이학석사 학위논문

The Role of AKAP6 in mouse ESCs

- Knock-down of AKAP6 promotes mESCs apoptosis, not differentiation -

마우스 배아줄기세포에서 AKAP6의 영향

- AKAP6 발현 억제로 인한 마우스 배아줄기세포의 세포 사멸 유발 -

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서울대학교 대학원

분자의학 및 바이오제약전공

김수연

Abstract

The Role of AKAP6 in mouse ESCs

- Knock-down of AKAP6 promotes mESCs apoptosis, not differentiation -

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Background

Researches focusing on Embryonic stem cells have the infinite possibility of cell based therapy for cure disease. Thus, it is important to know the regulating mechanism of cell signaling molecules in stem cells. Herein, we report the novel aspects of apoptosis in mouse embryonic stem cells, which was regulated by AKAP protein expression.

Methods and results

mouse Embryonic Stem Cells (mESCs) were differentiated into Embryoid bodies by hanging drop method and their RNA harvested at 1, 4, 7 day. EB represented more higher AKAP6 expression than undifferentiated ESCs. Using shAKAP6 plasmids, transient transfection experiments and making of stable knock-down cell lines were performed. To elucidate AKAP6 effect on mESC differentiation, stable AKAP6 knock-down cell lines were formed and then, knock-down cells were differentiated into EBs. Expression level of three germ lineage markers was checked by real-time PCR. However, stemness markers and three germ layer markers didn't show any reasonable changes, although AKAP6 expression was resonably decreased in shAKAP6 cells. Form these results, we concluded that AKAP6 didn't affect mESC differentiation. But, When culturing of stable knock-down cell lines, we observed morphological differences between control and shAKAP6 cells. Staining actin filaments for clarifying cell structural differences showed disrupted actin arrangement in AKAP6 knock-down cells. After, we performed transient knock-down of AKAP6 and observed that frequent membrane ruffling occurred in AKAP6 knock-down mESCs. Membrane ruffling is widely known as migration indicator and/or apoptosis indicator. Migration signaling molecules were detected. When AKAP6 was suppressed, migratory proteins were

decreased. Specially, FAK, which is generally known as anti-apoptotic factor, was decreased in AKAP6 knock-down mESCs. With these reasons, we assumed that AKAP6 knock-down induces apoptosis in mESCs. To confirm apoptotic characters, we performed Annexin V/PI FACS analysis and we detected cleaved caspase 3 expression. In AKAP6 knock-down mESCs, Annexin V/PI double positive populations were higher than control cells and also, increased cleaved caspase 3 expression was shown by immunofluorescence and westernblot analysis.

Conclusion

We demonstrated the effect of AKAP6 in mouse Embryonic Stem Cells. Particularly, we explained that knock-down of AKAP6 was not a differentiation factor. Our findings proposed that knock-down of AKAP6 was close to a potential apoptotic factor. These results suggest a novel therapeutic effects of stem cells in apoptosis related disease.

Keywords: mouse Embryonic Stem Cells, A-kinase Anchoring Protein 6, Apoptosis.

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Introduction

Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst, are pluripotent and capable of self-renewal. They can be expanded indefinitely and have the ability to differentiate into all adult cell lineages [1]. Mouse embryonic stem cells (mESCs) have been used to study the complexities of stem cell biology at the molecular level. Because of their unique properties, ESCs are considered a potential cell-based treatment for disease. There are several methods to induce mESC differentiation. Two common methods are 1) to plate mESCs as a monolayer onto specific matrix components, (called attached ESCs), or 2) to aggregated mESCs into Embryoid bodies (EBs). EBs have the capacity to spontaneously differentiate into all three germ layers [2]. Moreover, mESCs can be further differentiated by generating attached EBs, where aggregated EBs were attache to extra cellular matrix components. Although stem cell research has advanced in recent years, many questions about the molecular mechanisms driving stem cell differentiation and/or death remain. Recent studies suggested that subcellular signal transduction is mediated through scaffolding proteins such as A-kinase anchoring proteins (AKAPs) [3]. AKAPs are signaling modulators that are distributed in multiple cellular compartments.

They function to spatially and temporally organize the localization of signaling molecules [4]. Most AKAPs were named according to their molecular weight. However, an exception to this rule is AKAP6, widely known as muscle-AKAP (mAKAP). AKAP6 is a ~250kDa scaffolding protein that is localized to the nuclear envelope and the sarcoplasmic reticulum [5]. In a previous report, AKAP6 showed tissue-specific expression in differentiated cardiomyocytes and the skeletal muscles [6-7]. In this study, we report that AKAP6 is also expressed in differentiated mouse ESCs. We found that depletion of AKAP6 does not affect mESCs differentiation, however, our results suggest that it is involved in ESC apoptosis.

Materials and methods

Maintaining Mouse Embryonic Stem Cells

The cells were cultured as previously described [2]. C57BL/6-background mouse ESCs (accession no. SCRC-1002; ATCC) and E14 mouse ESCs were maintained on MEFs (Mouse Embryonic Fibroblasts, CEFOBIO #CB-CF1-002) feeder layer. MEFs were cultured in DMEM (Dulbecco's modified Eagle's medium; GIBCO) high glucose supplemented with 10% FBS (Fetal Bovine Serum; GIBCO), 1% antibiotic antimycotic (GIBCO). One day before subculturing mESCs, MEFs were treated with Mitomycin C (10ug/ml medium, Sigma-Aldrich). mESCs were cultured in DMEM with 10% FBS (Hyclone), 1% penicillin/streptomycin (GIBCO), 0.1mM β-mercaptoethanol (Sigma), 1% non-essential amino acids (GIBCO), 2mM L-glutamine (GIBCO) (ES media). In ES media, 1000 U/ml of ESGRO® LIF (leukemia inhibitory factor, Millipore) was added to maintain mESCs pluripotency. mESCs were dissociated with 0.05% trypsin (GIBCO) and subcultured on MEFs every 2-3 days.

