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석사학위청구논문

Change the potency of early stage embryo : possible role of β-catenin

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성신여자대학교 대학원

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이 논문을 석사학위논문으로 제출함

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인 준 서

백수경의 석사학위 논문으로 인준함.

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Master of Science

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논 문 개 요

난자는 수정 이후 난할 과정을 거쳐 2 세포기, 4 세포기, 8 세포기, 상실배, 포배의 순서로 발생하게 된다. 포유동물의 수정란과 초기 난할 시기의 할구는 개체로 형성되는데 필요한 모든 조직과 기관으로 분화 할 수 있는 전능성(totipotency) 을 유지하고 있으나, 난할 과정을 거치면서 포배를 형성하게 되면 이러한 능력이 감소되어 3개의 배엽으로 분화 할 수 있는 전분화능(pluripotency)을 갖게 된다. 포유동물의 초기 배아의 전능성은 8 세포기까지 유지된다고 알려져 있다. 이러한 발생 능력의 변화는 수정난이라는 한 개의 세포에서 다양한 기능을 갖도록 분화되고 또한 이들 기능이 통합되는 다세포로 구성된 온전한 개체로 발생하는 개체 발생 조절에 있어서 중요하나, 발생 시기에 따른 발생능력의 조절 기작과 그 양상이 아직까지 잘 밝혀져 있지 않다. 또한, 발생 능력을 판단하는데 사용될 수 있는 분자 수준의 표식자(marker)도 잘 알려져 있지 않다. 따라서 본 연구에서는 수정 이후 난할 시기 배아의 핵에 위치하는 것으로 알려져 있고 줄기 세포 특성 유지, 신체 축 형성 등의 발생 현상에 관여하는 β-catenin이 전능성의 변화에 어떠한 관련이 있을 것인지도 알아보고자 하였다. 이를 위해 2-세포기 배아를 세포 투과성 펩타이드에 결합된 β-catenin이 함유된 배양액에서 배양하면서 그 발생 현상을 조사하였고, 초기 배아시기에 수정란 유전체의 활성 시기 전후에 발현 정도가 달라지는 것으로 알려진 유전자군의 발현 양상이 β-catenin의 양 증가에 따라 어떻게 변화할 것인 가를 분석하였다. 세포 투과성 펩타이드(cell permeable peptide, CPP)를 매개로 표적 단백질이 초기 배아의 세포질 내로 들어가는 것을 확인하기 위하여 세포 투과성 펩타이드와 녹색 형광 단백질과 재조합(recombinant

CPP-eGFP)하여 초기 배아에 처리, 공촛점 현미경(confocal microscope)을 이용하여 관찰하였다. 결과적으로 세포 투과성 펩타이드가 초기 배아의 세포질 내로 이동하였고, 세포내로 이동하는 세포 투과성 펩타이드의 양은 농도에 따라 큰 차이가 없었다. 세포 투과성 펩타이드와 재조합 된 β-catenin을 20 ng/ml, 2 μg/ml 농도로 2-세포기 배아 시기부터 처리하여 포배까지의 발생을 조사하였다. 난할 하는 배아의 형태는 8-세포기 까지는 대조군과 비교하여 각 시기별 차이는 없었으나 밀착(compaction) 진행 단계부터 대조군과 형태적 차이가 관찰되기 시작했다. 한편, 포배로의 발생률의 유의하게 낮았고, 동일 시점 내 포배로의 발생 단계가 β-catenin 처리군에서 늦었다. 2-세포기부터 β-catenin이 포함된 배양액에서 배양된 포배로 발생한 배아에서 수정란 유전체 발현 전후 발현 양상이 뚜렷하게 구별되는 유전자들(Wnt pathway, BMP pathway, transcription regulator, volume related factor) 중 일부의 발현 양상이 변화되는 것이 관찰되었다. 좀 더 많은 연구가 필요하겠으나 이러한 결과로부터 β-catenin이 전능성과 관련된 발생 능력 변화에 관여할 것이라는 예측을 할 수 있다.

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INTRODUCTION

Until 8 cell stage, the fertilized egg and blastomere have totipotency in mammals (Mitalipov et al., 2009), which is the ability to divide and produce all the differentiated cells in an organism, including extra embryonic tissues. During organogenesis, the blastomere are limited in developmental potency. The cells of inner cell mass (ICM) show pluripotency, which is the potential to differentiate into any type of the three germ layers, ectoderm, mesoderm and endoderm. Such a restriction of developmental potency of blastomere is critical in the development of organisms. However, time point and restriction mechanisms in differentiation potency are not clarified.

Stemness is a unique character of stem cells to be developmentally paused, while retaining developmental potency to differentiate and self-renew. For example, embryonic stem (ES) cells can self-renew, while preserving developmental potency and are able to differentiate into many other cell types. It is well established that β-catenin pathway is critical in both processes, (Anton et al., 2007). β-catenin is a key molecule which involved in Wnt / β-catenin pathway. In the absence of Wnt stimulation, free β -catenin levels are kept low in cytoplasm by a β-catenin / APC / Axin complex. But, in the presence of Wnt signal leading to break down the complex and free β-catenin accumulation in cytoplasm.

And accumulated free β-catenin translocate into the nucleus. In nucleus, β-catenin activates its target genes by binding to sequence-specific transcription factors of the TCF (T Cell Factor) / LEF (Lymphocyte Enhancer Factor) family and controls target gene's transcription level.

Xie et al. (2008) showed that activate β-catenin are mostly localized in the nucleus of all embryonic cells before morula, and primarily in the TE (Trophectoderm) of blastocysts. Based on them, following questions are arises. Why active β-catenin is concentration in nucleus of early stage embryos? What is the role of active β-catenin in nucleus? In addition, similarity with β-catenin in stemness, is it one of the factors for totipotency?

There are a few suspicious candidate which could regulate totipotency. In here including maternal factors, zygotic factors, volume related factors, polarity related factors (Wang et al., 2004) and known stem cell factors.

A few groups tried to evaluate the totipotency marker, but so far, the totipotency marker is not available (Cauffman et al., 2009). Such as Oct3 / 4 and Sox2, function in the maintenance of pluripotency in both blastocyst and ES cells, express since 8 cell or morula stages. Klf4, contributed the long term maintenance of the ES cell phenotype (Takahashi et al., 2006), show similar expression profiles with those of Oct 4 since morula stage.

Control of potency in developing embryo is critical phenomenon

to conform the normal development. Pluripotency of blastomere can be achieved after restriction of totipotency. However the mechanisms of restriction of totipotency are not cleared. So in this study, the molecules which could be involved in potency were analyzed using β-catenin translocation embryos. In addition, the possible roles of β-catenin in totipotency was analyzed.

MATERIALS AND METHODS

Experimental animals

All experiment involving 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 Women's University. Animals were maintained under standard conditions at animals house in Sungshin Women's University with diurnal rhythm kept under the 14 L : 10 D 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 induction and sampling embryos

Female CD-1 mouse were superovulated by injection of 5 IU of pregnant mares serum gonadotrophin (PMSG) and followed by injection of 5 IU of human Chronic gonadotrophin (hCG) after 48 hr. And then these female mice were put in the cage of stud male (1 : 1). The next morning of finding a vaginal plug was defined day 1 of pregnancy. To get each stage embryos of in vivo, after 20 hr, 48 hr, 52 hr, 72 hr, 84 hr, 120 hr post hCG injection pronucleus (PN), 2-cell, 4-cell, 8-cell, morula, blastocyst were collected from oviduct or uterus by flushing with BWW containing 0.4% bovine serum albumin (BSA), respectively. And then embryos were frozen in liquid

nitrogen and kept at -80℃ until used.

To get late 2-cell stage embryos, the pregnant mice were sacrificed at 48 hr post hCG injection and flushed the oviduct. After flushing, the healthy 2-cell embryos were collected and used to study.

