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Lactic acid mediated blastocyst development competence

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Lactic acid mediated blastocyst development competence

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Master of Science

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Sungshin University

Graduate School

ABSTRACT

Lactic acid mediated blastocyst development competence

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Developing preimplantation embryos require appropriate microenvironment and specific gene expression in each stage to acquire proper developmental capacity. Carbohydrate as energy sources during early stage mouse embryos is well established. Until 8-cell stage, embryos use pyruvate as a main energy source. Specifically, glucose is utilized after 8-cell stage and produce lactate highly, although oxygen presence as enough. So far, lactate is suggested as end product of glycolysis in early stage embryo. However, recent studies suggest new role of lactate such as signaling molecule, gene regulation, and so on. In this study, the possible role of lactate was explored through the evaluate expression of lactate dehydrogenases (LDHs) that catalyze interconversion of lactate to pyruvate. The cultured embryo in lactate-free condition (L(-)) showed delayed development than control group. As *in vivo* or *in vitro* embryo development, relative mRNA ratio of *Ldha* that prefers catalyzing pyruvate to L-lactate increased than other LDHs. In

addition, ratio switching timing was later in lactate-free cultured embryos than in BWW-cultured embryos and *in vivo* embryos. Even at the protein level, as embryo development, relative ratio of LDHA increased than others. Also, such timing was later in L(-) cultured embryos than other groups. In *Ldh* knockdown experiments with siRNA microinjection, the L(-) cultured embryo with *Ldha*-siRNA injection showed low development rate to 8-cell, compacted morula and blastocyst. Based on these results, LDHs expression levels could be modified by the environment and be adapt for the development. Also, data suggests that appropriate production and use of lactate is required for normal embryonic development. These data suggested that lactate metabolism is essential in developmental competence during early embryo stages.

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INTRODUCTION

During embryogenesis, embryo is surrounded by oviductal fluid that provides suitable microenvironment such as stable temperature, optimal pH and dynamic secretomes for supporting embryo development (Li and Winuthayanon, 2017). This supports the successful early embryo development that depends on the embryo's ability to generate energy metabolites, substrates and building blocks through appropriate metabolic pathways at given specific times (Cheon, 2008).

One-cell zygotes prefer oxidative phosphorylation with utilization of pyruvate as a carbon source (Kailun and Yang, 2017). At around the time of compaction, preimplantation mouse embryos switch from pyruvate to a glucose-based metabolism (Gardner and Leese, 1986; Leese and Barton, 1984). Distinct from most cell types that use glucose, the blastocyst converts approximately half the glucose consumed to lactate, even in the presence of sufficient oxygen to support its complete oxidation (Gardner and Leese, 1990). In this regard, Leese and co-worker (1996) showed that during the transition of morula to blastocyst, in mice, lactate production increased along with increase of glucose consumption. In human embryo, the same pattern appeared (Gott et al., 1990).

Lactate dehydrogenase (LDH) catalyzes inter-conversion between pyruvate and lactate. There are four types of LDH gene: *Ldha, Ldhb, Ldhc* and *Ldhd* (Fig. 1). First two genes code LDHA or LDHB subunit, respectively, which form five distinct tetrameric isoenzymes, LDH-1 to 5 (Porporato et al., 2011). LDH-1 (A0B4) catalyzes conversion L-lactate to pyruvate preferentially and LDH-5 (A4B0) catalyzes vice versa direction. LDH-2 (A3B1), LDH-3 (A2B2) and LDH-4 (A1B3) have intermediate enzyme activity (Fig. 1). The *Ldhc* codes LDHC subunit forming LDH-C_4 that catalyzes oxidation of L-lactate to pyruvate. Unlike others, *Ldhd* codes LDHD subunit that consists dimer utilizing D-lactate (Drabkin et al., 2019).

Several studies are interested in the new role of lactic acid rather than recognizing it just as a product of the glycolysis. Lactate may contribute to maintaining mitochondrial maintenance in muscle cells through cAMP pathway inhibition (Sun et al., 2016). In neuron, it also contributes to modulating the expression of genes associated with neuroprotection and synaptic plasticity (Margineanu et al., 2018). The role as a signaling molecule material was also studied. In cancer, lactate promotes angiogenesis and cell growth under hypoxia through NDRG3-c-Raf-ERK1/2 axis (Lee et al., 2015). Besides, in recent study, lysine lactylation was proposed as a new type of histone mark (Di Zhang et al., 2019).

According to previous studies in our laboratory, the expression of *Ldhs* during cleavage was detected in limited fashion. On the other hand, the mechanism of lactate in early stage embryo is not much known. In this study, the possible mechanism of lactate in early embryo development was examined. Late 2-cell stage embryos were cultured in lactate-free medium. The quantitative RT-PCR, immunofluorescence, and knockdown technique were employed. The lactate metabolism during early embryo is essential for optimal development.

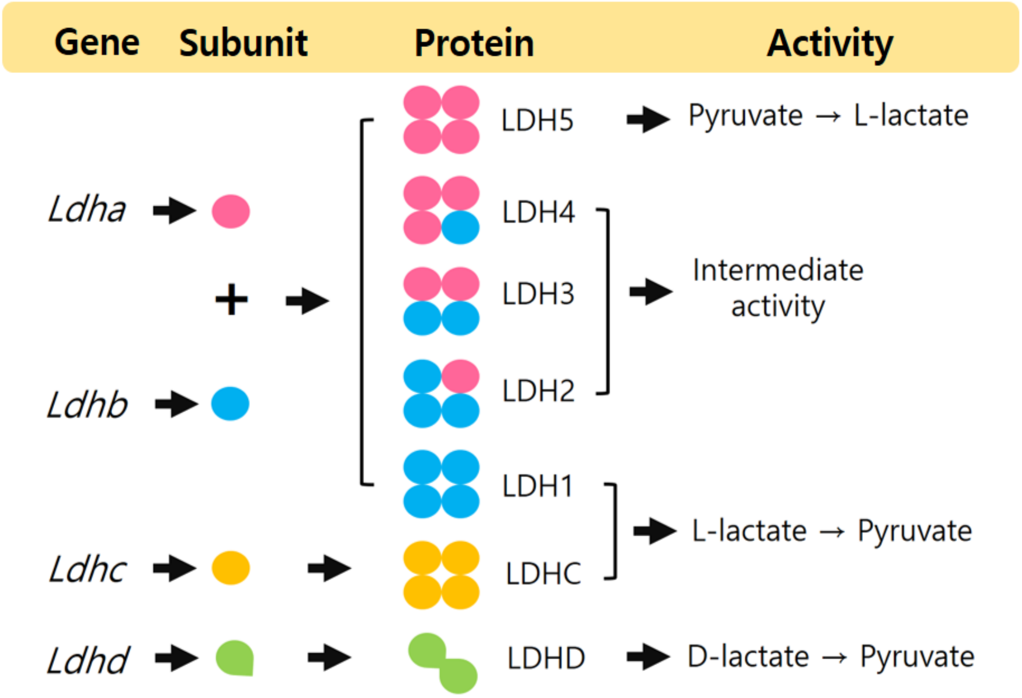


Figure 1. Composition of lactate dehydrogenases. Each type of LDH gene code LDHA, LDHB, LDHC and LDHD subunit. LDHA and LDHB subunit form five distinct tetrameric isoenzymes, LDH-1 to 5. The LDH-1 catalyzes conversion L-lactate to pyruvate preferentially and LDH-5 catalyzes vice versa direction. The LDHC which encoded by *Ldhc* catalyzes oxidation of L-lactate to pyruvate. Unlike other LDHs, LDHD catalyze oxidation D-lactate to pyruvate.

MATERIALS AND METHODS

Experimental animals

All experimental animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. All experiments were approved by the Institutional Animal Care and Use Committee in Sungshin Women's University (SSWIACUC-2019-011). These animals were maintained under standard condition at Sungshin University 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 after birth.

Superovulation and embryo sampling

To superovulate female mice, PMSG and hCG were diluted to 5mg/0.1ml with 0.85% saline. 6-8 weeks old female CD-1 mice were superovulated by PMSG (5IU / 0.1mL) intraperitoneal injection in followed 48hr later by hCG (5IU / 0.1mL). After then, females were placed with males of the same strain. Next morning, presence of vaginal plug was considered pregnant. Each stage of preimplantation embryo including unfertilized egg (UF), pronucleus (PN), 2-cell, 4-cell, 8-cell, morula, early or late blastocyst were collected from oviduct or uterus by flushing with BWW containing 0.4% bovine serum albumin (BSA) after 15, 21, 48, 56, 64, 80, 86, and 96 h of post-hCG injection, respectively. *In vitro* developed 4-cell, 8-cell, morula, early or late blastocyst were collected at 60, 72, 84, 96 and 120 h of post-hCG injection, respectively.

Culture Media preparation

Control medium is normal BWW medium. Lactate-free medium contained 21.58 mM (43.10 mOsM) NaCl and 1.71 mM (5.13 mOsM) $CaCl_2$ instead of 21.58 mM (43.10 mOsM) Sodium DL-lactate and 1.71 mM (5.13 mOsM) Calcium L-lactate, respectively. Medium composition listed in Table 1.

Total RNA extraction and first strand cDNA synthesis

Total RNA of 10 ea embryos was extracted using RNeasy® Micro kit (QIAGEN, USA) according to the manual of manufacture. To synthesize irst strand cDNA, we used Accuscript first strand cDNA synthesis kit (Stratagene, CA, USA) according to the manual of manufacture. Reaction reagents were total RNA of 10ea embryos, 5.0 μL Accuscript buffer (10X), 1.0 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. Mixture was incubated at 65°C for 5 min, placed at RT to anneal RNA with primers for 10 min, after then 0.4 μL DTT (100mM), 2.0 μl RNase block ribonuclease inhibitor (40 U/mL), and 1.0 μL Accuscript multiple temperature RT were added. The reaction mixture was incubated at 42°C for 1hr and 70°C for 15 min. Finally, the products were kept at -20°C before used.

Real-time RT-PCR analysis

Real-time PCR was performed by Thermal Cycler Dice Real Time system TP800 (Takara, Japan) according to thermal cycler schedule in Table 2. Each reaction was run in triplicate and consisted of 1 μL cDNA, 10 μL SYBR® Premix Ex Taq™ (Takara, Japan) and the specific primers listed in Table 3. To ensure amplification of a single product at appropriate melting temperature, dissociation curves were run on all reactions. The fold change in gene expression was calculated using the ∆∆CT method with the housekeeping gene, a ribosomal protein, 36B4(RPLP0), as the internal control.