In vitro Differentiation of mESCs

To induce differentiation, feeder elimination was needed. Feeder depletion was achieved by plating trypsinized mESCs on 100mm culture dish (Nunc) with DMEM / 10% FBS media in the absence of LIF for 30-60min at humidified 37℃, 5% CO_2 incubator. After 30min, mESCs without feeder (isolated mESCs) could be gotten by collecting only suspended cell. Embryoid body (EB) formation step was performed by hanging drop method [2-4]. The rounded droplet (350 cells per 20ul) on petri dish was maintained in humidified 37℃, 5% CO_2 incubator for 1 to 7days. On the specific day, EBs collected for total RNA, Protein isolation. Another step for differentiation was performed by attaching EBs onto 1.5% gelatin-coated 6well plate in DMEM / 10% FBS.

Transient Transfection of shRNA in mESCs

shRNA (MISSION® shRNA, SIGMA-Aldrich) was incubated with Metafectene® pro. (Biontex, Planegg, Germany) reagent in PBS for 20min at room temperature. After incubation time, complexes were dropwised to the cells. For knock-down of AKAP6, cells were prepared one day before transfection as monolayer mESCs without feeder cells. Non-targeting shRNA (pLKO) was used as control. The knock-down effect of shAKAP6 maintained approximately for 4days.

Formation of Stable Knock-down Cell Lines

For formation of stable AKAP6 knock-down cell lines, mESCs maintained on puromycin-resistant feeders (puro-MEFs, StemCell technologies) were transfected with AKAP6 shRNA or Non-targeting shRNA control (MISSION® shRNA) using Metafectene pro. The following day, cell medium was changed with 10ug/ml puromycin containing ES media, and daily replaced. Transfected mESCs were grown for 5-7days to stably generate AKAP6 shRNA or control shRNA. Multiple clones were selected and picked into newly prepared puro-MEF feeders. Clones were maintained with ES medium containing 1ug/ml puromycin and identified by Quantitative RT-PCR (qRT-PCR) and western Blotting. Puromycin was purchased from SIGMA-Aldrich.

Antibodies and Reagent

polyclonal muscle-AKAP (Covance); mouse monoclonal Anti-α-tubulin, Phalloidin-TRITC, 4′,6′-Diamidino-2-phenylindole dihydrochloride (DAPI) (SIGMA-Aldrich); Mouse polyclonal anti-FAK (BD); Rabbit polyclonal anti-phospho-FAK(Y397) antibody, Antibody diluent solution, Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 488 donkey anti-rabbit (Invitrogen); Rabbit polyclonal anti-Arp3, Rabbit polyclonal anti-cleaved caspase3, Rabbit monoclonal anti-ROCK1, Rabbit polyclonal

anti-Rac1/Cdc42, Rabbit polyclonal anti-Phospho-Rac1/cdc42 (Ser71), Rabbit monoclonal anti-WAVE-2 (Cell Signaling); Mouse monoclonal anti-RhoA (Santacruz); HRP-conjugated donkey anti-mouse and anti-rabbit immunoglobulins (Jackson Labs); HRP-conjugated goat anti-rabbit immunoglobulins (Santacruz).

RNA Preparation, Quantitative RT-PCR and Real-Time PCR Analysis

Total RNA was purified using RNeasy mini kit and QIAshredder (Qiagen, Inc.). 1ug of RNA was converted into cDNA by using High capacity RNA to cDNA kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was performed using TaKaRa Ex Taq (TaKaRa) with specific primers (Table. 1) and conducted in a Gene pro Thermal cycler, BIOER. Quantitative real-time RT-PCR run with FS Universal SYBR Green Master (Roche) and conducted in 7500 Fast Real-Time PCR system (ABI).

Western Blot Analysis

Cells were washed with cold PBS. After, cells were harvested and lysed with RIPA buffer (50mM Trish (pH8.0), 150mM NaCl, 1mM orthovanadate, 1% Triton X-100, 0.1% SDS, 0.1M NaF, 0.5% deoxycholic acid and protease inhibitor cocktail (GenDEPOT). Total proteins (20ug) were seperated by 6-15%

SDS-PAGE, transferred PVDF membranes, and immunoblotted with primary antibodies at 4℃. Blots were washed twice with 1x TBS/ 0.01% Tween20 (TBS-T) for 5min and incubated with HRP-conjugated secondary antibodies for 1hr. After, blots were washed for more than 1hr at room temperature. Chemiluminescence detection was performed using Novex® ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Science).

Immunofluorescence Staining

mESCs transfected with shAKAP6 were differentiated into EBs. After 1day, EBs were attached on 1.5% gelatin coated 35mm μ-Dish (ibidi, Germany). On the 3rd days, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washing with 1x Tris-buffered saline and permeabilizing with 0.05% Triton X-100 /PBS, blocking step was preceded with PBS containing 1% BSA. Cells were incubated with primary antibodies at 4℃ for overnight and then, cells were followed by fluorescent dye conjugated secondary antibodies or Phalloidin-TRITC. Nuclei was counterstained with DAPI. To obtain fluorescent confocal images, Dishes were placed on LSM 710 fluorescence microscope (Zeiss).

Annexin V/PI FACS Analysis for Apoptosis Detection

Attached EBs were trypsinized and washed with DPBS. Cells were stained with Annexin V FITC and Propidium Iodide according to the instructions. The fluorescence was detected by Flow cytometry using BD FACSCalibur. Annexin V FITC Apoptosis detection kit was purchased from BD.

