Integrated analysis of miRNA/mRNA network in placenta identifies key factors associated with labor onset of Large White and Qingping sows

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Labour onset is a very complex physiological process, and its mechanism is poorly understood. Here, we obtained the mRNA and miRNA expression profiles from the placentas of four groups of sows: Qingping sows 112 days after insemination with signs of labour onset (QS), Qingping sows 114 days after insemination with signs of labour onset (QL), Large White sows 114 days after insemination with signs of labour onset (LL) and Large White sows 112 days after insemination without signs of labour onset (LN). A set of differentially expressed genes, including 2164 mRNAs and 39 miRNAs, were found. A DAVID analysis of these differentially expressed genes revealed their critical roles in response to hormone stimulus, immune response. Cytoscape Network analysis of the functional genes found node mRNAs and that the regulatory network between the node mRNAs and miRNAs was established. A comparison of the sequencing data from the shorter gestation period (QS) and the normal gestation period (QL) indicated that these genes were responsible for the quicker and more sensitive reaction to the regulation of labour onset. This research not only detected the key factors that were involved in labour onset but also provided useful information for the research of gynaecological diseases.

Delivery indicates the end of the gestation period. Within a species, the duration of pregnancy always remains within a definite narrow range1, but the mechanism of labour onset and regulation remains obscure. Many factors are involved in the onset of labour, such as various hormones and inflammatory mediators during labour, which remain a dominant research topic in this field2–6. Progesterone is one of the most important hormones for sow pregnancy maintenance7, and the theory of Progesterone Withdrawal is the most popular yet proposed. The increase in prostaglandin (PG) reduces progesterone before term, followed by the initiation of labour8. Inflammatory cytokines and chemokines in the placenta have been extensively investigated in the context of normal and abnormal labours. These inflammatory cytokines regulate the release of uterotonics, such as prostaglandins9. It is urgent both to identify...
sensitive and specific genes and miRNAs that are associated with labour and to investigate the mechanism underlying labour.

As a compound tissue, the placenta, which consists of amnion, chorion and decidua, performs the functions of exchange for oxygen, nutrients, antibodies, hormones, and waste products between the mother and foetus and may carry valuable information about pregnancy. Pigs have a non-invasive placenta that is classified as diffuse, and almost the entire surface of the allantochorion is involved in the formation of the placenta.

MicroRNAs (miRNAs) are a family of approximately 22 nucleotide small RNA molecules that play important roles in regulating post-transcriptional translation by binding to the target mRNAs’ 3′-untranslated regions. miRNAs are involved in the maintenance of the myometrial quiescence and the timing of labour among hormonal, inflammatory and physical factors. Recent evidence suggests that pregnancy and labour in the myometrium, cervix and foetal membranes are influenced by miRNAs, including miR-200-12, miR-199a-3p, miR-214-14, miR-223, miR-34b, miR-34c15, miR-33816 and miR-22317. These studies indicate that miRNAs play key collaborative roles in the hormonal control of myometrial quiescence and contractility during pregnancy and labour. A comprehensive network of miRNA/mRNA expression profiles, which compares the quiescent pregnant mouse myometrium with the contractile one, has been established12. However, a comprehensive miRNA/mRNA regulatory network associated with pregnancy and labour in the placenta has not been constructed due to a lack of simultaneously profiled miRNAs and mRNAs across different pig breeds at labour. Pigs are closer to people in size, anatomy and physiology; therefore, pigs are considered an ideal model for human-related diseases. An increasing number of miRNAs have been identified from swine tissues, such as muscle and adipose tissue18–21, but fewer have been identified from the porcine placenta. Qingping pig is a Chinese local pig breed with black fur and has a shorter gestation length, but the mechanism of this trait remains to be elucidated.

