Journal of Reproduction and Development, Vol. 68, No 5, 2022

Original Article

Predicting of molecules mediating an interaction between bovine embryos and uterine epithelial cells

Tatsuo NOGUCHI[1)], Takeshi HAYASHI[2)], Yuki INOUE[1)], Shunsuke HARA[1)], Koumei SHIRASUNA[1)] and Hisataka IWATA[1)]

[1)]*Tokyo University of Agriculture, Kanagawa 243-0037, Japan*
[2)]*Fukuoka Agriculture and Forestry Research Center, Fukuoka 818-8549, Japan*

Abstract. Embryo-maternal reproductive tract interactions are pivotal for successful pregnancy. The present study predicted the molecules modulating embryo-uterine communication by comparing two sets of differentially expressed genes (DEGs): DEGs in uterine epithelial cells (UECs) collected from the uterus with and without blastocysts and DEGs between blastocysts developed *in vivo* and *in vitro*. Cows were subjected to super ovulation (SOV), followed by insemination or non-insemination at estrus (SOV + AI and SOV cows). Seven days after estrus, the uterus was flushed to collect UECs, and the presence of blastocysts in the uterus was confirmed. UECs were subjected to RNA-Sequencing (RNA-Seq) to identify DEGs. Publicly available RNA-Seq data of *in vivo* and *in vitro* developed bovine blastocysts were used to determine DEGs. Then, using ingenuity pathway analysis, activated- and inhibited-upstream regulators (USRs) for UECs in blastocysts were compared with those for blastocysts developed *in vivo*. RNA-Seq of UECs revealed that the DEGs were associated with immune response and cell adhesion pathways. The activated and inhibited USRs of UECs derived from SOV+ AI cows overlapped with the activated and inhibited USRs of blastocysts developed *in vivo*. Overlapping activated USRs include leukemia inhibitory factor, interleukin 6, fibroblast growth factor-2, transforming growth factor beta-1, and epidermal growth factor. In conclusion, the present study predicted the molecules that potentially mediate communication between the developing embryo and the uterus *in vivo* and prepare the uterus for pregnancy.

Key words: Blastocyst, Gene expression, Ingenuity pathway analysis, Upstream regulators, Uterine epithelial cells

(**J. Reprod. Dev. 68:** 318–323, 2022)

In mammals, oocytes are fertilized in the ampulla of the oviduct and descend to the uterus. In cows, day 5 embryos reach the uterine horn. However, embryo transfer is generally performed using day 7 embryos. Kimura *et al*. [1] reported that the transfer of day 13 embryos resulted in successful pregnancy, indicating that the interaction between early preimplantation embryos and the uterus is not an absolute condition for pregnancy. Molecules mediating early embryo development and conditions of the reproductive tract have not been extensively studied. Instead, interferon tau (IFNT), secreted from more advanced stage elongated embryos, has been the focus [2]. However, early developmental stage bovine embryos have been shown to secrete IFNT [3]. Microarrays of epithelial cells collected from the oviduct, including eight-cell stage embryos and unfertilized oocytes, revealed that the presence of bovine eight-cell stage embryos altered the gene expression profiles of oviductal epithelial cells [4]. The presence of blastocysts in the uterus alters the gene expression in the epithelial cells and endometrium of the uterine horn in pigs and cows [3, 5]. Moreover, uterine fluid composition changes in the presence of day 7 embryos [6]. These results suggest that molecules in the reproductive tract profoundly affect the early embryonic development. Reportedly, the developmental ability of *in vivo*-developed blastocysts is higher than that of *in vitro*-developed blastocysts based on their differential gene expression profiles and distinct embryo morphology [7–10], implincating a significant effect of maternal conditions on embryos. Furthermore, co-incubation of bovine embryos with oviductal epithelial cells improves embryo quality and changes its gene expression profiles [11]. In contrast, poor maternal conditions, such as nutrient restriction following artificial insemination (AI) affect embryonic equality [12], and half of the fertilized embryos in high-milk-producing cows degenerate before reaching the blastocyst stage [13]. Maternal response to embryos is believed to be mediated by molecules secreted into the uterine fluid in response to interactions between the reproductive tract and early developmental stage embryos [14]. However, the specific molecules that support early embryo development and the prepare the uterine cells for pregnancy remain unidentified. Most previous studies explored molecules from the milliard components of uterine fluids, but embryonic interaction information was not used to narrow down the number of candidates [6]. RNA-sequencing (RNA-seq) makes it possible to predict the upstream regulator molecules that causes the differential expression of hundreds of genes using information regarding the interactions between molecules and genes. Comparing the upstream regulators of genes differentially expressed (DEGs) in the uterine epithelial cells (UECs) due to the presence of embryos and those in the embryos *in vivo* suggests overlapping molecules that influence both UECs and embryos. In this study, we explored the molecules that regulate uterine cells and blastocysts using a combination of RNA-seq data from UECs and embryos.

