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석사학위 청구논문

FGFR expression based early embryonic developmental communication

; Possible role of FGF1 and its receptors in pre-implantation mouse embryo

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성신여자대학교 대학원

생 물 학 과

강 민 지

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이 논문을 석사학위논문으로 제출함.

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생 물 학 과

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FGFR expression based early embryonic developmental communication

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Submitted in partial fulfillment of the

requirements for the degree of master.

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2015년 11 월

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논 문 개 요

섬유아세포 성장인자(Fibroblast growth factor, FGF)는 세포의 증식과 분화 등 여러 가지 세포 활성을 조절하는 성장인자이다. 특히, FGF와 그 수용체를 통한 신호 전달을 통해서 측형성이나 구획(compartmentation)등의 현상을 조절함에 있어서 중요한 역할을 한다는 것이 많은 연구들을 통해서 밝혀져 왔다. FGF는 22개의 리간드와 조직특이적인 발현 양상을 갖고 리간드 특이적 결합력을 갖는 티로신 인산화효소 수용체가 동정되어 있다. 수용체들은 조직 특이적으로 발현하고 리간드와 복합체를 형성하게 되는데, 헤파린이나 헤파란황산이 이들의 결합을 조절한다고 알려져 있다. 헤파린은 또한 특정 위치의 세포와 직접적인 FGF의 농도 조절에도 관여한다. FGF family 중에서도 FGF-1은 모든 수용체와 높은 결합력을 가지고 있다. 한편 FGF는 치아 줄기세포의 증식 및 미분화 상태 유지, 눈의 발생에서 수정체기원판의 소포로의 분화, 진피유두 유래 FGF들의 모낭줄기세포 활성화, 신경판 유도, 사지싹 형성과 축 형성 등의 배아 발생에서의 역할이 잘 알려져 있다. 한편, 이전 연구에서 PCR 방법을 이용하여 생쥐의 착상 전 배아에서 FGF-1의 mRNA가 발현됨을 밝혔다. FGFR2와 FGFR3 단백질의 mRNA는 착상 전 배아의 특정 난할 단계 특이적으로 mRNA가 발현됨을 그리고 FGFR4 단백질의 mRNA는 착상전 배아의 모든 시기에 발현하고 FGFR1 단백질의 mRNA는 발현하지 않음을 밝혔다. 하지만 이들의 난할과정의 초기 배아 발생에 있어서의 역할은 아직 분명하게 밝혀진 바가 없다. 따라서 이들 FGF의 미수정란부터 포배까지의 시기에 어떤 역할이 있는지를 알아보기 위하여 실시간 역전사 중합효소 연쇄반응(real-time RT-PCR)을 이용하여 초기 배아 시기 동안 각각의 mRNA 발현 패턴을 분석하였다. 그리고 FGF-1, FGFR2, FGFR3의 단백질 발현을 알아보기 위해 면

역형광법(immune-fluorescence)을 수행하였다. 또한 FGF-1과 그 수용체들의 초기 배아에서의 기능을 분석하기 위해서 재조합 된 FGF-1과 FGFR의 길항제를 각각 농도 별로 처리하여 in vitro에서 배아를 배양하였다. 2-세포기 때 발현되는 양을 기준으로하여 상대적인 발현 정도를 분석하였다. 미수정란과 전핵 시기에서는 FGF-1과 수용체들의 mRNA양이 상대적으로 많았으나 2-세포기 이후에서는 보다 낮은 수치로 발현되었다. FGF-1은 in vivo와 in vitro그룹 모두 8-세포기에 mRNA 발현이 증가하였고, 이후 다시 감소하였다. FGFR2도 FGF-1과 마찬가지로 in vivo와 in vitro 그룹에서 8-세포기에서 높은 발현을 보였고, 4-세포기와 hatching 시기에도 높은 발현양상을 보였다. FGFR2의 1 이형체와 비교해서 2 이형체는 보다 낮은 수치로 발현되었다. FGFR3는 in vitro 그룹에서 상실배와 hatching 시기에 높게 나타났다. 단백질 수준에서는 FGFR2, FGFR3의 단백질은 *in vivo*와 *in vitro* 그룹 둘 다 모든 시기에 생쥐 배아에서 검출되었으나, FGF-1은 할구에 위치하지 않았다. FGF-1과 수용체의 초기배아 발생에 있어서의 역할을 확인하기 위해 재조합된 FGF-1과 FGFR의 길항제를 각각 처리하여 배아를 시간대별로 배양하였다. 재조합된 FGF-1를 처리한 경우에는 포배기로의 발생률과 hatching 비율이 증가하였다. FGFR의 길항제를 처리한 실험군에서는 상실배로의 발생률이 농도 의존적으로 유의적인 감소를 보였으나, 이후 시기에는 발생률의 유의적인 변화가 없었다. FGFR 활성 억제로 인한 발생률의 변화가 FGF-1에 의해 회복되는지를 확인하기 위해 FGFR 길항제와 재조합된 FGF-1을 같이 처리한 경우 8-세포기, 포배기로의 발생률 및 부화율 (hatching rate)이 FGFR 길항제 농도 의존적으로 억제되었다. 한편, 난할중인 배아가 수란관을 이동하는 기간에 수란관에서 FGF1이 발현되었다. 이러한 결과를 종합하여 볼 때 FGFR 발현은 FGF1의 자가 또는 측분비를 통하여 난할중인 배아의 발생을 조절하

는데 관여할 것으로 사료된다.

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INTRODUCTION

Multicellular stage of fertilized egg is accomplished through cleavage. During cleavage stage blastomers get polarity and construct trophoblast and inner cell mass. However they continuously communicate with their environment through stage specific competence. During travel the oviduct, the embryo suffer the maternal environmental factors such as carbohydrate and various growth factors, such as transforming growth factor β (TGF-β), endothelial growth factor (EGF), insulin-like growth factor (IGF), and fibroblast growth factor (FGF). These factors are influence embryo development. At the compacted morula stage, the inside cells are effectively isolated from the direct influence of materials surrounding the embryo. Functional role of growth factors has been demonstrated using various methods during the preimplantation period (Díaz-Cueto et al., 2001), but those are not enough to understand the early embryo development.

Fibroblast growth factors (FGFs) signaling is associated with diverse cellular events including cell proliferation, migration, and differentiation. During embryogenesis, FGFs express in the brachial arch units, somatic myotome, the apical ectodermal ridge of the developing limb bud, and the tooth bud. FGFs regulate the limb formation, tooth development, lens formation, neural stem cell proliferation, and so on (Niswander et al., 1992). FGFs are also control the patterning in developing embryo (Crossley et al., 1995).

In human and mouse, 22 FGF genes have been identified and the chromosomal locations of all are known (Ornitz et al., 2001). FGFs are grouped into subfamilies according to similar binding activity with their

receptors (Zhang et al., 2006).

FGF receptors (FGFRs) are 4 members related transmembrane receptors with intracellular tyrosine kinase domains and extracellular three immunoglobulin-like (Ig) domains. The Ig domains are alternative spliced; encoding IIIa, IIIb or IIIc isoforms which have different affinity with ligands. In some case, the IIIb and IIIc forms are expressed in different tissues during organogenesis. The IIIa form encodes a secreted extracellular protein which has no known signaling capability (Ornitz et al., 1996; Zhang et al., 2006).

FGFs bind to their receptors in the presence of heparan sulfate (HS), a linear and highly sulfated polysaccharide (Zhang et al., 2006; Wu et al., 2003). Interactions between FGF and heparin have been shown to stabilized FGFs to thermal denaturation and protect from proteolysis. In the absence of heparin, the FGF and FGFR signaling complex is lose stability. Diverse studies have shown that mutation in enzymes involved in heparan sulfate biosynthesis affect FGF signaling pathways during development (Ornitz, 2000).

FGFR2 role in the embryo was examined using target disruption mouse model (Arman et al. 1998). They suggested that FGFR2 is requiring for early postimplantation and implantation by FGF-4 signal. The two variants of FGFR2 are different localization pattern in organogenesis. It is suggest that FGFR2 variants regulate organogenesis in tissue specific manner (Urtreger et al. 1993). Rappolee et al (1998) shown that FGFR3 expressed at outgrowing and trophoblast giant cell in mouse blastocyst. This evidence suggested that FGFR3 is a candidate for transducing some of the signal of FGF in extraembryonic membrane such as endoderm and trophoblast giant cell. But, to make sure FGFR3 as a mitogenic signal factor need more studies. FGFR3

is also regulating skeletal formation during mouse embryogenesis. Targeted mutation of FGFR3 causes abnormal skeletal formation (Colvin et al. 1996). FGF-8 controls various organogenesis in mouse embryo development such as cardiogenesis, limb formation, brain development and morphogenesis of head and neck. These progresses occur through FGFR3 which has high affinity with FGF-8 (Crossely et al. 1995).

In previous study, FGF-1 mRNA is detected using reverse transcription polymerase chain reaction (RT-PCR) in preimplantation embryos. FGFRs are also stage specifically detected in preimplantation embryos. So far the roles of FGF-1 and FGFRs are proved in embryo development such as implantation, axis formation, organogenesis, and so on but not clear in cleavage embryo. Therefore, in this study, to evaluate the possible roles of FGFs and their receptors in the development during cleavage period, quantification of mRNA levels of FGF1, FGFR2, FGFR3 and FGFR4 using real-time RT-PCR and patterns of protein expression using immunofluorescence were analyzed. In addition, this study focused on the functional roles of FGF-1 in mouse early embryo development. To confirm the exogenous FGF-1 function, used recombinant mouse FGF-1 and AZD4547, a FGFR antagonist.