In the present study, we performed mRNA and miRNA profiling using Qingping and Large White placenta before and at labour onset and then constructed an interaction network of functional genes and an miRNA/mRNA regulatory network. The Database for the Annotation, Visualisation, and Integrated Discovery (DAVID) analysis of differentially expressed genes (DEGs) among LL, QL, QS and LN revealed a set of genes that are related to internal secretion, the extracellular matrix, the inflammatory response, the immune response and cellular apoptosis. The functional interaction network analysis of the DEGs indicated that FOS, APOE, MMP1, SMAD3, HSPA8, CXCR4 and PARK2 were the key node genes; then, the regulatory network between the node mRNAs and miRNAs was established. Moreover, a DAVID analysis of miRNA target DEG analysis highlighted the critical roles of five overlapping miRNA signatures (ssc-miR-132, ssc-miR-323, ssc-miR-19a, ssc-miR-664-3p and ssc-miR-296-5p) in the extracellular matrix, response to hormone stimulus, positive regulation of cell adhesion, focal adhesion, positive regulation of immune system process, and arachidonic acid metabolism. Additionally, a comparison of the sequencing data from Qingping pigs with a shorter gestation period of 112 days (QS) and a normal gestation period of 114 days (QL) revealed that the genes that are associated with immunity, signal peptide, and cell adhesion were up-regulated in QS pigs, which resulted in a quicker and more sensitive regulation of labour onset. These results have expanded the number of porcine miRNAs and suggest that pigs can be used as a model for human childbirth initiation to establish the biological basis for studying the role of miRNAs in regulating labour onset.

Results
mRNA and miRNA expression profiles in the placenta. To examine the genes that are related to the onset of labour, we determined the gene expression profiles in the placentas of LL, QL, QS and LN using Illumina HiSeq 2000. Compared with those in LN, among the 930 differentially expressed genes (DEGs) in LL (P ≤ 0.01, fold change ≤ 0.5 or fold change ≥ 2), 395 genes were up-regulated and 535 were down-regulated (Supplementary Table S1); among the 1591 DEGs in QL (P ≤ 0.01; fold change ≤ 0.5 or fold change ≥ 2), 690 and 901 genes were up- and down-regulated, respectively (Supplementary Table S2); and among the 1074 DEGs in QS (P ≤ 0.01; fold change ≤ 0.5 or fold change ≥ 2), 503 genes were up-regulated and 571 were down-regulated (Supplementary Table S3). Compared with the LN placenta, 9 up- and 10 down-regulated miRNAs were found in the LL placenta (P ≤ 0.01; fold change ≤ 0.5 or fold change ≥ 2), 13 up- and 10 down-regulated miRNAs were found in the QL placenta (P ≤ 0.01; fold change ≤ 0.5 or fold change ≥ 2), and 14 up- and 7 down-regulated miRNAs were found in the QS placenta (P ≤ 0.01; fold change ≤ 0.5 or fold change ≥ 2) (Supplementary Table S4). The unified set of differentially expressed genes containing a total of 2164 mRNAs and 36 miRNAs was found by the comparison of LL, QL and QS with LN. Remarkably, 182 genes were overlapping among the LL, QL and QS, 76 of which were up-regulated and 106 were down-regulated. In addition, 2 (ssc-miR-132, ssc-miR-323) and 3 (ssc-miR-19a, ssc-miR-664-3p and ssc-miR-296-5p) miRNAs were commonly detected in all of the regulated groups across the comparisons of LL, QL, and QS with LN. To validate the reliability of the mRNA and miRNA profiling results, RT-PCR was performed to detect the levels of 13 randomly selected differential mRNAs and 7 miRNAs from the differential expression data using the primer sequences listed in Supplementary Table S5. The RT-PCR results showed that most of the miRNAs were consistent with the miRNAs profiling results, except for miR-22 (Fig. 1). The RT-PCR results also showed that mRNAs agreed most with the mRNA profiling results (Table 1).
The Venn diagrams of the differentially expressed genes of the subgroups are presented in Fig. 2A (mRNAs) and 2B (miRNAs). Specifically, overlapping genes among the three groups accounted for 54%, 54% and 66% of the LL, QL, and QS mRNA diff-genes, respectively, which suggests the internal relationships of the subgroups in the number of genes within the unified diff-gene set. Additionally, 58%, 70% and 68% of LL, QL and QS miRNA diff-genes were overlapping with one another.