Received: April 7, 2022
Accepted: July 5, 2022
Advanced Epub: July 29, 2022

Correspondence: H Iwata (e-mail: h1iwata@nodai.ac.jp)

Materials and Methods

Animals

Three non-pregnant dry Holstein cows (aged 76, 94, and 149 months, respectively) (Table 1) were maintained on a farm and used for the collection of UECs. All cows displayed regular ovulation cycles, were fed a total mixed ration, and had ad libitum access to water. The present study was approved by the Ethical Committee for Animal Experiments of Tokyo University of Agriculture (No. 2020138).

Collection of UECs

Uterine flushing was performed seven days after estrus, using Ringer's solution containing bovine serum albumin (BSA, 1 mg/ml). UECs and embryos were collected from the uterine horn using a catheter, the collected medium was filtered through an Emcon embryo filter (75 μm; Immuno Systems Inc., Spring Valley, WI, USA), and the number of embryos was determined. UECs were collected from the flushing medium using a Pasteur pipette under a stereo microscope (Olympus, Tokyo, Japan). The UECs were centrifuged (300 *g*, 3 min) and RNA was extracted using an RNAqueous extraction kit (Life Technology, Carlsbad, CA, USA).

SOV

SOV was induced in cows using hormone treatment (Fig. 1). Briefly, cows were administered a controlled progesterone releasing device (CIDR 1900; Zoetis, Parsippany, NJ, USA), followed by treatment with estradiol benzoate (2 mg, intramuscular, Asuka Animal Health, Tokyo, Japan) on day 0. In addition, cows were treated with total follicle-stimulating hormone (FSH; Kyoritsu, Tokyo, Japan) (intramuscular, twice daily for three days in six decreasing doses; total: 30 AU). Forty-eight hours after the first FSH treatment, cows were treated with prostaglandin F2α (3 ml, intramuscular; Dalmazin, Fatro S.p.A., Bologna, Italy), and the CIDR was removed 12 h after PGF2α treatment. Twenty-four hours after CIDR removal, cows were treated with gonadotropin-releasing hormone (GnRH; Kyoritsu). Two days after CIDR removal, cows in estrus were artificially inseminated, and embryos and UECs were collected seven days after insemination.

RNA-Seq of UECs

RNA concentration and quality were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The average RNA integrity number (RIN) of the sample was 5.5 ± 0.7. The relatively low RIN value may be due to the collection of UECs from the flushing medium, but this method is noninvasive, and principal component analysis (PAC, Fig. 2) showed that the distribution of groups was clearly separated. The RNA samples were subjected to RNA-seq.

A cDNA library was created using a NEB Ultra RNA Prep Kit (New England Biolabs, Ipswich, MA, USA). To obtain raw date, 2 ng of cDNA was sequenced using HiSeq2500 (Illumina, San Diego, CA, USA). Then, adaptor sequences, ambiguous nucleotides, and low-quality sequences were filtered using the CLC Genomics Workbench software (Ver.21.0.3; Qiagen, Venlo, Netherlands). The data are registered in the DDBJ Read Archive as DRA011146.

Analysis of RNA-Seq data

Sequence data were aligned to the *Bos taurus* genome sequence (ARS-UCD1.2/bosTau9) to count sequence reads. Gene expression values were calculated using mapped sequence date, and statistical analysis of DEGs was performed ($P < 0.05$). This process was

Table 1. Number of blastocysts and total number of embryos collected

Cows ID	Months old	No. of collected	No. of blastocysts
1	76	24	9
2	94	35	4
3	145	10	1

No. of collected, numbers of embryos including degenerated embryos collected at flushing; No. of blastocyst, numbers of blastocysts collected at flushing.

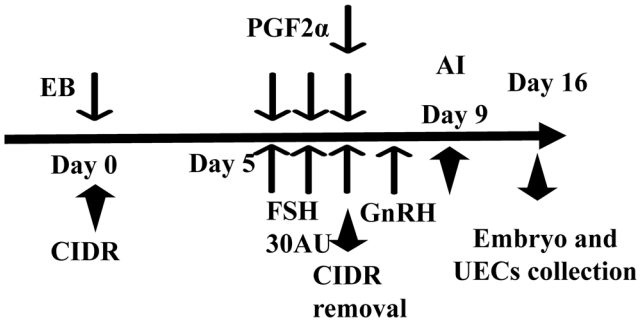

Fig. 1. The cows were administered a controlled internal drug-releasing (CIDR) device containing progesterone and estradiol benzoate (EB). Five days after treatment, the cows received follicular stimulating hormone (FSH, total 30 IU; six decreasing doses at 12 h intervals). Forty-eight hours after the first dose of FSH, prostaglandin F2a (1 ml, PGF2α) was administered intramuscularly, and 12 h after the PGF2α treatment, the CIDR was removed. One days after the removal of the CIDR, the cows were treated with GnRH. One day after the GnRH treatment, cows in estrus were artificially inseminated. Uterine flushing was performed seven days after insemination to collect the specimens for analysis.