Whole mount immunofluorescence of mouse embryo

Collected embryos were fixed in 4% paraformaldehyde in PBS 0.15% picric acid for 30 min at RT. Permeabilization performed 0.5% PBST (PBS containing 0.5% Triton X-100) for 3hr. Blocking was conducted by PBS containing 1% BSA. Then, embryos were incubated with goat polyclonal anti-LDHA (Santacruz, USA), rabbit polyclonal anti-LDHB (Abcam, UK), rabbit polyclonal anti-LDHC (Abcam, UK) or rabbit polyclonal antibody (Proteintech, USA) diluted 1:100 in 2% BSA/PBS containing 5% DMSO (Sigma-aldrich, USA) at 4°C overnight. Washed embryos were incubated with 2nd Cy3 conjugated goat anti-rabbit or rabbit anti-goat (Jackson ImmunoResearch, USA) diluted 1:80 for 2 h at RT and washed. Embryos were counterstained with DAPI (1:500) for 10 min at RT and mounted on dot slide using 20% glycerol. Embryos for negative control were followed above protocol without first antibody incubation. Stained embryos were observed with confocal microscope (Zeiss LSM 700 Laser scanning confocal microscope, Germany). The immunofluorescence intensity was measured by using Zen 2011 software.

siRNA microinjection

The *Ldh* siRNA duplexes were ordered from Bioneer. The sequence of antisense strand was introduced in Table 4. The 10 nmol siRNA were resuspended in 100 μL RNase-free water for 100 μM stock. Stock was diluted with 1X siRNA dilution buffer for 2μM. Working solution were aliquoted and stored at -20°C before used. Each blastomere of late 2-cell stage embryo injected 10 pL of 2 μM siRNA solution. The negative control group was injected same volume of negative control siRNA, FAM-labeled siRNA or siRNA dilution buffer. Injection pipettes with 1μm opening were produced using a mechanical pipette puller. Holding pipettes with ~10 μm inner and ~100 μm outer were produced using same puller (Fig. 8A). Microinjection was performed on an inverted microscope (Nikon Eclipse-Ti2; Japan) with the micromanipulation system (Nikon/NARISHIGE NTX-N4; Japan) at magnification 200X.

Statistics

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

Table 1. Medium composition

A. BWW medium (Total volume : 500 mL)

Component	Amount	Concentration(mM)
NaCl (Sodium chloride)	2.770 g	94.59
KCl (Potassium chloride)	0.178 g	4.78
KH2PO4 (Potassium phosphate)	0.081 g	1.19
MgSO4 (Magnesium sulfate)	0.0717 g	1.19
Glucose (D-(+)-glucose)	0.5 g	5.56
Ca-lactate	0.1865 g	1.71
Na-pyruvate	0.014 g	0.25
Na-lactate	1.9 ml	21.58
Antibiotics (x100 / x1000)	5 ml / 0.5 ml	-
Phenol red	1 ml	-
NaHCO3 (Sodium bicarbonate)	1.053 g	25.07

B. Lactate-free medium (Total volume : 500 mL)

Component	Amount	Concentration(mM)
NaCl (Sodium chloride)	2.770 g	94.59
KCl (Potassium chloride)	0.178 g	4.78
KH2PO4 (Potassium phosphate)	0.081 g	1.19
MgSO4 (Magnesium sulfate)	0.0717 g	1.19
Glucose (D-(+)-glucose)	0.5 g	5.56
~~Ca-lactate~~ → CaCl2•2H2O (Calcium chloride dihydrate)	0.1257 g	1.71
Na-pyruvate	0.014 g	0.25
~~Na-lactate~~ → NaCl (Sodium chloride)	0.6306 g	21.58
Antibiotics (x100 / x1000)	5 ml / 0.5 ml	-
Phenol red	1 ml	-
NaHCO3 (Sodium bicarbonate)	1.053 g	25.07

Table 2. Thermal cycler schedule

step		Temperature (℃)	Time
Hold	Hold	95	30 sec
3 step PCR (45 cycle)	Denaturation	95	1 min
	Annealing	59	30 sec
	Extension	72	1 min
Dissociation	Denaturation	95	15 sec
	Annealing	60	30 sec
	Extension	95	15 sec
Hold		4	Indefinitely

Table 3. Primers for genes

Gene	Symbol	NCBI gene reference	Primer sequence (5'-3')		Amplified length (bp)
Ribosomal protein, large, P0	Rplp0 (36B4)	NM_007475	S AS	CGACCTGGAAGTCCAACTACTTCCT GCACCTTATTGGCCAACAGCAT	303
Lactate dehydrogenase A	Ldha	NM_010699.2	S AS	ACTGTGTAACTGCGAACTCCAAGCT CTGCTTGTGAACCTCCTTCCA	452
Lactate dehydrogenase B	Ldhb	NM_008492.3	S AS	GCTCAACCTGGTGCAGAGAAA CTGTCCCCATTTCTGGATTCAG	344
Lactate dehydrogenase C	Ldhc	NM_013580.4	S AS	CACGGCAGTCTTTTCCTTAGCACT GAGTCCCCATGTTCTCCAAGAA	399
Lactate dehydrogenase D	Ldhd	NM_027570.3	S AS	ACGCAGGTGGGTATTGAGAGCTT CAAGGTGTTCCCATCATCCCA	183

Table 4. Antibodies information

Name	Cat. No	Company
LDHA - goat polyclonal	sc-27230	Santacruz
LDHB - rabbit polyclonal	ab75167	Abcam
LDHC - rabbit polyclonal	ab222910	Abcam
LDHD - rabbit polyclonal	14398-1-AP	Proteintech

Table 5. siRNA information

Name	Target gene	NCBI gene reference	Position of target sequence		Primer sequence (5’-3’)	Modification
Ldha-transcript1-siRNA-1	*Ldha*	NM_010699.2	460 - 481	S AS	CCUGGUCCAGCGAAACGU(dGdA) ACGUUUCGCUGGACCAGG(dTdT)	None
Ldha-transcript1-siRNA-2	*Ldha*	NM_010699.2	771 - 793	S AS	AGUCUCUUAACCCAGAACU(dGdG) AGUUCUGGGUUAAGAGACU(dTdC)	None
Ldha-transcript2-siRNA	*Ldha*	NM_001136069.2	344 - 367	S AS	GGACCAGCUGAUUGUGAAUC(dTdT) GAUUCACAAUCAGCUGGUCC(dTdT)	None
Ldhb-siRNA-1	*Ldhb*	NM_008492.2	328 - 350	S AS	UUGUGGCCGAUAAAGAUUA(dCdT) UAAUCUUUAUCGGCCACAA(dTdT)	None
Ldhb-siRNA-2	*Ldhb*	NM_008492.2	794 - 816	S AS	GAUGGUGGUGGACAGUGCC(dTdA) GGCACUGUCCACCACCAUC(dTdT)	None
Ldhc-siRNA-1	*Ldhc*	NM_013580	64 - 85	S AS	AGCUGAUUCAGAACCUAG(dTdT) CUAGGUUCUGAAUCAGCU(dGdC)	None
Ldhc-siRNA-2	*Ldhc*	NM_013580	673 – 695	S AS	CUCUGAAGUCACUGAACCC(dAdG) GGGUUCAGUGACUUCAGAG(dTdT)	None
Ldhd-siRNA-1	*Ldhd*	NM_027570.3	203 – 223	S AS	CCUCCUGAUGUUGUGGU(dTdT) ACCACAACAUCAGGAGG(dTdT)	None
Ldhd-siRNA-2	*Ldhd*	NM_027570.3	1083 - 1115	S AS	GUCCAGGAAGUAAGGCCUA(dTdT) UAGGCCUUACUUCCUGGAC(dAdC)	None

Negative control-siRNA	*None*	-	-	S AS	CGAGAUAUUACAAUCCGUACU(dUdU) AGUACGGAUUGUAAUAUCUCG(dUdU)	None
FAM labeled *Ldhb*-siRNA	*Ldhd*	NM_008492.2	328 - 350	S AS	UUGUGGCCGAUAAAGAUUA(dCdT) UAAUCUUUAUCGGCCACAA(dTdT)	5'FI (antisense)

RESULTS

Profiles of *Ldh* mRNAs and LDH proteins in mouse preimplantation embryo *in vivo*

To investigate *Ldh* mRNAs expression during early embryo development, quantitative real time PCR analysis was performed. In the 8-cell stage, the time of transition from pyruvate to glucose-based metabolism, the mRNA expression levels of *Ldha* and *Ldhb* were similar (Fig. 2A). On the other hand, *Ldhc* and *Ldhd* showed significantly lower expression than *Ldha*.

The *Ldha* mRNA expression was significantly higher in UF and 2-cell, and significantly lower in all embryonic stages except 4-cell (Fig. 2B). The mRNA level of *Ldhb* showed significantly higher fold changes in UF and PN, and slightly decreased in 2-cell stage, but still high (Fig. 2C). It showed vary low expression after 8-cell stage. The *Ldhc* mRNA expression was also high at UF and 2-cell, and decreased 4-cell (Fig. 2D). Like *Ldhb* mRNA expression pattern, it showed little expression after 8-cell stage. The mRNA level of *Ldhd* peaked on UF and rapidly decreased at 2-cell (Fig. 2E). After then, it continuously decreased during later stages.

The protein expression level of LDH subunits were identified using immunofluorescence. The intensity and localization of LDHA, B, C and D according to embryonic stage were shown in Figure 3A. LDHA expression showed similar to 8-cell throughout the stage, except for UF, morula, and early blastocyst, which showed significantly higher expression (Fig. 3B). Before 8-cell stage, the relative intensity of LDHB was high (Fig. 3C). After then, it peaked on early blastocyst again, but sharply decreased in late blastocyst. The relative intensity of LDHC showed a tendency to decrease throughout the stage (Fig. 3D). LDHD expression was significantly low in UF, PN and late blastocyst stage and peaked on 4-cell stage (Fig. 3E).