Results

AKAP6 is expressed in differentiated mosue ESCs.

AKAP6 is known to be expressed in brain, cardiomyocytes and skeletal muscles [5]. Therefore, we first checked whether AKAP6 is also expressed in mouse ESCs. Two types of ESCs, C57/BL6-background mESCs (abbreviation C57) and E14, were used to test expression levels (Figure 1A). Undifferentiated mESCs were aggregated into EBs, a well-known method for inducing spontaneous differentiation in ESCs [2,11]. C57 and E14 EBs were harvested at day 1, 4 and 7 (Figure 1B). We found that AKAP6 mRNA was more abundant in day 4 and 7 EBs than in undifferentiated mESCs. This indicated that AKAP6 is expressed in differentiated mESCs (Figure 1C). We also confirmed AKAP6 protein expression by western blotting (Figure 1D), and we found that this signal anchoring molecule is indeed expressed in differentiated mESCs.

Depletion of AKAP6 by shRNA does not affect mouse ESCs differentiation.

AKAP6 was expressed in differentiated mouse ESCs. Therefore, we hypothesized that AKAP6 expression may affect mESC differentiation. To explore this possibility, we induced AKAP6

loss-of-function using shRNA. Two kinds of shAKAP6 plasmids were chosen by binding sites of AKAP6 DNA coding sequences (Figure 2A). To determine knock-down efficiency of AKAP6 mRNA by the two shRNA plasmids, mESCs were transfected with shAKAP6 plasmid candidates and then differentiated into EBs. EBs transfected with shAKAP6 plasmid (#1) showed reduced AKAP6 mRNA levels. (Figure 2B). shAKAP6 plasmid (#1) was also able to decrease AKAP6 protein expression (Figure 2B). Using shAKAP6 plasmid (#1), we established stable AKAP6 knock-down cell lines as described in the materials and methods. To briefly explain this method, mouse ESCs cultured on puromycin-resistant MEFs were transfected with shAKAP6. Next, the cells were treated with puromycin for colony selection. As AKAP6 was expressed in differentiated mESCs, examination of AKAP6 knock-down involved assessing the extent of EB formation (Figure 2C-E). Numerous cell colonies were differentiated into EBs. Through analysis of AKAP6 mRNA and protein expression levels, we selected stable AKAP6 knock-down cell lines (Figure 2F). We hypothesized that depletion of AKAP6 affected differentiation of mESCs. Therefore we generated EBs using the stable shAKAP6 cell line (Figure 2G). We assessed their level of “stemness” by checking expression levels of germ-layer lineage markers by real-time PCR (Figure 2H). Stemness was determined by oct4 and nanog expression, the Endodermal markers Troma-1 and Sox17 [13-20], the Ectodermal

markers Nestin and NCAM [21-29], and the Mesodermal markers Desmin, SMA, and VE-cadherin [30-42]. However, although AKAP6 was significantly decreased in shAKAP6 EBs, their stemness and germ layer lineage markers did not significantly change. Based on this data, we concluded that knock-down of AKAP6 does not affect mESCs differentiation or lineage commitment.

Disrupted actin arrangement in AKAP6 knock-down cells.

Upon culturing stable AKAP6 knock-down cell lines, we observed morphological differences between control and shAKAP6 cells. Unlike control mESCs, undifferentiated shAKAP6 cells could not be maintained, and they eventually detached from feeder layer. shAKAP6 EBs also formed broken aggregates (Figure 3A). To clarify structural differences in shAKAP6 cells, we examined F-actin organization by phalloidin staining. Control cells maintained structural integrity and actin rigidity within plated EBs (Figure 3B). However, actin distribution was disrupted and actin rigidity was reduced in shAKAP6 cells (Figure 3C). AKAP6 knock-down cells could not be maintained indefinitely. Therefore, we transiently knocked-down AKAP6 in mouse ESCs (Figure 3D). Because transient transfection is used to accomplish short-term expression, we harvested transiently transfected mESCs at an earlier time point than the stable knock-down cells. We found that actin arrangement in transiently transfected cells

was similar to that of the stable knock-down cells (Figure 3E). Our results strongly suggest that AKAP6 knock-down in mESCs results in defective actin organization.

Down regulation of actin-related proteins and apoptotic induction in AKAP6 knock-down mouse ESCs.

Continuing experimental investigation of actin distribution, we observed that membrane ruffling frequently occurred in AKAP6 knock-down mESCs (Figure 4A). Membrane ruffling is generally considered an indicator of migration [43] or apoptosis [44,45]. Therefore, we wondered whether this ruffling event was a migratory phenomenon or if it was related to apoptosis. In control cells, migratory signaling molecules were detected at the protein level. However, when AKAP6 was suppressed, the level of actin organizing proteins such as FAK, Rac1/Cdc42, Arp3, and WAVE2 [43,46,47] were decreased (Figure 4B). Of these migration molecules, FAK is generally known as a cell survival and anti-apoptotic factor [48]. Because the levels of FAK decreased, we reasoned that AKAP6 knock-down may induce apoptosis in mESCs. To confirm apoptotic characters, we first performed an Annexin V/PI FACS analysis. AKAP6 knock-down cells showed higher Annexin V/PI double positive populations than control cells (Figure 4C). We also analyzed the expression of cleaved caspase 3, a commonly used marker for apoptosis, at the protein level (Figure 4D). In AKAP6 knock-down cells, we

observed that cleaved caspase 3 expression was increased. To ensure the evidence of apoptosis, we also performed immunostaining. As shown in Figure 4D, in AKAP6 knock-down cells, cleaved caspase 3 was stained increasingly, also Using a DAPI counterstain, we found evidence of DNA fragmentation in cleaved caspase 3 expressing cells (arrow head). Therefore, it is likely that membrane ruffling events were not a migratory phenomenon, but an apoptotic phenomenon caused by defective AKAP6 in mouse ESCs.