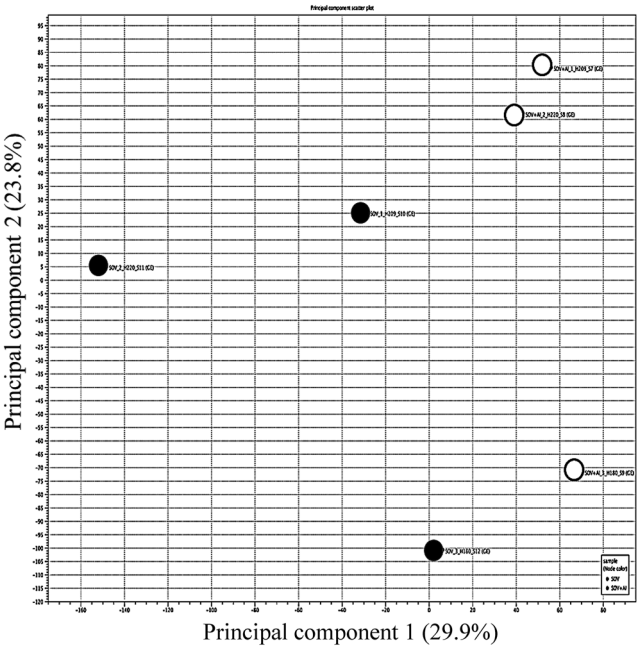

Fig. 2. PCA of the RNA-seq data of UECs from SOV with or without AI. Black circles represent SOV without AI and while circles represent SOV with AI.

conducted using the CLC Genomic Workbench software (Ver.21.0.3; Qiagen, Venlo, Netherlands). Pathways associated with the DEGs were predicted using DAVID bioinformatics resources 6.8 (https://david.ncifcrf.gov/tools.jsp) [15, 16], where *Bos taurus* was used as the species and background.

Publicly available RNA-Seq data

RNA-seq data registered in the DDBJ datebase DRA006210 were downloaded. Data were obtained from blastocysts developed *in vivo* and *in vitro*. For *in vitro* embryo production, oocytes were collected from Japanese Black cow ovaries and fertilized *in vitro* using Japanese Black bull sperms followed by seven days of culture in synthetic oviductal fluid medium. The detailed procedure for embryonic production is described in a previous report [10]. For *in vivo* embryo production, Japanese Black cows were subjected to SOV, and embryos were collected seven days after AI. The same lot of a Japanese Black bull sperms was used for both *in vivo* and *in vitro* embryo production.

Prediction of upstream regulators

Upstream regulators were predicted from the DEGs between SOV+AI and SOV and between *in vivo* and *in vitro* developed blastocysts using the upstream regulator function of the Ingenuity Pathway Analysis (IPA) software (Qiagen). This function can determine how many known targets of each transcriptional regulator were present in the list of DEGs. Overlapped P-values were calculated to measure statistically significant overlap using Fisher's extract test. This prediction uses accumulating information from human experiments regarding the relationship between molecules and gene expression. The activated-upstream regulators are expected to match the expected relationship direction with the observed gene expression (significantly up-regulated or down-regulated, $P < 0.05$), whereas inhibited-upstream regulators have an adverse expected relationship direction with the observed gene expression. Activated and inhibited z-scores: > 2.0 or < –2.0, respectively, indicate that the molecules were significantly activated or inhibited. If the dataset used for the prediction of upstream regulators contains bias, the bias is noted on the molecule's name. Using 1356 DEGs ($P < 0.05$), activated- and inhibited-upstream regulators of UECs in cows subjected to SOV and AI (SOV+AI group cows) were determined. In addition, using 2661 DEGs (FDR $P < 0.05$, absolute fold change > 3.0), activated- and inhibited-upstream regulators of *in vivo* developed blastocysts were determined.

