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석사학위 청구논문

# Correlation Work between Carbohydrate and Inflammation Factors in Early Embryo Development

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성신여자대학교 대학원

생 물 학 과

정 혜 진

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이 논문을 석사학위논문으로 제출함.

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Submitted in partial fulfillment of the

requirements for the degree of master.

November, 2015

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정혜진의 석사학위 논문으로 인준함.

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# 논 문 개 요

포유동물에서 수정란의 난할은 수란관을 이동하며 진행되는데 수란관 각 부위의 조건은 각 난할시기배아의 발생 능력을 획득하도록 도와주는 것으로 알려져 있다. 이는 내인성 및 외인성 인자들에 의한 것으로 탄수화물 기질도 그 중의 하나로 제안되어 왔다. 한편, 염증현상과 관련되어 있다고 알려진 prostaglandin-endoperoxide-synthase (PTGS)를 매개로 아라키돈산(arachidonic acid)산물인 $PGE_2$, $PGF_{2a}$, $PGD_2$, $PGI_2$, 트롬복세인 $A_2$가 착상 전 배아에서 발현된다고 알려져 왔다. 프로스타글란딘 유전자변형동물을 통해 프로스타글란딘이 배란, 수정, 부화(hatching), 착상 등에 관여하고 있음이 제안되었으나 난할 시기 동안의 역할은 밝혀진 바 없다. 초기배아의 물질대사 조건과 적응을 통한 배발생 조절을 위한 유전정보 활용은 포유동물에서 중요하다. 이러한 물질대사 적응현상에서 유전정보 활용의 변화가 수반되는데 일부 조직에서 PTGS매개 산물이 관련되어 있음이 제안되었으나 분명하게 밝혀지지 않았다. 포유동물의 초기 배아는 탄수화물을 이용한 물질대사 능력의 변화가 뚜렷하며 체외에서의 적응현상이 밝혀지고 있다. 따라서 이 연구는 이러한 탄수화물 또는 환경 변화에 적응하는 초기배아발생이 제공되는 탄수화물 종류에 대한 적응과정에서 프로스타글란딘과 관련된 정보가 이용되는 지를 알아보고자 하였다. 배란유도 후 미수정란, 수정란, 2세포기, 4세포기, 8세포기, 상실배, 포배를 얻어, 관련정보의 활용 정도를 확인하였다. 이를 근거로 기본 탄수화물과 특정이온을 함유한 단순배양액 BWW를 이용하여 2세포기를 배양하여 실험하였으며 제한적으로 탄수화물을 제공했을 때 각 관련 정보의 활용 정도를 조사하였다. 정량적 중합효소연쇄반응으로 *Ptgs*, 프로스타글란딘수용체, 그리고

발생표지인자를 분석하였고, 형광염색을 통하여 단백질의 발현을 분석하였다. *Ptgs1*의 mRNA양은 1세포기에 가장 많고, 2세포기 때 감소하고, 4세포기에 다시 증가했다. *Ptgs2*의 mRNA양은 1세포기까지 많게 유지되다가 2세포기에 감소하고, 다시 4세포기에 증가하다가 포배기에 감소를 보였다. 배양액 내의 탄수화물을 달리하였을 때 발생률은 glucose가 없을 때에 hCG 주사 후 72시간, 96시간, 120시간, 140시간에 각각 8세포기이상이 94.34%, 포배율이 49.61%, 후기포배율이 75.72%, 부화율이 70%로 모두 증가하였고, pyruvate와 glucose가 모두 없는 배양액에서는 72시간, 96시간 까지는 각각 8세포기 이상이 93.91%, 상실배율이 80.08%로 발생률이 증가하지만 140시간에는 hatching률이 유의적으로 감소했다. 이를 토대로 각각 다른 배양조건에서 *Ptgs1*과 *Ptgs2*의 mRNA 발현양상을 관찰한 결과 glucose가 없는 group에서 4세포기와 8세포기기에 두 가지 모두 발현량이 증가했고, glucose와 pyruvate가 모두 없는 배양조건에서는 8세포기에 두 가지 모두 발현량이 급증했고 hatching시기까지 점차 감소하였다. *Ptgfr* mRNA의 발현양상이 *Ptgs1*과 *Ptgs2*와 유사했으며, *Ptgir*과 *Ptger2*는 발현되지 않았다. 이러한 결과를 통하여 배양액 내의 이용할 수 있는 탄수화물 대사산물이 *Ptgs* 정보 활용 정도를 달리하여 난할시기의 배아에서의 PTGS의 발현 정도를 조절할 것이라고 사료된다.

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# INTRODUCTION

Successful early embryo development depend on the embryo's ability to generate energy metabolites, substrates and building blocks through appropriate metabolic pathways at given specific times (Cheon, 2008). Early studies on the development of preimplantation mouse embryos determined that the development of the zygote to the 2-cell stage has an absolute requirement of pyruvate (Biggers et al., 1967). Nutrient uptake studies showed that preimplantation mouse embryos switch from pyruvate to a glucose-based metabolism at around the time of compaction (Gardner and Leese, 1986; Leese and Barton, 1984). Lactate unable to support the first cleavage division alone, but lactate is readily oxidized by both the 1-cell and 2-cell mouse embryo (Wales et al., 1967, 1973). However, the effect of lactate on nutrient uptake has not been determined, nor has interaction of pyruvate and lactate utilization at all stages of development (Michelle et al., 2000).

In the absence of glucose the character decreasing pyruvate uptake after compaction does not occur. It means that the embryo is able to compensate for the absence of glucose by maintaining its uptake of pyruvate (Gardner and Leese, 1988). The embryo changes its carbohydrate preference as it develops (Eagle, 1959). Changing the ratio of certain medium components one can change the ratio of important intracellular regulators. For example, a gradient of lactate in the culture medium can have significant effect on mouse embryo viability (Gardner et al., 1993). From the various phenomena, it is suggested that the metabolites may be in developmental control.

Prostaglandin-endoperoxide synthase (PTGS) catalyzes a key step in the

conversion of arachidonic acid to prostaglandin $H_2$ ($PGH_2$), the immediate substrate for prostaglandin (PG) and thromboxane synthases. The cyclooxygenase exists under two isoforms PTGS1 and PTGS2 encoded by two separate genes, *Ptgs1* and *Ptgs2,* respectively, and differ mainly in their pattern of expression. PTGS1 is expressed in most tissues, whereas PTGS2 induced by numerous physiologic stimuli including cytokines, growth factors, mitogens, and tumor promoters (Smith et al., 2000). Prostanoids that synthesized from PTGS pathway are $PGE_2$, $PGF_{2a}$, $PGD_2$, $PGI_2$ and thromboxane$A_2$ ($TXA_2$). PGs were known to participate in female reproductive functions, including ovulation, fertilization, implantation, and decidualization (Lim et al., 1997; Helliwell et al., 2004).

The diverse actions of PGs are generally mediated through their G protein-coupled, cell-surface receptors that are linked to different signaling pathway (Negihi et al., 1995). Cell-surface receptor for $PGE_2$, $PGF_{2a}$, $PGD_2$, $PGI_2$, and $TXA_2$ have been cloned as PTGER, PTGFR, PTGDR, PTGIR, and TBXR, respectively (Narumiya et al., 1999). Moreover, $PGE_2$ has functionally distinct PTGER subtype, PTGER1, PTGER2, PTGER3 and PTGER4 (Coleman et al., 1994; Narumiya et al., 1999). Among the PTGER subtype, PTGER2 null female exhibit impaired reproductive functions, with smaller littersize (Hizaki et al., 1999; Tilly et al., 1999). Previous study suggests that PTGS2-derived $PGE_2$ is essential for ovulation via activation of PTGER2, and PTGS2-derived $PGI_2$ is involved in implantation and decidualization (Matsumoto et al., 2001). Blocking endogenous $PGI_2$ production by selective PTGS2 inhibitors retards embryo hatching (Huang et al., 2004a). And oviduct-derived $PGI_2$ enhance embryo development in a paracrine fashion, during transformation of 2-cell

embryos to morula (Huang et al., 2004b).

Some research suggest that the high glucose increase $PGE_2$ synthesis, which is controlled by the coupled activation of phospholipase $A_2$ ($PLA_2$)/arachidonic acid (AA) and PTGS2 via reactive oxygen species (ROS) (Kim et al., 2008). And previous study reported the prostanoids have been implicated in the regulation of liver metabolism (Brass et al., 1984; Bronstad et al., 1981; Curnow et al., 1972). However, whether carbohydrate metabolites and AA metabolites have correlation during embryo development or not is unknown. In this study, we tried to explore the correlation between carbohydrate metabolite and prostaglandins in embryo development.

## MATERIALS AND METHODS

### Experimental animals

All experiment animals were studied according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and under the Experimental Animals Committee of Sungshin University. Animals were maintained under the standard conditions at animals house in Sungshin University with diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 hr and clean room system. Animals were fed a standard rodent diet and water *ad libitum* from weaning at 21 days of age.