Conclusion

AKAP6 is a scaffolding protein, but its role in ESCs is still unclear. In this report, we first assessed if AKAP6 was expressed in mESCs, and we found that AKAP6 was expressed in differentiated mESCs. To further explore the role of AKAP6 during ESC differentiation, we used shRNA to induce an AKAP6 loss-of-function phenotype. We expected that decreased expression of AKAP6 would have an effect on mESC differentiation. However, contrary to our expectations, AKAP6 did not affect the differentiation of mESCs. We found that actin arrangement was perturbed in AKAP6 knock-down mESCs when compared to controls. We also observed frequent membrane ruffling events in AKAP6 knock-down cells. Therefore, we hypothesized that membrane ruffling was related to AKAP6 knock-down in mESCs. We found that the ruffling was a sign of apoptosis and that defective AKAP6 expression induced apoptosis in mESCs. Our novel results strongly suggest that AKAP6 is not a differentiation factor, but rather it is likely to act as an apoptotic factor in mESCs. This suggests that AKAP6 may be a promising protein to research in ESCs.

Discussion

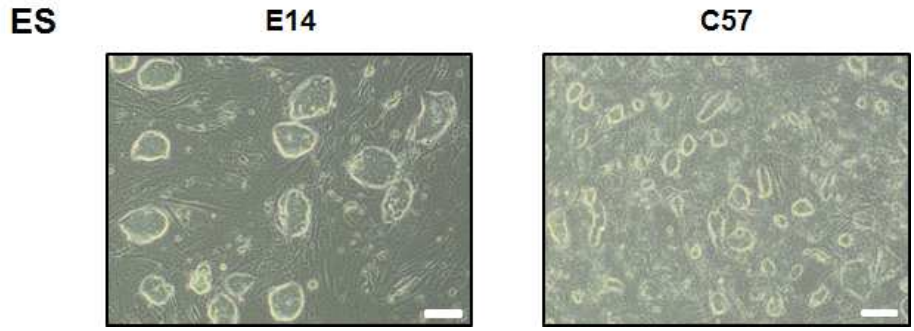
Intracellular and intercellular communication are sophisticated mechanisms regulated by many signaling process. During signal transduction, various signaling proteins have major roles in cellular differentiation, proliferation, and cell death. Anchoring proteins are known to compartmentalize signaling molecules. The typical anchoring proteins are known as A-kinase anchoring proteins (AKAPs). AKAPs are scaffolding proteins, and they spatially and temporally regulate multi-protein reaction platforms. The most important feature of AKAPs is their binding with PKA, a cAMP dependent-protein kinase A. PKA is a heterotetramer holoenzyme, and it binds with four cAMP molecules. Then the cAMP-PKA signaling pathway is anchored through interactions with AKAPs. As spatial regulators, AKAPs place their effectors close to substrates, and as temporal regulators, AKAPs control signaling pathways by assembling multi-protein complexes. In this report, we discussed AKAP6, known as muscle-AKAP (mAKAP). According to previous reports, AKAP6 is mainly expressed in cardiomyocytes and skeletal muscles, and it is localized to the perinuclear membrane. AKAP6 is a ~250kDa scaffolding protein and it organizes many proteins, including PKA, calcineurin, protein phosphatase 2A,

ERK5, PDE4D3, and others. AKAP6 directly binds to MEF2, a myogenic transcription factor, and this directs myoblast differentiation into myotubes [48]. According to our previous study, myogenin, a muscle specific transcription factor, binds the mAKAP promoter region thus promoting muscle differentiation and regeneration. This is the first report showing that AKAP6 associates with Embryonic stem cells. Initially, we assumed AKAP6 may have an effects on mESC differentiation. As we previously reported, EBs comprised of mESCs display hierachical differentiation. AKAP6 was expressed in differentiated mESCs (Figure 1). Hence, we first assessed the effect of AKAP6 on mESCs differentiation. We attempted to overexpress AKAP6, but the transfection efficiency was low. Therefore, we knocked-down AKAP6 using a shRNA system, and examined mESCs differentiation. We examined several differentiation methods, however, we concluded that AKAP6 did not function as a differenation factor. Interestingly, we discovered that AKAP6 depletion is associated with increased apoptosis in mESCs. When AKAP6 expression was supressed, depleted propagation and differentiation capabilities were observed (Supplementary Figure 1B, C). Through a transient knock-down process, we observed reduced actin distribution and increased membrane ruffling. Notably, we observed a decrese in FAK expression. FAK is commonly used as a marker for cell proliferation/migration or apoptosis. A previous report suggested that FAK has

anti-apoptotic effects [46], and it may determine cell survival or death in response to TNFα [47]. Thus, we speculated that AKAP6 knock-down in mESCs might affect apoptosis. We clarified this supposition by means of an Annexin V/PI FACS analysis and by detection of activated caspase 3. We also performed a TUNEL assay to analyze cell death (data not shown). Based on these data, we have concluded that AKAP6 does not affect mESC differentiation, however, knock-down of AKAP6 does have an effect on mESCs apoptosis. Although underlying mechanisms should be further explored, these novel findings have provided new insight into stem cell applications to cure disease.

Figures

A.