Total RNA isolation and first strand cDNA synthesis

Total RNAs of embryos were extracted using RNeasy® Micro Kit (QIAGEN, CA USA) according to the manual of manufacturer. Total RNA 28 μl were used to perform reverse transcription. First Strand cDNA Synthesis Kit (Agilent, CA, USA) according to the manual of manufacturer. Briefly, reaction reagents are total RNA 28 μl, 5.0 μl Accuscript buffer (10X), 6.0 μl oligo dT primer (0.5 μg/μl), 1.0 μl random primer (0.1 μg/μl), 2 μl dNTP mix (100 mM), 1 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 10 min, and then added 4.0 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 RT PCR

Transcripts of target gene were amplified using RT-PCR and the specific primers (Table 1). The primer parameters were 50% GC

contents, avoiding repeat base pair and lengthening 20-24 mer. For quantitative RT-PCR (qPCR) was performed using SYBR Premix Ex Taq™ (TaKaRa, Tokyo, Japan) (Table 2). Each reaction was run in triplicate and consisted of 1.0 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 genes, β-actin, Ppia and H2afz as the internal controls.

CPP-β-catenin construct

To evaluate over-expression of β-catenin can effect to early stage embryo, construct the recombinant CPP-β-catenin expression vector. CPPs have been shown to deliver oligo-nucleotides or small interfering RNA (siRNA) efficiently across cell. CPPs as kinds of KFGF, MAP-1, pVec, R7 and yPFY and in this experiment yPFY was used. As a control, recombinant CPP-eGFP was constructed, too. To purification a vector which is expression recombinant protein easily, β-catenin and eGFP were tagged with 6X His. The reason of 6X His tagging is that it is suspected there is no conformational change of the protein.

To get complement cDNA of β-catenin mRNA, total RNA was extraction from mouse liver, using TRIzol® Reagent (Molecular Research Center Inc., OH, USA) and cDNA synthesis using First

Strand cDNA Synthesis Kit (Agilent, CA, USA) according to the manual of manufacturer. TOPO vector (Invitrogen, CA, USA) was used to amplify the β-catenin cDNA. Amplified construct were purified using QIAprep® Miniprep Kit (QIAGEN, CA USA) and conformed the constructs with restriction mapping and sequencing. Recombinant CPP-β-catenin constructs were made with pET-20b(+) eXhoI and amplified β-catenin. Transfection the CPP-β-catenin construct to BL21 competent cell (Agilent, CA, USA). Aliquots 50 μl BL21 competent cell, add 1.7μl β-mercaptoethanol (Sigma-Aldrich, MO, USA) and incubation 10 min with swirling gently on ice. Add 1μl CPP-β-catenin DNA, incubation 30 min on ice and give heat shock 45 sec at 42℃. To terminate reactions, incubated 2 min on ice. After then added 0.95 ml pre-heated SOC medium (2 M Mg^{2+} stock, 2 M glucose, Bacto-Tryptone, Bacto-Yeast extract, NaCl, KCl) and incubation 1 hour at 37℃ with shaking 225 rpm. Centrifuge 10 min at 5000 rpm, discard the supplement, resuspend the pellet, spread on LB agar plate and incubation overnight at 37℃. Next day, pick the colony and culture with 5 ml LB Broth overnight at 37℃. Centrifuge 10 min at 5000 rpm, discard the supplement, resuspend the pellet and miniprep using QIAprep® Miniprep Kit (QIAGEN, CA USA) according to the manual of manufacturer. Sequencing the insertion to confirm the integrity of CPP and β-catenin.

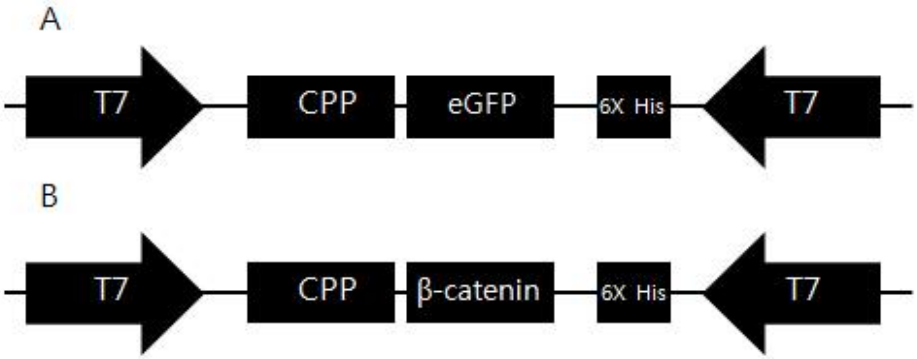


Figure 1. Diagram of the recombinant CPP protein expression vector.

Structure of recombinant (A) CPP-eGFP, (B) CPP-β-catenin protein expression vector.

Recombinant protein extraction and purification

CPP-β-catenin transformed cells were cultured and induced by 1 mM isopropyl-1-thio-β-galactopyranoside (IPTG, Sigma-Aldrich, MO, USA) for 8 hours at 37 ℃ in water shaker. Cells were lysed for 30 minutes on ice in a lysis buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea) and a lysozyme (QIAGEN, CA USA). Sonicate 30 sec and rest 30 sec on ice repeats 7 times. And the lysate was then separated into supernatant and pellets by centrifugation 30 min at 10,000g 4℃.

The soluble pellet was used to purify CPP-β-catenin-6His using Ni-NTA according to the manual of manufacturer. Briefly, add 5ml buffer B to pellet and incubate 40 min on RT. Centrifuge 30 min at 10,000g 4℃, get supernatant 4ml and add 1 ml Ni-NTA super flow® (QIAGEN, CA USA) slurry. Shaking for 60min at 4℃, centrifuge 30 min at 10,000g on cooling condition and discard supernatant. Add 1 ml NPI-20 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and gently mixed at least 10 times. Add 50 μl NPI-250 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) to elution and incubate 5 min on ice. Centrifuge 1 min at 12,000 rpm, get supernatant, add 200 μl NPI-250 and incubate overnight at 4℃.

Quantification of recombinant protein with Bradford method

The Bradford assay was followed by normalization based on the ratio of basic residues in each protein against BSA, the standard

protein. Firstly, a standard BSA assay curve was generated using various concentrations from 0 μg/mL to 2000 μg/mL. Used BSA's OD (Optical density) values were measure at 595 nm wave length. 20 μl of each recombinant proteins samples are reaction with 1 ml of Bradford reagent (Bio-Rad, CA, USA) for 5 min and measure OD values at 595 nm wave length 3 times. Each OD values are proportionally with standard BSA curve, define their concentration.

Recombinant CPP-β-catenin treatment

Collected 2-cell stage embryos were cultured for 72 hours and observed the development and analyzed a panel of genes. Recombinant CPP-β-catenin was treated in the concentration 20 ng/ml and 2 μg/ml CPP-β-catenin protein. To kept the concentration CPP-β-catenin protein was added into the media with 12 hr interval. Embryonic stages were evaluate every 24 hr.

Cleavage blocking

To evaluate whether CPP-eGFP and CPP-β-catenin translocated into the nucleus of blastomere, 20μM Aphidicolin (Sigma-Aldrich, MO, USA) was used for embryo's cleavage blocking (Samake and Smith, 1996). At late 2 cell, 4 cell, 8 cell stage embryos were collected and cultured with 20μM Aphidicolin during 24 hr. Confocal microscope was employed to analyze the nuclear translocation of CPP-eGFP.

Statistics

The t-test was used to evaluate the difference between each stages and experiment groups. Results were presented as mean ± SEM. A p-value less than 0.05 were considered to be significantly different.