MATERIALS AND METHODS

Experimental animals

All experiment involving animals were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Animals were maintained under standard condition at Sungshin Women's University diurnal rhythm kept under the 14L : 10D schedule with light-on an 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 induction and embryo collection

Female CD-1 mice were superovulated by injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma) followed 48h later by injection 5 IU of human chronic gonadotropin (hCG, Sigma). And then these female mice were placed with males of the same strain. The next morning of finding a vaginal plug was defined day 0.5 of pregnancy. Preimplantation mouse embryos were collected after 15 hr, 21 hr, 48 hr, 54 hr, 62 hr, 80 hr, 96 hr post hCG injection unfertilized egg (UF), pronucleus (PN), 2-cell, 4-cell, 8-cell, morula, and blastocyst were collected from oviduct or uterus by flushing with BWW medium containing 0.4% bovine serum albumin (BSA), respectively. Hatching embryos

and in vitro early stage embryos were cultured in the same medium, while checking with the naked eye.

Total RNA extraction and first strand cDNA synthesis

Total RNAs of 10 embryos were extracted using RNeasy® Micro Kit (QIAGEN, USA) according to the manual of manufacturer. First Strand cDNA Synthesis Kit (Agilent, USA) were used according to the manual of manufacturer. Briefly, reaction reagents are 28 μl total RNA, 5 μl Accuscript buffer (10X), 1 μl oligo dT primer (0.5 μg/μl), 1 μl random primer (0.1 μg/μl), 2 μl dNTP mix (100 mM), 6 μl RNase-free water. Reaction mixture was incubated at 65℃ for 5 min, placed the tube at RT to allow the primers to anneal to RNA for 5 min, and then added 4 μl DTT (100 mM), 1 μl Accuscript multiple temperature RT, 2 μl RNase block ribonuclease inhibitor (40 U/ml). The mixture was incubated at 42℃ for 1 hr and 70℃ for 15 min to terminate cDNA synthesis.

Real-time PCR analysis

For quantification of expression levels, transcripts of target genes were amplified using RT-PCR and the specific primers. Quantification RT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, Japan) and Thermal Cycler Dice Real Time System TP800 (TaKaRa, Japan). Each reaction was run in

triplicate and consisted of 1 μl cDNA. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. The fold change in gene expression was calculated using the ΔΔCt method with the housekeeping gene, a nuclear protein, H2afz, as the internal control.

Whole mount immunofluorescence of mouse embryo

Collected embryos were fixed in 4% paraformaldehyde in PBS containing 0.16% picric acid for 30 min at RT. Permeabilization was conducted by 0.5% PBST (PBS containing 0.5% Triton X-100) for 3 hr. The embryos were blocked in 0.5% PBST containing 10% normal serum for 1 hr at RT. After then, the embryos were incubated with rabbit polyclonal anti-FGF1 (Santacruz, USA), rabbit polyclonal anti-FGFR2 (Abcam, UK), rabbit polyclonal anti-FGFR3 (Santacruz, USA) at a 1:200 dilution at 4℃ overnight, respectively. Washed embryos were incubated with fluorescence 2nd antibody Cy3 conjugated AffiniPure (Jackson ImmunoReseach, USA) diluted 1:200 in 0.5% PBST for 2 hr at RT. The nuclear of embryos was staining with Hoechst33238 at 10 min. Dot slides were used for mounting. For negative controls, we deleted primary antibodies. Finally, fluorescent images were analyzed by Zeiss LSM 700 laser scanning microscope with ZEN software.

Embryo culture and chemical treatment

To get late 2-cell stage embryos, the pregnant mice were sacrificed at 48 hr post hCG injection and flushed the oviduct. After that, the healthy 2-cell embryos were collected and used to study. Recombinant FGF-1 (Prospecbio, Israel) was treated in the concentration 0, 5, 100, 500 ng/ml with or without 2 μg/ml heparin. To evaluate FGFRs effect in preimplantation embryo, AZD4547 (Selleckchem USA) was treated in 0, 10, 200, 400 nM. AZD4547 is known as FGFR antagonist, which targeting FGFR1/ 2/ 3 with IC50 of 0.2 nM/ 2.5 nM/ 1.8 nM, weaker activity against FGFR4 with IC50 of 165 nM. To determine the functions of FGFRs correlate with FGF-1, embryos were cultured in adding the recombinant FGF-1 and AZD4547 in BWW medium. AZD4547 was treated in the concentration 10, 400, 1200 nM. Recombinant FGF-1 was treated in 5 ng/ml with 2 μg/ml heparin. Embryonic stages were observed at 48 hr, 72 hr, 96 hr, 120 hr post hCG injection.

Statistics

The *t*-test for real-time PCR analysis was used to evaluate the difference between 2-cell embryo and other stage embryos. * indicate p-value <0.05 compared with control.

The chi-square test for in vitro culture analysis was used to evaluate the difference between control and experiment groups. At developmental stage to 8-cell, #, ## and ### indicate *P*-value < 0.05, < 0.01 and < 0.001,

respectively, compared with control. Other stages indicate the asterisks (*, ** and *** indicate *P*-value < 0.05, < 0.01, < 0.001, respectively.).

Table 1. Primer sequences for analyze the transcripts

		primer Primer sequence (5'-3')	NCBI reference	Amplified length (bp)
FGF1	S	ATG GCT GAA GGG GAG ATC ACA	NM_010197.3	210
	AS	ATA CAC TTC GCC CGC ACT TTC		
FGFR2-1 (IIIc)	S	CCA GAA GAG CCA CCA ACC AAA TAC	NM_010207.2	222
	AS	AGT ACA AGC ATA GAG GCC GGA GT		
FGFR2-2 (IIIb)	S	CAA CAC TGT GAA GTT CCG CTG	NM_201601.2	226
	AS	ATC GAG GTG GTA GGT GTG GTT G		
FGFR3	S	GTG CGT GTA ACA GAT GCT CCA	NM_008010.5	202
	AS	CTT TGC CAT TCT TCA GCC AGG		
FGFR4	S	CAC TGG CTC AAG GAT GGA CA	NM_008011.2	180
	AS	AGC ACA TCC AGG AGA TAG CTG TAG C		
H2AFZ (control)	S	TCC GGA AAG GCC AAG ACA AA	NM_016750.3	232
	AS	AGT GAC GAG GGG TCA TAC GCT TT		

Table 2. Real-time RT-PCR Thermal cycler schedule

Step		Temperature(℃)	Time	cycles
Hold		94	30 min	1
3 steps PCR	Denaturation	95	1 min	45
	Annealing	59	30 sec	
	Extension	72	1 min	
Dissociation	Denaturation	95	15 sec	1
	Annealing	60	30 sec	
	Extension	95	15 sec	
Hold		4	indefinitely	1

RESULTS

Profiling the FGF1, FGFR2, FGFR3 mRNA expression level in preimplantation mouse embryos

The expression patterns of FGF-1, FGFR2 and FGFR3 mRNA were analyzed by realtime RT-PCR. The relative quantify of FGF and its receptors mRNA were 2-cell stage embryo. FGF1 mRNA was high expressed in UF and PN stage embryos, but from 2-cell to hatching embryos had low expression pattern *in vivo* mouse embryos except 8-cell stage (Fig 1A). FGFR2 1 variant is known as IIIc isoform. Its mRNA pattern was also high level in UF and PN stage. From 4-cell to hatching stage was high level as contrasted with 2-cell (Fig 1B). FGFR2 2 variant is known as IIIb form. It is high level in UF and PN, but not more than 10 fold in UF as distinct from FGFR2 1 variant. FGFR2 2 variant mRNA expression was low level in later stage embryos (Fig 1C). FGFR3 was high expression in PN stage. After 2-cell stage, mRNA pattern was increased excluding hatching stage (Fig 1D). FGFR4 was high expression in UF, and from 2-cell to 8-cell, mRNA level was increased. The expression level decreased at morula stage, but return growth at blastocyst (Fig 1E). At the hatching stage, FGFR4 mRNA level is lower than 2-cell.

FGF-1 *in vitro* embryos were high expression in 8-cell stage (Fig 2A). FGFR2 1 variant was increased from 2-cell to 8-cell. Hatching stage was also high expressed (Fig 2B). FGFR2 2 variant was not increased at 8-cell and

hatching stages (Fig 2C). FGFR3 was increased at morula and hatching stages, and decreased at blastocyst (Fig 2D). FGFR4 was increased 4-cell and morula stage, but mRNA level in blastocyst was lower than 2-cell (Fig 2E).

