a "healthy reference" for future transcriptomic studies in early porcine embryos. 2) Characterization of the gene/gene networks and pathways that exhibited altered gene expression in embryos generated after *in vitro* manipulations such as parthenogenetic activation (PA) and somatic cell chromatin transfer (CT), and identifying critical gene networks/pathways that are associated with the deficiencies observed these *in vitro* manipulated embryos. 3) Assessment of the effect of induced ovulation with porcine luteinizing hormone (pLH) on the transcriptome of early porcine embryos. 4) Identification of potential gene markers for the embryo quality assessment in early porcine embryos.

8.1 EMPV1 microarray as an effective platform for transcriptomic analysis of early porcine embryos

As discussed in chapter 5 there have been efforts to characterize the gene expression profile of the developing porcine embryo (Smith et al., 2001; Whitworth et al., 2004; Blomberg et al., 2005; Blomberg et al., 2008), but a full description of the complete transcriptomic profile during pre-implantation development in the pig has not yet been

established. The EMPV1 microarray platform is the only pre-implantation embryo-specific microarray platform currently available for porcine (Tsoi et al., 2012). In the studies described in chapters 3 and 4, the EMPV1 microarray was shown to have high reproducibility and high efficiency in early porcine embryo transcriptomic profiling studies. Data presented in chapter 3 demonstrated that the EMPV1 microarray has a high degree of across-arrays correlation coefficient ($r2 \geq 0.97$), and high degree of correlation ($r^2 = 0.97$) between the Cy3 and Cy5 signals. In addition, data presented in chapter 3 confirmed that the EMPV1 microarray is enriched with transcripts related to developmental process-associated genes. Data presented in chapter 4 showed that the EMPV1 microarray has higher coverage for the day 5 porcine blastocyst embryo transcriptome than two commonly used porcine microarray platforms evaluated (SOMV2 and PigOligoArray). The findings from the studies reported in chapter 3 and 4 suggest that the EMPV1 is an efficient microarray platform for the transcriptomic profiling analysis of early porcine embryos. This platform will provide the foundation for future research into the *in vivo* and *in vitro* factors that affect the viability of porcine embryos, as well as the downstream effects of such factors on the live offspring that result from these embryos. Hence, the EMPV1 microarray platform was utilized in the studies described in chapter 5, 6 and 7.

8.2 "Normal" gene expression profile of early porcine embryo

In chapter 5, the global gene expression profiling of porcine oocytes and embryos representing 9 different developmental stages from GV to D11 (Germinal vesicle (GV), MII, 2-cell (2C), 4-cell (4C), 8-cell (8C), morula(M), early blastocyst (EB), expanded blastocyst (XB), hatched blastocyst (HB) and embryonic day 11 hatched blastocyst before elongation (D11)) have been performed. As discussed in chapter 2, oocyte-derived

mRNAs are degraded shortly after fertilization in mammals; hence, embryonic genome activation (EGA) and production of embryo-derived transcripts must occur during early embryonic development (Thompson et al., 1998; Schultz, 2002). Data presented in chapter 5 suggests that the molecular events associated with EGA in porcine pig embryos is probably initiated at or before the 4-cell stage, and the second wave of EGA probably peaks around the early blastocyst stage.

The total number of positively detected genes and embryo-activated genes with different molecular functions exhibit different trends from GV to D11 stage. Transcription factors, as the name would suggest, regulate the transcription of their target genes, and it is not surprising that the number of "embryo-activated genes" with "transcription factor" activity displayed a similar trend as the total number of positively detected genes and "embryo activated genes" from 8C to D11 stage. This result supports previous findings that the embryo-activated genes "take-over" the majority of the mRNA profile from the 8-cell stage onward. In the trophectoderm, the Na^{+}-K^{+} ATPase pump is involved in formation and maintenance of the blastocoel cavity (Krisher and Prather, 2012). In the present study, the number of "embryo-activated genes" with "ion channel activity" increased during blastocyst formation, and then decreased after blastocyst hatching. This result supports the proposed role of these genes in the formation and maintenance of the blastocoel cavity. Further comparative transcriptomic analysis among embryos from six different embryonic stages (4C, 8C, MOR, EB, XB, and HB) revealed eight major patterns of gene expression changes from 4C to HB stage (see Figure 5-5 in chapter 5), with the genes exhibiting expression pattern 1 being the largest group. The genes exhibiting expression pattern 1 displayed significant up-regulation from 4C to 8C stage and were then down regulated and remained at the lower levels from MOR to HB stage, which were similar to their expression levels at the 4C stage. This result indicates that the