Table 1. Sequences of primers

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Wnt3a	S	CTGCCACAAGAGCTTCCTGATT	208
	AS	TGGTGGGTGGATATAGCAGCAT	
Wnt7b	S	TGCACCCCCTTTTCAGTACCTT	212
	AS	ATCTGGAAGGGCCTGAGGAAAT	
Axin1	S	ACCAGGCACAGACAGAAATCCA	227
	AS	GCCATCATCCTCATCTGCATCT	
APC	S	AGCCTCTAAAAGCCCCAGTGAA	220
	AS	AATTGAGTTTCGCCCTGGAGAC	
Pou5f1	S	CGTGAAGTTGGAGAAGGTGGAA	236
	AS	GCCGCAGCTTACACATGTTCTT	
Sox2	S	CGAACTGGAGAAGGGGAGAGATTT	223
	AS	CCCCCAAAAAGAAGTCCCAAGA	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Klf4	S	ACCCAGTATACATTCCGCCACA	220
	AS	ACCGCCTCTTGCTTAATCTTGG	
Bmpr1b	S	GCTGGCACATTTCGTTTGGT	221
	AS	TTTTGACACAGCCCCTTTCC	
Smad2	S	TAGGTGGGGAAGTGTTTGCTGA	217
	AS	GTGCACATTCGGGTTAGCTGAT	
Acvr2b	S	CATGAACGACTTTGTGGCTGTG	224
	AS	ACGTGATGATGTTCCCCTTGAG	
Psen1	S	AAGAGTCGTATGGCGCTTGTTC	236
	AS	TGTTCTCCTCCAAGCTGCCTAA	
Hey1	S	GGAAAAGACGGAGAGGCATCAT	232
	AS	AACCCCAAACTCCGATAGTCCA	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Dtx2	S	CTCAGATTTGCCGGGTTCTTGT	210
	AS	TGGCCATCTTCTGCTTTCTCTG	
Celsr1	S	GCATCGTTAGTGGCAACCTGAA	222
	AS	GAAAGGGGCTGCTCACAAAGAT	
Cnih	S	CAATACCCTGAACCCTCTTGTCC	231
	AS	TTGCACCATCCTTCCTTCTGAC	
Cnih2	S	ACTTCAAGAACCCCATCGACCA	245
	AS	ATCATACATGACCTCAGAGCCATCC	
Dag1	S	TTGTCGGCACCTCCAGTTTATC	215
	AS	TCAGCTTGCCCTTCCTCTTCTT	
Pik3ca	S	CCACACTCGTCACCATCAAACA	213
	AS	TTTCTTCACGGTTGCCTACTGG	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Muc1	S	TTGTCTGTTGGGGTCTCCTTCTTC	227
	AS	AAAGTACCCTCCCGGAAAACCA	
Zmpste24	S	GCTGCCACATATCGCTTAGGAA	206
	AS	TGACTGGCCTGGAACTTGCTAT	
Degs1	S	GCTGGGCTAGGCATTTCATTGT	232
	AS	AAACTACCCATCACCAAGCCACAG	
Cln3	S	AGGACCCGGAAGTAAGAAGCAA	217
	AS	TCCTCCCTCTCAGAATCCTCAA	
Zeb1	S	CCTGCTGTCGTTCTTTGGATCA	213
	AS	CTCTTGCTCTGTGGGTTTTGGA	
Slbp	S	GAGAGGAAGTCATCATCGGGAAGT	226
	AS	ACCGCCGGCTGTACTTCTTAAA	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Tjp1	S	TTGCGACTAGCTGGTGGAAATG	227
	AS	ATGCGGCGATAAACGTCCTT	
Cnot4	S	CCCAGCCATCATCACAGGTATT	218
	AS	AGGGATTTGAAGGAGGTGGGTA	
β-actin	S	ACCAGGCACAGACAGAAATCCA	227
	AS	GCCATCATCCTCATCTGCATCT	
Ppia	S	AGCCTCTAAAAGCCCCAGTGAA	220
	AS	AATTGAGTTTCGCCCTGGAGAC	
H2afz	S	GCTGGCACATTTCGTTTGGT	221
	AS	TTTTGACACAGCCCCTTTCC	

Table 2. Termal cycler schedule

Step		Temperature (℃)	Time
Hold	Hold	94	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	5 min

RESULTS

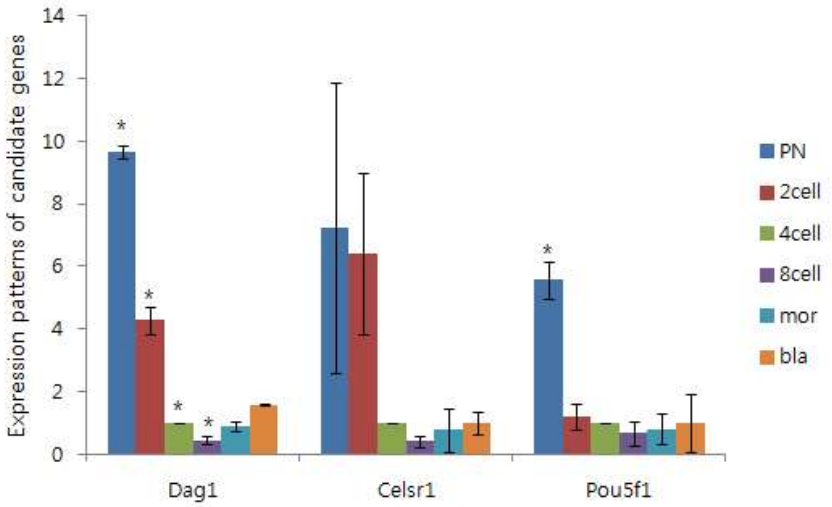
Expression profiles of candidate genes during early development

To evaluate the expression patterns of candidate genes during early stage embryos, the expression profiles were firstly analyzed using real-time PCR methodology. By the expression profiles the patterns could be divided into 8 clusters.

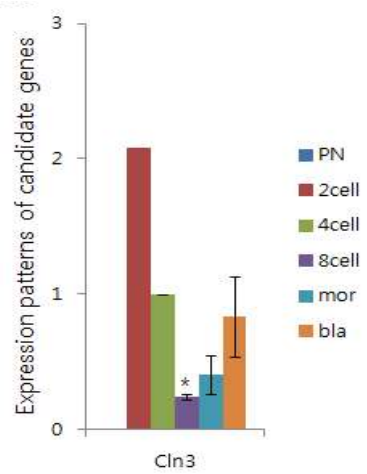
In cluster 1, mRNA were highly expressed at PN stage and then decreased but gradually increased at 8 cell stage (Fig. 2). *Dag 1*, *Celsr 1* and *Pou5f1* (also known as *Oct 4*) were involved in this cluster. In cluster 2, mRNA were not detected at PN stage and 2 cell stage, but gradually increased at 8 cell stage. *Wnt3a* and *Degs1* were involved in this cluster. In cluster 3, mRNA were not detected at PN stage, but gradually increased at 8 cell stage. *Cln 3* was involved in this cluster. In cluster 4, mRNA were peaked at PN stage and highly kept at 2 cell stage, but quickly decreased at 4 cell stage (Fig. 3). *Cnih*, *Pik3ca*, *Dtx 2* and *Axin* were involved in this pattern. In cluster 5, mRNA were peaked at PN stage and immediately decreased. After that almost not detected from 8cell stage (Fig. 3). *Slbp*, *Acvr2b*, *Hey 1*, *Bmpr1b*, *Smad 2*, *Tjp 1*, *Zeb 1*, *Gas 6* and *Sox 2* were involved in this pattern. In cluster 6, mRNA were high at PN stage and peaked at 2 cell stage. After that decreased at 4 cell stage (Fig. 4). *Muc 1*, *APC*, *Wnt7b* and Zmpste24 were involved in this pattern. In cluster 7, mRNA were low at PN stage and peaked at 2 cell stage. After that decreased at 4 cell stage (Fig. 4). *Cnih 2*, *Cnot 4*, *Psen 1* and *Klf 4*

were involved in this pattern. In cluster 8, mRNA were peaked at PN stage and after that fluctuated from 4 cell stage (Fig. 5).