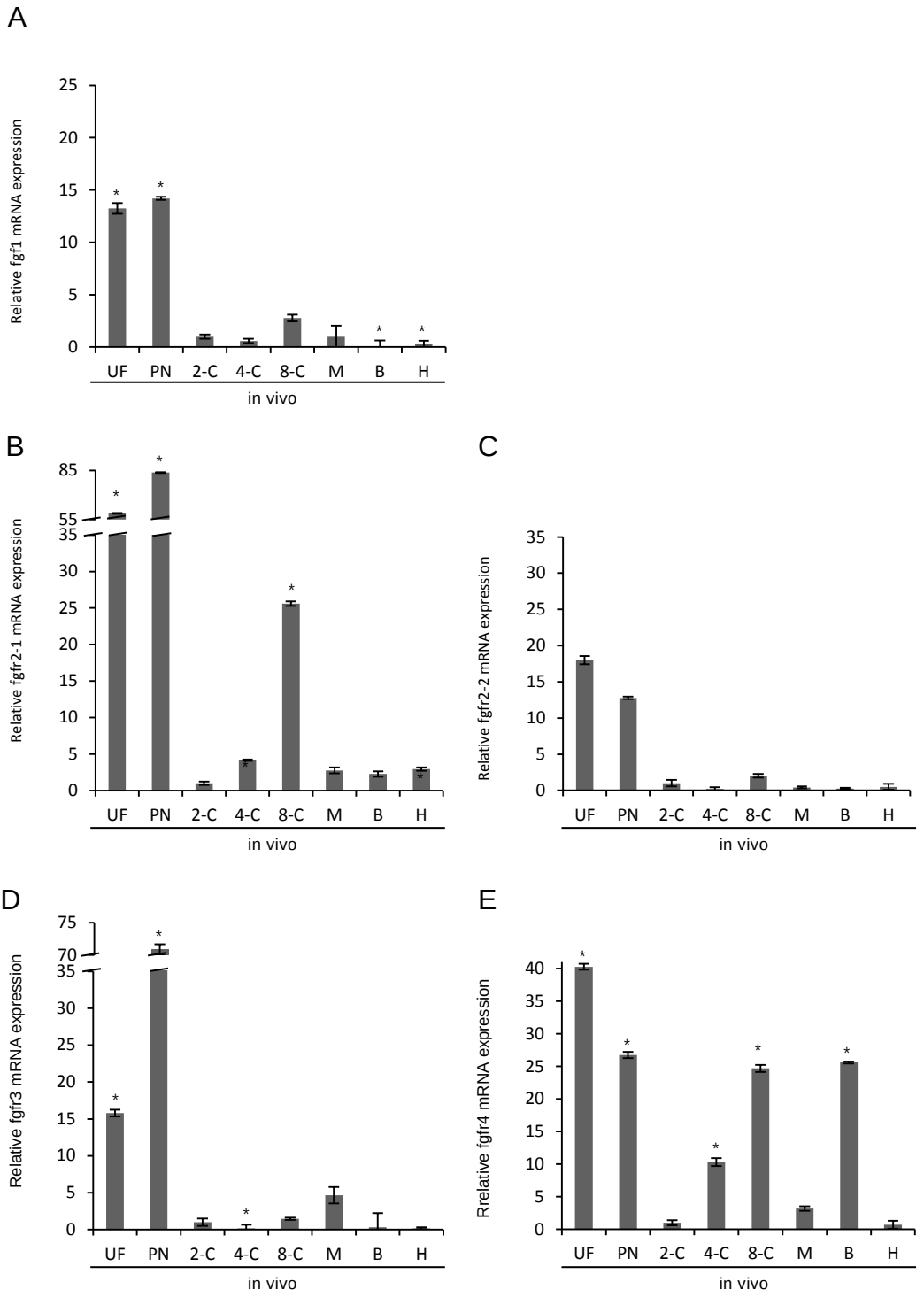

A
Relative fgf1 mRNA expression
25
20
15
10
5
0
UF PN 2-C 4-C 8-C M B H
in vivo
B
Relative fgfr2-1 mRNA expression
85
55
35
30
25
20
15
10
5
0
UF PN 2-C 4-C 8-C M B H
in vivo
C
Relative fgfr2-2 mRNA expression
35
30
25
20
15
10
5
0
UF PN 2-C 4-C 8-C M B H
in vivo
D
Relative fgfr3 mRNA expression
75
70
35
30
25
20
15
10
5
0
UF PN 2-C 4-C 8-C M B H
in vivo
E
Rrelative fgfr4 mRNA expression
40
35
30
25
20
15
10
5
0
UF PN 2-C 4-C 8-C M B H
in vivo

Figure 1. mRNA expression patterns of FGF-1 and FGFRs in preimplantation mouse embryos *in vivo*.

(A) FGF-1, (B) FGFR2 variant 1 (same IIIc form), (C) FGFR2 variant 2 (same IIIb form), (D) FGFR3 (E) FGFR4; UF: unfertilized egg, PN: pronucleus, 2-C: 2-cell, 4-C: 4-cell, 8-C: 8-cell, M: morula, B: blastocyst, H: hatching. *P-value < 0.05.

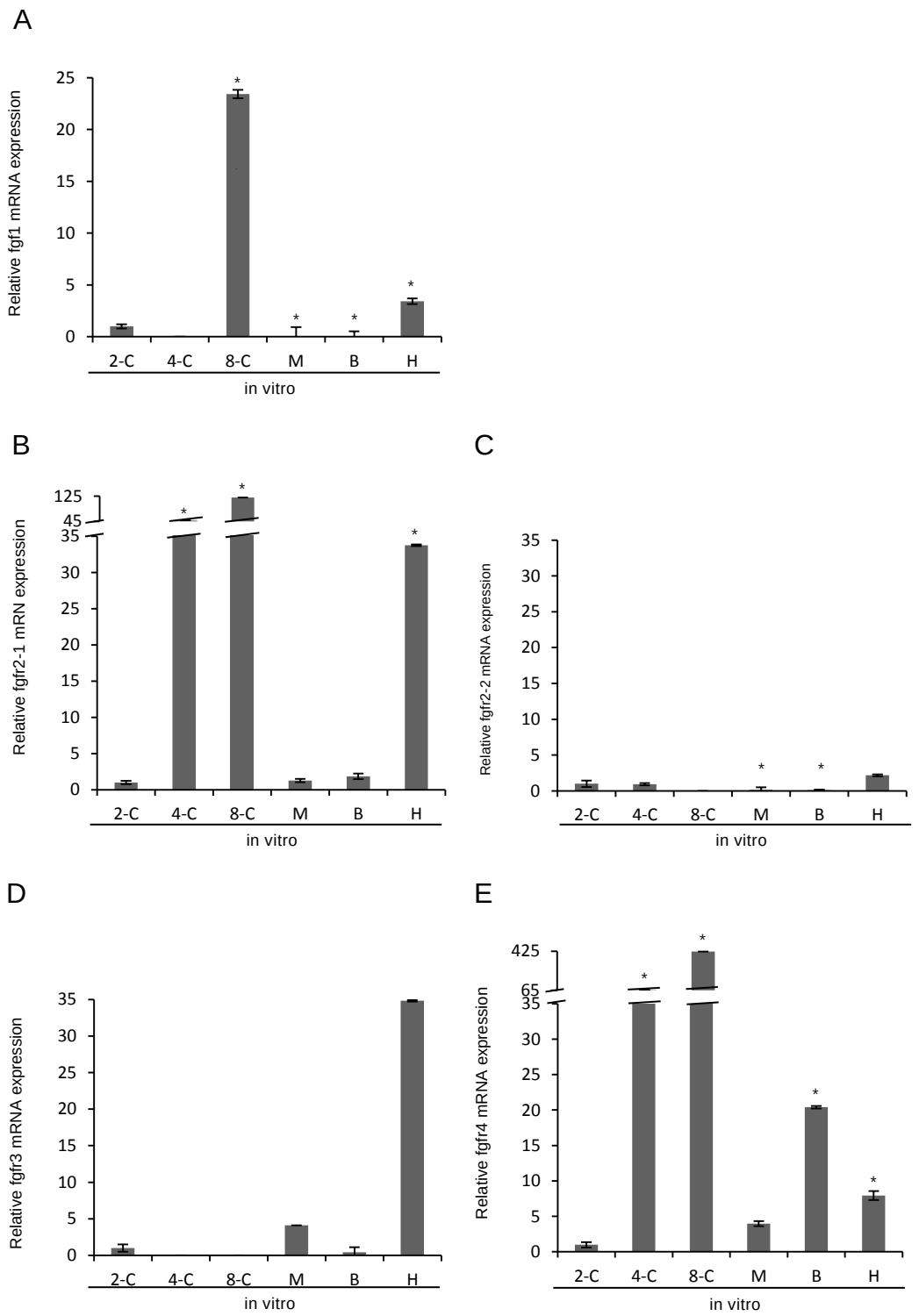

A
Relative fgf1 mRNA expression
25
20
15
10
5
0
2-C
4-C
8-C
M
B
H
in vitro
B
Relative fgfr2-1 mRN expression
125
45
35
30
25
20
15
10
5
0
2-C
4-C
8-C
M
B
H
in vitro
C
Relative fgfr2-2 mRNA expression
35
30
25
20
15
10
5
0
2-C
4-C
8-C
M
B
H
in vitro
D
Relative fgfr3 mRNA expression
35
30
25
20
15
10
5
0
2-C
4-C
8-C
M
B
H
in vitro
E
Relative fgfr4 mRNA expression
425
65
35
30
25
20
15
10
5
0
2-C
4-C
8-C
M
B
H
in vitro

Figure 2. mRNA expression patterns of FGF1 and FGFRs in preimplantation mouse embryos *in vitro.*

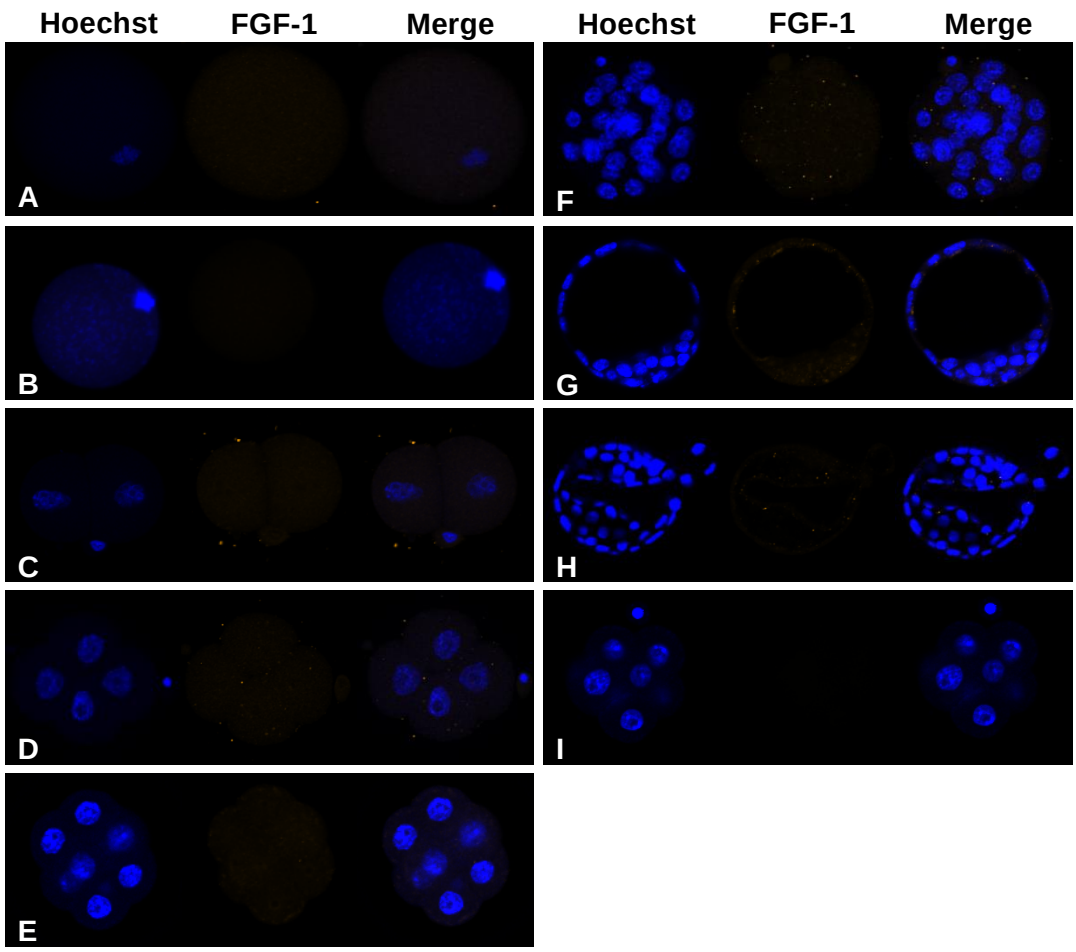
(A) FGF-1, (B) FGFR2 variant 1 (same IIIc form), (C) FGFR2 variant 2 (same IIIb form), (D) FGFR3 (E) FGFR4; 2-C: 2-cell, 4-C: 4-cell, 8-C: 8-cell, M: morula, B: blastocyst, H: hatching embryo in vitro. *P-value < 0.05.