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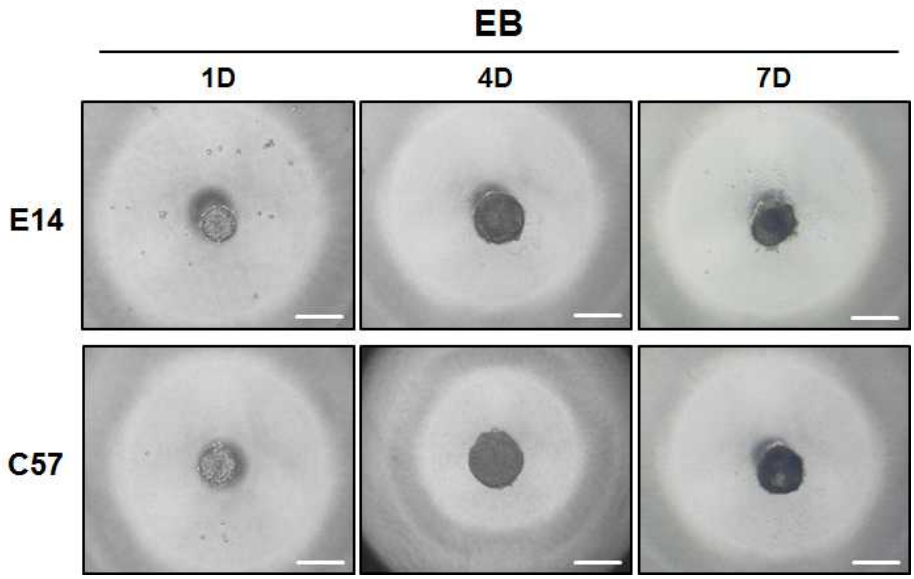
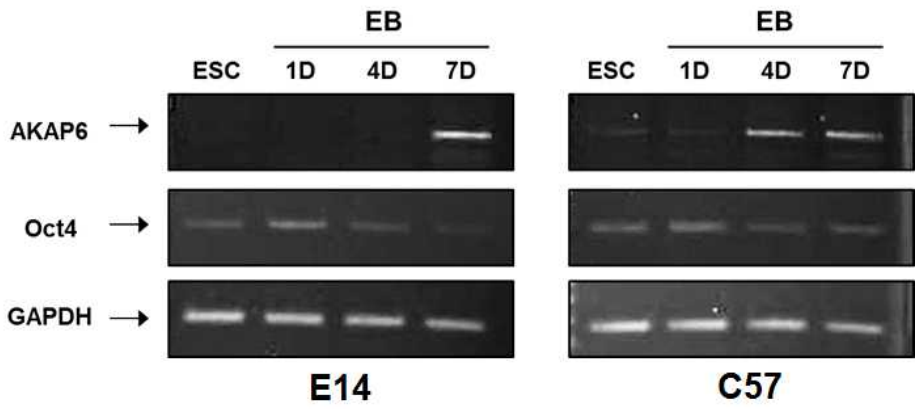


C.


EB
EB
ESC
1D
4D
7D
ESC
1D
4D
7D
AKAP6
Oct4
GAPDH
E14
C57

D.

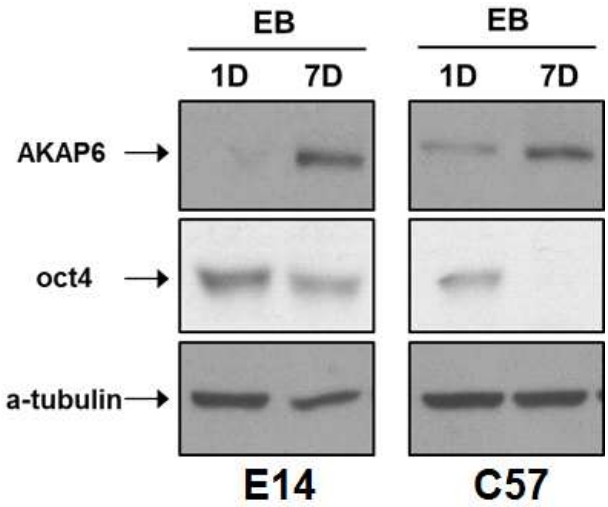

EB
EB
1D
7D
1D
7D
AKAP6
oct4
a-tubulin
E14
C57

Figure 1. AKAP6 is expressed in differentiated mosue ESCs.

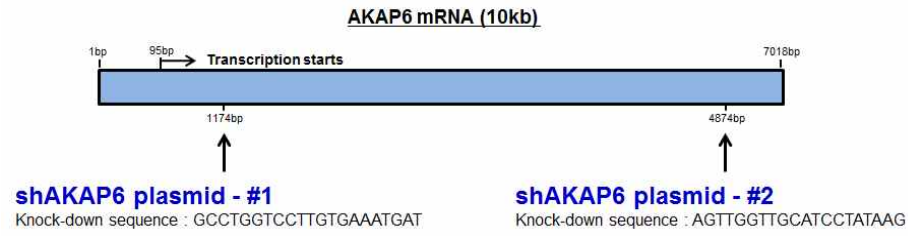
(A) Undifferentiated E14 and C57 mouse Embryonic Stem Cells were observed by phase-contrast microscope. Scale bar: 200μm

(B) For differentiation of mESCs, Embryoid Bodies (EBs) were formed by hanging drop method, and harvested at day 1, 4 and, 7. The morphology of EBs was observed by phase-contrast microscope. Scale bar: 200μm

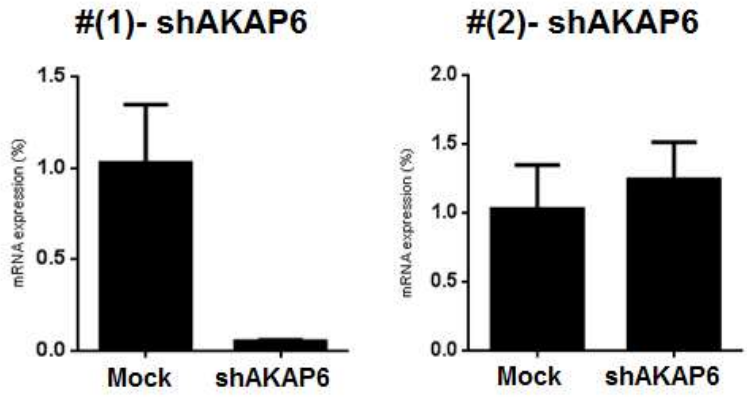
(C) We observed that AKAP6 was expressed in differentiated mESCs by RT-PCR