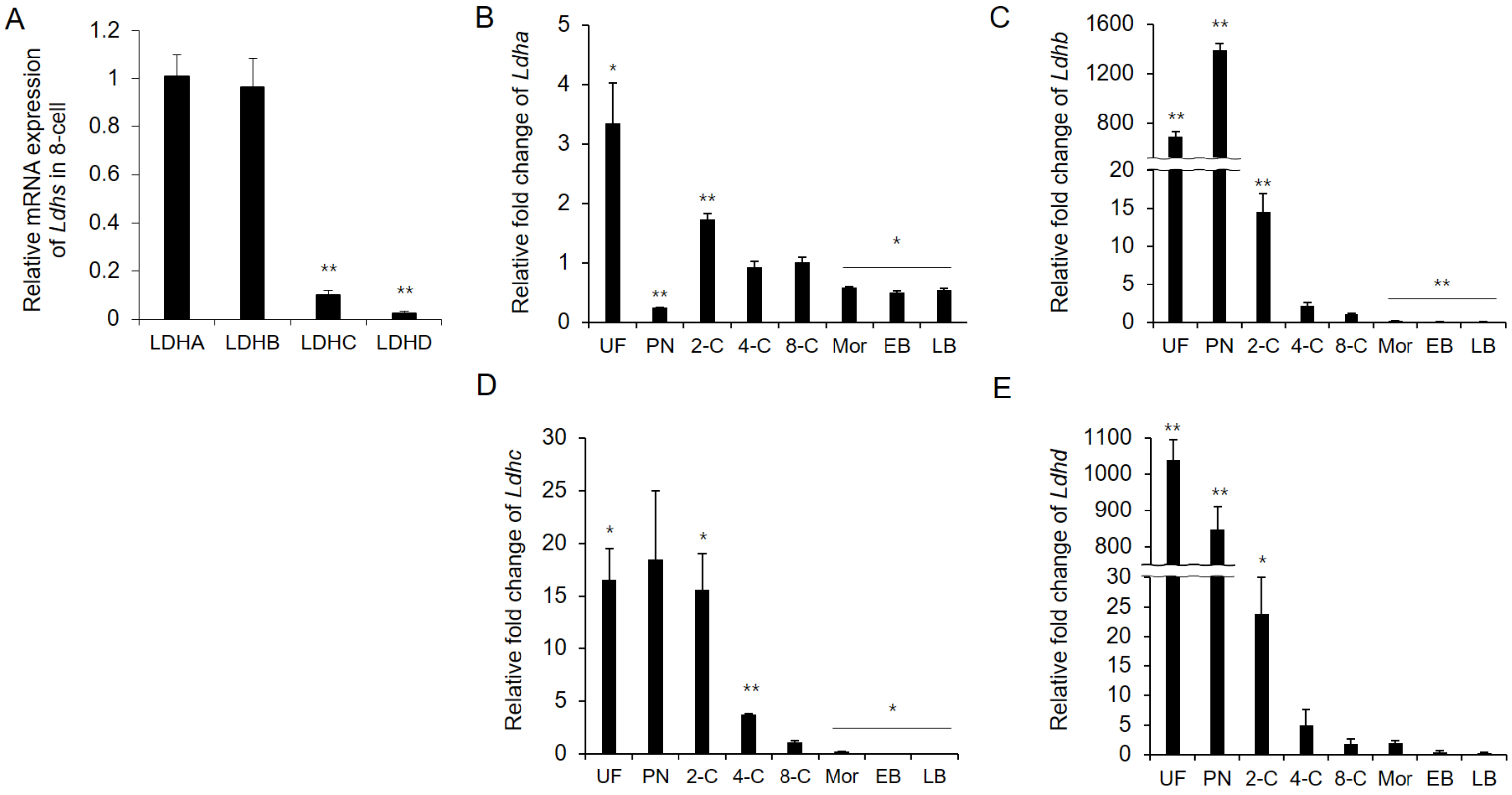
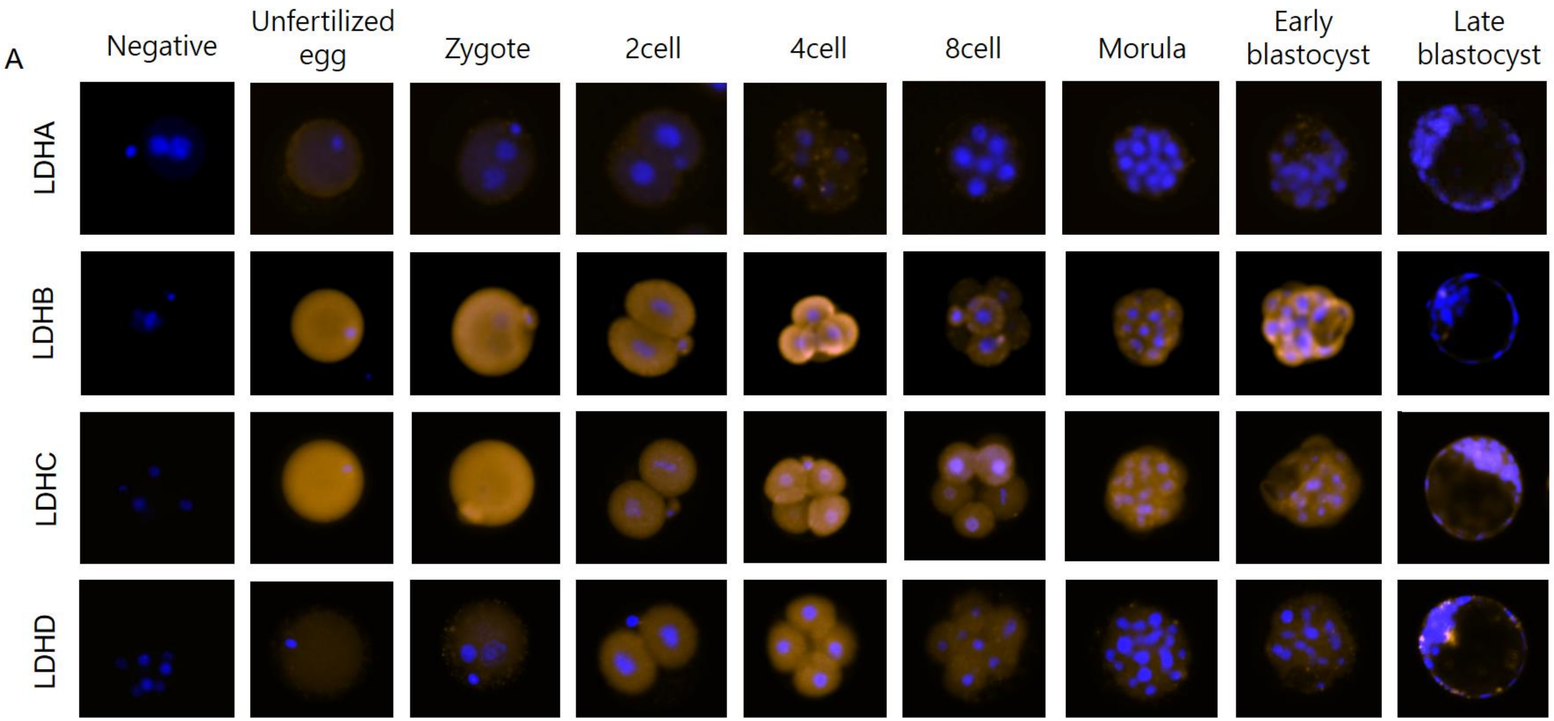


Figure 2. *Ldh* mRNAs expression in preimplantation embryo *in vivo*. A: Each *Ldhs* mRNA expression contrast to *Ldha* in 8-cell. B, C, D, E: The relative quantity of *Ldha, b, c* and *d* mRNA, respectively. The quantitative RT-PCR was performed

using SYBR green, primer for each gene, and cDNA of each stage embryos. Values represent the mean±SEM. *$P < 0.05$ and **, $P < 0.005$ vs. 8-cell.


A
Negative
Unfertilized egg
Zygote
2cell
4cell
8cell
Morula
Early blastocyst
Late blastocyst
LDHA
LDHB
LDHC
LDHD