Experimental design

SOV was induced in three Holstein cows; at estrus, the cows were artificially inseminated, and seven days after AI, the uterus was flushed to collect embryos and UECs. The number of embryos and blastocysts is presented in Table 1. After two months (83, 84, and 85 days after the first flushing), the same cows were subjected to SOV without AI, and UECs were collected seven days after estrus. These UECs were used for RNA-Seq to identify DEGs (Fig. 3). DAVID functional annotations and IPA were used to predict associated pathways and upstream regulators. Publicly available RNA-Seq data of *in vivo*- and *in vitro*-developed blastocysts were downloaded, and the DEGs were determined and analyzed using IPA with the latest database to identify upstream regulators important for *in vivo* embryonic development. Two sets of upstream regulators were compared to determine overlapping factors likely to be involved in the mediation of embryo-uterine interactions (Fig. 3).

Statistical analysis

The P values of the upstream regulators following IPA analysis were determined using Fisher's exact test. In the DAVID analysis, Fisher's exact test was used to measure gene enrichment in the annotation terms. Statistical significance was set at $P < 0.05$.

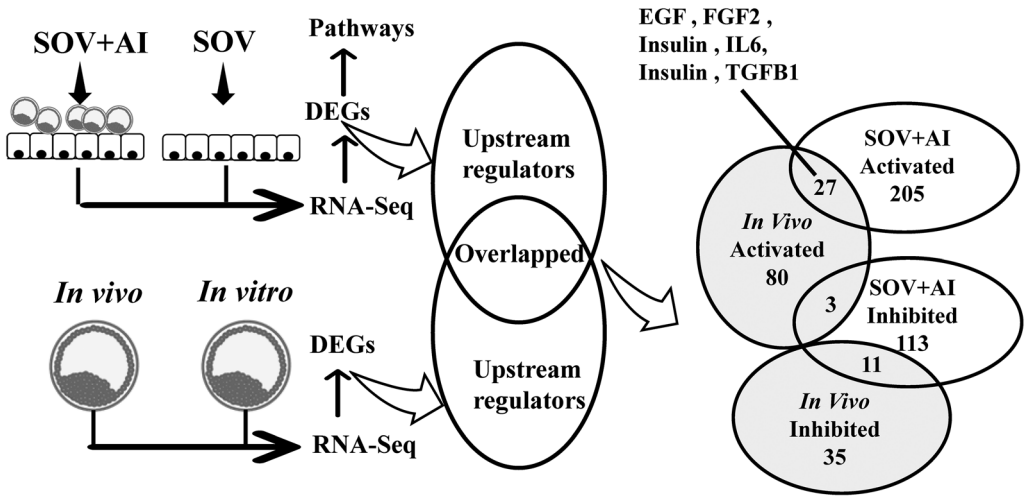

Fig. 3. Design of experiments. Cows were subjected to super ovulation followed by artificial insemination (SOV + AI) or not (SOV). The two groups of uterine epithelial cells (UECs) were subjected to RNA-Seq. Differentially expressed genes (DEGs) and associated pathways were determined. In addition, upstream regulators of UECs of SOV + AI were predicted using Ingenuity Pathway Analysis (IPA). Upstream regulators of *in vivo*-developed blastocysts were predicted from DEGs obtained from publicly available RNA-Seq dataset of *in vivo*- and *in vitro* developed blastocysts. Comparison of the two groups of upstream regulators predicted the molecules potentially involved in embryo-uterine interactions. For example, 27 molecules including EGF, FGF2, Insulin, IL6, Insulin, and TGFB1 were predicted as overlapped activated upstream regulators. Number of overlapped activated- and inhibited upstream regulators was described as a ven diagram.

Results

Gene expression of UECs derived from SOV+AI and SOV cows

RNA-Seq revealed 1355 significant DEGs between UECs from SOV+AI and SOV cows. Functional annotation of the DEG using DAVID bioinformatics resources revealed 22 pathways (Table 2; excluding 11 pathways related to disease), including pathways related to leukocyte transendhothelial migration, T cell receptor signaling pathway, tumor necrosis factor (TNF) signaling pathway, natural killer cell mediated cytotoxicity, primary immunodeficiency, and B cell receptor signaling pathway. IPA was used to predict upstream regulator molecules of the DEGs, including miRNAs and transcription factors, cytokines, and growth factors that potentially cause changes in gene expression. Among the 205 activated and 113 inhibited upstream regulators of the UECs of SOV+AI cows, molecules categorized as cytokines and growth factors are listed in Table 3. RNA-Seq data showed that among the interferon response genes (*IFNA2, IRF6, MX1, MX2, OAS1, ISG15, ISAD2,* and *IFI6*), the expression levels of *IFI6* and *ISG15* were significantly increased in the UECs of SOV+AI cows (1.69-, and 1.68-fold, respectively, $P < 0.05$). In addition, *IL1A*, and *IL1B1* were downregulated (−2.0- and −2.8-fold, respectively), whereas *IL6* was increased (3.6-fold) in UECs of SOV+AI cows compared with those of SOV cows.