### Superovulation and embryo sampling

Female CD1 mice were superovulated by injection of 5IU of pregnant mare's serum gonadotropin (PMSG) and 5IU of human chronic gonadotropin (hCG) after 48hr of PMSG injection. After the hCG injection, females were placed with males of the same strain. Mating was indicated by the presence of a vaginal plug the following morning. To get each stage embryos, unfertilized (UF), pronucleus (PN), 2-cell, 4-cell, 8-cell, morula, blastocyst, and hatching were collected from oviduct or uterus by flushing with BWW containing 0.4% bovine serum albumin (BSA) after 15 hr, 20 hr, 48 hr, 56 hr, 64 hr, 80 hr, and 96 hr post hCG injection, respectively. *In vitro* developed 4-cell, 8-cell, morula, blastosyst., and hatching embryo were collected at 60 hr, 72 hr, 96 hr, 120 hr, and 140 hr of hCG injection, respectively.

**Media preparation**

Collected 2-cell embryos were cultured in 5 groups of BWW media. Control media is normal BWW media. Pyruvate free media contained 0.25mM NaCl more than control media without 0.25mM (0.50mOsM) Na-pyruvate. Glucose free media contained 5.56mM (5.56mOsM) D-(+)-Raffinose pentahydrate instead of 5.56mM (5.56mOsM) Glucose. Pyruvate and Glucose free media contained 0.25mM (0.50mOsM) NaCl and 5.56mM(5.56mOsM) D-(+)-Raffinose pentahydrate without 0.25mM (0.50mM) Na-pyruvate and 5.56mM (5.56mOsM) Glucose. Lactate free media contained 21.58mM (43.10mOsM) NaCl and 1.71mM (5.13mOsM) $CaCl_2$ instead of 21.58mM (43.10mOsM) Na-lactate and 1.71mM (5.13mOsM) Ca-lactate. D-(+)-Raffinose pentahydrate is not transported by mammalian cells and impermeableness to cell membrane.

**Total RNA extraction and first strand cDNA synthesis**

Total RNA of embryos was extracted using RNeasy® Micro kit (QIAGEN, CA USA) according to the manual of manufacture. Total RNA of 10ea embryos were used to perform reverse transcription. First strand cDNA was synthesized using Accuscript first strand cDNA synthesis kit (Stratagene, CA, USA) according to the manual of manufacture. Briefly, reaction reagents were total RNA of 10ea embryos, 5.0 μl Accuscript buffer (10x), 1.0 μl oligo dT primer (0.5 μg/μl), 1.0 μl random primers (0.1 μg/μl), 2 μl dNTP mix (100 mM), RNase-free water. Reaction mixture was incubated at 65℃ for 5min, placed the tube at RT to allow the primers to anneal to the RNA for 10 min, after then added 4.0 μl DTT (100 mM), 2.0 μl RNase block ribonuclease inhibitor (40 U/ml), 1.0 μl Accuscript

multiple temperature RT. The mixture was incubated at 42℃ for 1 hr and 70℃ for 15 min. And kept at -20℃ before it used.

**Real-time PCR analysis**

Real-time PCR was performed using SYBR Premix Ex Taq™ II and Thermal Cycler Dice Real Time System TP800 (TaKaRa, Tokyo, Japan). Each reaction was run in triplicate and consisted of 1.0 μl cDNA, 10 μl SYBR Premix Ex Taq™ and the specific primers listed in Table 1. The fold change in gene expression was calculated using the ΔΔCt method with the housekeeping gene, a ribosomal protein, H2afz, as the internal control: $\Delta\Delta Ct = \Delta Ct\ (Target_{sample}\text{-}H2afz_{sample}) - \Delta Ct\ (Target_{2\text{-}cell}\text{-}H2afz_{2\text{-}cell})$.

**Whole-mount Immunofluorescence**

Embryos were fixed with 4%paraformaldehyde in PBS containing 0.15% picric acid for 1 hr at RT and kept in PBS at 4℃ before it used. Permeabilization performed with 0.5% PBST for 3 hr at RT. Then blocked in 10% normal goat serum in PBS for 1 hr at RT and incubated with rabbit-anti cyclooxygenase 2 polyclonal antibody(dilution 1:200; Bioss antibodies, USA) in PBS containing 5% dimethylsulfoxide (DMSO) for overnight at 4℃. And washed three times for 5min each in PBST and incubated with 2nd Cy3 conjugated goat-anti rabbit antibody for 2hr at RT and washed. Then embryos were counterstained with Hoechst for 10min at RT and mounted with 20% glycerol on dot slide. Stained embryos were observed with confocal microscope (Zeiss LSM 700 Laser scanning confocal microscope, Germany). The mean immunofluorescence intensity was measured

by using ZEN 2011 software.

**Statistics**

The *t*-test was used to evaluate the difference between control and experiment group. Results were presented as mean ± SEM. Values of $P < 0.05$ were considered significant. The $\chi^2$ test was used to evaluate the development rate and experiment group development rate. Values of $P < 0.05$ were considered significant.

Table 1. Sequence of primers for identify and quantification of transcripts

| Gene | Symbol | | Primer sequence(5'-3') | Amplified length(bp) |
|---|---|---|---|---|
| Prostaglandin-endoperoxide | ptgs1 | S | CCT CAC CAG TCA ATC CCT GTT GTT | 195 |
| synthase 1 | | AS | GTG TCA GCA GGA AAT GGG TGA A | |
| Prostaglandin-endoperoxide | ptgs2 | S | CCC ACA GTC AAA GAC ACT CAG GT | 195 |
| synthase 2 | | AS | TAG TTG CTC ATC ACC CCA CTC A | |
| Prostaglandin E receptor 2 | Ptger2 | S | TCT TTA GTC TGG CCA CGA TGC T | 211 |
| (subtype EP2) | | AS | AAG CAC CAT GTC CCA GGA CAG TA | |
| Prostaglandin F receptor(FP) | Ptgfr | S | CCT GGA GAT GAT CAT TCA GCT CC | 192 |
| | | AS | CGT AGC AGA ATA TAG ACC CAG GGA | |
| Prostaglandin I receptor(IP) | Ptgir | S | TTC GCC ATG ACG TTC TTC G | 205 |
| | | AS | AAT ACT GCT GAT GCT CGC CCA | |
| H2A histone family, member z | H2afz | S | TCC GGA AAG GCC AAG ACA AA | 232 |
| | | AS | AGT GAC GAG GGG TCA TAC GCT TT | |

Table 2. Thermal cycler schedule

| Step | | Temperature (℃) | Time |
|---|---|---|---|
| Hold | Hold | 95 | 30sec |
| 3 step PCR (45 cycles) | Denaturation | 95 | 1min |
| | Annealing | 59 | 30sec |
| | Extension | 72 | 15sec |
| Dissociation | Denaturation | 95 | 15sec |
| | Annealing | 60 | 30sec |
| | Extension | 95 | 15sec |
| Hold | | 4 | 5min |

## RESULTS

### Profiles of *Ptgs1* and *Ptgs2* mRNA and PTGS protein in mouse embryo during early embryo development

To investigate the quantitative of *Ptgs1* and *Ptgs2* mRNA during early embryo development, real time PCR analysis was performed. The relative quantity of *Ptgs1* mRNA peaked on PN stage and decreased on the other stages except morula stage. *Ptgs2* mRNA level was highly expressed at UF and PN stage, and rapidly decreased at 2-cell and increased again at 4-cell stage and gradually decreased (Fig. 1).

To evaluate the expression of PTGS2 protein on each stage of preimplantation embryo, whole-mount immunofluorescence was performed with cyclooxygenase2 specific antibody incubated with Cy3 conjugated $2^{nd}$ antibody and counterstained with Hoechst 33258. Until compaction, PTGS2 was mainly localized in cytoplasm (Fig. 2A-F). From blastocyst stage, it is expressed in trophoectoderm and inner cell mass (Fig. 2G,H). The intensity of PTGS2 protein peaked at PN stage and increased at morula stage and decreased at hatching stage compare to 2-cell stage embryo.