The hierarchical clustering results revealed that the subgroups could be well differentiated from our unified set of differentially expressed mRNAs, except for QS1 (Fig. 3A), which suggests significant differences among subgroups and the good generality of our diff-genes, though they were identified from relatively small datasets. miRNA diff-genes performed less accurately than did mRNA diff-genes (Fig. 3B).

Because the miRNA and mRNA data shared the same sample set, this difference was likely due to the complexity of the conversion from miRNA expression to the phenotypic differences among different pig subgroups, which is mediated and influenced by many factors, such as posttranscriptional regulation and generally a large number of varieties of miRNA gene targets. Additionally, the difference could also be attributed to the effects of the small sample size.

Functional analysis of differentially expressed genes. To study the DEmRNA functions, we performed a GO analysis of LL, QL, QS and overlapping DEGs. The top 10 GO terms are shown in Fig. 4.

Table 1. Quantitative RT-PCR validation of differentially expressed genes. qRT-PCR-based validation of GGTA1, LIPG, CXCL14, APOE, RERG, HSPA4, IGFBP2, TNFRSF12A, RAMP1, HBB, HSP70, SERPINA3-2, MMP1, CYP2C36 and PGHS-2 expression levels in LN, LL, QS and QL.
“response to oestrogen stimulus” and “response to steroid hormone stimulus”, demonstrating the key roles of various hormones in the onset of labour, which is consistent with the previous theory (8,22). In addition, some representative functional terms are involved in immune function, such as “immune response”, “defence response”, and “inflammatory response”, and some enriched terms are connected with the triglyceride metabolic process, cytokine activity and regulation of cell growth. Cyclooxygenase 2 (COX2), also known as prostaglandin G/H synthase 2 (PGHS-2), is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs). According to the RNA-seq data, there is a 4-fold expression in LL compared with that in LN, and the high expression of COX2 leads to an increase in PGs. The relative quantitative expression of COX2 is significantly higher in LL than that in LN, even over 8-fold for amnion (Fig. 5A), which was confirmed by immunohistochemistry (Fig. 5C).

To identify the commonly influenced mechanisms of the miRNAs and mRNAs, miRNA putative targets and DEGs were analysed using DAVID bioinformatics resources according to GO terms. Because miRNAs tend to downregulate target mRNAs, the expression of a genuine target mRNA is expected to be anticorrelated with miRNA expression. As a result, a total of 161, 189 and 340 inversely correlated genes were obtained by a comparison of LL, QS and QL with LN (Supplementary Table S7) and were defined as DEmiRNA targets. To further understand the miRNA regulatory function of labour onset in different pig breeds, five overlapping DEmiRNAs (ssc-miR-132, ssc-miR-323, ssc-miR-19a, ssc-miR-664-3p and ssc-miR-296-5p) were used for the gene ontology and pathway analyses in the DAVID software. For the overlapping DEmiRNA target genes, the annotation cluster with the highest score was involved in hormone stimulus, including “response to organic substance”, “response to endogenous stimulus”, “response to steroid hormone stimulus” and cell adhesion. The KEGG pathway analysis showed that most enriched pathways are associated with the inflammation response and hormone metabolism, such as “MAPK signalling pathway”, “p53 signalling pathway”, “cytokine-cytokine receptor interaction”, “androgen and oestrogen metabolism”, and “steroid hormone biosynthesis”. In addition, apoptosis and the adherens junction pathway are enriched. Interestingly, the overlapping DEmiRNA target-enriched GO terms and KEGG pathways were similar to the results that were obtained from the differentially expressed gene profile, indicating that the miRNA regulation of gene targets may have a tremendous effect on the overall gene signature in terms of hormone stimulus and immune response to labour onset.