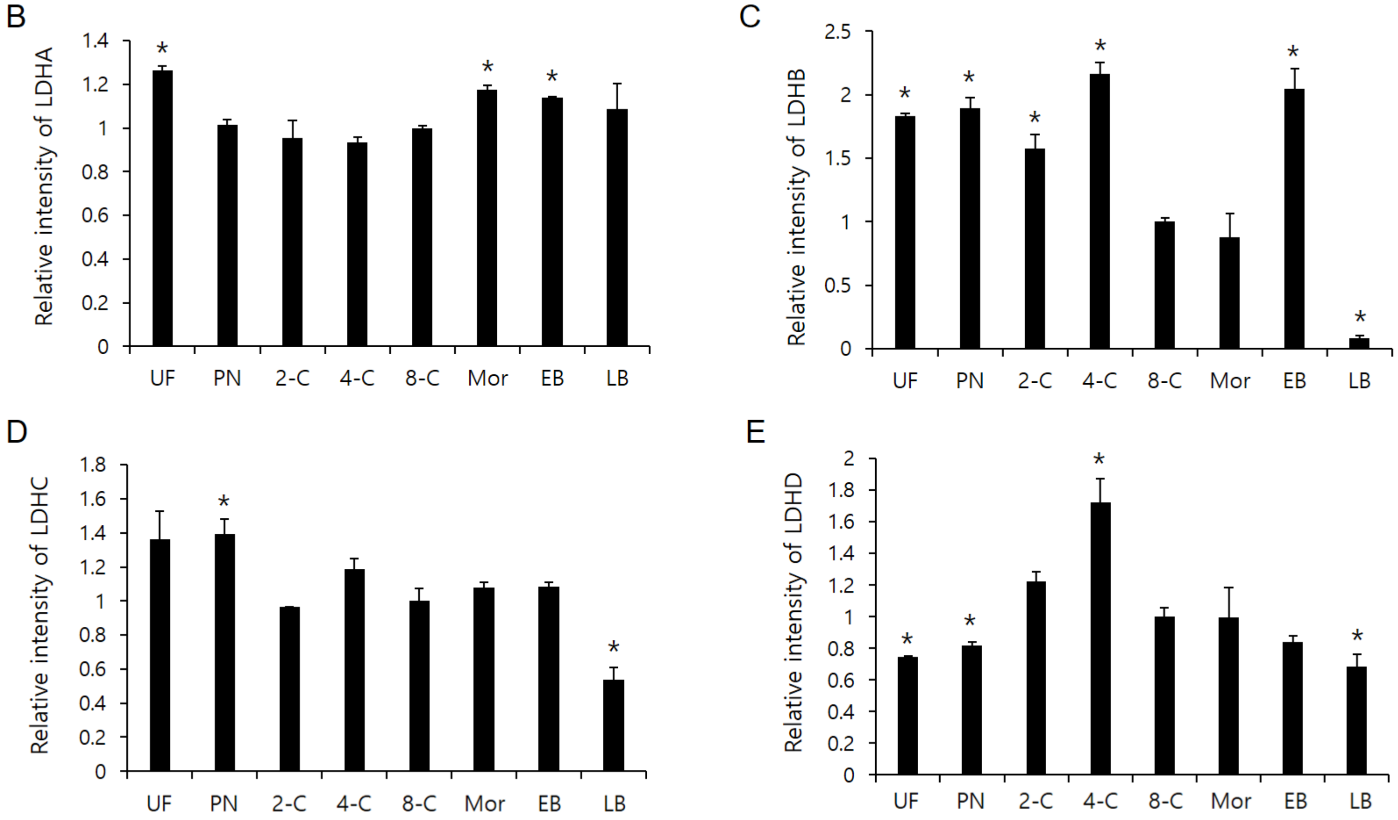


Figure 3. Profiles and localization of LDH in preimplantation stage embryo *in vivo*. Whole immunofluorescence of each LDH subunit in preimplantation embryo in vivo. Each stages of embryo were collected from oviduct or uterus by

flushing. They were immunostained with specific antibodies for LDHA, B, C and D and counterstained with DAPI. A: Representative images of LDHA, B, C, and D (light orange) and DAPI (blue). Magnification; 200X. B, C, D and E: Relative intensity of LDHA, B, C, and D subunit, respectively. Values represent the mean±SEM. *, $P < 0.05$ vs. 8-cell.

Effect of lactate on early embryo development *in vitro*

To investigate the role of lactate in preimplantation embryo development, we cultured late 2-cell stage embryo with or without lactate and developmental rates were checked at 60, 72, 84, 96, 120 and 144 h post-hCG (Fig. 4). At post-hCG 60h, the development rate to 4-cell or more than was no difference between the two groups (Fig. 4A). Embryonic development to 8-cell was delayed significantly in L(-) cultured embryos. L(-) embryos reached above 8-cell stage (83.6%) were significantly fewer than control embryo (70.5%) (Fig. 4B). At post-hCG 84hr, the ratio of embryo which reached above compacted morula was significantly lower in L(-) group (Fig. 4C). At 48 or 72 h after culture (Fig. 4D and 4E), the percent of blastocyst was 20.2% or 75.72% in L(-) group, which was significantly lower than control embryo (38.98% or 86.44%, respectively). However, at post-hCG 144h, the number of embryos reached above hatching was not significantly difference (Fig. 4F).

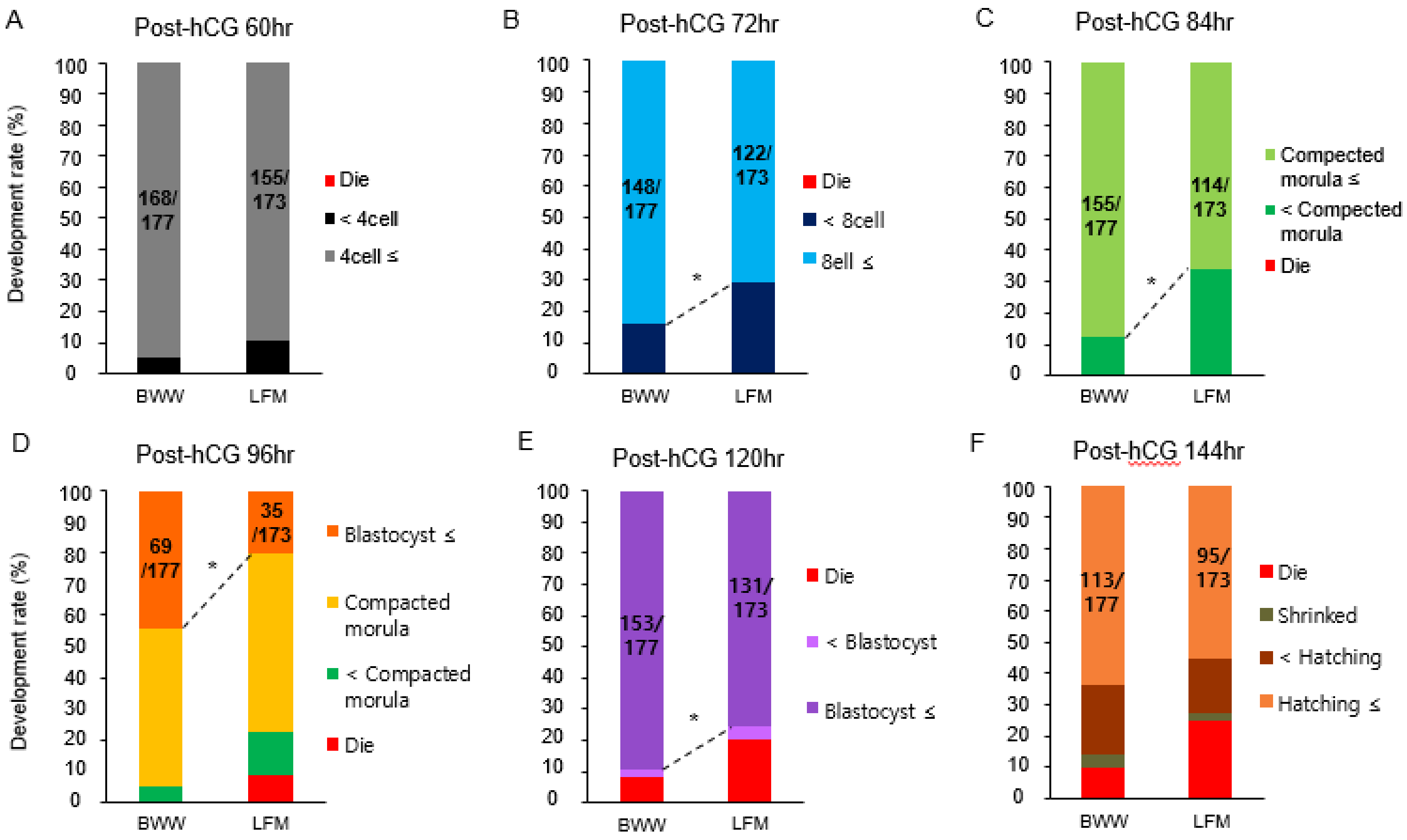


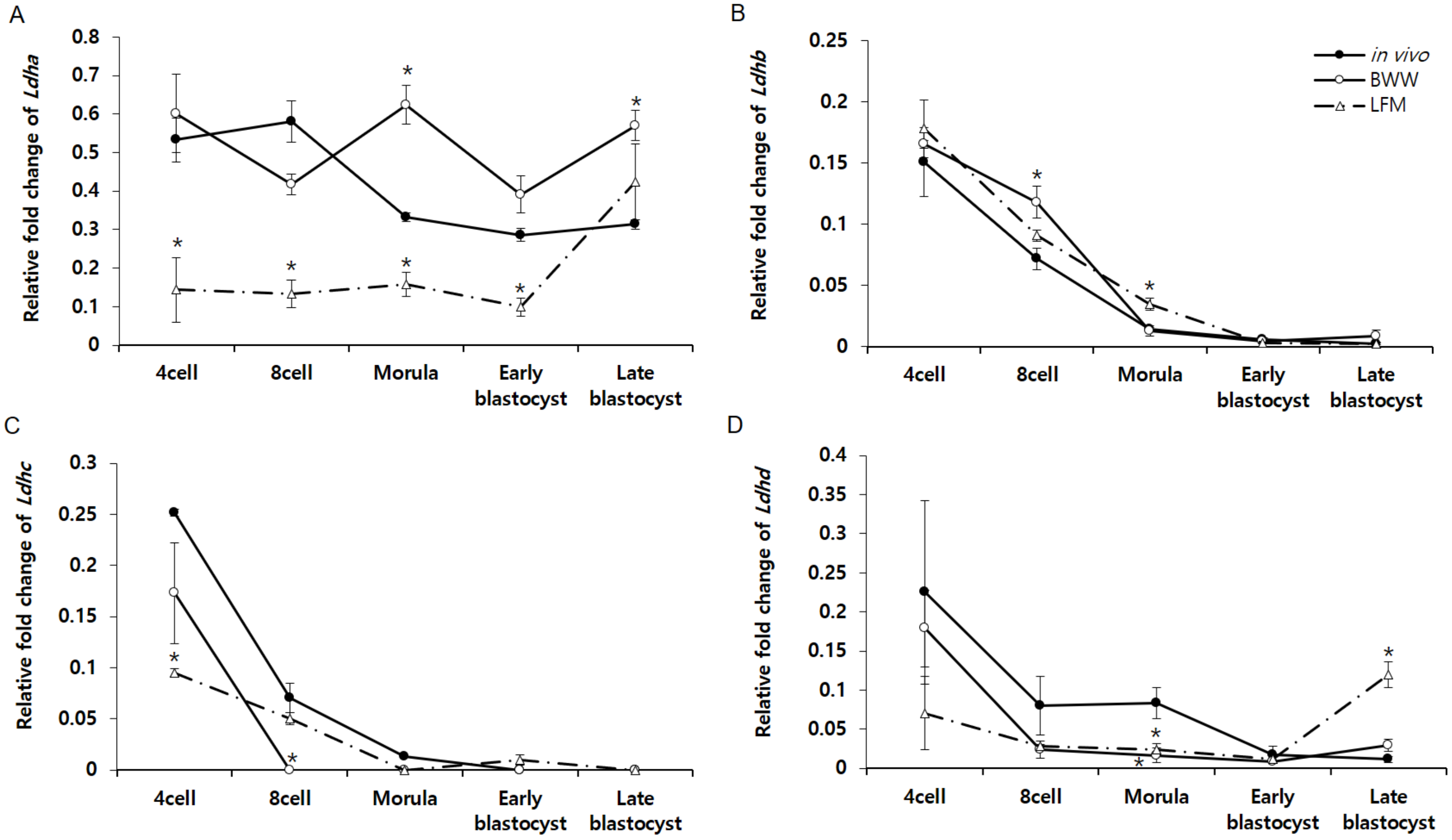
Figure 4. Development ratio of embryo in control BWW or lactate-free BWW medium. A-F: Developmental ratio of control (BWW) and lactate-free(L(-)) cultured embryos at each observation time. Late 2-cell stage embryos were collected

from oviduct at post-hCG 48 h and ramdomly divided to two group : BWW medium-cultured group (control) or lactate-free medium (LFM) cultured group. Developmental rate was checked at 60, 72, 84, 96, 120 and 144 h post-hCG. *, $P < 0.05$ control vs. L(-) of the same time pointy.