Profiling the protein expression of FGF-1, FGFR2, and FGFR3 in preimplantation mouse embryos

The protein expression of FGF-1 and its receptors were identified using immunofluorescence. FGF-1 was expressed in 8-cell and morula stage embryos, but other stages are low level (Fig 3). FGFR2 was detected in all stages preimplantation mouse embryos in vivo (Fig 4). FGFR-3 was also strongly detected in all stages preimplantation mouse embryos in vivo, but the protein level decreased at the after morula stage (Fig 5).

In vitro group was in accord with results of *in vivo*. FGF-1 was low expression level except 8-cell and morula stage (Fig 6). FGFR2 and FGFR3 were detected in all stage embryos *in vitro* (Fig 7, Fig 8).

This suggested that FGF-1 and FGFRs are regulating the preimplantation embryo development.


Hoechst
FGF-1
Merge
Hoechst
FGF-1
Merge
A
B
C
D
E
F
G
H
I

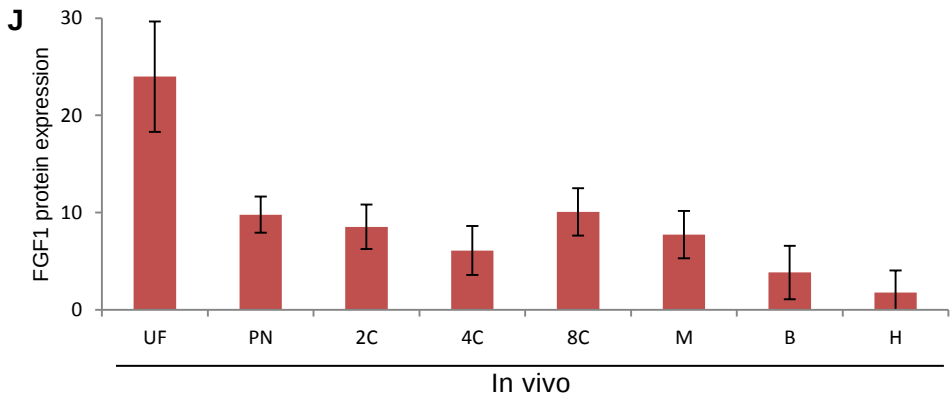
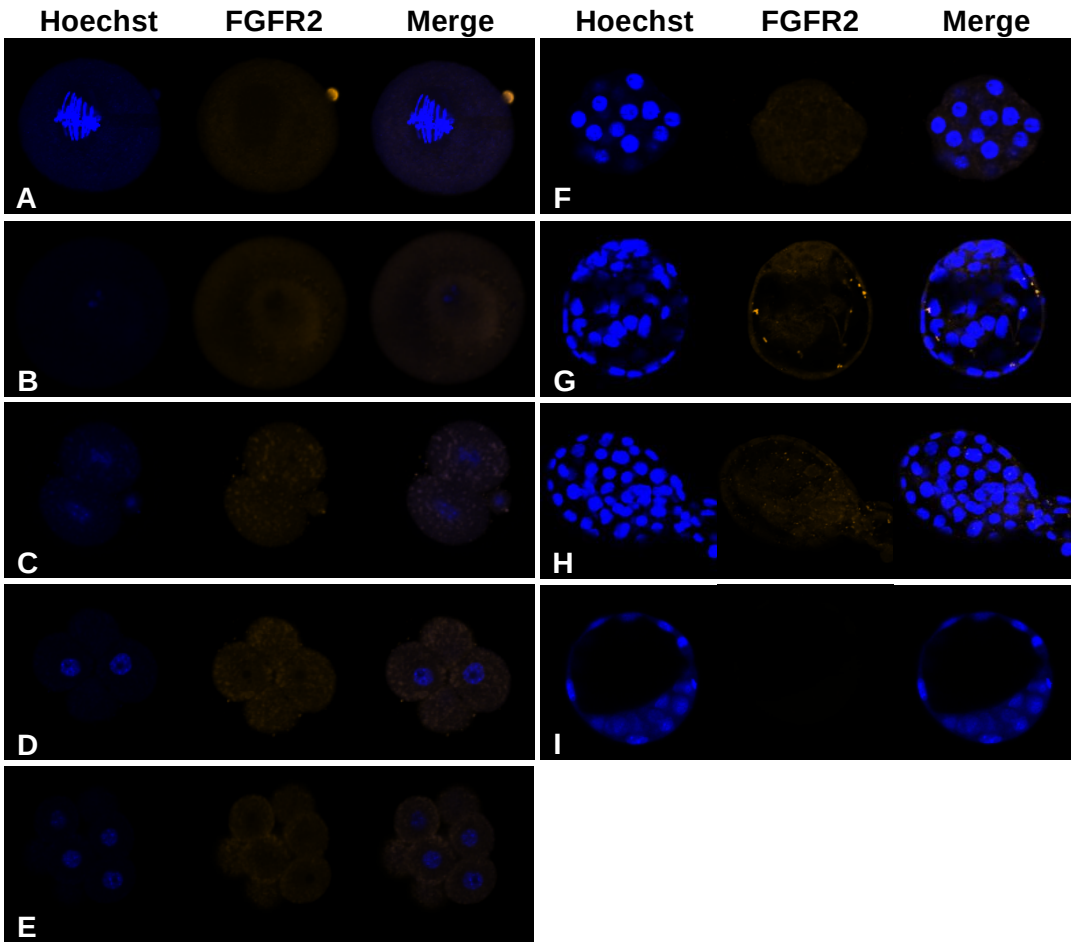

J
FGF1 protein expression
30
20
10
0
UF
PN
2C
4C
8C
M
B
H
In vivo

Figure 3. Immunofluorescence of FGF-1 in preimplantation mouse embryos in vivo.