**Network analysis of differentially expressed genes.** The Reactome plug-in in the Cytoscape software was used to visualise the functional interaction (FI) network of DEGs and to further identify major functional genes. The FI network of DEGs is shown in Fig. 6. A number of differentially expressed genes, such as FOS, SMAD3, HSPA8, CXCR4, EGR1, HNPF4, CTNNA2, APOE and PARK2, were found to be key node genes. Then, the network was constructed using the enriched genes that are involved in hormone stimulus and the immune response (Supplementary Fig. S1-2), with FOS, SMAD3, HNPF4, HSPA8 and CXCR4 as the central node genes. The regulatory direction of these node genes was also checked in QS and QL, where FOS and HNPF4 were altered in the same direction in QL, LL and QS and SMAD3 was altered in the same direction in LL and QS. SMAD3 proteins are signal transducers and transcriptional regulators that mediate multiple signalling pathways. The HSPA8 gene encodes a member of the heat shock protein 70 family, which can activate the MAPK signalling pathway. FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. The relative quantitative expression of the FOS gene was tested by qPCR and showed a significant difference
Figure 3. The mRNA and miRNA expression patterns of a unified set of differentially expressed genes and miRNAs. (A) Hierarchical clustering of a unified set of differentially expressed genes. (B) Hierarchical clustering of a unified set of differentially expressed miRNAs.

Figure 4. The Top 10 GO (biological process) Term analyses of DEGs of LL, QL, and QS, compared with LN placenta and overlapping.
Figure 5. COX-2 and FOS expression in the amnion, chorion and decidua of LL and LN. (A) COX-2 mRNA level in the amnion, chorion and decidua of LL and LN as determined by qRT-PCR. (B) The expression levels of FOS in the amnion, chorion and decidua of LL and LN were detected by qRT-PCR. (C) COX-2 immunohistochemical results in the amnion, chorion and decidua of LL and LN. Original magnification, 400×. (D) FOS immunohistochemical result in the amnion, chorion and decidua of LL and LN. Original magnification, 400×. All of the error bars indicate SD. *P < 0.05, **P < 0.01.
between LN and LL in the chorion and decidua tissue (Fig. 5B). The relatively high expression in LL was also confirmed by immunohistochemistry (Fig. 5D).

Construction of the miRNA/mRNA network. To further elucidate the interactive relationship between the node mRNAs and miRNAs, we searched for potential regulating miRNAs. The inversely correlated miRNAs were identified by comparing the miRNAs from target prediction with those from miRNA expression profiling, and miRNA/mRNA networks were constructed using the Cytoscape software. For further research, each miRNA/mRNA pair was scored to examine the target relationship and negative correlation of miRNA/mRNA networks (Fig. 7), and we found that miR-345-5p and miR-326 correlate with targets HNF4A, FOS and SMAD3, whereas miR-193a-3p, miR-664-3p and miR-122 correlate with target SMAD3. These results indicate that miRNA/mRNA networks indirectly reflect the expression patterns of node mRNAs and miRNAs and that understanding the structure of the miRNA/mRNA networks could provide more insight into labour onset.

Identify the essential factors that are associated with a shorter gestation period. The essential factors that are associated with a shorter gestation period were identified by a functional classification of the 544 DEGs from QS/QL (P ≤ 0.01; fold change ≤ 0.5 or fold change ≥ 2). Among the 544
DEGs, 307 genes were up-regulated, and 237 were down-regulated (Supplementary Table S6). These DEGs were analysed using DAVID bioinformatics resources according to GO terms. The up-regulated DEGs derived from QS/QL are enriched in immune-related terms. For example, CRP, CCL21, C5, ESR2, PENK, REG3G and SCG2 are genes that are involved in the initiation of the inflammatory response and in the effective stage of the inflammatory response. The level of immunoglobulin (lgG) in Qingping pig is remarkably higher than that detected in other species\(^\text{23}\). The expression of selected genes, including PTPRD, NTRK2 and PTPRS, is enriched in the term “immunoglobulin”.

**Discussion**

To our knowledge, this is the first comprehensive report about the miRNA and mRNA signatures in the placenta, and the overall findings in the study will facilitate our understanding of the molecular events underlying the transition of the placenta from a refractory to a contractile state, the roles of microRNAs, their targets, and their transcriptional and hormonal regulation.