A



B



C

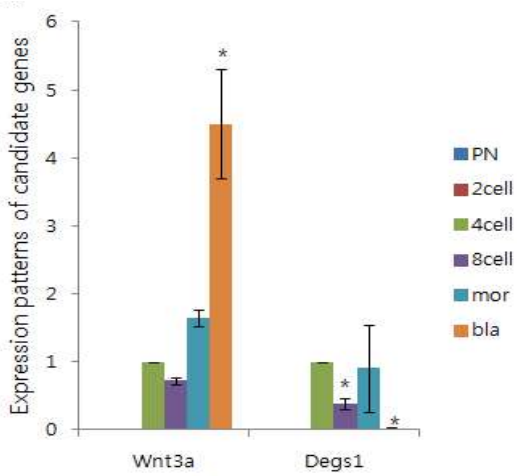
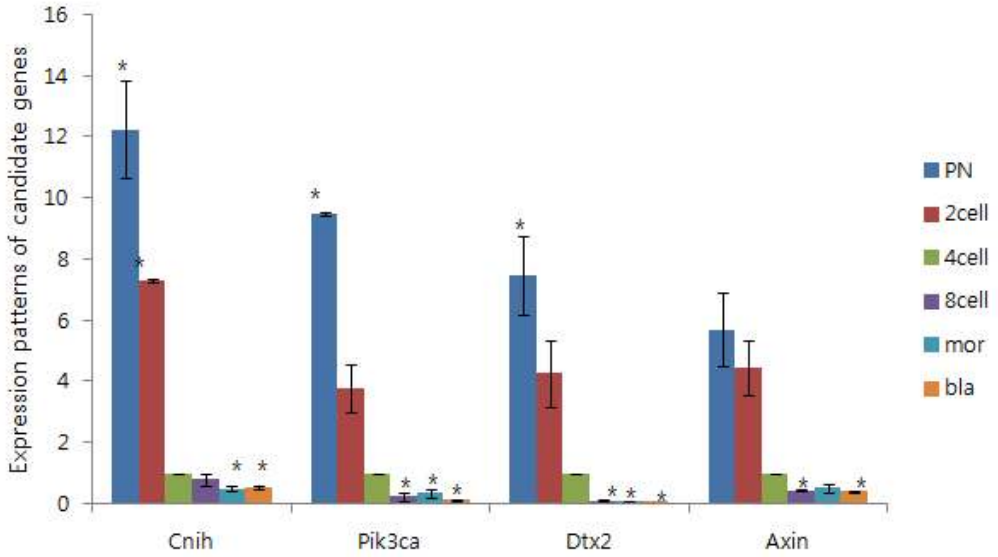


Figure 2. Expression pattern of cluster 1, 2 and 3.

(A) cluster 1, (B) cluster 2, and (C) cluster 3. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: P < 0.05 4-cell vs other stages.

A



B

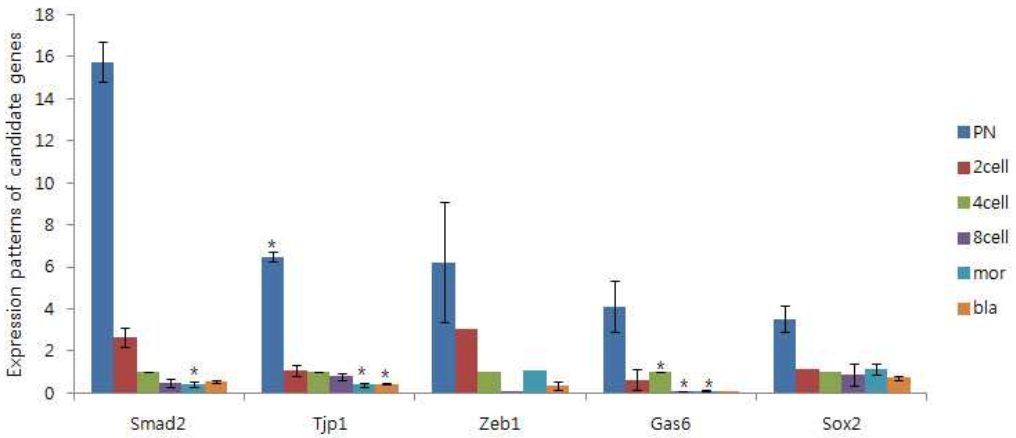
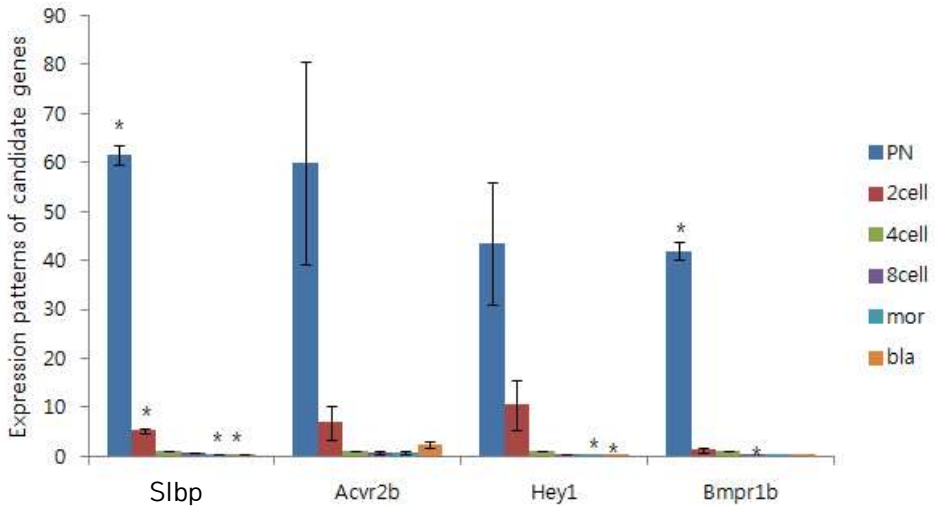


Figure 3. Expression pattern of cluster 4 and 5.

(A) cluster 4 and (B) cluster 5. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: $P < 0.05$ 4-cell vs other stages.

A



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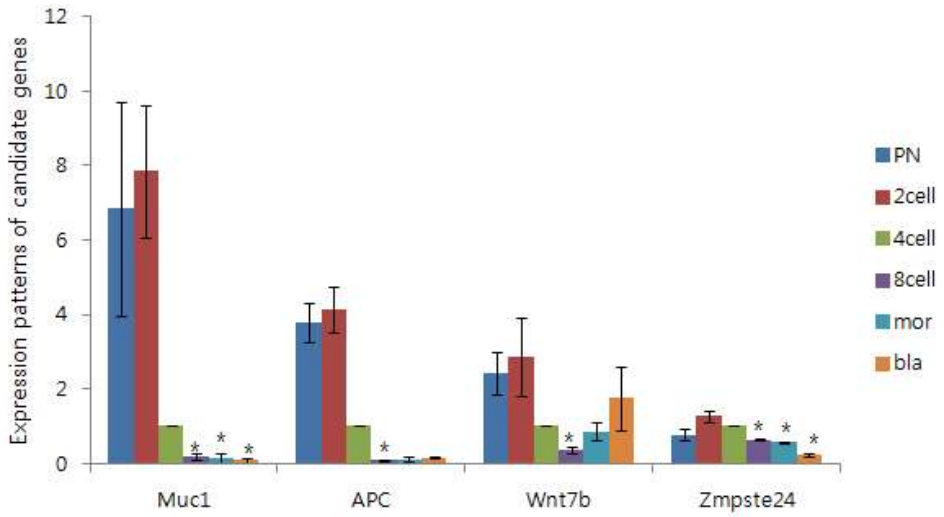


Figure 4. Expression pattern of cluster 6 and 7.