(A) Unfertilized egg, (B) Pronucleus, (C) 2-cell, (D) 4-cell, (E) 8-cell, (F) morula, (G) blastocyst, (H) hatching stage embryo. (I) Negative control (8-cell embryo). (J) Protein intensity of FGF-1. Magnification; 200X.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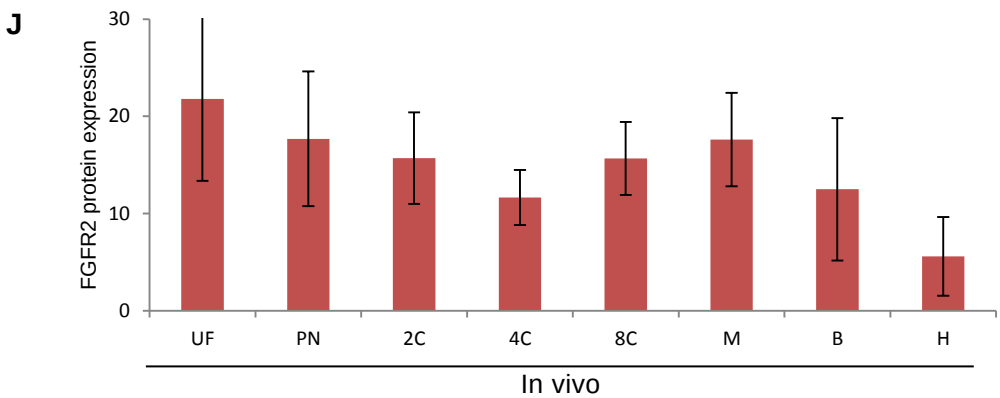
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FGFR2 protein expression
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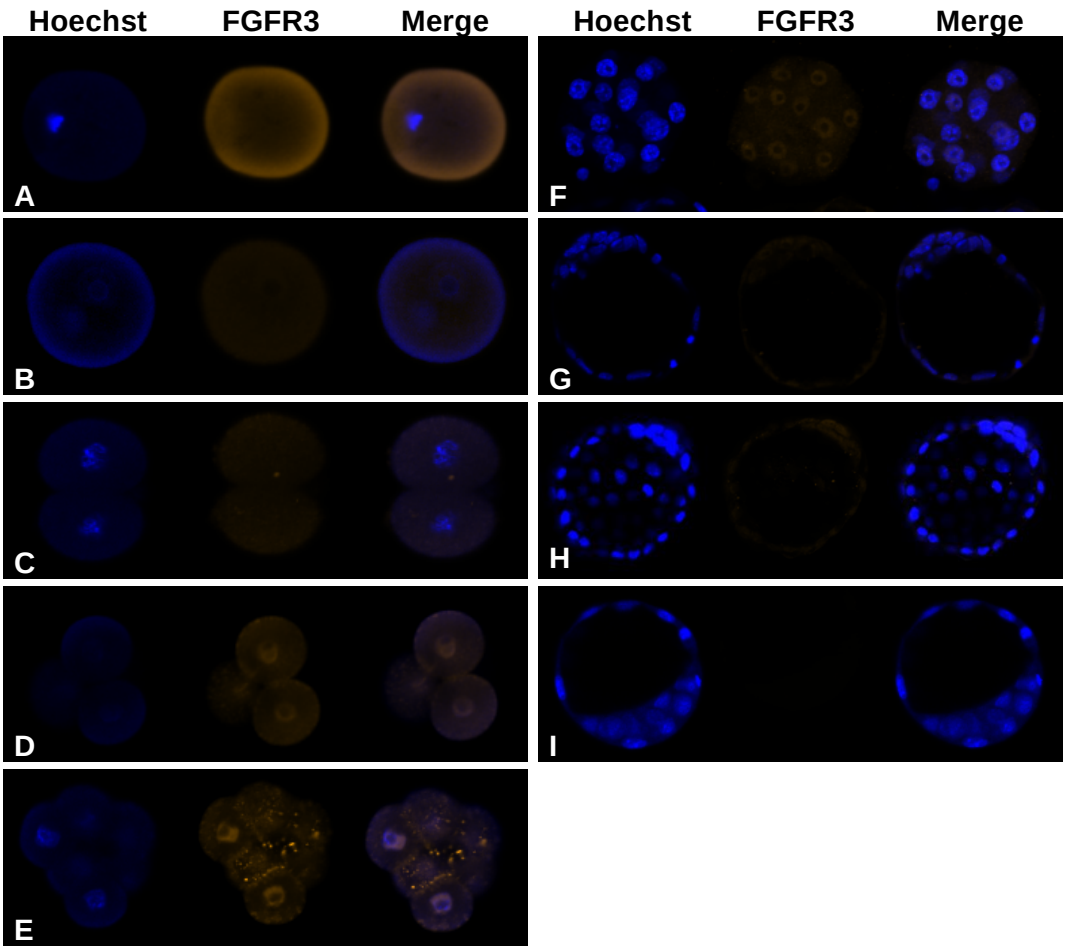
In vivo

Figure 4. Immunofluorescence of FGFR2 in preimplantation mouse embryos in vivo.

(A) Unfertilized egg, (B) Pronucleus, (C) 2-cell, (D) 4-cell, (E) 8-cell, (F) morula, (G) blastocyst, (H) hatching stage embryo. (I) Negative control (blastocyst). (J) Protein intensity of FGFR2. Magnification; 200X.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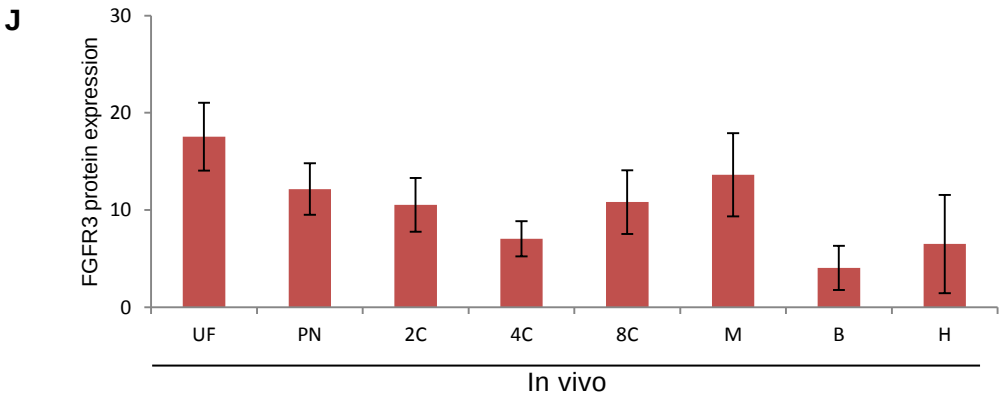
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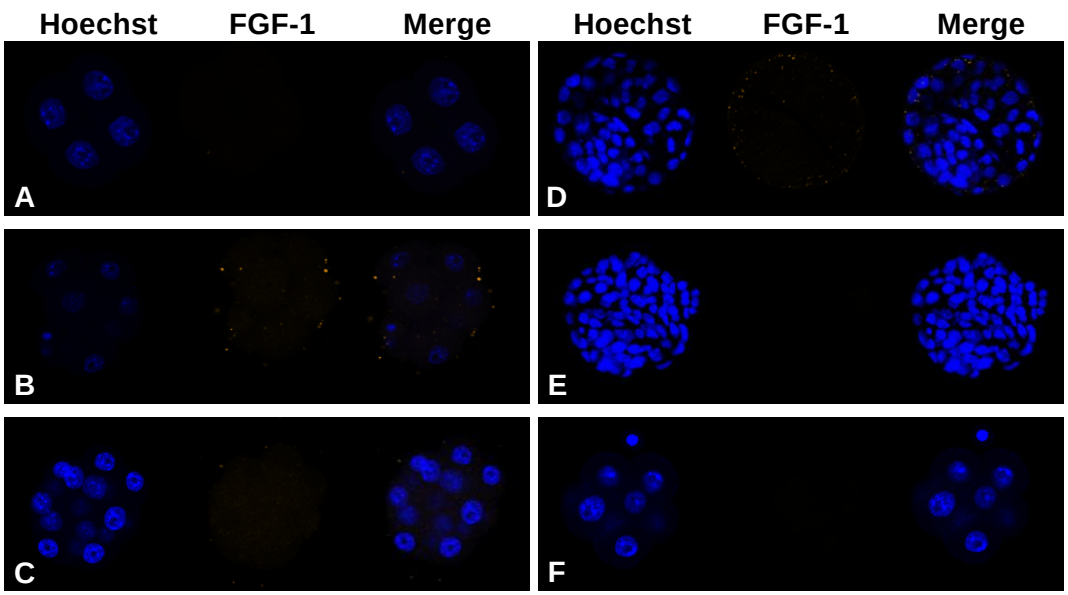
In vivo

Figure 5. Immunofluorescence of FGFR3 in preimplantation mouse embryos in vivo.

(A) Unfertilized egg, (B) Pronucleus, (C) 2-cell, (D) 4-cell, (E) 8-cell, (F) morula, (G) blastocyst, (H) hatching stage embryo. (I) Negative control (blastocyst). (J) Protein intensity of FGFR3. Magnification; 200X.



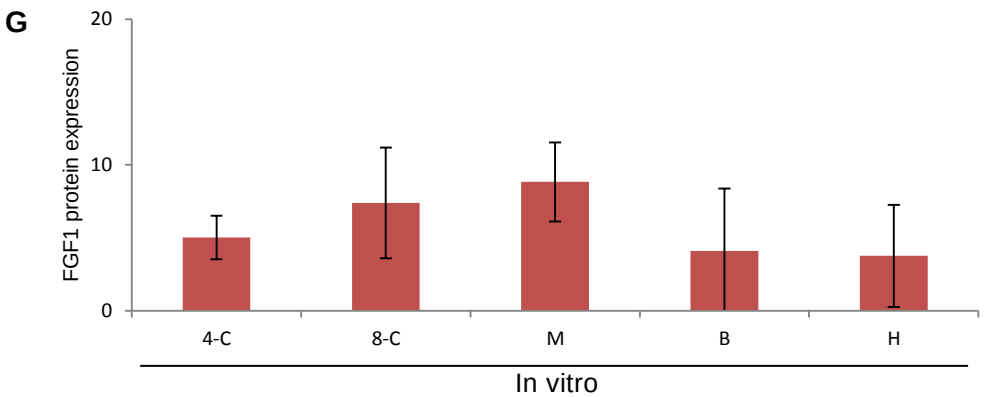
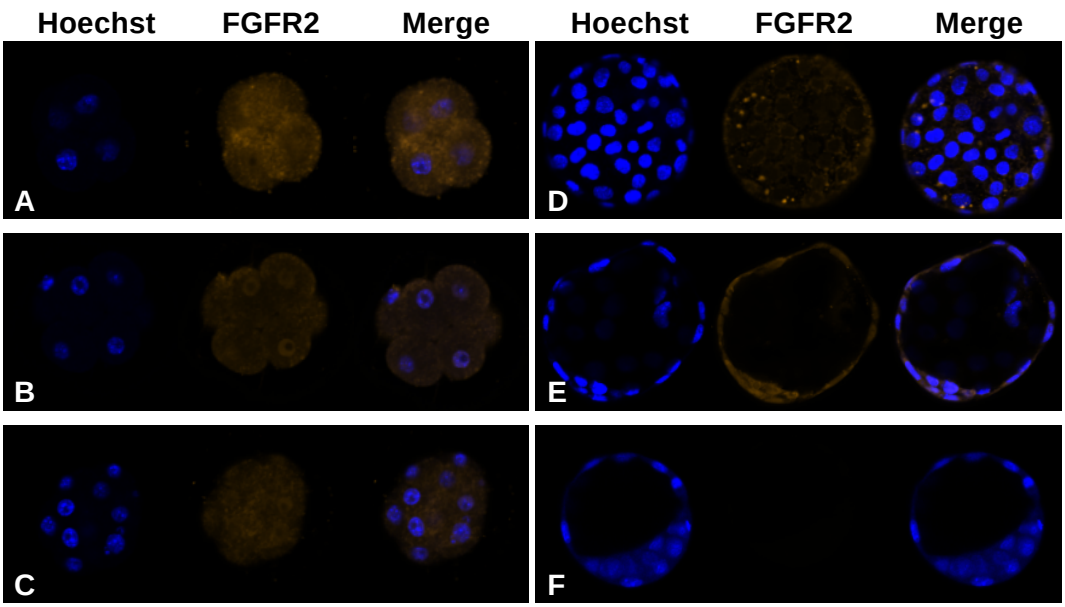


Figure 6. Immunofluorescence of FGF-1 in preimplantation mouse embryos in vitro.