Prediction of molecules involved in the interaction between embryos and UECs using IPA

Factors mediating the interactions between the maternal reproductive tract and early preimplantation embryos might be diffused in the uterine fluid because direct contact between the blastocyst and uterus is not required to affect gene expression [14]. This prompted us to filter and reduce the number of upstream regulators of UECs in SOV + AI cows. The public data registered in the DDBJ (DRA006210) are RNA-Seq data of bovine *in vivo*- and *in vitro*-developed blastocysts. IPA of the DEGs (FDR $P < 0.05$, absolute fold change >3.0) identified upstream regulators that caused changes in the gene expression profiles of *in vivo*-developed blastocysts. When the activated- and inhibited-upstream regulators of *in vivo*-developed embryos were compared with those of UECs from SOV+AI cows (Fig. 3), several molecules overlapped between the two sets of activated upstream regulators and between those of inhibited upstream regulators (Fig. 3 and Table 4). The overlapping factors categorized as cytokines and growth factors without bias were leukemia inhibitory factor (LIF), interleukin 6 (IL6), fibroblast growth factor 2 (FGF2), transforming growth factor beta 1 (TGFB1), and epidermal growth factor (EGF) (Table 4).

Discussion

The present RNA-Seq date showed that gene expression in UECs collected from the uterine horn on day 7 after estrus was affected by the presence of embryos, and the genes affected were associated with interferon response, immune response, and cell adhesion. In addition, the combination of IPA and RNA-Seq data derived from UECs and blastocysts showed that *LIF*, *IL6, FGF2, TGFB1,* and *EGF* are potential mediators between the uterus and early preimplantation embryos.

A previous report using quantitative real-time PCR revealed that bovine endometrial explants exposed to day 8 blastocysts had altered expression of genes involved in interferon signaling (*MX1*, *MX2*, *OAS1*, *ISG15*, and *RSAD2*) [17]. In the present study, RNA-seq of UECs collected from the uterine horn flushing showed 1355 differential gene expression profiles between the SOV+AI and SOV groups.

Table 2. Pathways significantly enriched by differentially expressed genes in bovine uterine epithelial cells between SOV+AI and SOV groups

Term	Count	P Value
Ribosome	61	4.07805E–34
Cell adhesion molecules (CAMs)	32	9.77704E–08
Leukocyte transendothelial migration	26	4.48439E–07
Hematopoietic cell lineage	22	1.39848E–06
T cell receptor signaling pathway	22	1.29348E–05
TNF signaling pathway	22	2.03368E–05
Natural killer cell mediated cytotoxicity	23	2.25268E–05
Primary immunodeficiency	12	2.61825E–05
Chemokine signaling pathway	29	0.000114141
Cytokine-cytokine receptor interaction	32	0.000162524
B cell receptor signaling pathway	13	0.003675118
Platelet activation	18	0.008715247
NF-kappa B signaling pathway	14	0.012230956
Chagas disease (American trypanosomiasis)	16	0.015394733
Osteoclast differentiation	17	0.02912761
Mineral absorption	8	0.034992874
Toll-like receptor signaling pathway	14	0.0359839
Complement and coagulation cascades	11	0.036881797
Antigen processing and presentation	11	0.039954575
Measles	17	0.041359224
Spliceosome	16	0.041531247
Tight junction	12	0.044963824
ECM-receptor interaction	12	0.044963824

Table 3. Cytokine and growth factor upstream regulators of differentially expressed genes of super ovulated and artificially inseminated cows (SOV+AI) compared with super ovulated cows without AI

Upstream regulator	Molecule type	Predicted activation state	Activation z-score
IL1A	cytokine	Activated	2.114
CNTF	cytokine	Activated	2.173
IL1B	cytokine	Activated	2.203
CSF3	cytokine	Activated	2.224
WNT1	cytokine	Activated	2.63
EDN1	cytokine	Activated	3.237
LIF	cytokine	Activated	3.264
IL6	cytokine	Activated	3.37
OSM	cytokine	Activated	3.744
IGF1	growth factor	Activated	2.116
FGF2	growth factor	Activated	2.155
CSHL1	growth factor	Activated	2.375
GH1	growth factor	Activated	2.566
TGFB2	growth factor	Activated	2.645
EGF	growth factor	Activated	2.735
TGFB1	growth factor	Activated	3.134
ANGPT2	growth factor	Activated	3.161
GDF2	growth factor	Activated	3.278
FLT3LG	cytokine	Inhibited	–2.828
IL21	cytokine	Inhibited	–2.74

Table 4. Overlapped upstream regulators predicted from UECs and *in vivo* developed blastocysts