Transcriptome analysis of sows’ placentas. Generally, the transcriptome includes all of the RNA transcripts in the cell. The transcriptome reflects the genes and sRNAs that are actively expressed at any given time. The placental RNAs and sRNAs of sows before labour onset and during labour were sequenced in a novel way. The original sequencing images were converted into raw reads by base calling, and clean reads were then obtained from the raw reads. In the present study, 12 libraries of miRNAs and mRNAs were created from high-throughput sequencing data, and the molecular mechanisms and signalling pathways controlling the onset of labour were investigated by analysing the transcriptome sequencing data of sows’ placentas, which, in theory, could be a powerful tool for explaining the pivotal factors that are involved in the shorter gestation period of Qingping sows and could be considered the models of choice for research into human obstetrics. However, why Qingping sows have the unique characteristic of a shorter gestation period remains to be further elucidated and could provide new clues for preterm delivery.

An alteration in the hormone level is associated with the onset of labour. Hormone metabolism plays an essential role in the initiation of labour. Because of its relaxation effect, progesterone is considered one of the primary hormones that are responsible for the maintenance of pregnancy\(^\text{7}\). It has been widely accepted that progesterone withdrawal caused by the regression of the corpus luteum through a high level of prostaglandins is associated with the initiation of labour. The GO and KEGG pathway analyses of the DEGs and DEMiRNAs provided substantial evidence that DEGs and DEMiRNAs can alter the hormone level associated with the onset of labour. Numerous genes, including CPS1, CA2, CFTR, LDLR, OXTR and FOS, are enriched in the DAVID term of hormone stimulus. In LL, the expressions of COX2, IL-1\(\beta\), IL-6 and IL-8 are markedly increased. It is noteworthy that the type-2 cyclo-oxygenase enzyme is the expression product of COX2, which suggests that the up-regulation of COX2 corresponds to the increasing concentration of PG. TNF-\(\alpha\) and TGF-\(\beta\), which are both highly expressed in the placenta, regulate the secretion of hormones as related to labour\(^\text{9}\). Arachidonic acid is the precursor to assemble PGs and is inextricably intertwined with the initiation of labour. The DEGs of CBR3, CYP2E1, GPX3 and GPX5 are enriched in the function of arachidonic acid metabolism. As previously reported, PGF2\(\alpha\) induces P450c17 expression in the corpus luteum\(^\text{24}\). Because P450c17 stimulates the transformation from progesterone to androstenedione, increased P450c17 prompts the synthesis of oestrogen followed by the initiation of labour. Obviously, the transformation from progesterone to androstenedione leads to a reduction in the progesterone concentration in the serum\(^\text{25}\), which has an inductive effect on the initiation of labour. In previous studies, miR-122, miR-132, miR-137 and miR-151-5p have been shown to affect progesterone production\(^\text{26}\), and P4 could regulate the expression of DEMiRNAs, such as miR-345-5p and miR-193a-3p\(^\text{27}\). miR-19a and miR-19b are influenced by E2\(^\text{28}\). Our results show that a
A key molecule that mediates TGF-beta activity, which leads to inflammation and apoptosis. Two theo-
mopathies have been suggested regarding the initiation of the apoptotic mechanism: the TNF-induced (tumour
actor interaction" , also indicate their immune function in placenta, which strengthens in the early stage of
process of labour, resulting in a shorter gestation period compared with that of other species. Therefore,
shorter gestation period can be partly explained by the stronger immune system, which is well supported
by the fact that Qingping pigs are less vulnerable to various diseases in practice. The stronger immune
system intensifies the immune response that is linked to the initiation of labour and accelerates the initial
process of labour, resulting in a shorter gestation period compared with that of other species. Therefore,
more effective functions in the immune response are crucial factors that are associated with the shorter
gestation period of Qingping sows.

One decade ago, the shorter gestation period of the Qingping sow was considered one of its phys-
ological characteristics; we can now reveal the internal factors at the molecular level. From this point
of view, these factors that are associated with shorter gestation period can serve as hints for the patho-
genesis of preterm labour in humans when the inflammation or immune response play a leading role in
preterm labour. Accordingly, a stronger inflammation or immune response can increase susceptibility
to preterm labour.