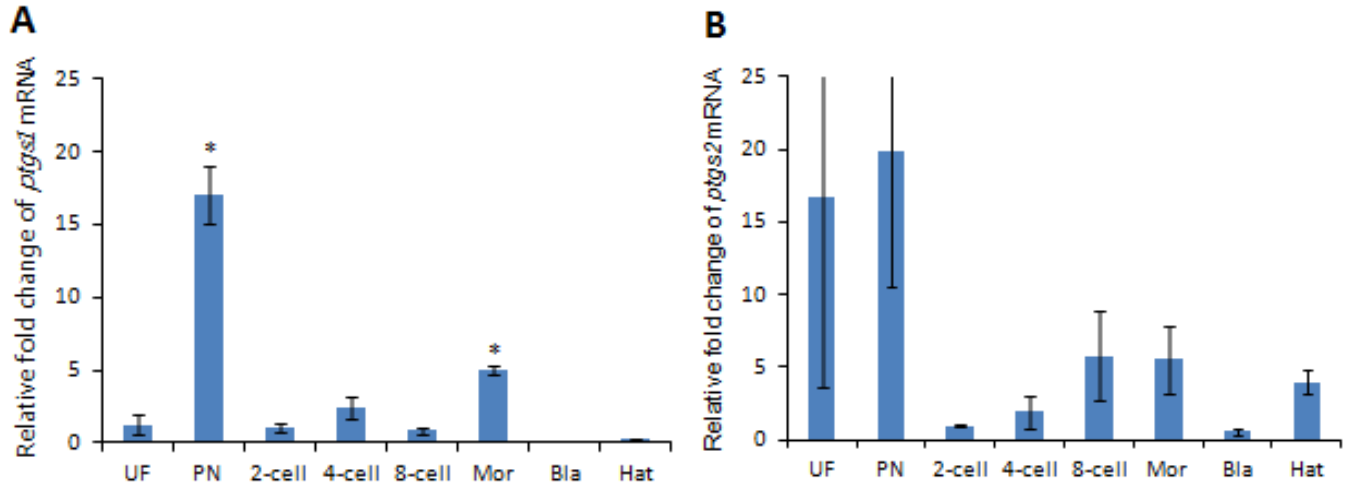
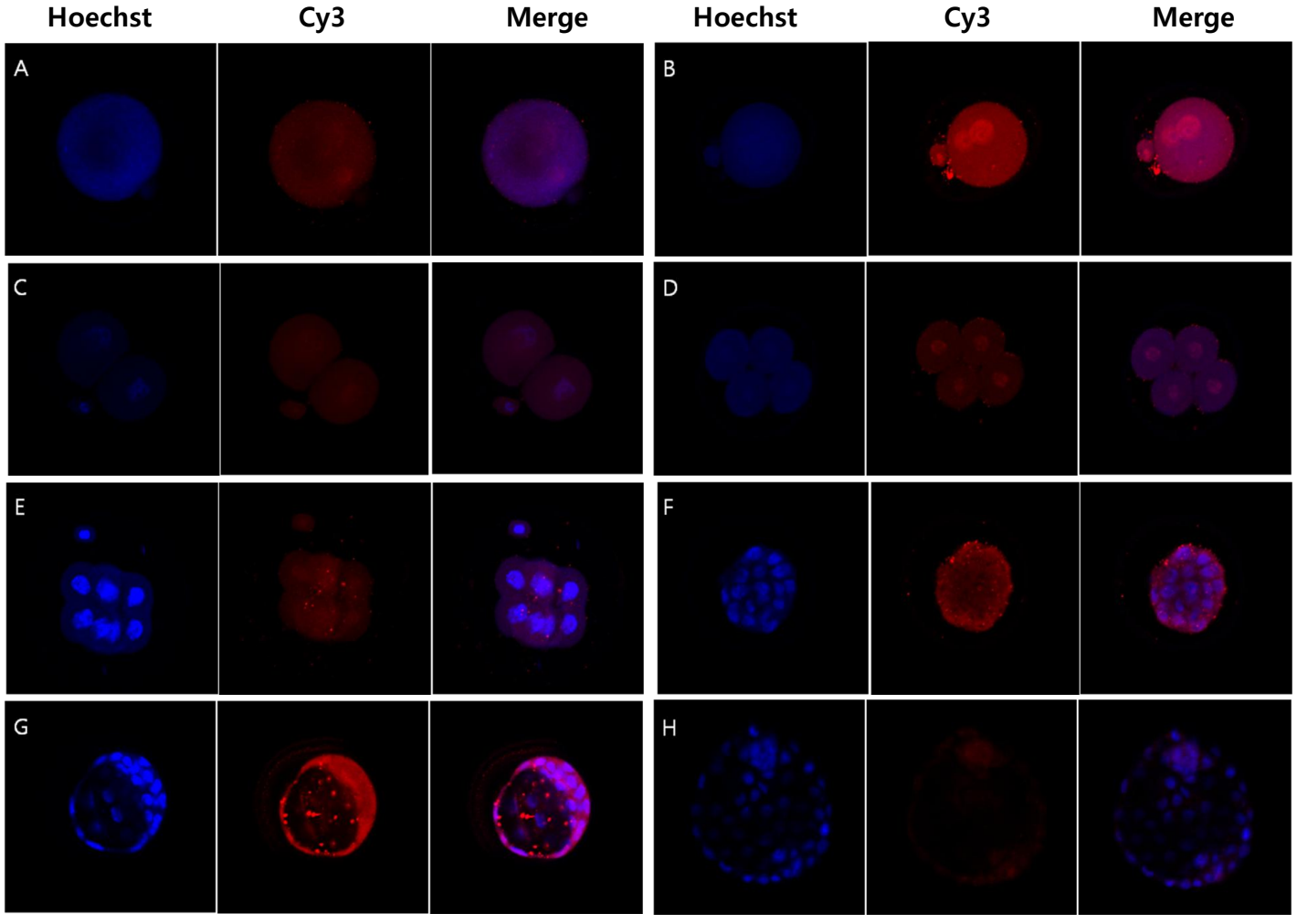



**Figure 1. mRNA expression profiles of *Ptgs1* and *Ptgs2* in mouse embryo during early embryo development.**

The relative quantity of *Ptgs1* and *Ptgs2* mRNA. Total RNA was extracted from each stage of embryo *in vivo* and relative fold change was normalized with 2-cell stage embryo. Values represent the mean±SEM. *$P < 0.05$ vs. 2-cell.


Hoechst
Cy3
Merge
Hoechst
Cy3
Merge
A
B
C
D
E
F
G
H

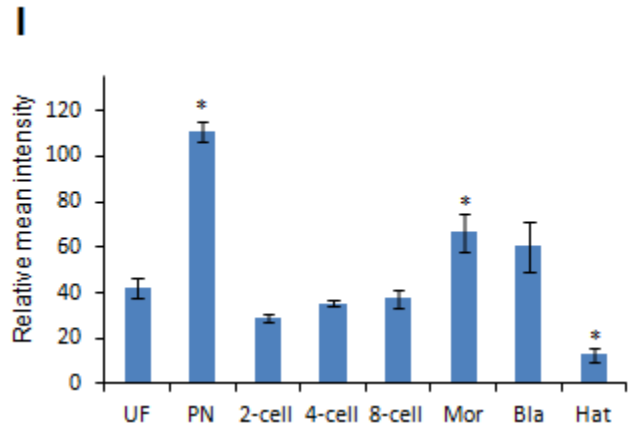



**Figure 2. Profiles and localization of PTGS2 protein in preimplantation embryo.**

The localization of PTGS2 protein in preimplantation embryo was examined by whole-mount immunofluorescence. A: UF, B: PN, C: 2-cell, D: 4-cell, E: 8-cell, F: Morula, G: Blastocyst, H: Hatching, I: intensity of PTGS2. PTGS2 is red(Cy3) and nucleus is blue(Hoechst 33258). Values represent the mean±SEM . * $P$< 0.05 vs. 2-cell.

**Effect of Carbohydrate metabolite on early embryo development**

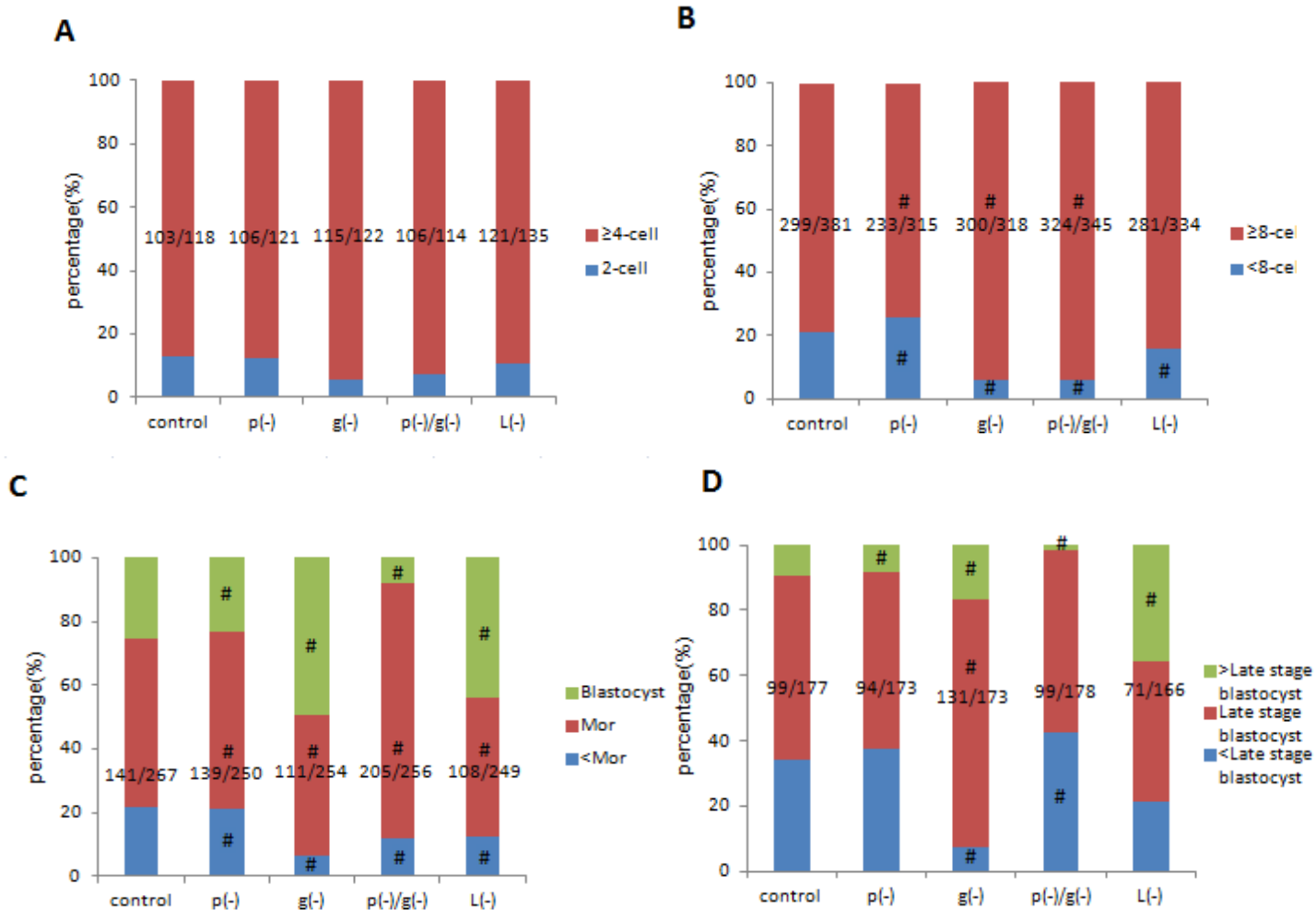
To investigate the effect of carbohydrate metabolite on early embryo development *in vitro* culture, developmental rate was observed at 60 hr, 72 hr, 96 hr, 120 hr, 140 hr post hCG (Fig. 3). At 60 hr post hCG, post 4-cell stage rate have no significant change in experimental group (Fig. 3A). At 72 hr post hCG, post 8-cell stage rate increased in g(-) and p(-)/g(-) group and decreased in p(-) group (Fig. 3B). At 96 hr post hCG, morula stage rate increased in p(-) and p(-)/g(-) group and decreased in g(-) and l(-) group, but post morula stage rate increased in g(-) and l(-) group and decreased in p(-) and p(-)/g(-) group (Fig. 3C). At 120 hr post hCG, late blastocyst stage rate increased only in g(-) group. And pre-late blastocyst stage rate decreased in g(-) and increased in p(-)/g(-) group. Post late blastocyst stage rate increased in g(-) and l(-) group and decreased in p(-) and p(-)/g(-) group compare to control (Fig. 3D). At 140 hr post hCG, hatching rate increased in g(-) and l(-) group and decreased in p(-)/g(-) (Fig. 3E).