Name	Categories	SOV+AI	Score	VIVO	Score
actinomycin D	biologic drug	Inhi.	–2.48	Inhi.	–2.55
selenomethylselenocysteine	chemical - endogenous mammalian	Inhi.	–2.24	Inhi.	–2.45
LY294002*	chemical drug	Inhi.	–2.93	Inhi.	–2.71
sirolimus	chemical drug	Inhi.	–2.35	Inhi.	–5.07
U0126*	chemical drug	Inhi.	–2.25	Inhi.	–2.72
NR4A1	ligand-dependent nuclear receptor	Inhi.	–2.34	Inhi.	–2.04
miR-29b-3p*	mature microRNA	Inhi.	–2.91	Inhi.	–2.42
miR-199a-5p*	mature microRNA	Inhi.	–2.49	Inhi.	–2.36
let-7*	microRNA	Inhi.	–4.44	Inhi.	–2.18
RICTOR	other	Inhi.	–5.90	Inhi.	–5.42
BTNL2	transmembrane receptor	Inhi.	–2.33	Inhi.	–2.14
hydrogen peroxide	chemical - endogenous mammalian	Acti.	3.88	Acti.	4.12
phenylephrine	chemical drug	Acti.	2.24	Acti.	2.39
dalfampridine	chemical drug	Acti.	2.42	Acti.	3.61
cisplatin	chemical drug	Acti.	2.65	Acti.	2.19
lipopolysaccharide	chemical drug	Acti.	3.13	Acti.	2.22
daidzein	chemical drug	Acti.	2.36	Inhi.	–2.84
forskolin	chemical toxicant	Acti.	2.03	Acti.	2.53
thioacetamide*	chemical toxicant	Acti.	2.67	Acti.	2.95
LIF	cytokine	Acti.	3.01	Acti.	3.26
IL6	cytokine	Acti.	3.37	Acti.	3.37
AGTR2	G-protein coupled receptor	Acti.	2.21	Acti.	2.21
Tgf beta	group	Acti.	2.68	Acti.	2.77
ERK*	group	Acti.	3.44	Acti.	2.70
Insulin	group	Acti.	4.55	Acti.	2.33
FGF2	growth factor	Acti.	2.09	Acti.	2.16
ANGPT2*	growth factor	Acti.	2.34	Acti.	3.16
TGFB1	growth factor	Acti.	2.87	Acti.	3.13
EGF	growth factor	Acti.	2.96	Acti.	2.74
GDF2*	growth factor	Acti.	3.22	Acti.	3.28
VGLL3	other	Acti.	2.00	Acti.	2.45
F2*	peptidase	Acti.	3.34	Acti.	2.63
SMAD2	transcription regulator	Acti.	2.08	Acti.	2.54
SMAD4	transcription regulator	Acti.	2.52	Acti.	3.28
TP53	transcription regulator	Acti.	3.33	Acti.	2.01
YAP1*	transcription regulator	Acti.	3.37	Acti.	3.12
STAT3	transcription regulator	Acti.	4.18	Acti.	3.04
ATF4*	transcription regulator	Acti.	4.36	Acti.	2.57
IRF2	transcription regulator	Acti.	2.02	Inhi.	–2.12
SPI1	transcription regulator	Acti.	2.22	Inhi.	–3.22
IGF1R*	transmembrane receptor	Acti.	2.71	Acti.	2.01

SOV+AI, Uterine epithelial cells of super ovulated and artificially inseminated cows; VIVO, *in vivo* developed blastocysts; Acti, Activated upstream regulators (Acti); Inhi, Inhibited upstream regulators (Inhi). * Prediction of the molecules containing bias factors.

In addition, the DEGs included interferon response genes such as *ISG15* and *IFI6*. Publicly available data showed that both *in vivo* and *in vitro*-produced bovine blastocysts express *IFNT,* indicating that the upregulation of interferon-responsive genes occurred in response to IFNT from embryos. Functional annotation showed that the DEGs between SOV+AI and SOV were significantly associated with immune-responsive pathways, including T-cell receptors, TNF signaling, B-cell receptors, nuclear factor kappa B (NF-kB), and toll-like receptors. These findings support the reports that the presence of embryos in the uterus induces downregulation of immune-responsive genes in pigs and cows [5, 18]. Furthermore, Muñoz *et al*. [19] reported that IL1B and TNFA levels were lower in the uterine fluid sourced from embryo transfer heifers than in that from sham transfer heifers. In another study, the protein levels of TNFA in the endometrium at day 8 increased in response to the embryo transfer, but mRNA levels did not differ [20]. Consistent with Muñoz *et al*. [19], in the present study, the mRNA expression levels of *IL1B* decreased in the SOV+AI group; however, no significant changes were observed in the *TNFA* levels. The levels of TNFA in UECs in response to the embryos need to be examined in future studies.