Immunity is involved in the process of labour onset. During pregnancy, the function of maternal
immunity changes within the uterus, where innate, pro-inflammatory immune responses are advanta-
geously regulated to prevent immunological resistance of foetal allograft. Immunity is related to the ini-
tiation of term and preterm labour. The destruction of the balance of cytokines by bacteria or other
factors increases the production of pro-inflammatory cytokines at the placenta and activates the process
of delivery. In the present study, a number of immune-related differentially expressed genes are identi-
fied, and among them, interleukin gene family (IL) and chemokine (CCL, CXCR, CXCL) are differentially
expressed. Tissue macrophages synthesise neutrophil chemoattractants CXCL1/CXCL2 in response to
inflammatory stimulation, with CXCL1/CXCL2 neutrophil recruitment as an important early step in
controlling tissue infections or injury. Inflammatory cytokines may affect the activity of receptors in the
placenta and uterus, which results in prenatal environment reversion. As a critical factor mediating the
process of immunity and inflammation, TGF-β induces the combination of FOS and JUN to form the
protein complex and exerts its influence on proliferation, differentiation and apoptosis. Interestingly,
many immune- and inflammatory-related DEGs and DEmiRNAs have a high expression. The enriched
GO term of genes, such as immune response, immune cell activation and immunoglobulin, and KEGG
pathways, such as "MAPK signalling pathway", "p53 signalling pathway", and "cytokine-cytokine recep-
tor interaction"; also indicate their immune function in placenta, which strengthens in the early stage of
labour onset.

Cell adhesion molecules (CAMs) are proteins that are located on the cell surface and bind either
with other cells or with the extracellular matrix in a process called cell adhesion. Several cell adhesion-related genes are up-regulated in both LN and LL, including cadherins (CDH20, CDHR4, PCDH19, and CDHB16) and selectins (SELL). In fact, CAMs are signals in the inflammatory response. DEmiRNA targets are also enriched in the adherent junction pathway and cytokine-cytokine receptor interaction. Pro-inflammatory cytokines, such as TNF-α and IL6, are released by neutrophils and macrophages to induce the expression of adhesion molecules and to recruit leukocytes. Simply put, CAMs induce labour onset, through which the inflammatory response is mediated.

Apoptosis is related to the onset of labour. Apoptosis is the process of programmed cell death and
may occur in multicellular organisms. This process is regulated by conserved genes, such as Bcl-2 family,
caspase family, oncogene C-myc and anti-oncogene P53, some of which were detected as DEGs in LN/LL,
with the BCL2-associated athanogene 3 (BAG3) and FOS genes being down-regulated. Moreover, BAG3,
SMAD3, CXCR4, NR4A2 and TNFRSF12A are enriched in the term "regulation of cell death". SMAD3 is
a key molecule that mediates TGF-beta activity, which leads to inflammation and apoptosis. Two theo-
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34. Inflammatory cytokines may affect the activity of receptors in the placenta and uterus, which results in prenatal environment reversion. As a critical factor mediating the process of immunity and inflammation, TGF-β induces the combination of FOS and JUN to form the protein complex and exerts its influence on proliferation, differentiation and apoptosis. Interestingly, many immune- and inflammatory-related DEGs and DEmiRNAs have a high expression. The enriched GO term of genes, such as immune response, immune cell activation and immunoglobulin, and KEGG pathways, such as "MAPK signalling pathway", "p53 signalling pathway", and "cytokine-cytokine receptor interaction"; also indicate their immune function in placenta, which strengthens in the early stage of labour onset.

Essential factors for the shorter gestation period. According to the DEGs and the specific functions
mentioned above, two distinct gene-expressing models have been developed from the two groups of
Qingping sows with different gestation periods. The up-regulated DEGs derived from QS/QL are enriched
in immune-related terms. For example, CRP, CCL21, C5, ESR2, PENK, REG3G and SCG2 are
genes that are involved in the initiation of inflammatory response and the effective stage of the inflamma-
tory response. Functional studies reveal that CRP, one of the marker genes of inflammation, is capable of
activating the NF-κB signalling pathway and stimulating the secretion of interleukin IL-6 and monocyte
chemoattractant protein (MCP-1), which lead to the activation of platelet and induction of apoptosis.