Profiles of *Ldh* mRNA in mouse preimplantation embryo *in vitro*

To investigate *Ldh* mRNAs expression in vitro cultured embryo, quantitative real time PCR analysis was performed. In L(-) cultured embryo, the mRNA expression of *Ldha* was significantly lower than *in vivo* at all stages except late blastocyst (Fig. 5A). In all groups, *Ldhb* and *Ldhc* mRNAs expression showed tendency to decrease as embryos developed (Fig. 5B and 5C). The mRNA expression of *Ldhd* peaked on late blastocyst significantly compared to that level of *in vivo* embryos (Fig. 5D).

Among the various *Ldh* genes, only *Ldha* codes for a subunit that prefers oxidation lactate to pyruvate, so we compared relative expression ratio of other genes contrast to *Ldha* (Fig. 5E-G). In all group, *Ldhb* expression of 4-cell stage embryo was more than 2 times higher than *Ldha* and decreased as the preimplantation embryo development. *In vivo*, BWW-cultured, and L(-) cultured embryos were found to reverse the expression ratios of *Ldha* and *Ldhb* at 8-cell (Fig. 5E), morula (Fig. 5F), and morula to blastocyst transition (Fig. 5G) phases, respectively. *Ldhc* and *Ldhd* showed lower expression ratio than *Ldha* in all groups and all stages.


A
Relative fold change of *Ldha*
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4cell
8cell
Morula
Early blastocyst
Late blastocyst
*
B
Relative fold change of *Ldhb*
0.25
0.2
0.15
0.1
0.05
0
in vivo
BWW
LFM
C
Relative fold change of *Ldhc*
0.3
0.25
0.2
0.15
0.1
0.05
0
D
Relative fold change of *Ldhd*
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

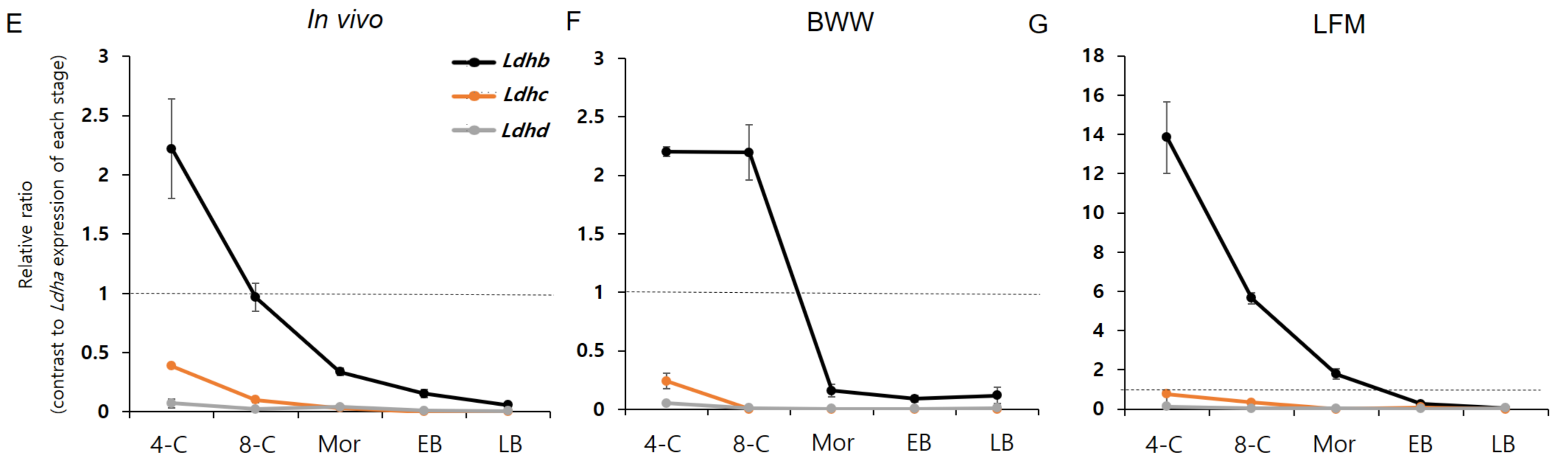


Figure 5. Relative *Ldh* mRNAs expression in cultured embryo. Gene expression was evaluated by RT-qPCR. The mRNA expression of *Ldha*(A), *Ldhb*(B), *Ldhc*(C) and *Ldhd*(D) in preimplantation embryo *in vitro*. Values represent the mean±SEM. *, P < 0.05 *in vivo* vs. *in vitro* of the same stage. E: *in vivo*, F: control (BWW), G: lactate-free cultured group. Dashed line indicate the point of same expression some *Ldh* level with *Ldha*.

Localization and profiles of LDH protein in mouse preimplantation embryo *in vitro*

LDH proteins in cultured embryo were identified using immunofluorescence. The intensity and localization of LDHA, B, C and D in BWW or L(-) cultured embryo were shown in Figure 6A and B, respectively.

The relative intensity of LDHs contrast to in vivo 2-cell was presented in Figure 6C-F. In L(-) cultured group, the relative intensity of LDHA was significantly lower than that of *in vivo* in all stage (Fig 6C). This pattern is consistent with mRNA expression pattern except for late blastocyst stage (Fig. 5A). LDHB intensity decreased according to stage in L(-) cultured embryo (Fig. 6D). In BWW-cultured embryo and *in vivo* embryo, relative intensity of LDHC showed a similar pattern except 4-cell stage (Fig. 6E). In comparison of relative intensity of LDHD, *in vitro* cultured embryos showed lower intensity in 4-cell and higher intensity in 8-cell than that of *in vivo* (Fig 6F).

Among the various LDH subunits, only LDHA prefers oxidation lactate to pyruvate, so we compared relative expression ratio of other subunits contrast to LDHA (Fig. 7A-C). *In vivo*, BWW-cultured, and L(-) cultured embryos were found to reverse the expression ratios of LDHA and LDHB at 4-cell to 8-cell stage (Fig. 7A), 8-cell to morula stage (Fig. 7B), and late blastocyst stage (Fig. 7C), respectively.

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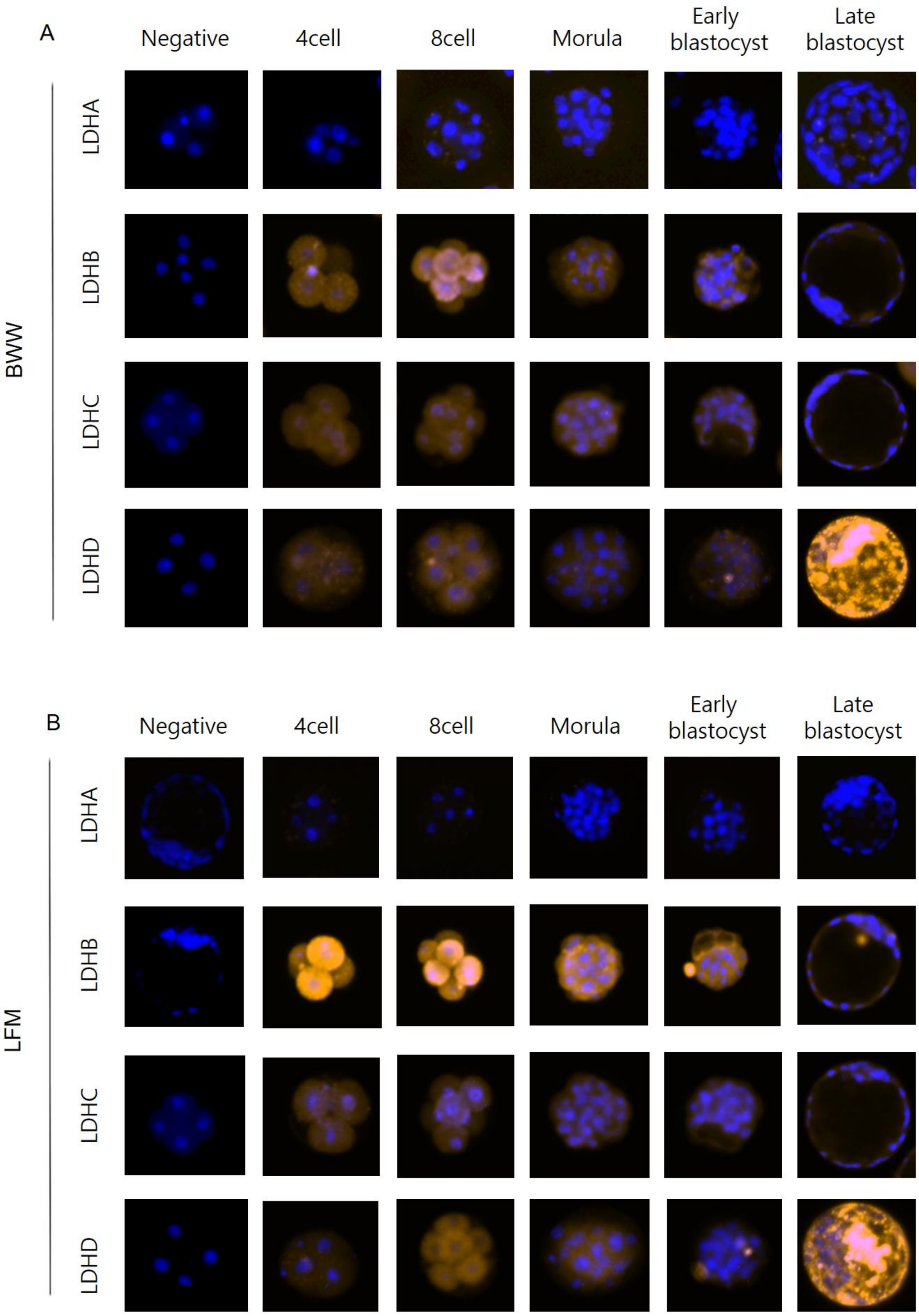