(A) cluster 6 and (B) cluster 7. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: $P < 0.05$ 4-cell vs other stages.

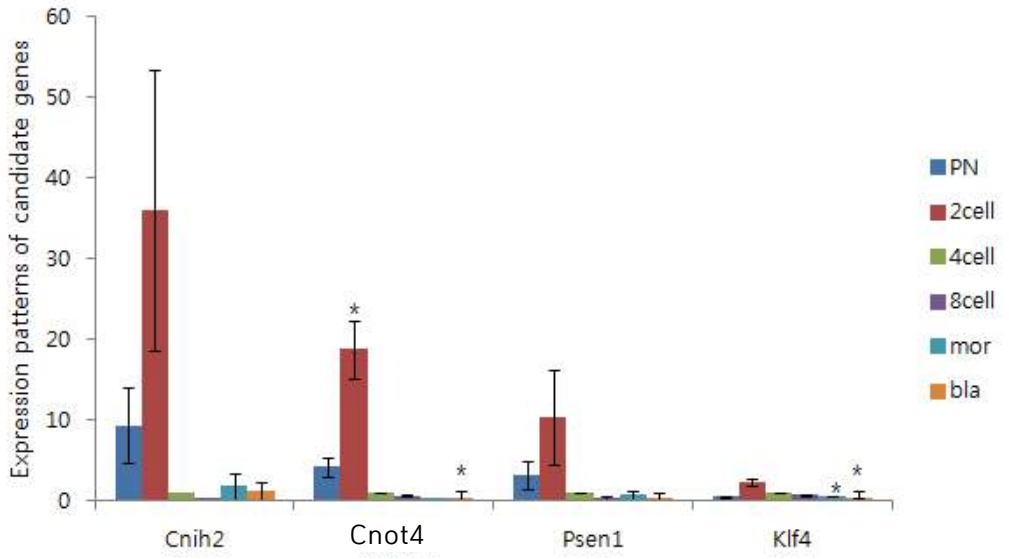


Figure 5. Expression pattern of cluster 8.

Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: P < 0.05 4-cell vs other stages.

CPP-eGFP trasnlocated into the early stage embryos

To confirm CPPs enters to embryos, confocal microscope was employed. After 6 hr of treatment, eGFP could be detected in the blastomere. yPFY-eGFP was detected in the all stage embryos after treatment (8 cell stage ~ blastocyst stage, Fig. 6).

2 cell stage embryos were flushed from oviduct and culture with or without purified recombinant yPFY-eGFP in BWW medium containing 0.4% BSA. The morphology of each time points was same between control and treated embryos (Fig. 7). The developmental rates to the blastocyst stage were not different between control and yPFY-eGFP groups (Table 3).

Developmental patterns after cell permeable peptide yPFY-β-catenin treatment

Most of the embryos cultured in the media containing recombinant yPFY-β-catenin developed to the blastocyst but the stage of blastocyst was lower than the control at the same time points. The developmental rates to blastocyst stage were decreased in the yPFY-β-catenin treated embryos. In 20 ng/ml treated group, the rates of blastocyst were significantly lower in both 20 ng/ml (lower 16.7% than the control) and 2 μg/ml (lower 18.7% than the control) groups. However, there was no difference between treated groups (Table 4). Until early 8-cell stage, developmental stage of embryos were same between control and treated groups but not sam after late 8-cell stage (Fig. 8).

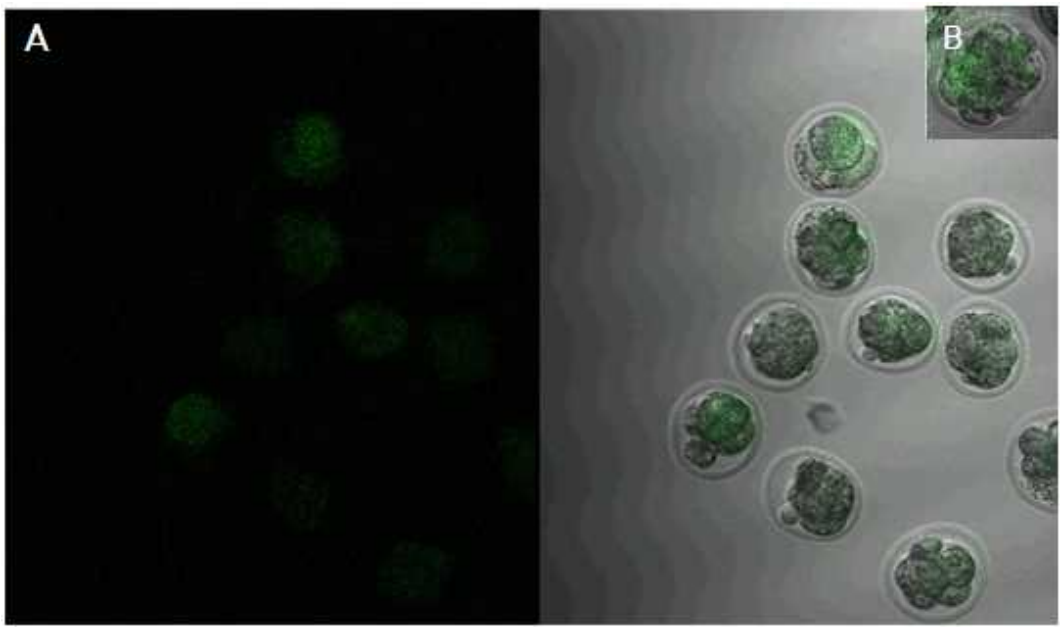


Figure 6. Confocal image of yPFY-eGFP treated early stage embryos.

2 μg/ml yPFY-eGFP was treated and analyzed the translocation with confocal microscope after 48 hours of culture. Box showd the 20 ng/ml yPFY-eGFP treated embryo. Magnification : X100.