(A) 4-cell, (B) 8-cell, (C) morula, (D) blastocyst, (E) hatching embryo. (F) Negative control (blastocyst). (G) Protein intensity of FGF-1. Magnification; 200X.



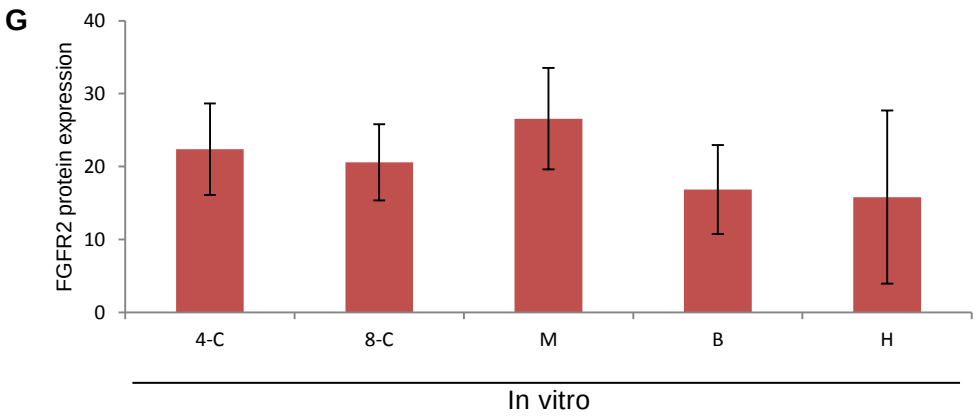


Figure 7. Immunofluorescence of FGFR2 in preimplantation mouse embryos in vitro.

(A) 4-cell, (B) 8-cell, (C) morula, (D) blastocyst, (E) hatching embryo. (F) Negative control (blastocyst). (G) Protein intensity of FGFR2. Magnification; 200X

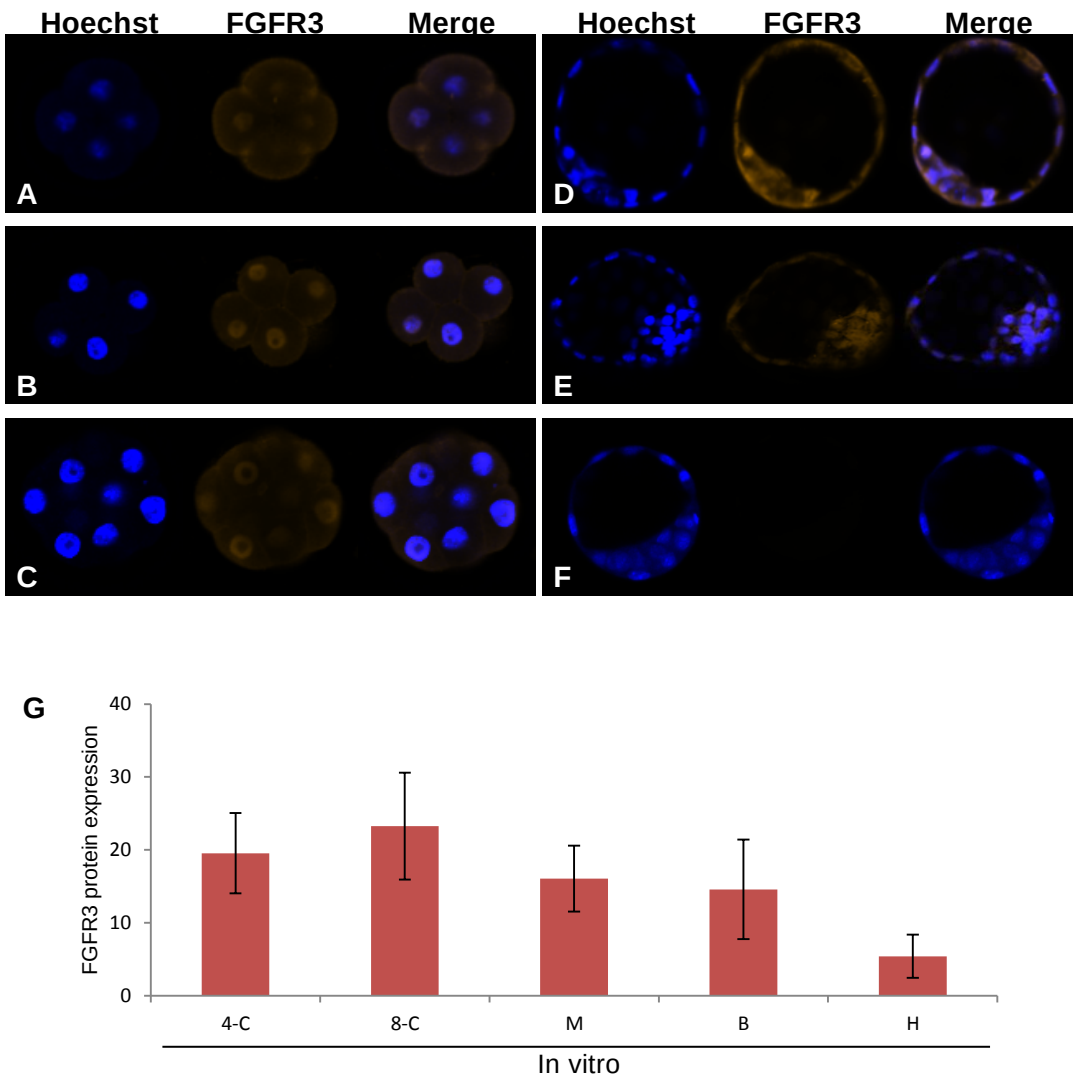


Figure 8. Immunofluorescence of FGFR3 in preimplantation mouse embryos in vitro.

(A) 4-cell, (B) 8-cell, (C) morula, (D) blastocyst, (E) hatching embryo. (F) Negative control (blastocyst). (G) Protein intensity of FGFR3. Magnification; 200X

Determination of FGFRs effect in preimplantation mouse embryo by blocking FGFR activity.

To determine FGFR function in mouse embryo development, embryos were cultured *in vitro* treated AZD4547 which known as FGFR antagonist. For FGFR2, 3 blocking, AZD4547 was treated 10 nM and for repression of FGFR4 activity AZD4547 was treated 200, 400 nM. At the 72 hr post hCG injection, treated group's developmental rate was slower than control. Developmental rate to 8-cell stage decreased significantly (Fig 9A; 0 nM; 65.12% vs 10 nM; 41.67%, #p-value<0.05). Embryonic development to morula also reduced in 400 nM treated group (0 nM; 56.57% vs ***400 nM; 31.58%, ***p-value<0.001). But, embryonic development at the other stage was not significantly different (Fig 9B, C).

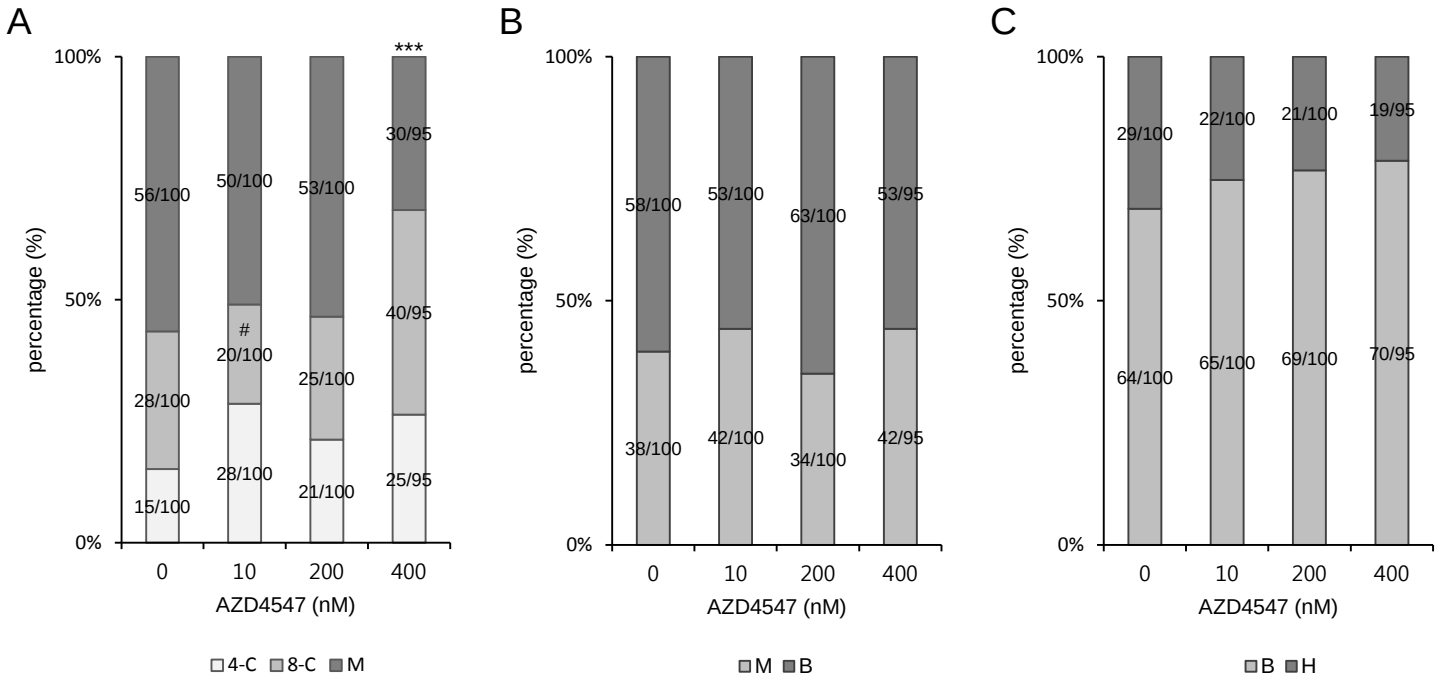


Figure 9. Effects of FGFR antagonist on the embryo development.