To estimate the *Ptgs1* and *Ptgs2* mRNA level change of early embryo in different carbohydrate metabolite culture condition, real time PCR analysis was performed. In control culture condition, 4-cell embryo mRNA expression higher slightly than 2-cell stage embryo and blastocyst stage mRNA expression level is the lowest. In p(-) group, *Ptgs1* mRNA expression level increased in 4-cell. And at the blastocyst and hatching stage, *Ptgs1* mRNA significantly decreased compare to *in vitro* control group. In g(-) group, *Ptgs1* mRNA expression level significantly increased at 4-cell, 8-cell and morula stage and significantly decreased at

blastocyst and hatching stage compare to *in vitro* control group. In p(-)/g(-) group, *Ptgs1* mRNA peaked on 8-cell stage and gradually decreased after 8-cell stage to hatching stage, but significantly higher than control group at 8-cell, morula, and blastocyst stage. In l(-) group, *Ptgs1* mRNA expression significantly increased at hatching stage and significantly decreased at blastocyst than in vitro control group (Fig. 4A). In p(-) group, *Ptgs2* mRNA expression level increased at 4-cell stage and gradually decreased after. In g(-) group, *Ptgs2* mRNA expression level peaked at 4-cell stage and significantly decreased than *in vitro* control group at morula stage. P(-)/g(-) group embryo's *Ptgs2* mRNA expression level significantly increased than *in vitro* control at 8-cell stage and rapidly decreased after 8-cell stage. In l(-) group, *Ptgs2* mRNA expression was high at 4-cell, 8-cell and hatching stage and low at morula and blastocyst stage (Fig. 4B).

To investigate the change of localization and intensity of PTGS2 protein in different culture condition, whole-mount immunofluorescence was performed. The localization of PTGS2 protein was same as *in vivo* embryo. Until compaction, the protein localized in cytoplasm and since blastocyst, localized in trophoectoderm and inner cell mass (Fig. 5-10). Intensity of PTGS2 protein obtained using ZEN 2011 software. At all *in vitro* group, PTGS2 protein intensity decreased than 2-cell stage but only the PTGS2 protein of morula stage in p(-)/g(-) group was significantly increased than 2-cell stage. In p(-) group, PTGS2 protein significantly increased at 4-cell and hatching stage than *in vitro* control group. In g(-) group, PTGS2 protein significantly increased at morula and blastocyst stage than *in vitro* control group. In addition, PTGS2 protein intensity of p(-) group and g(-) group increased than *in vitro* control group at all stages of

embryos. PTGS2 protein intensity of p(-)/g(-) group significantly increased at morula stage than *in vitro* control. In l(-) group, PTGS2 protein intensity increased at blastocyst stage than 2-cell embryo (Fig. 10).


A
100
80
60
40
20
0
percentage(%)
103/118
106/121
115/122
106/114
121/135
control
p(-)
g(-)
p(-)/g(-)
L(-)
≥4-cell
2-cell
B
299/381
233/315
300/318
324/345
281/334
#
≥8-cel
<8-cel
C
141/267
139/250
111/254
205/256
108/249
Blastocyst
Mor
<Mor
D
99/177
94/173
131/173
99/178
71/166
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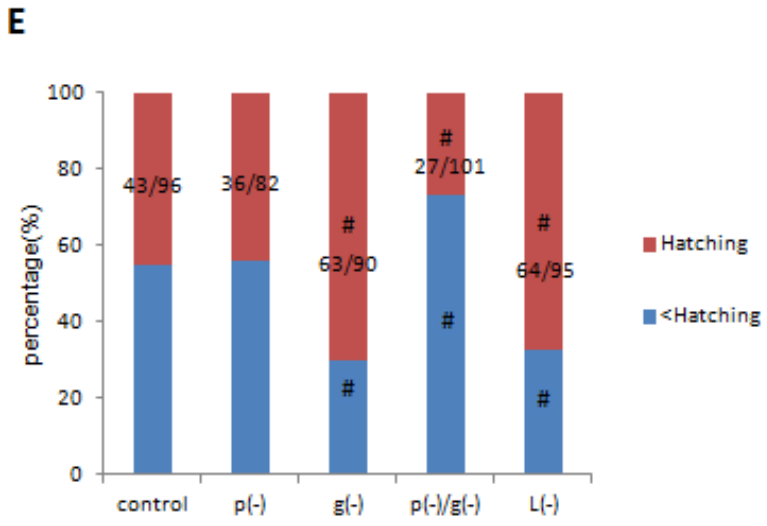


**Figure 3. Development ratio of embryo in different culture condition.**

Developmental ratio of control(BWW), pyruvate free; p(-), glucose free; g(-), pyruvate and glucose free; p(-)/g(-), and lactate free; L(-) culture condition. A: 60 hr, B: 72 hr, C: 96 hr, D: 120 hr, E: 140 hr. #$P < 0.05$.

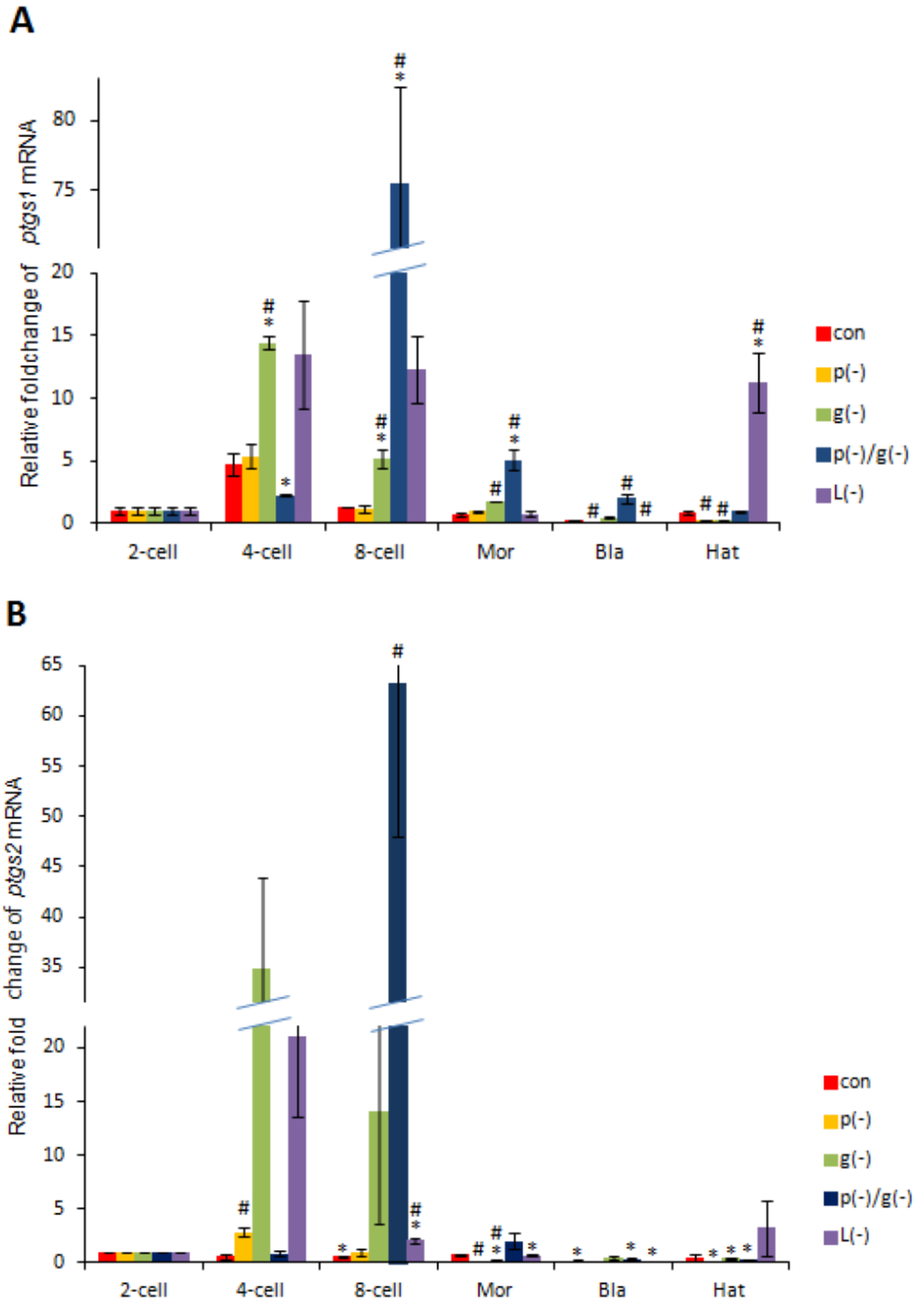
A
Relative foldchange of ptgs1 mRNA
80
75
20
15
10
5
0
2-cell
4-cell
8-cell
Mor
Bla
Hat
con
p(-)
g(-)
p(-)/g(-)
L(-)
B
Relative fold change of ptgs2 mRNA
65
60
55
50
45
40
35
20
15
10
5
0
2-cell
4-cell
8-cell
Mor
Bla
Hat
con
p(-)
g(-)
p(-)/g(-)
L(-)