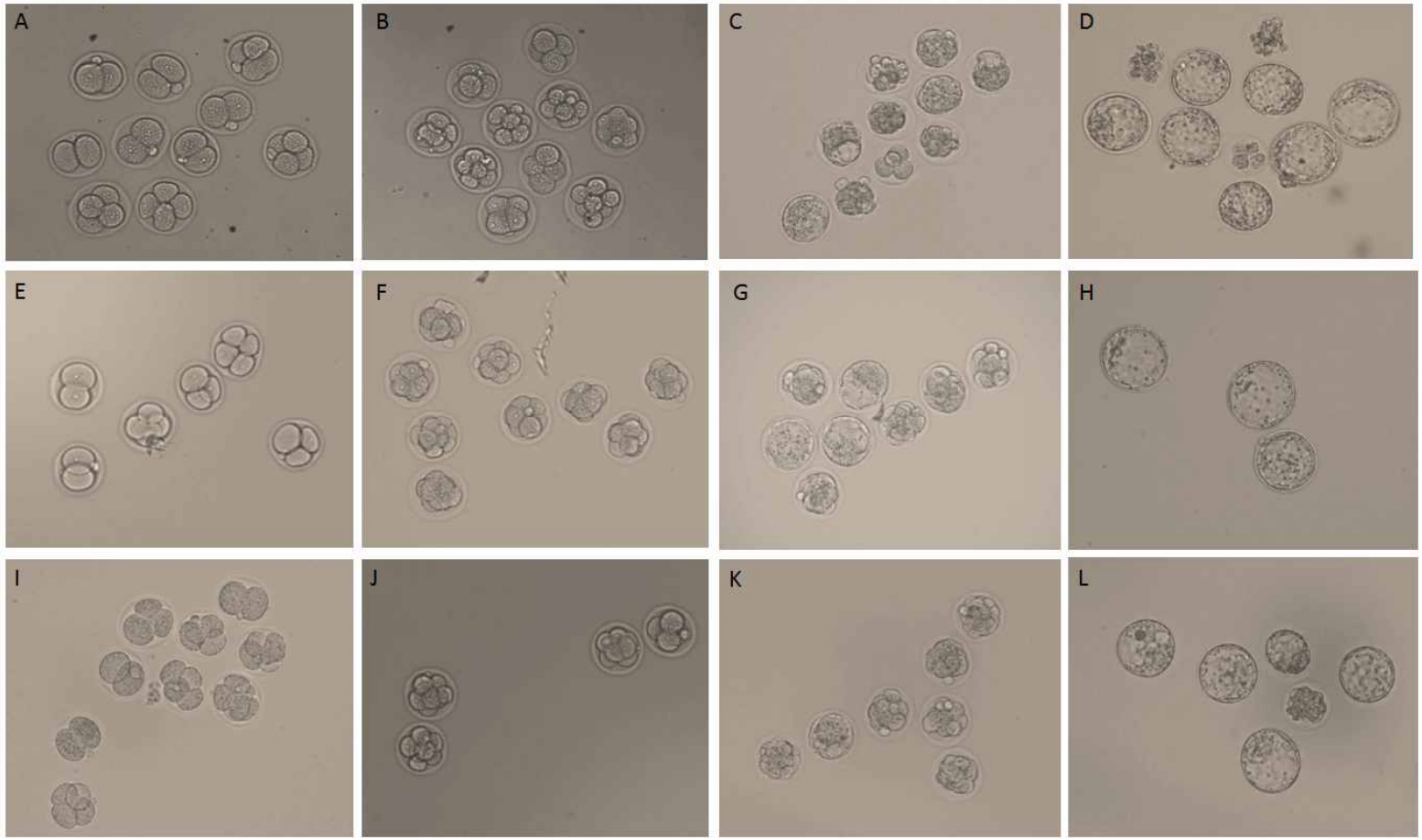


Figure 7. Photomicrograph of control and yPFY-eGFP treated early stage embryos.

A-D : control, E-H : yPFY-eGFP 20 ng/ml, I-L : yPFY-eGFP 2 μg/ml. hCG injection after A, E, I 54 hours; B, F, J 72 hours; C, G, K 96 hours; D, H, L 120 hours. Magnification : X40.

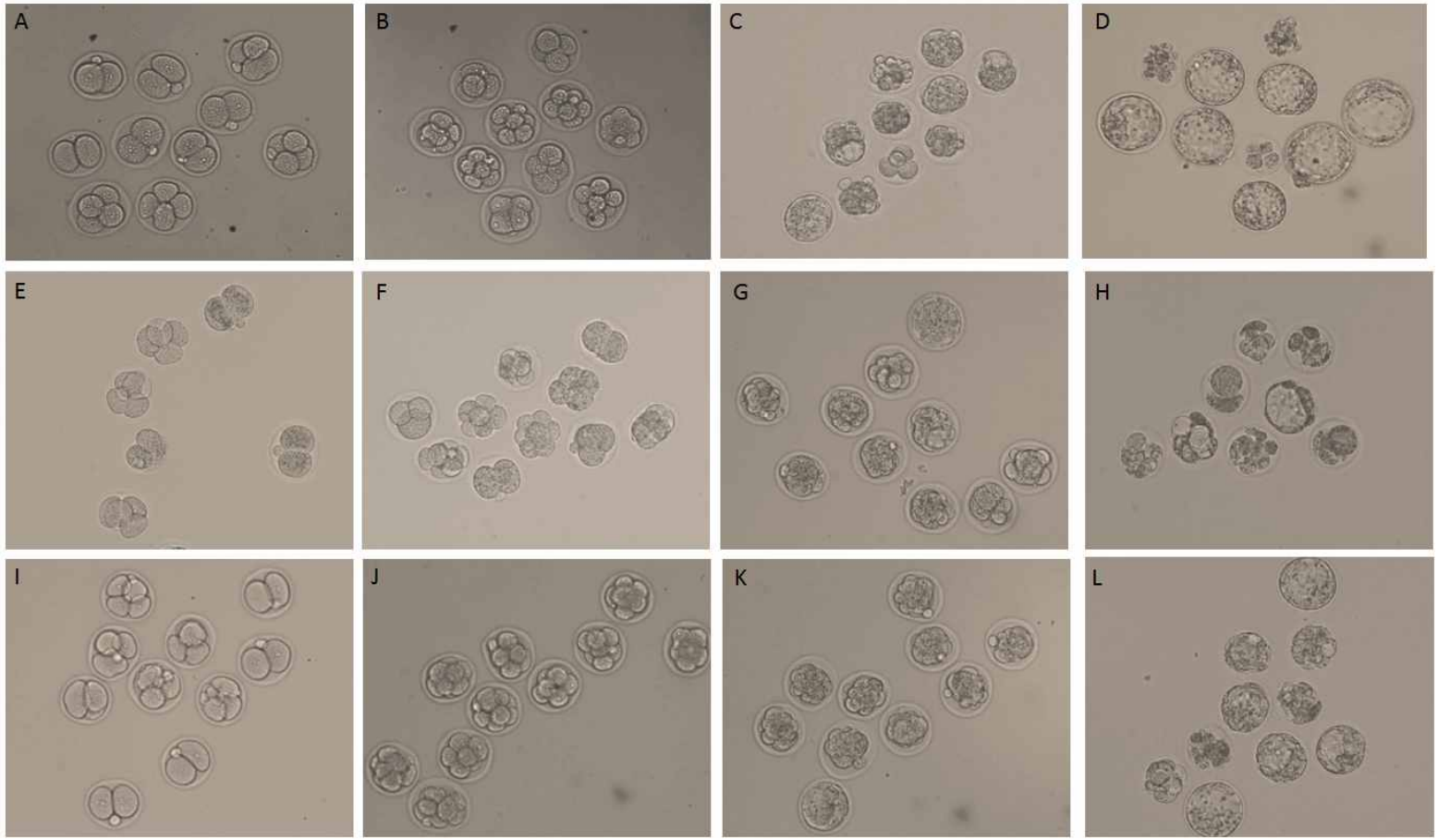


Figure 8. Photomicrograph of control and yPFY-β-catenin treated early stage embryos.

A-D : control, E-H : yPFY-β-catenin 20 ng/ml, I-L : yPFY-β-catenin 2μg/ml. hCG injection after A, E, I 54 hours; B, F, J 72 hours; C, G, K 96 hours; D, H, L 120 hours. Magnification : X40.

Table 3. Effects of yPFY-eGFP on embryo development

Treated protein	Concentration	48 hr after hCG (ea)	120 hr after hCG (ea)	Developmental rate (%)
Control		150	130	86.7
yPFY-eGFP	20 ng/ml	150	121	80.7
	2 μg/ml	150	118	78.7

Table 4. Development of 2 cell embryo treated with yPFY-β-catenin protein

Treated protein	Concentration	48 hr after hCG (ea)	120 hr after hCG (ea)	Developmental rate (%)
Control		150	130	86.7
yPFY-β-catenin	20 ng/ml	150	106	70.7
	2 μg/ml	150	103	68.7

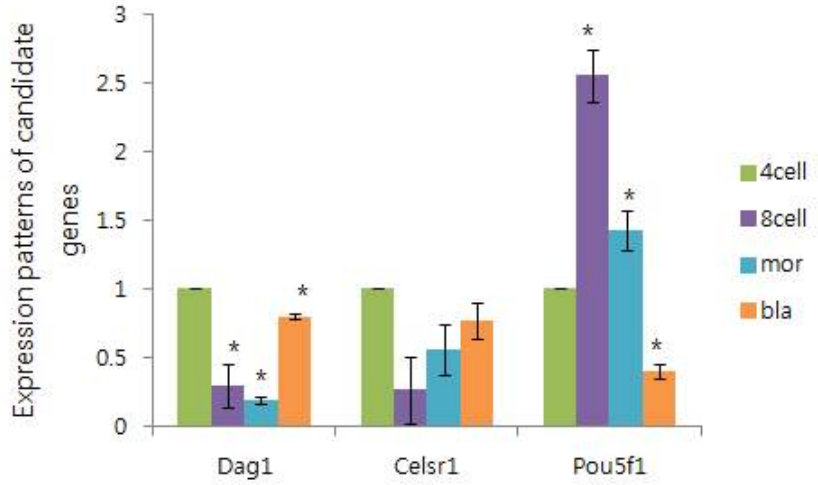
Expression profiles of candidate genes during early development after treatment CPP-β-catenin recombinant protein

To evaluate the expression pattern after treatment with yPFY-β-catenin during early stage embryos, the expression profiles of the candidate genes were analyzed using real-time PCR analysis.