(A) 72 hr (B) 96 hr (C) 120 hr post hCG injection; 4-C: 4-cell, 8-C: 8-cell, M: morula, B: blastocyst, H: hatching stage embryo. $^{\#}P<0.05$ (control vs 10nM; 4-cell to 8-cell), $^{***}P<0.001$ (control vs 400nM; 8-cell to morula).

Determination of FGF-1 effects in preimplantation mouse embryo by recombinant FGF-1.

To determine FGF-1 function in mouse embryo development, embryos were cultured *in vitro* treated mouse recombinant FGF-1 with heparin. At the 72 hr post hCG injection, embryonic development to 8-cell was significantly different correspond with the control group (Fig 10A; 0 ng/ml; 57.15% vs 100 ng/ml; 65.01%, *p-value<0.05). At the 96 hr post hCG injection, embryonic development to blastocyst was also significantly different with control group (Fig 10B; 0 ng/ml; 60.42% vs 5 ng/ml; 76.00%, 100 ng/ml; 87.21%, 500 ng/ml; 86.52%, ***p-value<0.001). Likewise, hatching rate was significantly different (Fig 10C; 0 ng/ml; 31.18% vs 5 ng/ml; 26.25%, 100 ng/ml; 48.24%, 500 ng/ml; 46.51%, **p-value<0.01).

Heparin was related to regulation of FGF-FGFR complex. To address this hypothesis, embryos were cultured *in vitro* without heparin. This condition changed the developmental rate. Development to 8-cell stage increased at the FGF-1 treated group, but to morula stage decreased (Fig 11A). At the blastocyst stage, developmental rate showed relatively low significance (Fig 11B). In addition, development at hatching stage was not significantly (Fig 11C).

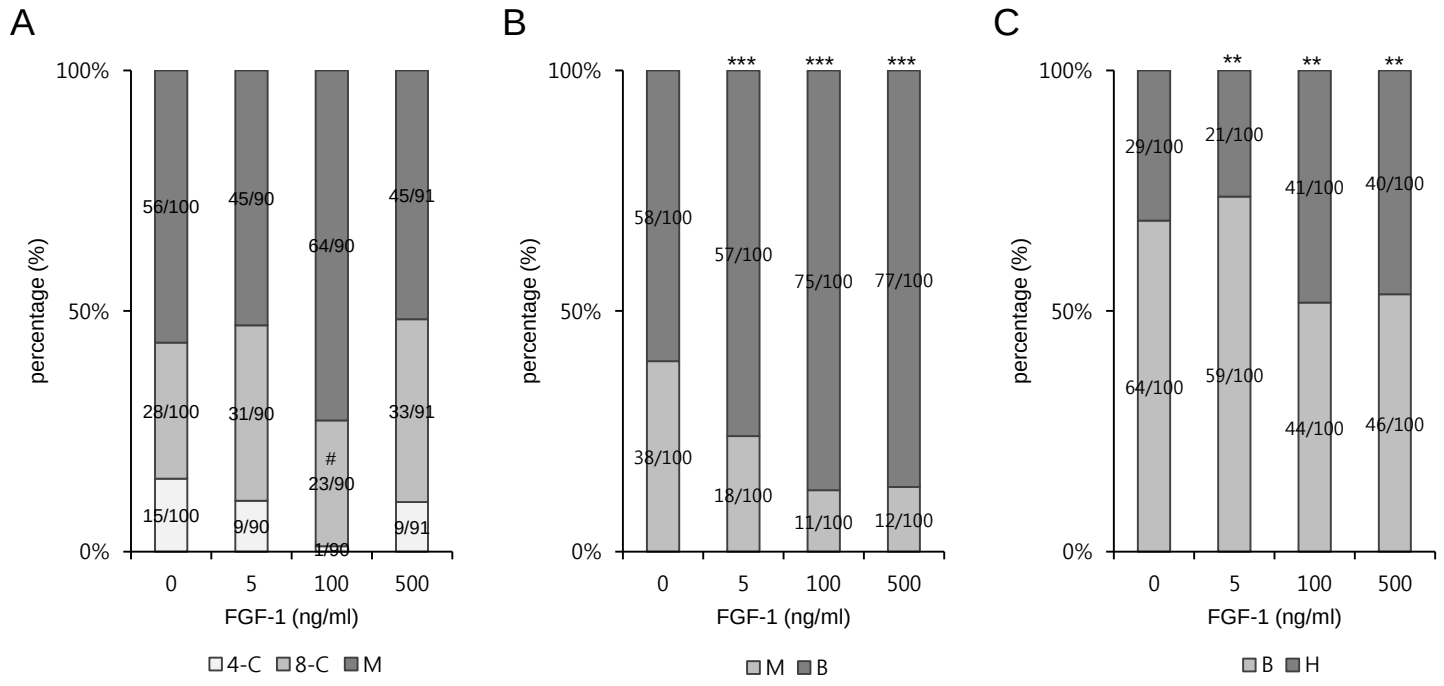


Figure 10. Effects of recombinant FGF-1 on the embryo development with heparin.

(A) 72 hr (B) 96 hr (C) 120 hr post hCG injection; 4-C: 4-cell, 8-C: 8-cell, M: morula, B: blastocyst, H: hatching stage embryo. $^{\#}P<0.05$ (control vs 100ng/ml; 4-cell to 8-cell), $^{**}P<0.01$, (control vs treated group; morula to blastocyst), $^{***}P<0.001$ (control vs treated group; blastocyst to hatching)

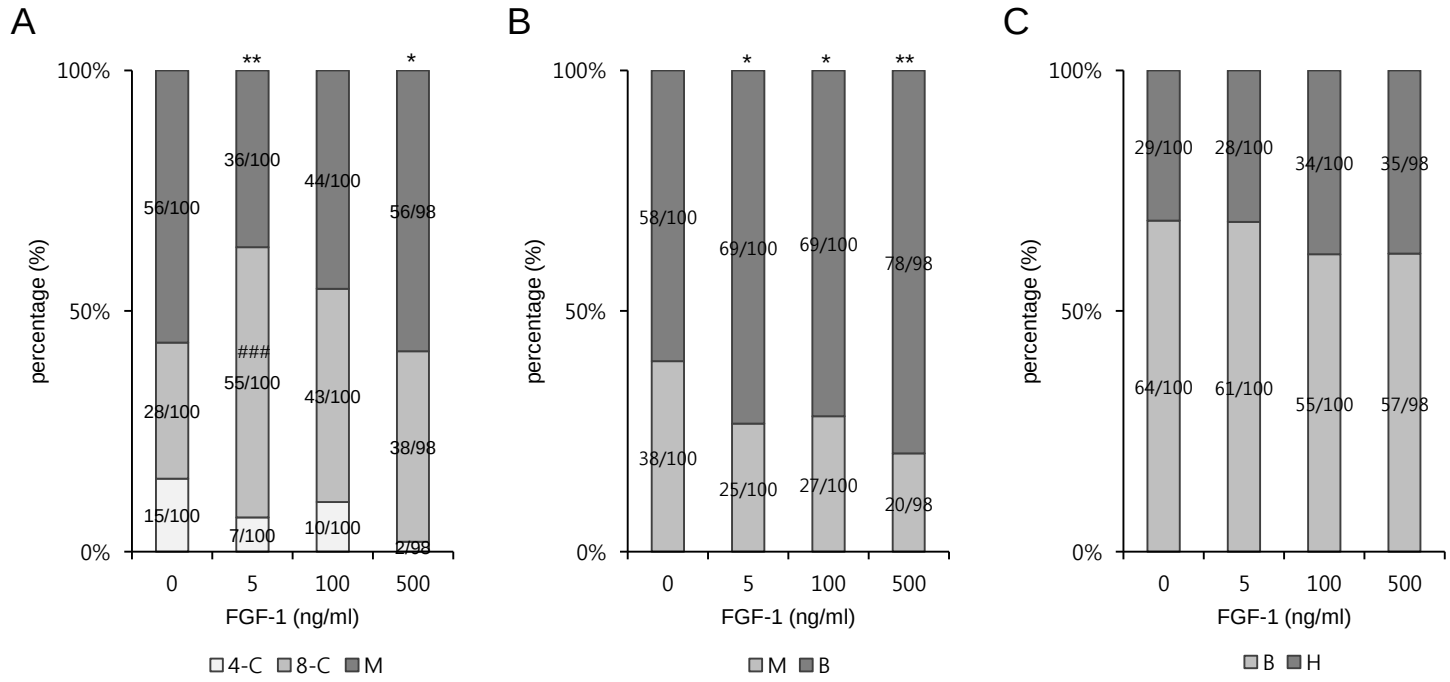


Figure 11. Effects of recombinant FGF-1 on the embryo development without heparin.

(A) 72 hr (B) 96 hr (C) 120 hr post hCG injection; 4-C: 4-cell, 8-C: 8-cell, M: morula, B: blastocyst, H: hatching stage embryo. $^{###}P<0.001$ (control vs 5ng/ml; 4-cell to 8-cell), $^{*}P<0.05$, $^{**}P<0.01$ (control vs treated group; 8-cell to morula, morula, to blastocyst)

Determination of FGF-1 and FGFRs correlation by co-treated recombinant FGF-1 and FGFR antagonist

To examine the role of FGF-1 in mouse embryo development is correlated with roles of FGFRs, we cultured the embryos in vitro treated with AZD4547 and recombinant FGF-1 as mentioned in Materials and Methods. At the 72 hr post hCG injection, developmental rate changed compared with group of treated AZD4547 alone. Development from 4-cell to 8-cell was increased when adding recombinant FGF-1. As the AZD4547 increase, however, developmental rate decreased. Development to morula stage was decreased in co-treated group (Fig 9A vs Fig 12A). At the 120 hr post hCG injection, developmental rate of co-treated group decreased in a dose dependent manner different with treated AZD4547 alone (Fig 9C vs Fig 12C). Developmental rate to blastocyst was not significantly (Fig 12B).