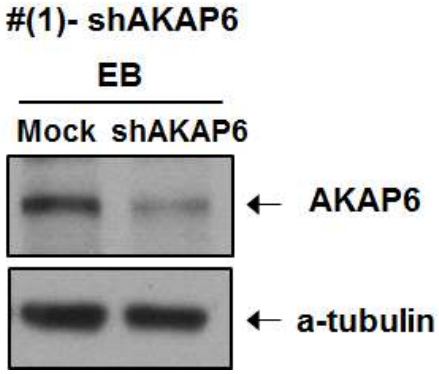
(E) Day 1 and 7 EBs were collected and harvested for Western blotting of AKAP6.

A.

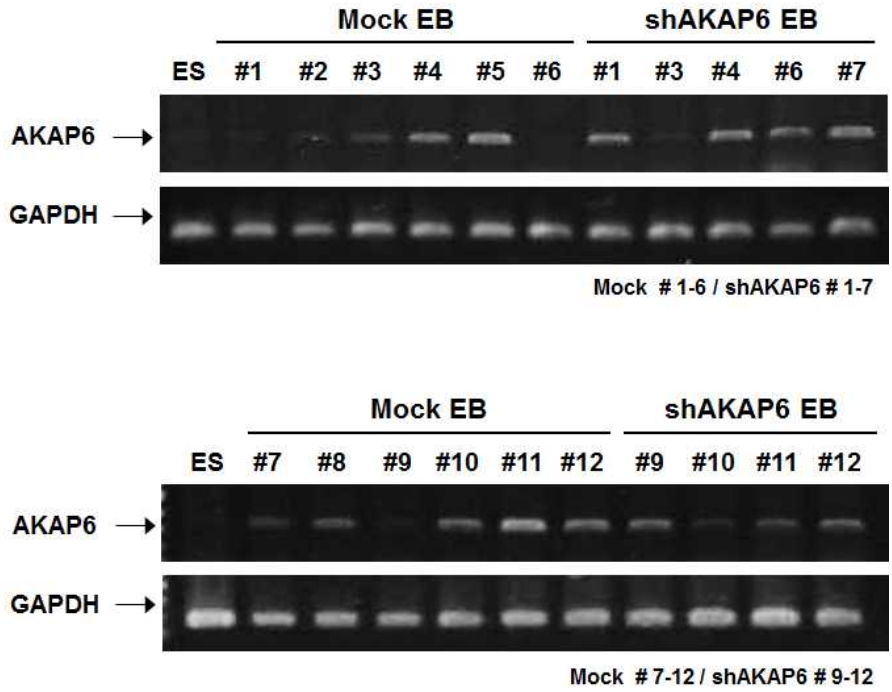

AKAP6 mRNA (10kb)
1bp
95bp
Transcription starts
7018bp
1174bp
4874bp
shAKAP6 plasmid - #1
Knock-down sequence : GCCTGGTCCTTGTGAAATGAT
shAKAP6 plasmid - #2
Knock-down sequence : AGTTGGTTGCATCCTATAAG

B.

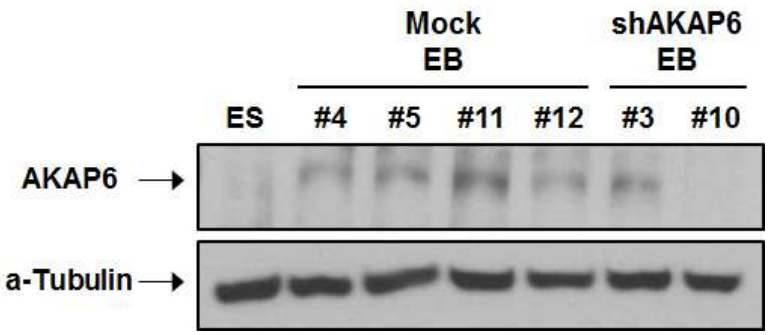

#(1)- shAKAP6
#(2)- shAKAP6
mRNA expression (%)
1.5
1.0
0.5
0.0
2.0
1.5
1.0
0.5
0.0
Mock
shAKAP6
Mock
shAKAP6


#(1)- shAKAP6
EB
Mock shAKAP6
AKAP6
a-tubulin

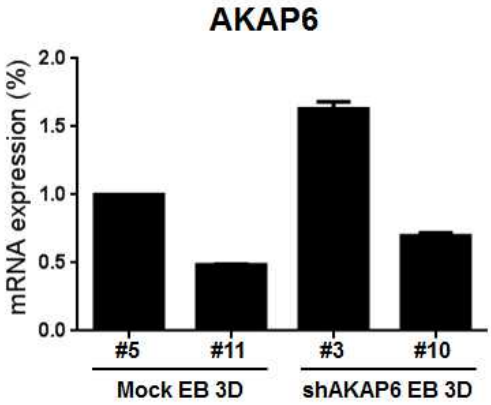
C.