genes with pattern 1 expression are necessary for the embryonic development at the 8C stage. PANTHER over-representation analysis showed that the genes with the pattern 1 expression profile were enriched with nucleic acid (specifically RNA) binding, metabolic and splicing-associated gene. Our results from the global mRNA profiling analysis suggest that the embryonic-activated genes probably "take-over" the majority of the mRNA profile from 8C stage. Hence, the pattern1 genes associated with nucleic acid (specifically RNA) binding, metabolic and splicing probably are involved in the degradation of maternal mRNA stocks and the splicing of the embryonic mRNA produced from the EGA.

Comparative transcriptomic analysis among embryos from six different embryonic stages (4C, 8C, MOR, EB, XB, and HB) also revealed 24 upstream regulators (Table 5-3, chapter 5) that were predicted to be significantly activated or inhibited from the 4C to HB stages. Specifically, five of these transcription factors (MYCN, MYC, NRF1, HSF2, and PPARGC1A) were predicted to be activated in all the stages from 8C to HB, in comparison with 4C stage. Transcription factors MYC and MYCN play important roles in maintenance of pluripotency, self-renewal, and cell cycle control in embryonic stem (ES) cells (Rahl et al., 2010; Varlakhanova et al., 2010; Chappell et al., 2013). In addition, MYC plays a key role in the regulating the transcriptional elongation by RNA polymerase II (Pol II) in ES cells (Rahl et al., 2010). Data reported in chapter 5 shows that MYC and MYCN were predicted to be the most significantly activated transcription regulator in all embryonic stages from 8C to HB, in comparison with 4C stage. This result suggests that the MYC and MYCN may play important roles during early porcine embryonic development.

HSF2 is one of the major transactivators of heat shock protein genes in response to stress (Wang et al., 2003). HSF2 is considered to be involved in embryonic development, brain

development, and gametogenesis (Kallio et al., 2002; Wang et al., 2003). In our studies, HSF2 was predicted to be activated at the 8C stage and remain activated from the 8C to HB stages, which is consistent with previous finding in mice (Mezger et al., 1994).

The degradation and *de novo* synthesize mechanism of NRF2 is part of the cellular protection system that protects the cells against oxidative and electrophilic stresses, prevents apoptosis, and promotes cell survival (Kobayashi et al., 2006; Kaspar et al., 2009). NRF1 and NRF2 have overlapping functions with each other, mostly in the regulation of antioxidant genes, during early embryonic development (Leung et al., 2003; Motohashi and Yamamoto, 2004). In the study presented in chapter 5, the "NRF2-mediated oxidative stress response" pathway was significantly altered from the 8C to HB stages, in comparison with 4C stage. In addition, NRF1 is predicted to be significantly activated from the 8C to HB period. These results suggest that the NRF1 and NRF2 mediated cellular protection system against oxidative stress is activated from the 8C stage in pig embryos.

Although the embryo samples utilized in the study reported in chapter 5 contain a relatively complete set of embryos from different embryonic stages, the 2C stage embryos were not evaluated. Based on the findings in chapter 5, the 2C to 4C stage is a very important period for the determination of the exact timing of the molecular mechanism behind EGA process in porcine embryos. In addition, the 4C stage is a complicated embryonic developmental stage, which could be further classified into early 4C, 4C, and late 4C stages. Hence, further studies with porcine embryos samples from 2C, early 4C, 4C, and late 4C stages will help to determine the exact timing and identification of key regulators of the molecular mechanism behind EGA process in the pig.