In cluster 1, *Celsr1* and *Dag1* expression patterns were similarly with before treatment but *Pou5f1* expression patterns were changed (Fig. 9A). In cluster 2, *Cln 3* was not detected after treatment (Fig. 9B). In cluster 3, *Wnt3a* and *Degs1* expression patterns were changed (Fig. 10A). In cluster 4, *Pik3ca*, *Dtx 2* and *Axin* expression patterns were similarly with before treatment but *Cnih* expression patterns

were changed (Fig. 10B). In cluster 5, *Tjp 1* expression patterns was similarly with before treatment but *Smad 2* expression patterns was changed (Fig. 11A). And *Zeb 1*, *Gas 6* and *Sox 2* were not detected after treatment. In cluster 6, *Acvr2b* and *Hey1* expression patterns were similarly with before treatment but *Slbp* expression patterns was changed (Fig. 11B). And *Bmpr1b* was not detected after treatment. In cluster 7, *Muc1, APC, Wnt7b* and *Zmpste24* expression patterns were similarly with before treatment (Fig. 12A). In cluster 8, *Cnih2, CNOT4, Psen1* and *Klf4* expression patterns were similarly with before treatment (Fig. 12B).

A

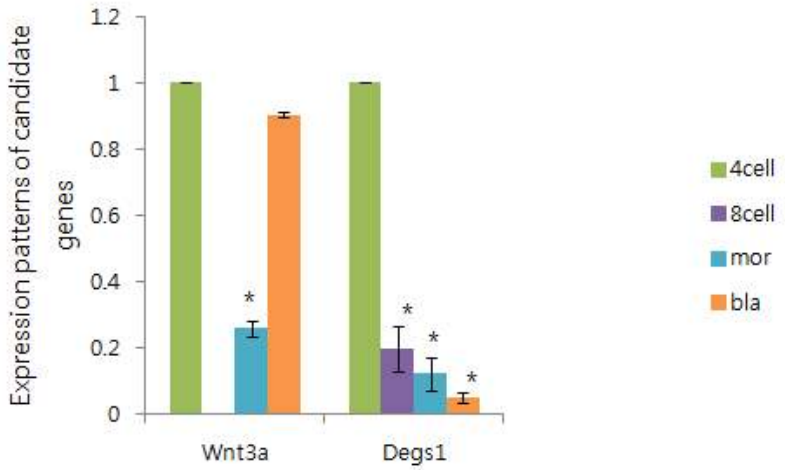


B

Figure 9. Expression pattern of cluster 1 and 2 after treatment β-catenin.

Expression of cluster 1 (A) and cluster 2 (B) genes was modified by yPFY-β-catenin. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM.*: P < 0.05 4-cell vs other stages.

A



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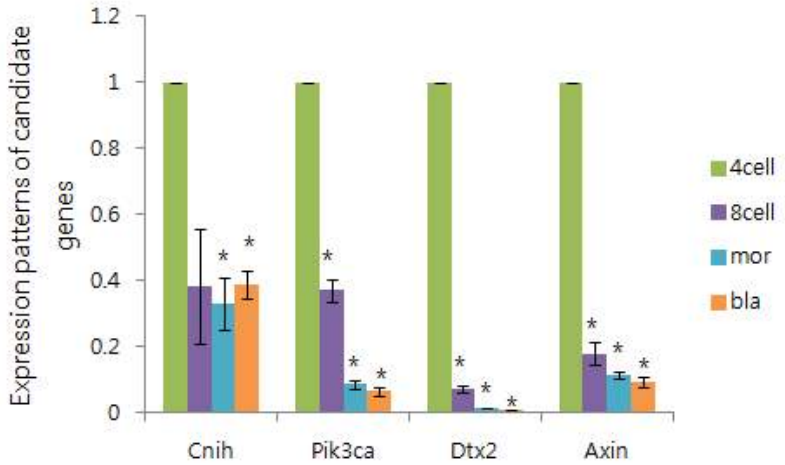
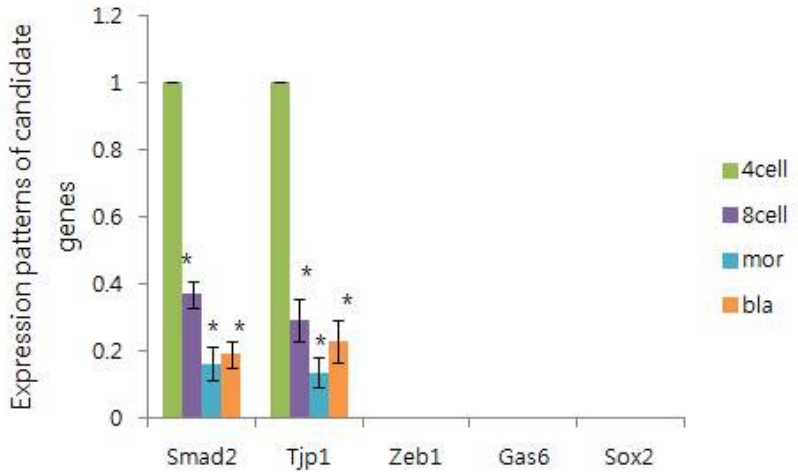


Figure 10. Expression pattern of cluster 3 and 4 after treatment β-catenin.

Expression of cluster 3 (A) and cluster 4 (B) genes was modified by yPFY-β-catenin. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: $P < 0.05$ 4-cell vs other stages.

A



B

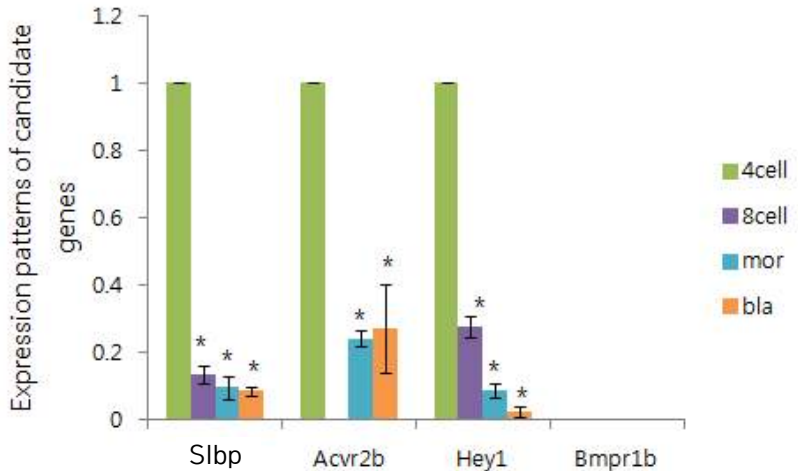
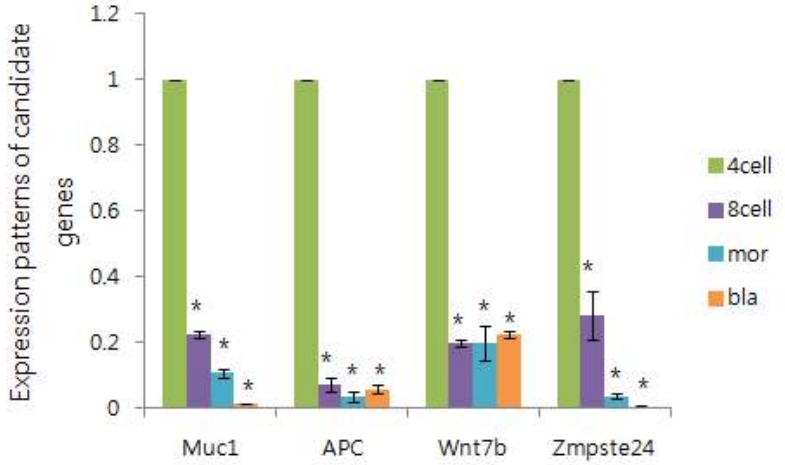