**Figure 4. Expression pattern of *Ptgs1* and *Ptgs2* mRNA in different carbohydrate culture condition.**

A: *Ptgs1* mRNA level *in vitro*, B: *Ptgs2* mRNA level *in vitro*. Values represent the mean±SEM. **P* < 0.05 vs. *in vivo* 2-cell, #*P* < 0.05 vs. *in vitro* control.

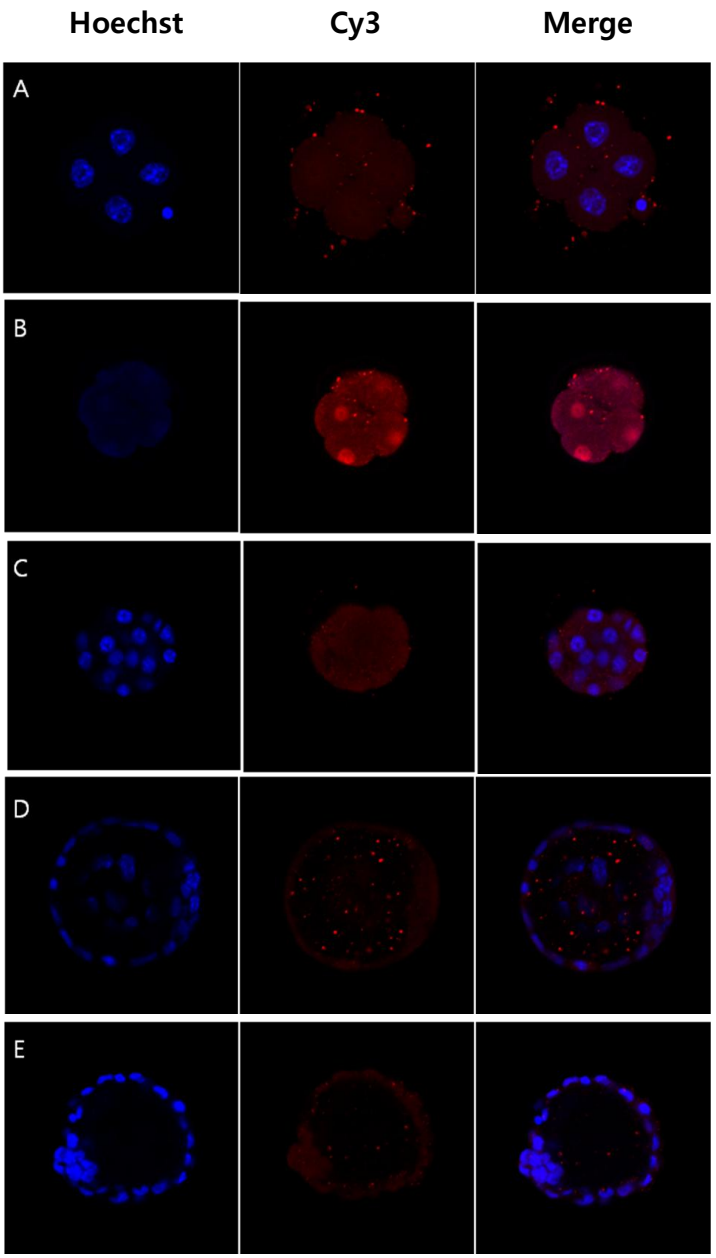


**Figure 5. Profiles and localization of PTGS2 protein *in vitro* control embryos.**

A: 4-cell, B: 8-cell, C: Morula, D: Blastocyst, E: Hatching

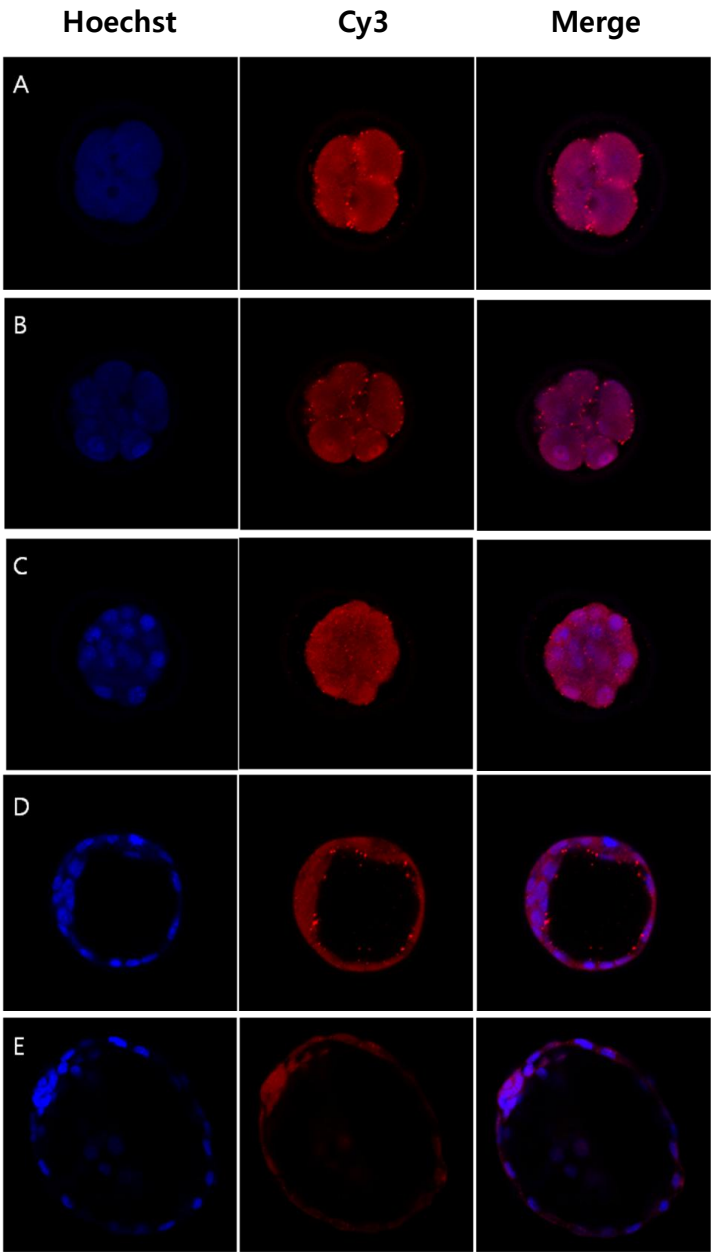


**Figure 6. Profiles and localization of PTGS2 protein *in vitro* p(-) embryos.**

A: 4-cell, B: 8-cell, C: Morula, D: Blastocyst, E: Hatching.