8.3 Effect of somatic cell chromatin transfer (CT) and parthenogenetic activation (PA) on early porcine embryo transcriptome

As discussed in chapter 6, somatic cell nuclear transfer (SCNT) has great potential applications in basic and biomedical research. However, the application of SCNT is limited by a low embryonic survival rate and the high incidence of abnormalities in individuals that develop to term, which are believed to be associated the incorrect or incomplete nuclear reprogramming (Wang et al., 2011; Mesquita et al., 2013). Somatic cell chromatin transfer (CT) is a cloning technology that was designed to facilitate the reprogramming process (Sullivan et al., 2004; Rodriguez-Osorio et al., 2009). It involves *in vitro* remodeling of the donor nuclei prior to their transfer into enucleated oocytes to remove nuclear components that may interfere with nuclear remodeling (Sullivan et al., 2004). It is considered that the embryos generated from CT are more competent than embryos produced from traditional SCNT. Although promising results have been reported using chromatin transfer (CT), the embryos generated still exhibit abnormalities similar to those observed following conventional SCNT (Sullivan et al., 2004; Mesquita et al., 2013). In chapter 6, significant differential expression of 103 genes was observed in the CT HB embryos in comparison with *in vivo* HB stage embryos. This number was less than previously reported gene expression profile differences between SCNT and IVV-derived porcine blastocysts (Whitworth et al., 2011). This result supports the hypothesis that the gene expression profile of CT embryos are more similar to *in vivo* embryos and, therefore, are likely to be more competent than conventional SCNT embryos.

As discussed in chapter 6, embryos derived from parthenogenetic activation (PA) are valuable for studies on gene imprinting (Naturil-Alfonso et al., 2012) and are a potential alternative source of embryonic stem cells (Brevini and Gandolfi, 2008; Naturil-Alfonso et al., 2012). However, PA generated embryos are subject to severe development failure

(Hao et al., 2004). Several previous studies in different species have reported a large number of differentially expressed genes between PA and *in vivo* pre-implantation embryos (Whitworth et al., 2005; Liu et al., 2010; Naturil-Alfonso et al., 2012; Isom et al., 2013). In comparison with *in vivo* HB, comparative microarray analysis in chapter 6 revealed 1492 significantly differentially expressed genes in PA-derived HB, which is consistent with previous findings. In addition, approximately half of the differentially expressed genes in CT HB were also differentially expressed in PA HB and displayed the same direction of expression (up- or down-regulation) changes. As described in chapter 6, CT and PA embryos were generated following the same oocyte *in vitro* maturation (IVM) processes and embryo *in vitro* culture (IVC) conditions. Hence, the differential expression of these "commonly differentially expressed genes" in both PA and CT HB embryos probably are associated with these common *in vitro* manipulation processes. Although the gene expression profile of HB stage embryos derived from CT are more similar to their *in vivo* counterparts than the conventional SCNT embryos, significant differential expression of critical genes and gene networks were still observed in the CT HB. NOTCH is an important regulator of development in many animal species (Shepherd et al., 2009), and participates in many critical biological processes including cell fate specification, differentiation, proliferation, apoptosis, migration, and angiogenesis (Bolos et al., 2007). Small perturbations in NOTCH activity may lead to numerous developmental defects and diseases (Shepherd et al., 2009). Differential expression of three "notch signalling" pathway-associated genes (*HES1*, *PSEN2*, and *JAG1*) in CT HB was observed in the study reported in chapter 6. These "notch signalling" pathway-associated genes displayed significant and more dramatic differential expression in PA HB than CT HB. These results suggest that the "notch signalling" pathway is dysregulated in both PA and CT HB, and that this dysregulation of "notch signalling"

pathway in PA HB is more profound than in CT HB. The altered regulation in Notch signalling probably contributes to the impaired development of both PA and CT-derived embryos.