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Signalling during labour. The observation of the DEGs that are involved in isolated pathways and the gene functional interaction network indicate that the synergic interaction of these genes plays a vital role in the efficiency of their functions. When the maternal nutrient provision can hardly meet the foetus’ requirements, which occurs with the development of the foetus during the latter gestation period, the stress response is triggered, which in turn leads to the secretion of signalling factors, such as “stress” hormone and chemotactic factors. Through blood circulation, signalling factors are transported to the placenta, where some hormone receptors and unspecific receptors are located. The expressions of certain transcriptional factors and signalling transduction molecules are regulated when receptors are aware of the alteration in the concentration of signalling factors. The differential expression of FOS, SMAD3, HNF4a and TNF-α is observed between LN and LL, which is consistent with our RT-PCR results. The synthesis and release of TNF-α and TGF-β by foetal membranes at term gestation are regulated by several hormones that are potentially involved either in the maintenance of pregnancy or in the parturition process. Therefore, it can be concluded that inadequate maternal nutrient supply is likely to be the original signal that is responsible for the initiation of labour.

In conclusion, a significant number of commonly dysregulated mRNAs and a subset of commonly dysregulated miRNAs are obtained by comparing the mRNA and miRNA expression profiles of labour onset. These dysregulated mRNAs and miRNAs are involved in the response to hormone stimulus, immune response, and apoptosis and may thus represent good candidate targets for future research. The overall results of this study facilitate our understanding of the mechanism underlying labour onset by expanding the number of porcine miRNAs and establishing the biological basis for the study of miRNAs in regulating labour onset.

Materials and Methods

Animal and tissue collection. Four groups (n = 3) of multiparous sows with a parity of three were selected for this study. Group one consisted of Large White sows 112 days after insemination with no sign of labour (LN); group two was composed of Large White sows 114 days after insemination with distinct signs of labour and before the birth of the first cub (LL). In addition, two groups of Qingping sows 112 (QS) and 114 (QL) days after insemination with the same signs of LL were selected. After the sows described above were slaughtered, the amnion, chorion and decidua tissues were separated and collected from twelve sows. All of the studies involving animals were conducted according to the regulation (No. 5 proclamation of the Standing Committee of Hubei People’s Congress) approved by the Standing Committee of Hubei People’s Congress, P. R. China. The sample collection was approved by the Ethics Committee of Huazhong Agricultural University with the permit number No. 30700571 for this study. The animals were allowed access to feed and water ad libitum under the same normal conditions and were humanely sacrificed as necessary to ameliorate suffering. The methods were carried out in accordance with the approved guidelines.

RNA extraction. The total RNA was extracted from each of the three parts of the placenta using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s recommendations. The total RNA was treated with RQ1 DNase (Promega, USA) to remove DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A260/A280) using SmartSpec Plus (Bio-Rad, USA). The RNA integrity was further verified by 1.5% agarose gel electrophoresis. The total RNA from the amnion, chorion and decidua of the same sow was pooled equally.

mRNA sequencing and statistical analysis. For each of the twelve pooled samples, 10 μg of RNA was used for RNA-seq library preparation. Polyadenylated mRNAs were purified and concentrated with oligo(dT)-conjugated magnetic beads (Invitrogen, USA) before being used for directional RNA-seq library preparation. The purified mRNAs were iron-fragmented at 95°C, followed by end repair and 5′ adaptor ligation. Then, reverse transcription was performed with an RT primer harbouring a 3′ adaptor sequence and a randomised hexamer. The cDNAs were purified and amplified by PCR, followed by purifying and quantifying the PCR products corresponding to 200–500bp. For high-throughput sequencing, paired-end 100-bp sequencing of the cDNAs was performed on the Illumina HiSeq 2000 system (Illumina, USA). The obtained sequence reads (Fastq files) were checked by FastQC software. Clean reads with a minimum length of 16 nt were obtained by trimming the raw reads and deleting the low-quality reads, and then the clean reads were mapped to the porcine reference genome sequence (Susrofa10.2) with 2-nt mismatch using the TopHat software. We estimated the gene expression level using RNA-Seq by the reads per kilobase of genes per million mapped reads (RPKM). EdgeR59 was used to filter differentially expressed genes (DEGs). An integrated analysis of different functional databases was performed using the “functional annotation clustering” tool of the database for annotation, visualisation, and integrated discovery. For gene interaction network analysis, the Reactome plug-in in the Cytoscape software was used to visualise the general relationship between differentially expressed genes.