IPA can be used to predict upstream regulators causing changes in gene expression using publicly available information about the genes. Our IPA of DEGs identified candidate molecules that regulate gene expression in UECs collected from SOV+AI cows. Previously,

Rodríguez-Alonso *et al*. [14] suggested that the interaction between early preimplantation embryos and the uterus/oviduct involves diffusible molecules because the maternal tissues response does not require direct interaction. Therefore, we selected molecules that could be categorized as growth factors and cytokines. Furthermore, we compared the activated- and inhibited-upstream regulators of UECs derived from SOV+AI and those of *in vivo*-developed blastocysts, on the premise that growth factors or cytokines present in the uterine environment affect both UECs and embryos. Interestingly, the comparison revealed that activated-upstream regulators of UECs derived from SOV+AI overlapped with activated-upstream regulators of *in vivo*-developed blastocysts, and this was the same for inhibited-upstream regulators (Table 4 and Fig. 3). The overlapping significantly activated upstream regulators predicted without the bias data set: LIF, IL6, FGF2, TGFB1, and EGF. LIF, IL6, FGF2, TGFB1, and EGF are pivotal factors in the uterine environment, supporting embryo development and preparing the uterus for pregnancy [21–25]. A previous report indicated a potential association between early embryonic development and the expression of the anti-inflammatory cytokine TGFB1 in the uterine endometrium on days 7 after insemination [21] and the expression of FGF2 and FGF2R in the reproductive tract of cows [22–25]. In addition, Campanile *et al*. [26] reported that LIF and IL6 secreted from the embryo and uterus play pivotal roles in maternal and uterine interactions. Based on the expression values of these genes in blastocysts and UECs derived from the RNA-Seq data, *TGFB1* and *IL6* were highly expressed in embryos, and *LIF* and *IL6* were highly expressed in UECs. Therefore, we hypothesized that these factors are crucial molecules mediating successful embryonic development and preparing the UECs for pregnancy. However, the actual effects should be examined in future studies.

In conclusion, the present study identified genes in UECs responsive to the presence of embryos and found that EGF, TGFB1, FGF, insulin, LIF, and IL6 are potential molecules mediating successful embryonic development in the uterus.

Conflict of interests: We declare no conflicts of interest in this study.