In addition, significant down-regulation of pluripotency regulator (*NANOG*) and TE development-associated genes (KRT18, GATA2) were also observed in both CT and PA HB, in comparison with *in vivo* HB. The altered regulation of these genes and gene networks probably contributes to the impaired development of CT and PA-derived embryos.

TP53 (tumor protein p53) is a well-known cell-cycle regulator and apoptosis mediator gene (Molchadsky et al., 2010). Data reported in chapter 6 showed that approximately one fourth (23 genes) of all the differentially expressed genes in CT HB were regulation targets of the transcription factor TP53. A total of 136 TP53 regulation target genes were differentially expressed in PA HB, which is more than 5 times of the differentially expressed TP53 targets in CT HB, in comparison with *in vivo* HB stage embryos. Further results from the IPA upstream regulator analysis suggest that the TP53 is significantly activated in both CT HB and PA HB. In addition, *ANXA8* (annexin A8) is a member of the annexin (ANXs) family, which is a group of Ca^{2+}-dependent phospholipid-binding proteins involved in many important biological processes including vesicle trafficking, calcium signalling, cell growth, cell cycle, and apoptosis (Hata et al., 2012). Over expression of *ANXA8* has been reported to be associated with cancer and apoptosis (Hammond et al., 2006). In our studies, *ANXA8* displayed significantly higher expression in PA HB than CT HB, and no detectable expression of *ANXA8* was observed in IVV HB. These results indicate an activated apoptosis process in both PA and CT derived HB, and the activation of this apoptosis process appears to be greater in PA HB than in CT HB.

Data reported in chapter 5 suggest that "eIF2 signalling", "mitochondrial dysfunction", "regulation of eIF4 and p70S6K signalling", "protein ubiquitination", and "mTOR signalling" pathways all displayed significant changes from 4C to HB stage *in vivo*, and these pathways are considered to play important roles during early porcine embryonic development. Data reported in chapter 6 showed that these pathways were the five most significantly changed canonical pathways in PA HB in comparison with IVV HB, and most of the differentially expressed genes associated with these pathways were down-regulated in PA HB (Figure 5-2). In addition, the transcription factor MYC and MYCN were predicted to be the most significantly activated transcription regulators in all embryonic stages from 8C to HB, in comparison with 4C stage. This result suggests that the MYC and MYCN may play important roles during early porcine embryonic development. On the other hand, the transcription factor MYC and MYCN were both predicted to be significantly inhibited in PA HB. In addition, significant down-regulation of pluripotency regulator (*NANOG*) and TE development-associated genes (KRT18, KRT8, and GATA2) were also observed in PA HB. All of these results suggest that porcine embryos derived from PA experience significant dysregulation in critical gene networks and regulators of early embryonic development

Data reported in chapter 6 showed that significant differential expression of 31 genes were observed during the "normal" blastocyst hatching process in IVV embryos. However, all of these 31 genes were not properly regulated in PA embryos during blastocyst hatching process including several critical pluripotency, trophoblast development, and implantation-associated genes (*NANOG*, *GATA2*, *KRT8*, *LGMN*, and *DPP4*). In addition, altered regulation of "notch signalling"-associated genes were also observed during the blastocyst hatching process in PA embryos. Failing in regulate the expression of these critical genes during the hatching process probably contributed to the

delayed and less efficient development of PA embryos and their reduced ability to hatch and undergo implantation.

In the studies described in chapter 6, only CT-derived HB and PA-derived XB and HB stage embryos were examined. Future studies with CT and PA-derived embryo samples from earlier or later embryonic developmental stages should help to identify the key factors that affect the competence of CT and PA embryos and the molecular mechanisms that are responsible for the deficiencies in CT and PA embryos.