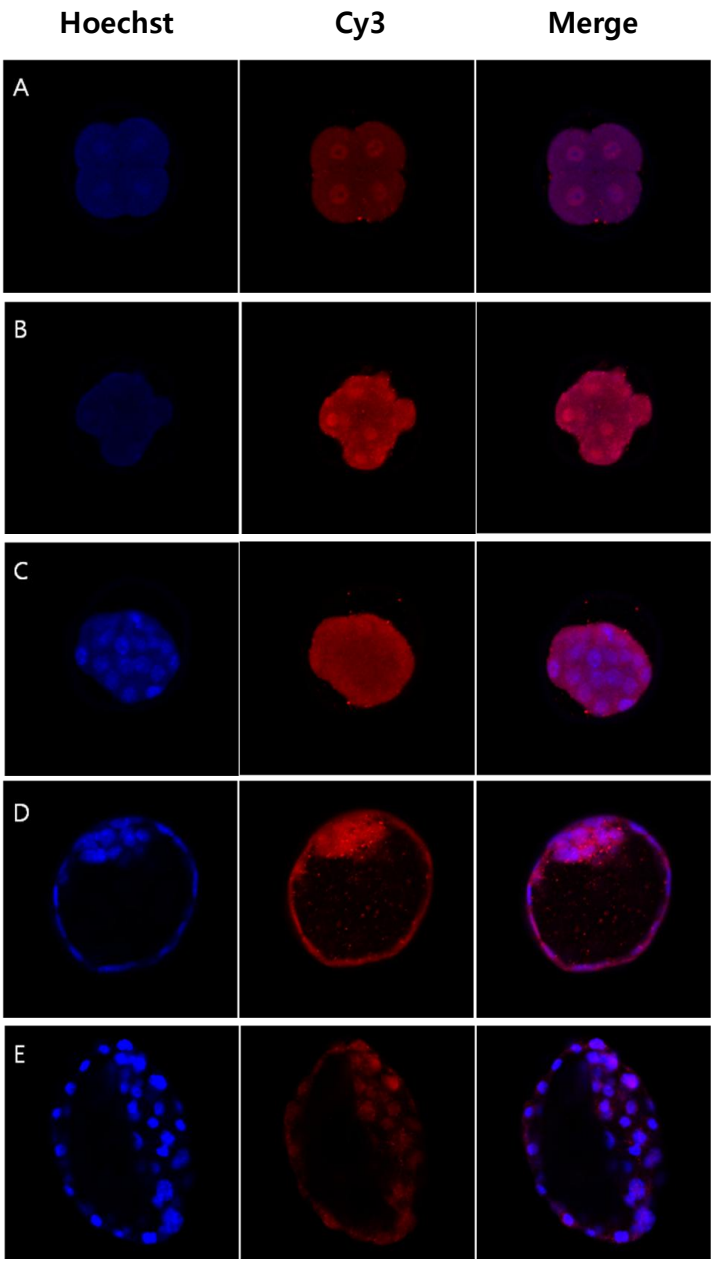


**Figure 7. Profiles and localization of PTGS2 protein *in vitro* g(-) embryos.**

A: 4-cell, B: 8-cell, C: Morula, D: Blastocyst, E: Hatching.

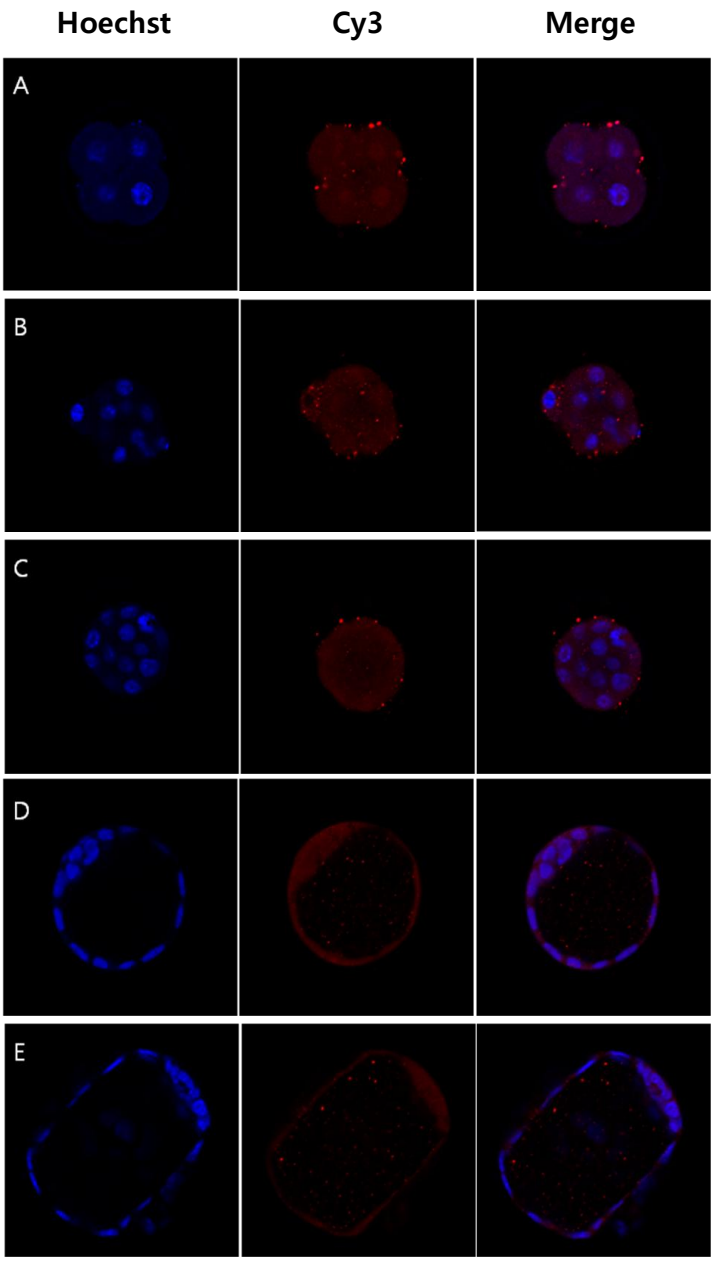


**Figure 8. Profiles and localization of PTGS2 protein *in vitro* p(-)/g(-) embryos.**

A: 4-cell, B: 8-cell, C: Morula, D: Blastocyst, E: Hatching.

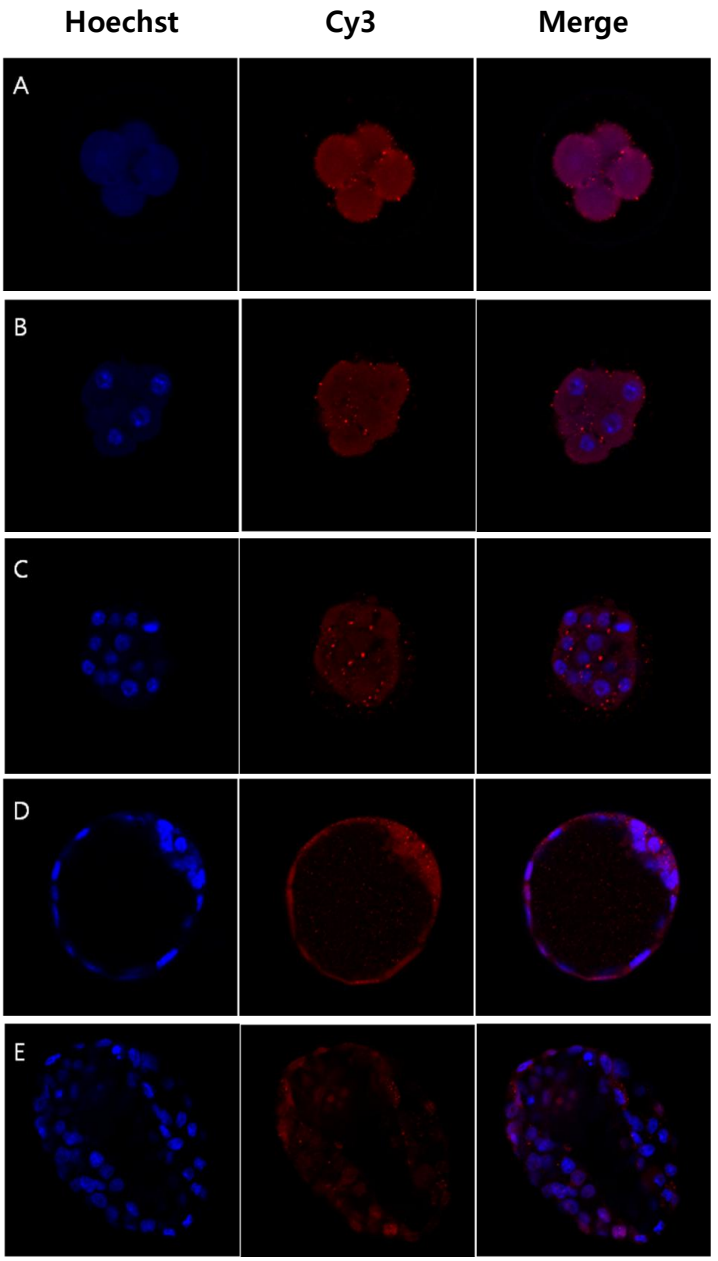


**Figure 9. Profiles and localization of PTGS2 protein *in vitro* L(-) embryos.**

A: 4-cell, B: 8-cell, C: Morula, D: Blastocyst, E: Hatching.