A
Negative
4cell
8cell
Morula
Early blastocyst
Late blastocyst
BWW
LDHA
LDHB
LDHC
LDHD
B
Negative
4cell
8cell
Morula
Early blastocyst
Late blastocyst
LFM
LDHA
LDHB
LDHC
LDHD

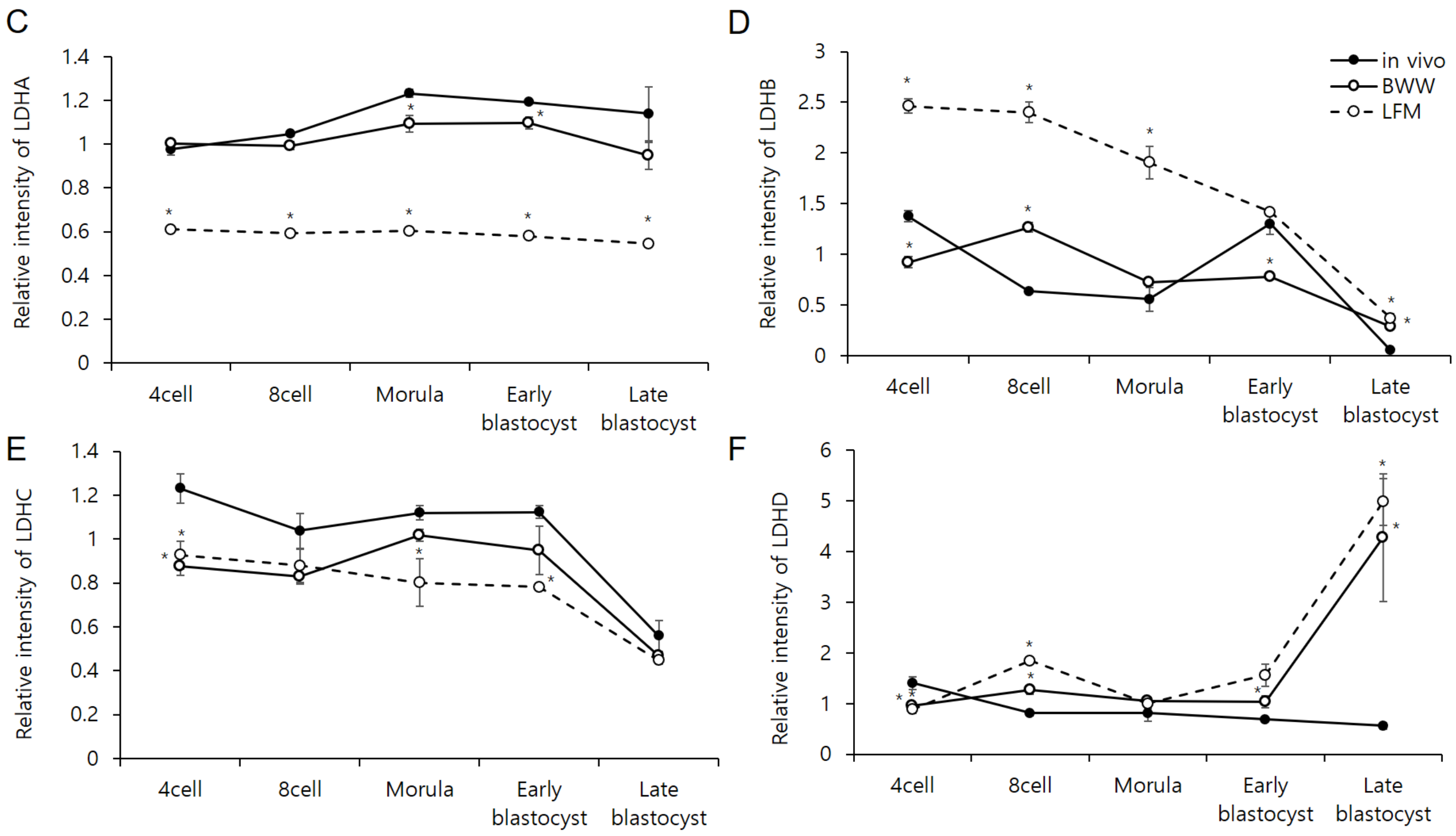


Figure 6. Profiles and localization of LDH protein in preimplantation stage embryo *in vitro*. Whole mount immunofluorescence of each LDH subunit in cultured embryo with or without lactate. Each stages of embryo were collected

at 12 (4-cell), 24 (8-cell), 36 (compacted morula), 48 (early blastocyst) and 72 h (late blastocyst) after culture. They were immunostained with specific antibodies for LDHA, B, C and D and counterstained with DAPI. A, B: Immunostained control and L(-) cultured embryo, respectively. Representative images of LDHA, B, C, and D (light orange) and DAPI (blue). Magnification; 200X. Relative intensity of LDH protein contrast to *in vivo* 2-cell. C, D, E and F: relative intensity of LDHA, B, C and D, respectively. Values represent the mean±SEM. *, $P < 0.05$ vs. *in vivo* 2-cell.

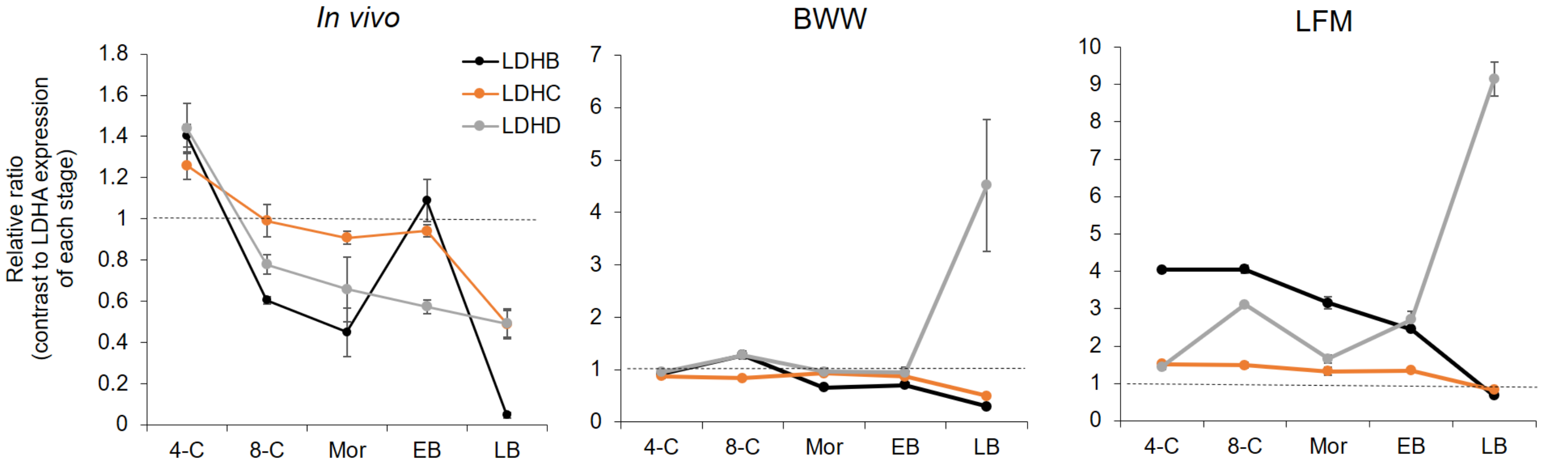


Figure 7. Relative ratio of LDH subunits in cultured embryo. The relative ratio of LDH subunits was evaluated by whole mount immunofluorescence. A: *in vivo*, B: control (BWW), C: lactate-free cultured group. Dashed line indicate the point of same expression some LDH level with LDHA.

Inhibition of lactate utilization and production by *Ldh* siRNA microinjection

To investigate the role of lactate in preimplantation embryo development, siRNA was used to block the use and production of lactate. Late 2-cell stage embryos were injected *Ldh* siRNA and developmental rate was observed (Fig. 9). First, we evaluated the efficiency of injected siRNA (Fig. 8). Each blastomere of late 2-cell embryo was injected FAM-labeled *Ldhb* siRNA and evaluated FAM intensity in each time of post-microinjection. Over time, fluorescence intensity decreased, but 78% of the fluorescence remained until 96 hours after injection (Fig. 8B-C). After siRNA injection, embryos were cultured and collected at 4-cell, 8-cell and morula stage to evaluate *Ldh* mRNA expression levels (Fig. 8D-G). No significant differences in *Ldh* mRNA levels were detected between non-injected (control) and negative-siRNA injected embryos (negative control). The *Ldha* mRNA levels in *Ldha*-siRNA injected embryos significantly decreased compared with control and negative control group (Fig. 8D). In *Ldhb*–siRNA injected group, knockdown efficiency decreased 97.5% to 70.2% at 4-cell 8-cell stage as the embryo develops (Fig. 8E). Because *Ldhc* mRNA expressed rarely in 8-cell and morula stage, knockdown efficiency was evaluated only at 4-cell stage. The percent of knockdown was 92.9 - 95.2% in *Ldhc* or *Ldhd*-siRNA injected embryos (Fig. 8F-G).

Late 2-cell stage embryos were collected from oviduct at post-hCG 48h and randomly divided to each group and injected 10 pl of 2μM *Ldh*-siRNA solution (experimetal group) or 1X siRNA dilution buffer (control group). Specially, *Ldha* siRNA injected embryos were randomly divided to BWW medium-cultured group (*Ldha*-BWW) or lactate-free medium cultured group (*Ldha*-LFM). The developmental rate of siRNA injected embryos was checked at 60, 72, 84, 96, 120 and 144 h post-hCG (Fig. 9). At post-hCG 60h, the development rate to 4-cell or more than was no difference between all groups (Fig. 9A). Likewise, at post-hCG 72 or 84 h, there was no difference in the percentage of embryos that developed above 8-cell or compacted morula between all groups except *Ldha*-LFM group (Fig. 9B-C). At 96 h post-hCG (Fig. 9D), the percent of blastocyst was 12.5% in *Ldha*-LFM group, which was significantly lower than control embryo (48.4%). Meanwhile, *Ldhc*-siRNA injected group showed high development rate at that time, temporarily (Fig. 9D). At 96 hours after culture (Fig. 9E), the percent of blastocyst was significantly lower in *Ldha*-LFM group (60.0% vs 87.1 in control group). Hatching rate decreased significantly in all group except *Ldhc* or *Ldhd*-siRNA injected group (Fig. 9F).