8.4 Effect of hormone (pLH)-induced superovulation on early porcine embryo transcriptome

Control and synchronization of ovarian follicular development and induction of ovulation with exogenous hormones can provide practical advantages in livestock management and application of assisted reproductive technology (ART) such as embryo transfer (Degenstein et al., 2008). As outlined in chapter 7, previous studies with gilts have shown that induction of ovulation with porcine luteinizing hormone (pLH) followed by fixed-time artificial insemination can reliably synchronize the ovulation and shorten the period between artificial insemination (AI) and ovulation without detrimentally influencing pregnancy rate or litter size in cyclic gilts (Degenstein et al., 2008; Cameron et al., 2010). It is also found that the diameter of the largest follicles prior to ovulation was smaller in pLH-induced animals than control animals (Degenstein et al., 2008). As discussed in chapter 2 and chapter 6, embryos with molecular deviations may display similar morphological characteristics as "normal" embryos in pre-implantation stages (Nánássy et al., 2008; Rodriguez-Osorio et al., 2009). This has raised questions regarding the effect of this ovulation induction protocols on factors such as the quality of embryos and the health of offspring resulting from these procedures

Data reported in chapter 7 showed that pLH-induced superovulation in gilts has limited influence on the gene expression profile of blastocyst stage porcine embryos, since only 11 genes showed differential expressions between pLH and control blastocyst. This limited number of differentially expressed genes only accounts for 0.08% of all the genes detected in control and pLH BL embryos (13373 genes). However, several of the 11 differentially expressed genes in pLH BL are considered to play important roles during the early embryonic development such as ion, lipid, and amino acid homeostasis, trophoblast development, cell cycle, growth, and migration.

A review of the literature would indicate that the induction of ovulation in pigs does not generally affect key fertility parameters such as pregnancy rates and litter sizes (Draincourt, 2013). More specifically, induction of ovulation with pLH followed by fixed-time insemination has been shown to not affect fertility by our research group (unpublished) and others (Cassar et al., 2005).

8.5 Implications

During the pre-implantation period of embryonic development, the porcine embryo exhibits dramatic morphological changes and many key developmental events take place, such as cleavage, morula compaction, EGA, blastocyst formation, and hatching (Oestrup et al., 2009; Sirard, 2012). Although the morphological steps involved in these key developmental events are well documented, the molecular mechanisms underlying these events have not been fully understood. The “normal” gene expression profile of early porcine embryos that has been characterized in chapter 5 provides useful baseline information for the determination of the key regulators of the molecular mechanisms associated with important embryonic developmental events such as the EGA process, morula compaction, blastocyst formation, and hatching. In addition, this “normal” gene

expression profile of early porcine embryos can also be used as a "healthy reference" profile for future transcriptomic profiling analysis with early porcine embryos generated by a variety of different ART manipulations.

As discussed in the chapter 2, to date, morphological characteristics and blastocyst formation rates remain as two of the major parameters commonly used in embryonic developmental competence assessment (Lonergan, 2007). Findings from the study described in chapter 6 showed that embryos produced after *in vitro* ART manipulations such as PA and CT could develop into expanded blastocyst and hatched blastocyst stage, even with dysregulations in critical pathways and gene networks. Hence, the morphological criteria and blastocyst development rate alone are insufficient to determine the competence of embryos generated from ART (such as PA and CT). In addition, the dysregulated critical genes observed in CT and PA embryos could serve as potential candidate genes for the embryo quality marker gene selection and verification.

Real-Time Quantitative PCR (QRT-PCR) is widely utilized for quantifying mRNA and cDNA due to its sensitivity and repeatability (Jamnikar Ciglenečki et al., 2008), and is considered to be the "Gold standard" for verification of the expression level of genes of interest after transcriptomic analysis using microarrays or next generation sequencing platforms. Stable endogenous control genes are necessary for the reliable relative quantification analysis of QRT-PCR. However, differential expression of many commonly used "housekeeping" genes, such as *ACTB*, *GAPDH*, and *S18* were observed among *in vivo* embryos from different developmental stages during early embryonic development in chapter 4 and 5. In addition, findings reported in chapter 5, 6 and 7 showed that the expression levels of these commonly used "housekeeping" genes can also be affected by hormone (pLH) induction in gilts and different *in vitro* ART manipulations processes such as PA and CT. All these results suggest that the commonly used