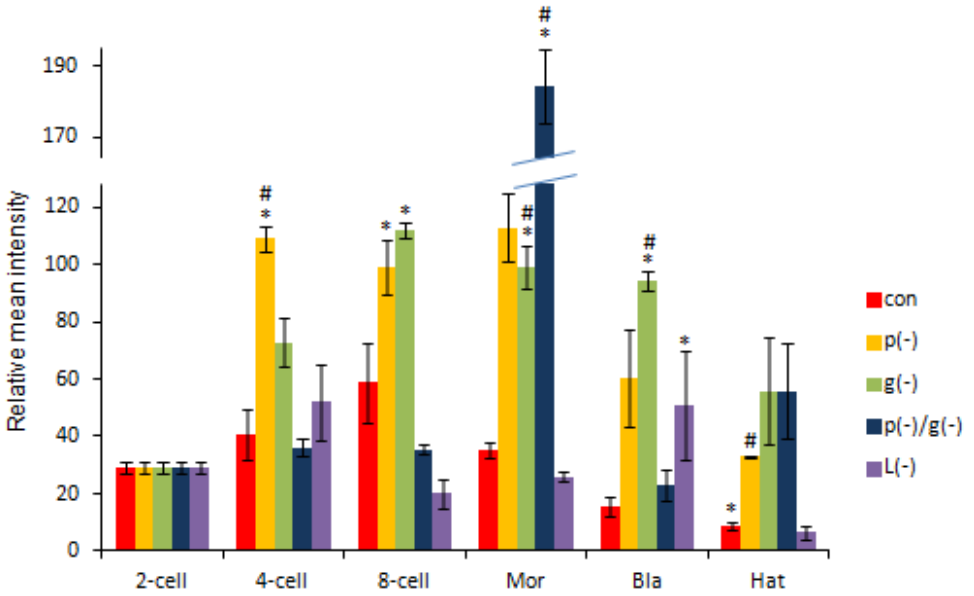


**Figure 10. Intensity of PTGS2 protein *in vitro*.**

Values represent the mean±SEM. *$P < 0.05$ vs. *in vivo* 2-cell, #$P < 0.05$ vs. *in vitro* control.

**Amount of prostaglandin receptor mRNA in early embryo development and *in vitro* cultured embryo**

To investigate which prostaglandin acts on preimplantation embryo development, prostaglandin receptor mRNA was analyzed by real time PCR analysis. In early embryo development, *Ptgfr* mRNA expression increased in UF, PN and hatching stage and decreased in morula and blastocyst (Fig. 11A). When embryo cultured *in vitro*, *Ptgfr* mRNA increased in g(-) and l(-) conditioned 4-cell embryo. At 8-cell stage, *Ptgfr* mRNA increased in g(-), p(-)/g(-) and l(-) and decreased in p(-) condition. At morula stage, *Ptgfr* mRNA level increased in p(-)/g(-) and decreased in control, p(-) and g(-) condition compare to 2-cell embryo. At blastocyst stage, *Ptgfr* mRNA decreased in all group, and hatching stage *Ptgfr* mRNA decreased in all group except l(-) (Fig. 11B).

*Ptgir* and *Ptger2* mRNA expression level is very low at both *in vivo* and *in vitro* embryo (Fig. 12,13). It seems to *Ptger2* mRNA increase at *in vivo* PN stage a little, but it was not significant (Fig. 13A).

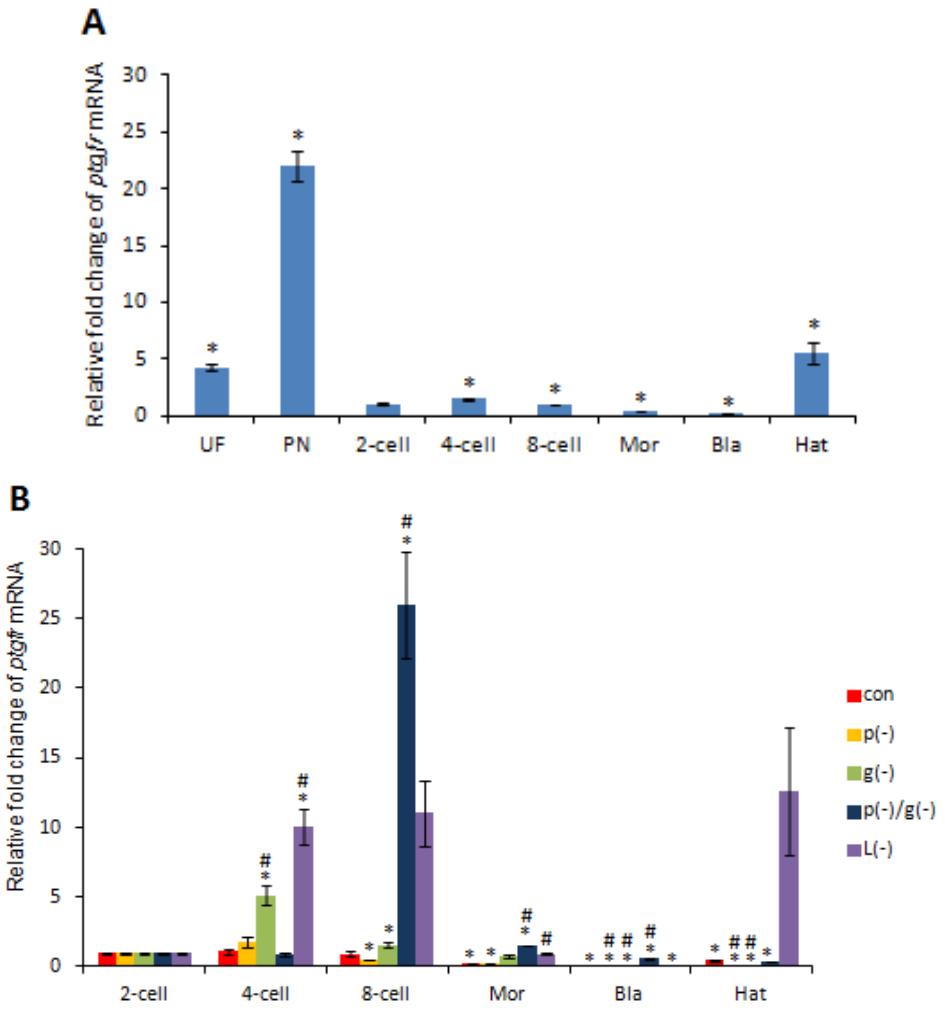


**Figure 11. Profiles of prostaglandin $F_{2a}$ receptor mRNA levels in preimplantation embryo *in vivo* and *in vitro*.**

A: The relative quantity of *Ptgfr* mRNA of *in vivo* embryo, B: The relative quantity of *Ptgfr* mRNA of in vitro embryo. Values represent the mean±SEM. **P* < 0.05 vs. *in vivo* 2-cell, #*P* < 0.05 vs. *in vitro* control.

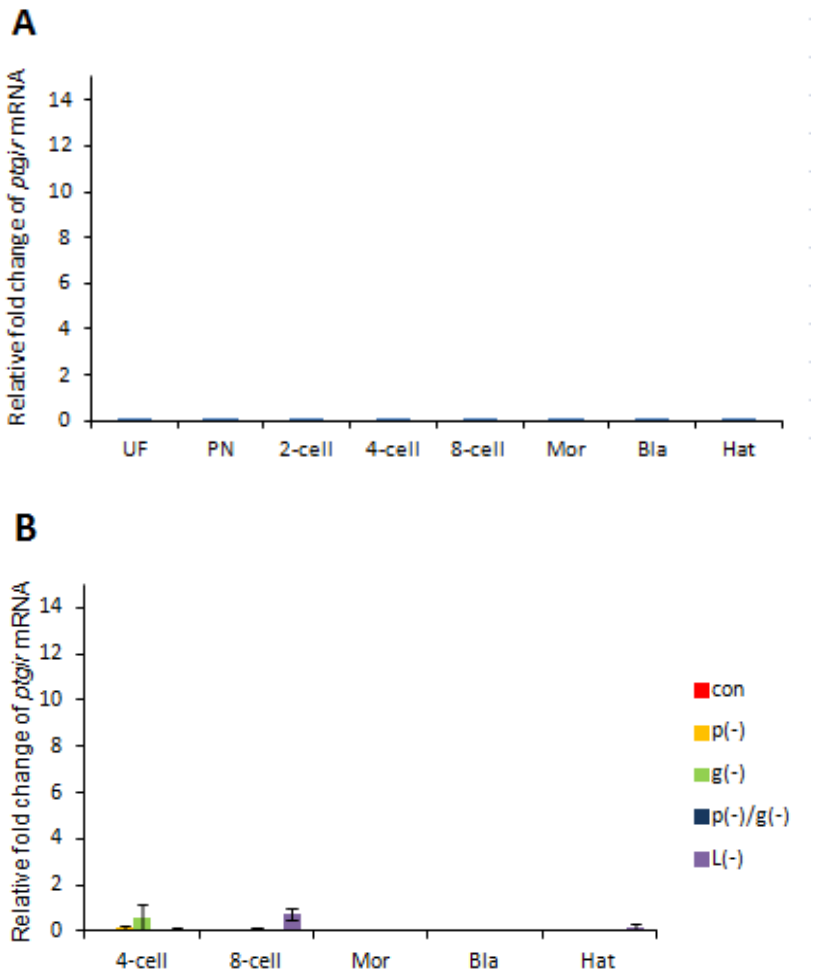


**Figure 12. Profiles of prostaglandin $I_2$ receptor mRNA levels in preimplantation embryo *in vivo* and *in vitro*.**

A: The relative quantity of *Ptgir* mRNA of *in vivo* embryo, B: The relative quantity of *Ptgir* mRNA of *in vitro* embryo. Values represent the mean±SEM. *$P < 0.05$.