miRNA profiling and statistical analysis. A total of 3 μg of total RNA was used for small RNA cDNA library preparation with the Balancer NGS Library Preparation Kit for small/microRNA (GnomeGen) by following the manufacturer’s instructions. Briefly, RNAs were ligated to 3′ and 5′ adaptors sequentially, reverse transcribed to cDNA and then PCR amplified. The entire library was tested by 10% native PAGE
miRNA target predictions and DAVID analysis. Because the online target gene prediction software contains no pig genes and cannot predict miRNA potential target genes, we used the miranda software to find the DEMiRNA potential target genes in all of the pigs (+ CDS to the back of the exon were observed as 3′UTR; - to the CDS front of exon were observed as 3′UTR). KEGG pathway and GO Term analyses of these target genes were performed using DAVID bioinformatics resources.

mRNA and miRNA analysis by synthesis. To identify the functional regulations from miRNAs to mRNAs, we combined the computational target predictions at the sequence level and the inverse expression relationships between miRNAs and mRNAs in context. Specifically, functional regulations were detected using a subset of miRNAs and genes that was characterised by (i) both miRNAs and genes being associated with labour onset, (ii) a regulatory relationship according to computational predictions, and (iii) expression profiles were strongly negatively correlated. Then, the miRNA/miRNA regulatory network was constructed using the popular Cytoscape software, and KEGG pathway and GO Term analyses of the target genes in the miRNA/mRNA network were performed to explore the functional roles of miRNAs using the DAVID bioinformatics resources.

qRT-PCR analysis of miRNAs and mRNAs. RNAs were first reverse transcribed to cDNAs with the Thermo Scientific Maxima First Strand cDNA Synthesis Kit K1622 (Thermo, USA). Quantitative real-time PCR (qPCR) reactions were performed by Bio-RAD CFX Connect (Bio-RAD, USA) with twelve biological replicates, and each reaction was performed in triplicate. U6 RNA and β-actin were used as endogenous internal controls, and the relative expression levels were calculated with the 2−ΔΔCT method.

Immunohistochemistry. The three parts of the placenta were stored in 4% paraformaldehyde, dehydrated and embedded in paraffin. Then, 5-μm sections were cut on the microtome and placed onto clean, positively charged microscope slides, followed by heating the slides in a tissue-drying oven for 45 minutes at 60°C, washing them with xylene 3 times for 5 minutes and hydrating them separately in 95%, 70%, 50%, and 30% ethanol for 2 minutes. Incubation with the primary antibodies (Santa Cruz Biotechnology, FOS: sc-253, COX2: sc-1746) was performed at room temperature for 2 h. DAB was used for visualisation after incubation with the secondary antibody (IgG), and the sections were then counterstained with haematoxylin and mounted for microscopic examination.

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Acknowledgments
We are grateful to Dr. Hong Xiang and Yan Li for paper suggestion and edition, Professor Hanchang Zhu for help in language editing. The authors also acknowledge all the members of the laboratory for their help in sampling. This work was supported by the National Key Technology Support Program of 530 China (Grant number 2011BAD28B01 and 2013BAD20B06), Hi-Tech Research and Development Program of China (2013AA102502), Hubei Province Technology Support Program of China (Grant number 2013BBB14), Hubei Province Major Technology Innovation Program of China (Grant number 2014ABA025), Natural Science Foundation of Hubei Province of China (2015CFB267).

Author Contributions
Conceived and designed the experiments: R.Z., J.C., F.L., J.P. and S.J. Performed the experiments: H.L. and B.W. Analysed the data: H.L., B.W. and S.J. Contributed reagents/materials/analysis tools: H.L., B.W., J.G. and J.Z. Wrote the paper: H.L.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep
Competing financial interests: The authors declare no competing financial interests.
How to cite this article: Li, H. et al. Integrated analysis of miRNA/mRNA network in placenta identifies key factors associated with labor onset of Large White and Qingping sows. Sci. Rep. 5, 13074; doi: 10.1038/srep13074 (2015).
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