"housekeeping" genes in somatic tissues probably are not suitable to be used as "housekeeping" genes in studies of early porcine embryos. As described in chapter 4 to 7, an equal amount of a synthetic RNA transcript was added to each reverse transcription (RT) reaction in this research to serve as an external reference gene for the QRT-PCR analysis, and as a positive control for reverse transcription, in order to assess variability resulting from any RT or PCR inhibitors. Results from the present research suggest that this strategy produced reliable QRT-PCR results with *in vivo* early porcine embryos from different embryonic stages and embryos produced after different treatments (such as PA, CT, and pLH induction in gilts). Hence, this strategy is probably a reliable option for QRT-PCR analysis in future studies with early porcine embryos.

8.6 Strengths and limitations of the present research

This section presents an overall summary of the strengths and limitations of the research described in this thesis. The microarray experiments described in chapter 5 to 7 were all performed with the EMPV1 microarray platform. In the studies described in chapter 3 and 4, the EMPV1 microarray platform was demonstrated to be an effective platform for early porcine embryo transcriptomic profiling analysis, and has better coverage of the early porcine transcriptome than several commonly used porcine microarray platforms. Although the EMPV1 has good coverage of the early porcine embryo transcriptome, as a gene expression microarray, its efficiency is still limited by the prior gene sequence knowledge of the porcine genome. Data presented in chapter 4 showed that after re-annotation of the EMPV1 probes with the most updated porcine genome database (Sus scrofa10.2 genome), approximately 75% of all the probes on the EMPV1 platform were characterized. Hence, there were still 25% of the probes included in the EMPV1 array that are without annotation, and the expression data of these 25% probes cannot be

utilized in further downstream analysis such as gene ontology analysis. More complete porcine genome mapping and annotation is necessary for the characterization of these 25% probes in the EMPV1 microarray. Unfortunately, the porcine genome mapping and annotation is an on-going process and remains incomplete at this moment.

The reference design used for microarray comparative transcriptomic analyses described in chapter 5 and 6, allowed for the reliable and flexible assessment of each comparison among all the groups examined in the analysis. The same reference aRNA pool generated from10 different oocytes and early embryonic stages (GV, MII, 2C, 4C, 8C, MOR, EB, XB, HB, and D11 HB) was used as the reference sample in all of these microarray comparative transcriptomic analyses. All experimental samples were labeled with Cy5 dye and compared with a Cy3 dye labeled reference aRNA pool. Hence, there was no dye effect among different experimental samples. This specific microarray experimental design and analysis strategy allows for the reliable and flexible analysis not only among different groups in the same analysis, but also among different treatment groups from different analyses. With the same experimental design and strategy, it is possible to compare freely among the transcriptomic profiling data reported in chapter 5 and 6 of this thesis and transcriptomic profiling data from future studies. As described in chapter 5, when applied to the EMPV1 platform, the reference aRNA pool produced reference signals (signals that were higher than the average signal of negative controls) for 95% of all the genes spotted on the microarray. However, because there were still 5% of all the genes spotted on EMPV1 microarray that were not covered by the reference aRNA pool sample, it was not possible for the expression data of these 5% genes to be carried forward to the downstream analyses.

The results reported in chapter 5 suggest that the 2C to 4C stage is a very critical period for the determination of the molecular mechanism behind EGA initiation process in

porcine embryos. In addition, as previously mentioned in chapter 5, the 4C stage is a complicated embryonic developmental stage, which could be further classified into early 4C, 4C, and late 4C stages. Although the embryo samples utilized in the study reported in chapter 5 contain a relatively complete set of embryos from different early embryonic stages, the 2C, early 4C, and late 4C stage embryos were not available. Hence, further studies with porcine embryos samples from 2C, early 4C, 4C, and late 4C stages will help to determine the molecular mechanism behind the initiation of EGA in pig.