References

1. **Kimura K, Matsuyama S.** Successful nonsurgical transfer of bovine elongating conceptuses and its application to sexing. *J Reprod Dev* 2014; **60**: 210–215. [Medline] [CrossRef]
2. **Roberts RM, Chen Y, Ezashi T, Walker AM.** Interferons and the maternal-conceptus dialog in mammals. *Semin Cell Dev Biol* 2008; **19**: 170–177. [Medline] [CrossRef]
3. **Sponchiado M, Gomes NS, Fontes PK, Martins T, Del Collado M, Pastore AA, Pugliesi G, Nogueira MFG, Binelli M.** Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. *PLoS One* 2017; **12**: e0175954. [Medline] [CrossRef]
4. **Maillo V, Gaora PÓ, Forde N, Besenfelder U, Havlicek V, Burns GW, Spencer TE, Gutierrez-Adan A, Lonergan P, Rizos D.** Oviduct-embryo interactions in cattle: two-way traffic or a one-way street? *Biol Reprod* 2015; **92**: 144. [Medline] [CrossRef]
5. **Almiñana C, Heath PR, Wilkinson S, Sanchez-Osorio J, Cuello C, Parrilla I, Gil MA, Vazquez JL, Vazquez JM, Roca J, Martinez EA, Fazeli A.** Early developing pig embryos mediate their own environment in the maternal tract. *PLoS One* 2012; **7**: e33625. [Medline] [CrossRef]
6. **Sponchiado M, Gonella-Diaza AM, Rocha CC, Turco EGL, Pugliesi G, Leroy JLMR, Binelli M.** The pre-hatching bovine embryo transforms the uterine luminal metabolite composition *in vivo*. *Sci Rep* 2019; **9**: 8354. [Medline] [CrossRef]
7. **Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P.** Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced *in vivo* or *in vitro*. *Mol Reprod Dev* 2002; **62**: 320–327. [Medline] [CrossRef]
8. **Rizos D, Clemente M, Bermejo-Alvarez P, de La Fuente J, Lonergan P, Gutiérrez-Adán A.** Consequences of *in vitro* culture conditions on embryo development and quality. *Reprod Domest Anim* 2008; **43**(Suppl 4): 44–50. [Medline] [CrossRef]
9. **Maillo V, Lopera-Vasquez R, Hamdi M, Gutierrez-Adan A, Lonergan P, Rizos D.** Maternal-embryo interaction in the bovine oviduct: Evidence from *in vivo* and *in vitro* studies. *Theriogenology* 2016; **86**: 443–450. [Medline] [CrossRef]
10. **Noguchi T, Aizawa T, Munakata Y, Iwata H.** Comparison of gene expression and mitochondria number between bovine blastocysts obtained *in vitro* and *in vivo*. *J Reprod Dev* 2020; **66**: 35–39. [Medline] [CrossRef]
11. **Asaadi A, Kafi M, Atashi H, Azari M, Hostens M.** Frozen-thawed ampullary cell monolayer improves bovine embryo *in vitro* development and quality. *Zygote* 2019; **27**: 337–346. [Medline] [CrossRef]
12. **Kruse SG, Bridges GA, Funnell BJ, Bird SL, Lake SL, Arias RP, Amundson OL, Larimore EL, Keisler DH, Perry GA.** Influence of post-insemination nutrition on embryonic development in beef heifers. *Theriogenology* 2017; **90**: 185–190. [Medline] [CrossRef]
13. **Sartori R, Bastos MR, Wiltbank MC.** Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. *Reprod Fertil Dev* 2010; **22**: 151–158. [Medline] [CrossRef]
14. **Rodríguez-Alonso B, Sánchez JM, González E, Lonergan P, Rizos D.** Challenges in studying preimplantation embryo-maternal interaction in cattle. *Theriogenology* 2020; **150**: 139–149. [Medline] [CrossRef]
15. **Huang W, Sherman BT, Lempicki RA.** Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44–57. [Medline] [CrossRef]
16. **Huang W, Sherman BT, Lempicki RA.** Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009; **37**: 1–13. [Medline] [CrossRef]
17. **Passaro C, Tutt D, Mathew DJ, Sanchez JM, Browne JA, Boe-Hansen GB, Fair T, Lonergan P.** Blastocyst-induced changes in the bovine endometrial transcriptome. *Reproduction* 2018; **156**: 219–229. [Medline] [CrossRef]
18. **Ott TL.** Symposium review: Immunological detection of the bovine conceptus during early pregnancy. *J Dairy Sci* 2019; **102**: 3766–3777. [Medline] [CrossRef]
19. **Muñoz M, Corrales FJ, Caamaño JN, Díez C, Trigal B, Mora MI, Martín D, Carrocera S, Gómez E.** Proteome of the early embryo-maternal dialogue in the cattle uterus. *J Proteome Res* 2012; **11**: 751–766. [Medline] [CrossRef]
20. **Correia-Álvarez E, Gómez E, Martín D, Carrocera S, Pérez S, Peynot N, Giraud-Delville C, Caamaño JN, Balseiro A, Sandra O, Duranthon V, Muñoz M.** Early embryonic and endometrial regulation of tumor necrosis factor and tumor necrosis factor receptor 2 in the cattle uterus. *Theriogenology* 2015; **83**: 1028–1037. [Medline] [CrossRef]
21. **Talukder AK, Rashid MB, Takedomi T, Moriyasu S, Imakawa K, Miyamoto A.** Day-7 embryos generate an anti-inflammatory immune response in peripheral blood immune cells in superovulated cows. *Am J Reprod Immunol* 2019; **81**: e13069. [Medline] [CrossRef]
22. **Yang QE, Johnson SE, Ealy AD.** Protein kinase C delta mediates fibroblast growth factor-2-induced interferon-tau expression in bovine trophoblast. *Biol Reprod* 2011; **84**: 933–943. [Medline] [CrossRef]
23. **Okumu LA, Forde N, Mamo S, McGettigan P, Mehta JP, Roche JF, Lonergan P.** Temporal regulation of fibroblast growth factors and their receptors in the endometrium and conceptus during the pre-implantation period of pregnancy in cattle. *Reproduction* 2014; **147**: 825–834. [Medline] [CrossRef]
24. **Tríbulo P, Siqueira LGB, Oliveira LJ, Scheffler T, Hansen PJ.** Identification of potential embryokines in the bovine reproductive tract. *J Dairy Sci* 2018; **101**: 690–704. [Medline] [CrossRef]
25. **Chiumia D, Schulke K, Groebner AE, Waldschmitt N, Reichenbach HD, Zakhartchenko V, Bauersachs S, Ulbrich SE.** Initiation of conceptus elongation coincides with an endometrium basic fibroblast growth factor (FGF2) protein increase in heifers. *Int J Mol Sci* 2020; **21**: 1584. [Medline] [CrossRef]
26. **Campanile G, Baruselli PS, Limone A, D'Occhio MJ.** Local action of cytokines and immune cells in communication between the conceptus and uterus during the critical period of early embryo development, attachment and implantation - Implications for embryo survival in cattle: A review. *Theriogenology* 2021; **167**: 1–12. [Medline] [CrossRef]