Figure 11. Expression pattern of cluster 5 and 6 after treatment β-catenin.

Expression of cluster 5 (A) and cluster 6 (B) genes was modified by yPFY-β-catenin. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: P < 0.05 4-cell vs other stages.

A



B

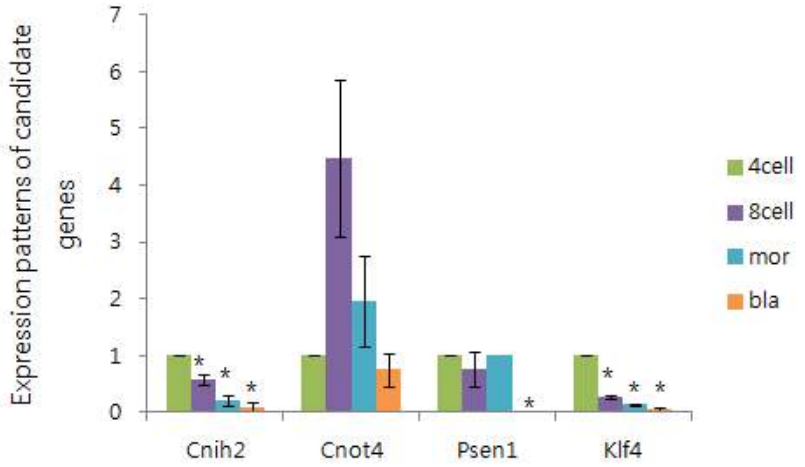


Figure 12. Expression pattern of cluster 7 and 8 after treatment β-catenin.

Expression of cluster 7 (A) and cluster 8 (B) genes was modified by yPFY-β-catenin. Total RNA was extracted from embryos of various stages. Real-time PCR was performed and repeated three times. Values represent the mean ± SEM. *: P < 0.05 4-cell vs other stages.

Discussion

Roles of β-catenin in cells are very variety; stemness maintaining, body axis formation, cell adhesion, and gastrulation (Anton et al., 2007; De Varies et al., 2004; Haegel H et al., 1995). β-catenin is a key factor which involved in Wnt / β-catenin pathway and in the presence of Wnt signal leading to break down the complex and free β-catenin accumulation in cytoplasm (Hitoshi, 2011). And accumulated free β-catenin translocate into the nucleus (Xie et al., 2008). In nucleus, β-catenin activates its target genes by binding to sequence - specific transcription factors of the TCF / LEF family and controls target gene's transcription level (Watanabe and Dai, 2011). Until now, known β-catenin target genes in mouse embryo are *Cdx1* and *Brakyury*, but there roles are not cleared (Sebastian J et al., 2000).

Wnt3a have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis. And *Wnt7b* play important roles in the development (Hac et al., 2008). *Wnt3a* and *Wnt7b* expression level at morula and blastocyst stage are decreased after treatment β-catenin. Similarly, *Smad2*, a member of the TGF - β family and regulates cell proliferation, apoptosis, and differentiation and *Acvr2b*, a regulator of BMP signaling which involved in growth, cell differentiation, and homeostasis (Galvin et al., 2010) expression level at morula and blastocyst stage are decreased after treatment β-catenin. It is suggested that Wnt pathway and BMP pathway are

related with β-catenin and affect to development process after morula stage.

Muc1, which acts as an adhesion protein, *Cnih*, which involved in the selective transport and *Degs1*, responsible for inserting double bonds into specific positions in fatty acids composing cell membrane expression level at morula and blastocyst stage are decreased after treatment β-catenin. It is suggested that β-catenin can affect to cell adhesions and cell membrane after morula stage. As show as Fig 8, β-catenin treated embryo development process are suppressed. After compaction, embryos have problem with formation normally morula and blastocyst. By the contrast, *Cln3*, involved in lysosome function was not detected after treatment β-catenin. By these result, β-catenin affect to cell adhesion and it caused to problem with formation normally blastomere.

On the contrary, *Pou5f1* also known as *Oct4*, a transcription factor and *Cnot4*, a subunit of the a global transcriptional regulator expression level at 8 cell stage are increased after treatment β -catenin. By the contrast, *Zeb1,* which plays a role in transcriptional repression was not detected after treatment β-catenin. It is suggested that β-catenin affect to transcription level of embryo, maintain stemness and it made embryo development process suppression.

After treatment β-catenin, *Bmpr1b,* a receptor of BMP signaling, *Gas6,* involved in the stimulation of cell proliferation and *Sox2,* a transcription factor that is essential for maintaining pluripotency were not detected in early stage embryos. It's mechanism should be

studied, too.

β-catenin treated embryos were developed to the blastocyst stage but the level of developmental stage was lower than the control. Morphology of each stages was same with control until 8-cell stage but it was little different between control and treated groups after late 8-cell stage. It is suggested that β-catenin can affect to the embryo development after late 8-cell stage.

On the other, the developmental rates to the blastocyst stage were significantly decreased by the treatment of yPFY-β-catenin. It means that increased free β-catenin was the reason of decreased the developmental rate of 2-cell embryo to blastocyst.

In summary, by the treatment recombinant cell permeable peptide yPFY-β-catenin the developmental rates of 2-cell embryo to blastocyst were decreased dramatically by the increase of β-catenin in the blastomere. And the expression profiles of each cluster were changed. It was included the genes which involved in Wnt signaling, BMP signaling, volume control factors and some of the transcription factors. Based on these results, it is suggested that β-catenin may be one of the potency regulating factors in early stage embryos.

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Abstract

Change the potency of early stage embryo : possible role of β-catenin

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After fertilization by the cleavage, embryos develop to 2 cell, 4 cell, 8 cell, morula and blastocyst. In mouse early stag embryos have totipotency until 8 cell stage, which is the ability to divide and produce all the differentiated cells in an organism, including extra embryonic tissues. But the blastomere of ICM have pluripotency, which is the potential to differentiate into any type of the three germ layers. These change of developmental potency is critical in the development of organisms, but time points and losing mechanisms are not examined. Also, molecular markers of decide the potency are not clarified. In this study, the role of β-catenin, which localized in early stage embryo nucleus, and related in maintain stemness and body axis formation, in developmental potency change. And analyzed

of early stage embryo related genes expression pattern. Real-time RT PCR technology, recombinant CPP-β-catenin protein method, purification and quantification of recombinant protein method and treatment recombinant protein to embryos were employed to explore the roles of β-catenin. The embryos group of treated β-catenin are suppressed development after compaction but their morphology are normal. And the several kinds of gene expression patterns are changed in culture with β-catenin embryos. There are more researches are needed, based on this study it is suggested that β-catenin affect to change of developmental potency.