In chapter 7, comparative transcriptomic profiling analysis was performed between blastocyst stage embryos produced from control and pLH- induced gilts. Although several of the differentially expressed genes in pLH BL are considered to play important roles during the early embryonic development, it is not conclusive whether the pLH-induced ovulation has effect on the embryonic quality of early porcine embryos. Hence, further studies with embryos from later embryonic stages and placentas from pLH-induced gilts would assist in determining if indeed pLH treatment in gilts had perturbing effects on embryonic quality.

8.7 Future studies

Although the results from the present research have proven that the EMPV1 microarray platform is an effective tool for the transcriptomic profiling studies with early porcine embryos, the EMPV1 array does not have full coverage of the complete transcriptome of early porcine embryos. Hence, future transcriptomic profiling studies with different stages of early porcine embryos produced from different *in vivo* and *in vitro* sources using NGS technologies could help to identify more new (or unique) transcripts that expressed in porcine embryos, generate a more detailed and complete transcriptomic profile of early porcine embryos. NGS analyses could also help to characterize the complete

transcriptomic changes that result from different ART technologies. In addition, it would be interesting to separate the male and female embryos during the comparative transcriptomic profiling analyses and characterize their different transcriptomic profile changes in response to different environmental stresses and ART manipulations.

Research presented in chapter 5 suggesting that the lipid metabolism associated gene networks, including the transcription factor *SREBF1* and *SREBF2*, were playing important roles during the hatching process of porcine blastocysts. In addition, the research presented in chapter 5 identified several lipid metabolism associated transcription factors (such as *PPARGC1A* and *PPARGC1B*) that playing important roles during *in vivo* early porcine embryonic development. Further studies of these lipid metabolism associated transcription factors and their key regulation targets in both mRNA and proteins levels with embryos from different developmental stages, along with the detailed studies on the amount of different lipid contents in each embryonic stages, could help to characterize their function during the early embryonic development in porcine. In addition, these studies could also help to identify the roles that different lipid contents storage in porcine embryos were playing during the early porcine embryonic development.

The research presented in chapter 5 suggested that the "oxidative stress responses"-associated genes (including the transcription factors *NRF1* and *NRF2*) were significantly regulated during the *in vivo* early porcine embryonic development. It is possible that the regulation of these oxidative stress responses associated genes is a self-protection mechanism of the embryos to cope with environmental changes (such as the environment changes from oviduct to uterus). Studies of these "oxidative stress responses"-associated genes in both mRNA and protein levels with embryos from different developmental stages, along with "knock in" and/or "knock out" studies of *NRF1*, *NRF2*, and the key

regulation targets of *NRF1* and *NRF2* under in vitro systems, will help to characterize their detailed functions during the porcine early embryonic development.

Although the research presented in chapter 7 identified differential expression of several critical embryonic developmental-associated gene in blastocysts produced from PLH-induced gilts, previous studies performed by our research group (unpublished) and others (Cassar et al., 2005) suggest that the PLH-induction does not have significant effect on the fertility of gilts. Hence, further studies of embryos from later stages and embryos produced from the next generations of PLH-induce animals will be necessary to determine if the PLH-induction have any effect on embryo qualities and the fertility of the offspring.

8.8 Overall conclusions

Overall, the results from the present research suggest that the molecular events associated with EGA in porcine pig embryos is probably initiated at, or before, the 4-cell stage, the embryo-activated genes "take-over" the majority of the mRNA profile from 8-cell stage, and the second wave of EGA probably peaks around the early blastocyst stage. In addition, embryos produced from PA and CT could develop into expanded blastocyst and hatched blastocyst stage, even with dysregulations in critical pathways and gene networks. Hence, the morphological criteria and blastocyst development ratio are insufficient to determine the competence of embryos generated from ART (such as PA and CT). The dysregulated critical genes observed in CT and PA embryos could serve as potential candidate genes for the embryonic competence gene markers selection and verification.

8.9 References

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Appendices A: Animal ethics statement

All animal studies were conducted in accordance with the Canadian Council on Animal Care (CCAC) Guidelines and Policies with approval from the Animal Care and Use Committee: (Livestock) for the University of Alberta (Permit Number: DYCK-2006-56).