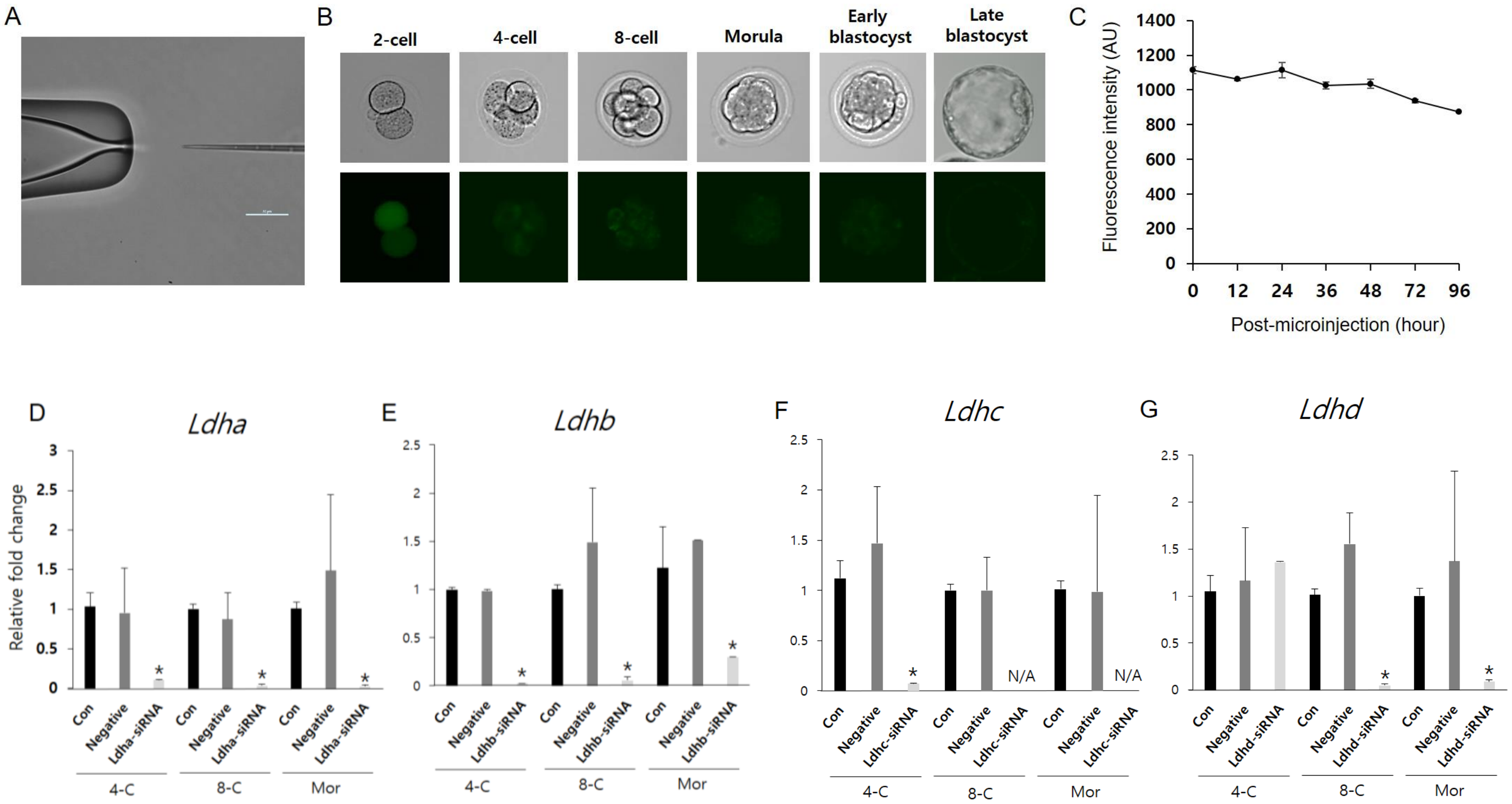
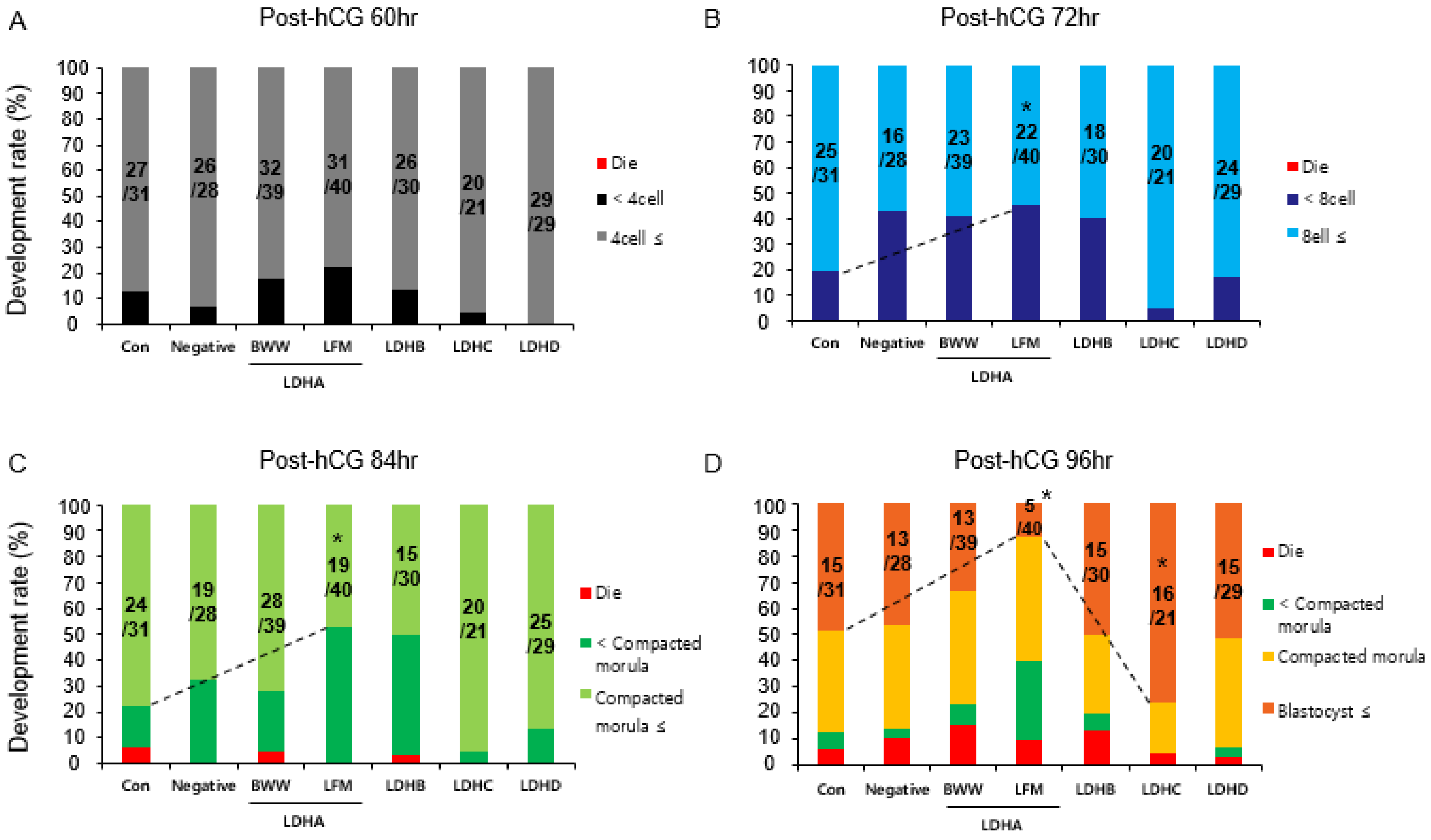


Figure 8. Stability of siRNA until blastocyst, which were injected at 2-cell. siRNA-FAM was injected to each blastomere of 2-cell embryo and analyzed the intensity of injected siRNA. Using confocal microscope and qRT-PCR. A:

microinjection pipette and holding pipette. Magnification; 400X. Scale bar indicate 50μm. B: Representative images of injected siRNA-FAM at each stage embryo. C: Fluorescence intensity of injected siRNA-FAM in each time of post-microinjection. D-G: The relative mRNA expression of *Ldh* in negative control group (negative) and each *Ldh* siRNA injected group (*Ldha, Ldhb, Ldhc* or *Ldhd*) contrast to non-injection group (con). *, $P < 0.05$ non-injected group vs. each siRNA-injected group of the same stage. N/A; not available.