Mock EB
shAKAP6 EB
ES #1 #2 #3 #4 #5 #6 #1 #3 #4 #6 #7
AKAP6
GAPDH
Mock # 1-6 / shAKAP6 # 1-7
Mock EB
shAKAP6 EB
ES #7 #8 #9 #10 #11 #12 #9 #10 #11 #12
AKAP6
GAPDH
Mock # 7-12 / shAKAP6 # 9-12

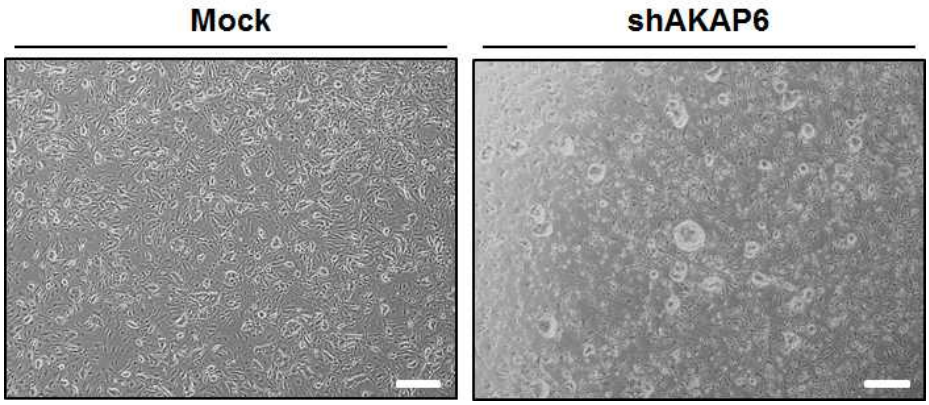
D.


Mock EB
shAKAP6 EB
ES #4 #5 #11 #12 #3 #10
AKAP6
a-Tubulin

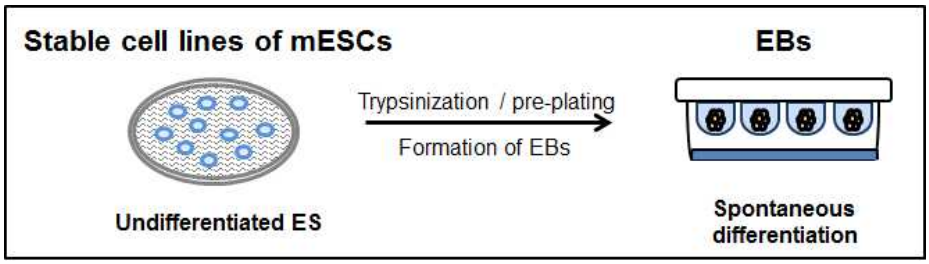
E.

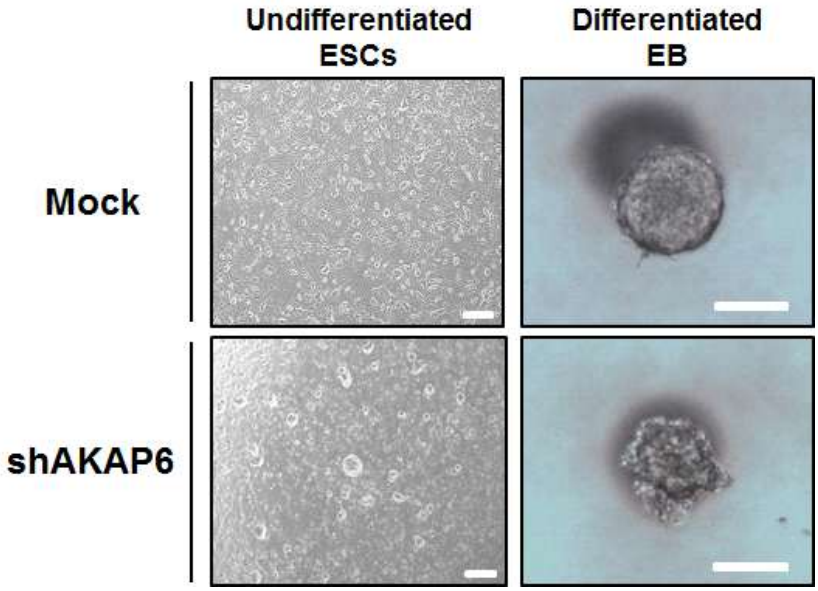

AKAP6
mRNA expression (%)
2.0
1.5
1.0
0.5
0.0
#5
#11
#3
#10
Mock EB 3D
shAKAP6 EB 3D

F.


Mock
shAKAP6

G.


Stable cell lines of mESCs
EBs
Trypsinization / pre-plating
Formation of EBs
Undifferentiated ES
Spontaneous differentiation


Undifferentiated ESCs
Differentiated EB
Mock
shAKAP6

H.