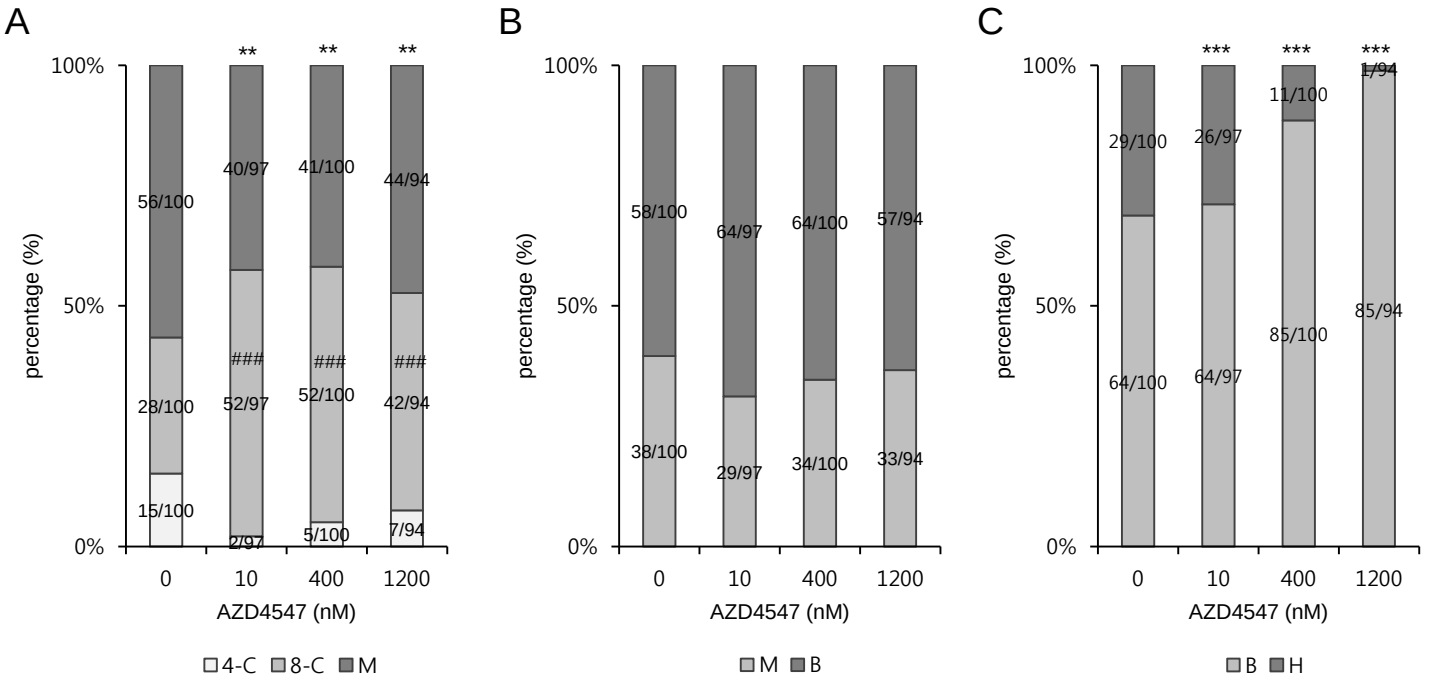


Figure 12. Determine of FGF1 (5ng/ml) and FGFRs correlation in embryo development

(A) 72 hr (B) 96 hr (C) 120 hr post hCG injection; 4-C: 4-cell, 8-C: 8-cell, M: morula, B: blastocyst, H: hatching stage embryo. $^{###}$p<0.001 (control vs treated group; 4-cell to 8-cell), $^{**}P$<0.01 (control vs treated group; 8-cell to morula), $^{***}P$<0.001 (control vs treated group; blastocyst to hatching).

DISCUSSION

FGFs are essential for embryo development in animal development including *Drosophila, C. elegans*, mouse, and human. Various evidences supported these. The FGF-4 mRNA is expressed at all stages of preimplantation mouse embryos from 1-cell through blastocyst, it is temporally and spatially overlapping expression pattern of Oct4, a trans-activation factor. These factors are important for preimplantation embryonic development (Rappolee et al., 1994). FGF-2 and FGF-7 that are derived from the endometrium act to promote the implantation of the embryo by enhancing the spreading and invasion of the trophectoderm as paracrine factors (Taniguchi et al., 1998). These factors are not expressed in preimplantation embryos. FGF-8 expresses in mouse embryo during formation of primitive streak. This factor regulates the limb development (Crossely et al. 1995). These functions by FGFs occur through their receptors.

FGF-FGFR signaling has two pathways. One is the PI3-kinase/Akt pathway. Chen et al (2000) show that all FGFRs are expressed in the embryoid body. They suggested the FGF signaling through FGFRs-PI3K-Akt is essential for embryoid body differentiation. Another is the MAPK/ERK pathway, the major signaling pathway. Wang et al (2004) show that all MAPK pathway molecules are expresses in preimplantation mouse embryos. They show that FRS2 and GRB1, the targets of FGFR, are present in mouse embryos. It is suggested

that MAPK/ERK signaling pathway through FGFR is require for embryo development. Yamanaka et al (2010) show that stochastic and progressive specification of epiblast and primitive endoderm lineages occur during maturation of the blastocyst in FGF/MAPK signal dependent manner. FGFRs activate these signaling pathways during embryogenesis. Despite the functions of FGFRs in postimplantation were proved through a lot of research, there are not cleared in preimplantation mouse embryos.

So, we tried to understand the functions of FGFRs. In previous study, we defined that FGFRs expressed in preimplantation embryos, and FGF-1, has high affinity against all FGFRs, was also detected. We perform real-time RT-PCR to confirm the mRNA expression patterns of FGF-1, FGFR2, FGFR3 and FGFR4. These factors were high expression in unfertilized egg and 1-cell stage. FGF-1 mRNA was increased at the 8-cell stage. FGFR2-1 variant mRNA was increased in the 4-cell, 8-cell and hatching embryos. FGFR3 mRNA was increased in morula and hatching embryos. It can be predicted that FGF-1, FGFR2 1 variant and FGFR3 are related to early embryo development. Protein expression was determined by immunofluorescence. Receptors are expression in all stage embryos. But, FGF-1 expressed low level in all stages. It is suggested that FGF-1 acts as paracrine factor through FGFRs. It can be predicted as a result that FGF-1 expressed in early pregnant mouse oviduct (data not shown). To confirm the function of FGFRs in preimplantation embryos, we cultured the embryos with AZD4547 which known as FGFRs

antagonist. Developmental rate to morula was reduced in treated group compared with the control group. It is correspond with the FGFR2 variant 1 mRNA expression pattern.

To confirm the function of FGF-1 in embryo development, the embryos were cultured with mouse recombinant FGF-1. Developmental rate was significantly improved to morula, blastocyst, and hatching compared with the control group. It is correspond with the FGF-1 mRNA expression pattern. It is suggested that FGF-1 involved in embryonic development to morula, blastocyst and hatching. In the case of without heparin, developmental rate was changed. Although development to 8-cell and morula stage was increased, embryonic hatching was not significantly compared with heparin-treated group. Therefore, heparin is important to embryo development as a regulator of FGF-FGFR complex.

To examine the role of FGF-1 in mouse embryo development is correlated with roles of FGFRs, treated AZD4547 and recombinant FGF-1 simultaneously. Developmental rate increased in early stage compared with treated AZD4547 alone. And hatching was not significantly in co-treated group. It is suggested that blocking of FGFR activity by AZD4547 rescue through recombinant FGF-1.

Based on these results FGF-1 regulates preimplantation mouse embryo development through signaling pathway of its receptors.

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ABSTRACT.

FGFR expression based early embryonic developmental communication; possible role of FGF1 and its receptors in pre-implantation mouse embryo

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Fibroblast growth factors (FGF) are involved in several cellular events including cell proliferation, migration and differentiation. In particular, lots of studies suggested that FGF-FGFR signaling pathway is essential for embryo development in mammals including mouse and human. FGFs are identified 22 members and have different affinity with tyrosine kinase FGFR in tissue specific manner. Their receptors formed the complex with FGFs. At this time, heparin or heparan sulfate regulate that FGFs bind to FGFRs. In previous studies, we identified FGF-1 mRNA was expressed in preimplantation mouse embryos. FGFR2 and FGFR3 mRNA were also expressed in specific time.

FGFR4 mRNA was expressed in all stage embryos, but FGFR1 mRNA is not detected in early embryo. However, FGFRs functions in early mouse embryos are not cleared. To confirm this, we analyzed the mRNA and protein expression patterns in preimplantation mouse embryos using real-time RT-PCR and immunofluoro-chemistry assay. Maternal FGF and its receptors transcripts were detected with relatively high level. FGF1 mRNA levels were increased at 8-cell stage but after that decreased. FGFR2 and FGFR3 were expressed in all stages mouse embryo. These results are similar to in vitro group. In the case of FGF-1, it was not almost detected in blastomeres. To understand the function of FGF-1 and receptors, we cultured the mouse embryo with recombinant FGF-1 or FGFR antagonist, AZD4547. AZD4547 inhibited embryo development in a concentration dependent manner. Recombinant FGF-1 helps the development. Taken together, it is exposed that FGF-1 and FGFRs are involved in cleavage stage embryo development. In addition, it suggests that FGF-1 may work as auto- and paracrine factor.