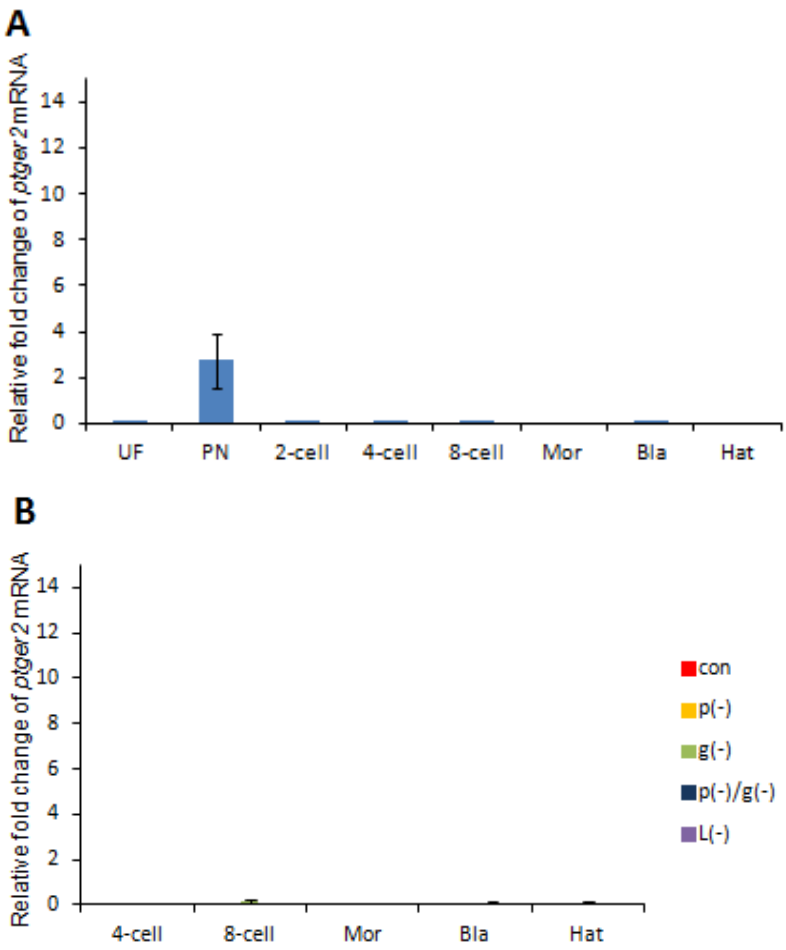


**Figure 13. Profiles of prostaglandin $E_2$ receptor mRNA levels in preimplantation embryo *in vivo* and *in vitro*.**

A: The relative quantity of *Ptger2* mRNA of *in vivo* embryo, B: The relative quantity of *Ptger2* mRNA of *in vitro* embryo. Values represent the mean±SEM. *P < 0.05.

## DISCUSSION

The *Ptgs1* mRNA relative quantity increased before 2-cell stage and become basal level at 2-cell stage. It suggests that maternal transcript of *Ptgs1* work during oocyte maturation or fertilization. PTGS2 was mainly localized in submembrane cytoplasm. These results may be support previous studies, metabolic products produced by PTGS are involved in ovulation (Joyce et al., 2001).

Whole-mount immunofluorescence was performed to investigate the localization and expression pattern in preimplanation embryo. There is many studies research the immunohistochemical localization of PTGS. PTGS was detected in cytoplasm of 2-cell, 8-cell embryo, trophoblast of blastocyst, zona pellucida of 4-cell embryo unspecifically (Van der Weiden et al., 1996), cytoplasm of Morula (Marshburn et al., 1990), trophoectoderm and inner cell mass of blastocyst (Huang et al., 2004a; Parkrasi et al., 2007). Our results represent the same pattern in immunofluorescence. Until morula stage, mouse PTGS2 protein detected in cytoplasm of embryo and detected trophoectoderm and inner cell mass in blastocyst and hatching stages.

We cultured 2-cell embryo with various carbohydrate metabolite condition and observed development rate and analyzed *Ptgs* and receptor of PG mRNA relative quantity level. Mouse embryo development is identically inhibited by raise osmolarity (Dawson et al., 1997). So we use D-(+)-Raffinose pentahydrate for

osmolytes as it is not transported by mammalian cells and does not permeate membranes. When glucose not supplied to culture media, development rate of 8-cell at 60 and 72 hr post hCG and blastocyst at 96 hr and 120 hr post hCG and hatching stage at 140 hr were increased. It may result from the glucose was known as a toxic factor at *in vitro* culture of embryo (Nagao et al., 1994). Likewise, pyruvate and glucose free conditioned embryo's development rate of 8-cell at 60 and 72 hr post hCG and the rate of morula at 96 hr post hCG increased, but development rate of pyruvate and glucose free group decreased from 120 hr post hCG and hatching rate at 140 hr post hCG significantly decreased. These result suggest that both glucose and pyruvate deficient culture condition disrupt the hatching of embryo.

Expression pattern and relative quantity of *Ptgs1* and *Ptgs2* mRNA was investigated in different condition of *in vitro* embryo. *Ptgs1* mRNA expression in BWW cultured embryo peaked in 4-cell embryo and decrease from 8-cell to blastocyst stage. *Ptgs1* and *Ptgs2* mRNA expression in glucose free conditioned embryos significantly increased at 4-cell, 8-cell and morula stage and decreased at hatching stage compare to *in vitro* control group. *Ptgs1* and *Ptgs2* mRNA expression in pyruvate and glucose free conditioned embryo significantly high in 8-cell stage and decrease since morula stage. These results suggest the correlation between development rate and *Ptgs* mRNA expression level. The high level of *Ptgs1* and *Ptgs2* mRNA may support development rate of preimplantation

embryo.

Prostaglandin receptor mRNA expression was analyzed to investigate what prostaglandins acts on development. *Ptgir* and *Ptger2* were not expressed during *in vivo* or *in vitro* development. *In vivo* embryo's *Ptgfr* mRNA expression peaked at PN stage like *Ptgs1* mRNA expression pattern. This suggests that PTGS acts on preimplantation embryo by mediating $PGF_{2a}$. At *in vitro* conditioned embryos, *Ptgfr* mRNA expression pattern was similar to *Ptgs1* and *Ptgs2* mRNA expression. The *Ptgfr* mRNA expression level peaked at 8-cell of pyruvate and glucose free group. In glucose free group, *Ptgfr* mRNA expression level increased at 4-cell and 8-cell stage compare to *in vitro* control. It supports *in vivo* data that PTGS may acts by mediating $PGF_{2a}$. *Ptgir* and *Ptger2* mRNA expression were not detected both *in vivo* and *in vitro* preimplantation embryos.

In summary, the expression profiles of the *Ptgs* and *Ptgsr* were changed by the kinds of carbohydrate. In addition, the stage specific expression was detected in carbohydrate specific manners. This result may suggest the carbohydrate metabolites have potential role in PTGS expression regulation and developmental regulation mediating $PGF_{2a}$.

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# ABSTRACT

## Correlated Work between Carbohydrate and Inflammation Factors In Early Embryo Development

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In mammalian animals, cleavage of fertilized eggs was progressed with migrating trough oviduct. Suitable condition of oviduct support the embryo to gain developmental ability by various factors including endogenous controller and exogenous factors, and carbohydrate was well known regulator. On the other hand, arachidonic acid (AA) metabolites such as $PGE_2$, $PGF_{2a}$, $PGD_2$, $PGI_2$, and $TXA_2$ derived from prostaglandin-endoperoxide-synthase (PTGS), known as inflammation factor, are known to expressed in preimplantation embryo. Diverse knockout mouse studies reported the effect of prostaglandin in ovulation, fertilization, hatching and implantation but role of prostaglandin in cleavage stage was not known. Some report proposed that metabolites and inflammation factors have correlation in some tissues, but it is not known clearly. In this study, we tried to investigate whether carbohydrate metabolite regulates the prostaglandin or not

in early embryo development. Female CD1 mouse were superovulated and each stage of embryos were collected from oviduct and uterus at specific time for analyze the natural condition embryo's related information. And we studied using specific carbohydrate conditioned BWW medium for culture of 2-cell embryo and analyzed the data when carbohydrate were restricted. Real time quantitative PCR was performed to *Ptgs* and receptors of PG, and investigated the protein expression using whole-mount immunofluorescence. *Ptgs1* mRNA expression *in vivo* embryo peaked at 1-cell stage and low at 2-cell stage, and increase at 4-cell stage again. *Ptgs2* mRNA expression was high until 1-cell stage, and decreased at 2-cell and increased at 4-cell again, and decreased at blastocyst stage. When restricted the carbohydrate metabolites, development rate of glucose free group increased at 72 hr, 96 hr, 120 hr and 140 hr post hCG. In pyruvate and glucose free group, development rate of 72 hr, 96 hr post hCG increased but decreased from 120 hr to 140 hr post hCG. *Ptgs1* and *Ptgs2* mRNA expression level of *in vitro* embryo increased at 4-cell and 8-cell of glucose free group. In pyruvate and glucose free group, *Ptgs1* and *Ptgs2* mRNA expression rapidly increased at 8-cell stage and gradually decreased to hatching stage. From this result, we suggest the *Ptgs* have potential role in preimplantation embryo development, and investigated prostaglandin receptor mRNA expression to know what prostaglandin may have role. *Ptgfr* mRNA expression pattern was similar to both *Ptgs1* and *Ptgs2* mRNA expression. And *Ptgir* and *Ptger2* mRNA expression were not detected. This result may suggest the potential role of carbohydrate metabolites in PTGS expression regulation and developmental regulation mediating $PGF_{2a}$.