A
Post-hCG 60hr
Development rate (%)
100
90
80
70
60
50
40
30
20
10
0
27 /31
26 /28
32 /39
31 /40
26 /30
20 /21
29 /29
Con
Negative
BWW
LFM
LDHB
LDHC
LDHD
LDHA
Die
< 4cell
4cell ≤
B
Post-hCG 72hr
25 /31
16 /28
23 /39
*
22 /40
18 /30
20 /21
24 /29
Die
< 8cell
8ell ≤
C
Post-hCG 84hr
24 /31
19 /28
28 /39
*
19 /40
15 /30
20 /21
25 /29
Die
< Compacted morula
Compacted morula ≤
D
Post-hCG 96hr
15 /31
13 /28
13 /39
5 /40
*
15 /30
*
16 /21
15 /29
Die
< Compacted morula
Compacted morula
Blastocyst ≤

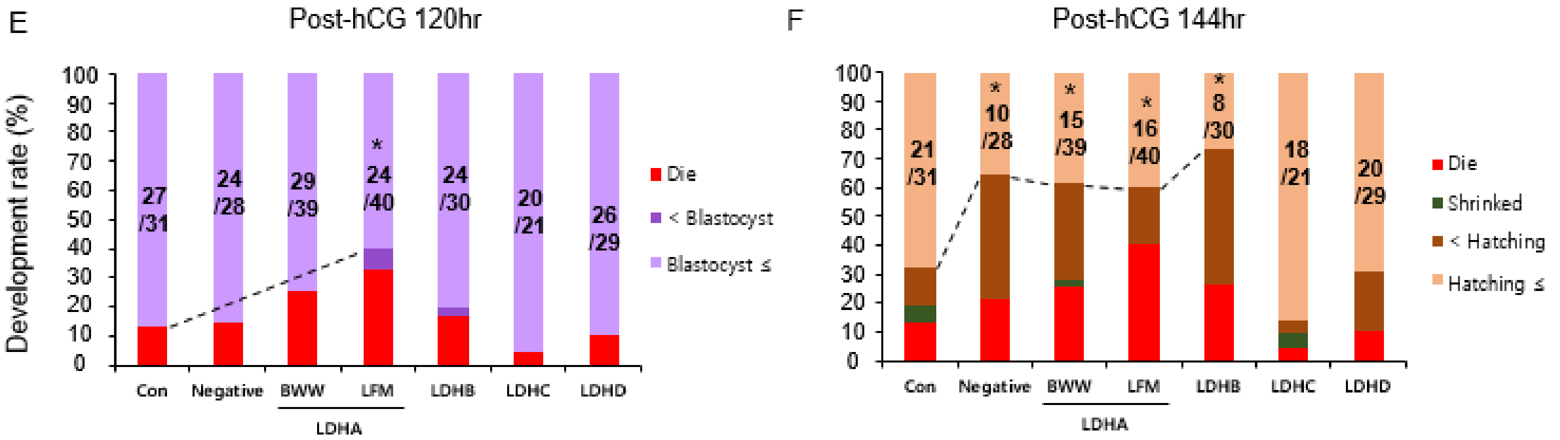


Figure 9. Assessment of developmental competence of embryos injected with 2μM *Ldh* siRNA. A-F: Developmental ratio of *Ldh* siRNA injected embryos at each observation time. Late-2cell stage embryos were collected from oviduct at post-hCG 48hr and randomly divided to each group and injected 10 pl of 2μM *Ldh*-siRNA solution (experimetal group) or 1X siRNA dilution buffer (control group). Specially, *Ldha* siRNA injected embryos were randomly divided to two group : BWW medium-cultured group (*Ldha*-BWW) or lactate-free medium cultured group (*Ldha*-LFM). Developmental rate was checked at 60, 72, 84, 96, 120 and 144 h post-hCG. *, P < 0.05 control vs. L(-) of the same time pointy.

Discussion

During preimplantation embryo development, the carbohydrate metabolism shifts from oxidative phosphorylation to glycolysis between morula and blastocyst stages (Hu and Yu., 2017). In many mammals including mice, human, pig and bovine, lactate production increased along with increase of glucose consumption (Fabrice Guerif et al., 2013; Gott et al., 1990; Leese et al., 1996; Sturmey, 2003). In embryo development, the reason and role of lactate increased at such time are still unknown.

To determine the role of lactic acid in embryonic development, lactate dehydrogenase expression was examined at the mRNA and protein levels. Since the high expression ratio of LDHA suggests a high conversion of pyruvate to lactate, the expression ratio of other LDHs to LDHA was examined. In addition, we compared the development rate of embryos cultured in BWW or lactate-free BWW medium (LFM), and examined the change in development rate when limiting conversion between pyruvate and lactic acid by knockdown of *Ldhs* with siRNA.

As embryos develop *in vivo*, the mRNA expression of *Ldhb, Ldhc*, and *Ldhd* decreases dramatically after 8-cell stage, whereas mRNA expression of *Ldha* little bit decreased but maintained certain levels until blastocyst stage. The *Ldhs* expression patterns of in vitro cultured embryos were similar to that in vivo, but the timing of change in *Ldhb* to *Ldha* expression ratio were slower than *in vivo* at 8-cell to morula in the BWW group and morula to early blastocyst in the LFM group. Even when comparing the expression ratio of LDH subunit by the whole mount immunofluorescence, the timing of change in LDHB to LDHA expression ratio were the earliest between stage 4-cell and 8-cell in *in vivo* group and the slowest between stage early blastocyst and late blastocyst in LFM-cultured group. Meanwhile, LFM-cultured group showed slow transition rate to 8-cell, morula and blastocyst at 72 h, 84 h and 96 h post-hCG, respectively. These results suggest that the conversion of pyruvate to lactic acid is required at certain developmental periods and this may affect developmental rates.

To block the use and production of lactate and investigate its impact on embryo development, *Ldh*-knockdown using siRNA was performed. Among knockdown groups, to limit the exogenous lactate and production of lactate, part of *Ldha*-siRNA injected embryos were cultured in LFM. Unlike other group, only they showed low development rates throughout the culture period except 60h post-hCG. Also, in *Ldhc*-siRNA injected embryos showed significantly higher rate of development to blastocyst at 96h post-hCG compared to control group. This suggests that appropriate production and use of lactic acid is required for normal embryonic development.

In this study, the subunit of LDH, an enzyme involved in the production and the use of lactic acid, was profiled during embryo development. In order to more directly identify the role of lactate in preimplantation embryo development, it is necessary to confirm the enzyme activity and actual LDH protein amount according to the subunit combination.

Emerging evidence suggests new roles for lactic acid, not just by-products of glycolysis, such as signaling molecule in neuron (Magistretti and Allaman, 2018), histone hyperacetylation by inhibit HDACs (Wagner et al., 2015) and lysine lactylation as a type of histone mark (Di Zhang et al., 2019). To understand specific mechanism of the role of lactate in early embryo development, subsequent studies are needed.

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논문개요

초기 배아 발생 동안, 적절한 대사 활동은 환경에 대한 적응 및 건강한 배아 발생을 위해 요구된다. 이 과정에서 생산되는 대사 물질에 대한 연구는 배아 발생을 이해하는데 중요하다. 초기 마우스 배아 발생 과정에서, 배아는 8-세포기까지 피루브산을 주된 에너지원으로 사용한다. 흥미롭게도 8-세포기 이후에 피루브산-기반 신진대사는 포도당-기반 신진대사로 전환되며, 산소가 풍부한 환경에서 젖산의 생산이 급격히 증가한다. 과거에 젖산은 해당과정의 최종 산물로 여겨졌다. 이에 대해, 최근 연구들은 신호전달 물질, 유전자 조절 등의 새로운 젖산의 역할을 제안하고 있지만, 초기 배아 내 젖산의 역할에 대한 연구는 매우 제한되어 있다. 본 연구에서는 젖산과 피루브산 간 전환에 관여하는 젖산탈수소효소(LDH)의 발현을 살펴봄으로써, 초기 배 발생에서의 젖산의 역할을 알아보고자 하였다. 젖산을 제거한 배양액(L(-))에서 배양된 배아는 단순 배양액(BWW)에서 배양된 배아에 비해 발생이 지연되는 모습을 보였다. *In vivo* 또는 *in vitro* 배 발생 동안, 피루브산의 환원 작용을 선호하는 Ldha의 상대적인 mRNA 비율은 다른 Ldh보다 증가하는 모습을 보였다. 다른 Ldh에 대한 Ldha의 상대적인 비율이 증가하는 시기는 L(-) 조건에서 배양된

배아에서 가장 늦게 나타났다. 면역형광법을 통해 LDH subunit의 단백질 수준을 비교했을 때에도, 배아가 발생함에 따라, LDHA의 상대적인 비율이 다른 LDH subunit 보다 증가하는 모습을 보였다. siRNA 미세 주입을 통한 Ldh knockdown 실험에서, Ldha-siRNA가 주입된 배아를 L(-) 환경에서 배양했을 때, hCG 주사 후 72시간, 84시간, 96시간 째에 8-세포기, 상실배, 포배로의 발생률이 대조군에 비해 낮았다. 이러한 결과를 종합해보면, 젖산탈수소효소(LDH)의 발현은 환경에 대한 적응과 배아 발생에 적합하도록 달라질 수 있으며, 젖산의 적절한 생산과 이용이 초기 배아 발생에 있어 요구된다고 제안할 수 있다. 초기 배아 발생에서 젖산의 필수 역할을 확인하기 위한 추가 연구가 필요하지만, 이러한 데이터는 젖산 대사가 초기 배아 발달 능력 획득에 필수적이라는 것을 암시한다.

감사의 글

학위 과정 동안 잘 배울 수 있도록 이끌어주신 모든 분들에게 감사드립니다. 대학교 2 학년 겨울방학에 우연히 접하게된 연구실에서 석사까지 많은 것을 배울 수 있어서 좋았습니다.

먼저, 학위 과정을 잘 마칠 수 있도록 많은 것을 가르쳐주시고 좋은 방향으로 이끌어주신 교수님께 감사드립니다. 논문심사에서, 좋은 논문이 될 수 있도록 여러 조언을 해주신 서울여대 김해권 교수님과 성신여대 전민영 교수님께도 감사의 인사를 드립니다.

실험이 안되서 힘들어할 때 많은 조언을 해주고 같이 고민해주었던 순영언니, 흔들리면서 불안해할 때마다 언니 덕분에다시 의지를 갖고 해낼 수 있었고 결국 지금까지 이렇게 잘 마무리할 수 있었어요 :)! 함께 프리온 프로젝트를 했던 연정언니, 실험을 잘 모를 때 마다 다가와서 알려주고 동기들과 힘들어할 때 어려운 일들을 함께 해주어 고마웠어요! 그리고 항상 먼저 나서서 연구실 일을 뚝딱 해결하는 민영아, 박사과정 잘 보내고 앞으로 실험도 원하는 방향으로 잘 풀리길 바랄께^^! 같이 졸업하는 희선이랑 주혜야, 백지장도 맞들면 낫다고 어려운 일들도 다같이 해결해나가면서 서로 많은 힘이 되었던 것 같아, 고마워 친구들아, 졸업하고나면 여행도 가고 휴식도 가지면서 앞으로의 일들도 잘 헤쳐나가자!

이번에 신입생으로 입학한 주은이랑 태은아, 서로 의지하면서 공부하고 실험도 열심히 잘 나가길 바래! 마지막으로 전화로 투정부려도항상 제 편이 되어주셨던 부모님과 항상 응원해준 친구들에게도 감사하고 고맙다는 말을 전하고 싶습니다 :)!

모두 좋은 결과가 있길 바라고, 힘들때 마다 서로 많은 도움이 되길 바래요! 감사합니다 :)