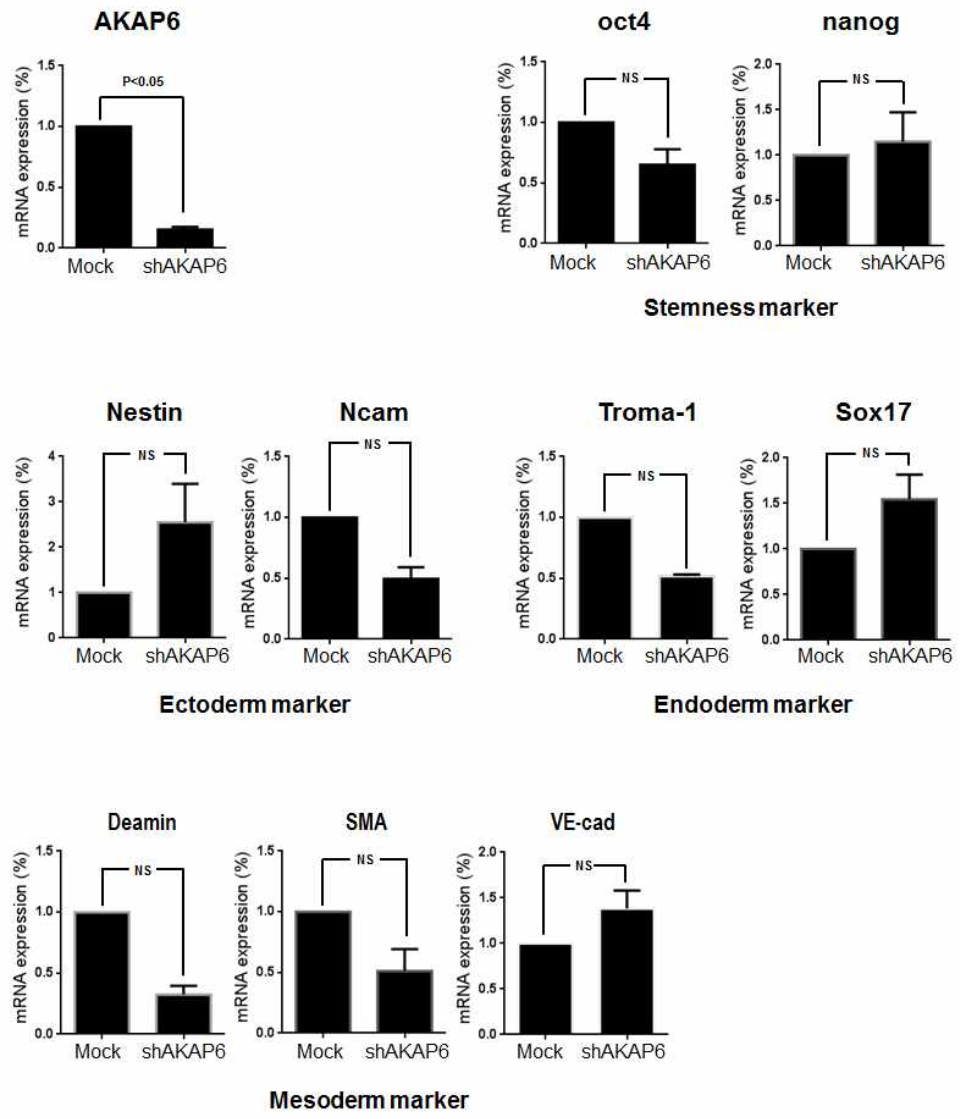

AKAP6
P<0.05
mRNA expression (%)
Mock
shAKAP6
oct4
nanog
NS
Stemness marker
Nestin
Ncam
Troma-1
Sox17
Ectoderm marker
Endoderm marker
Deamin
SMA
VE-cad
Mesoderm marker

Figure 2. Depletion of AKAP6 by shRNA does not affect mouse ESCs differentiation.

(A) Two kinds of shAKAP6 plasmids were chosen by AKAP6 CDS binding region.

(B) One shAKAP6 plasmid which binds AKAP6 CDS forward region was selected by confirming successful working. (n=2)

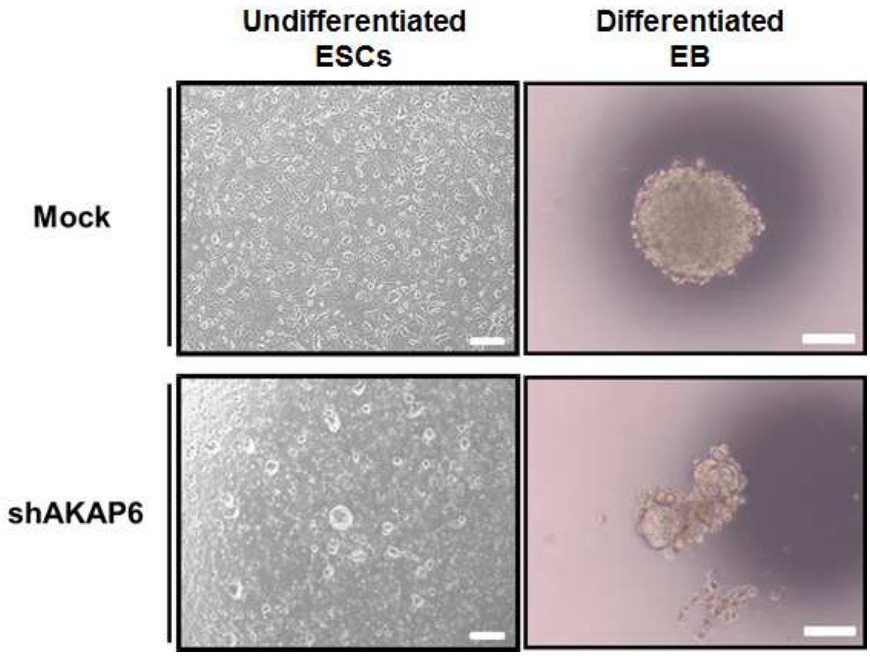
(C)-(E) The procedure for stable knock-down colony selection.

(F) Stable AKAP6 knock-down cell lines. Control cells and shAKAP6 cells were observed by phase-contrast microscope. Scale bar: 200μm

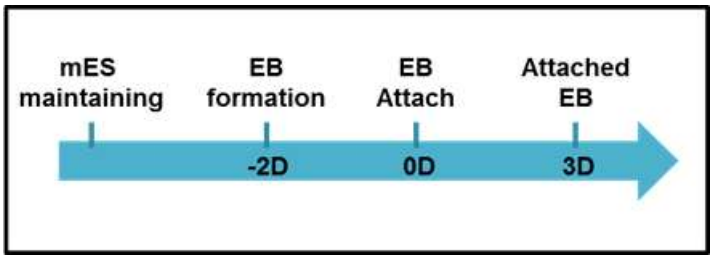
(G) Schematic image for differentiation of stable knock-down cells.

(H) Expression level of AKAP6, Stemness markers and embryo three germ-layer markers was detected by real-time PCR analysis. (n=3)

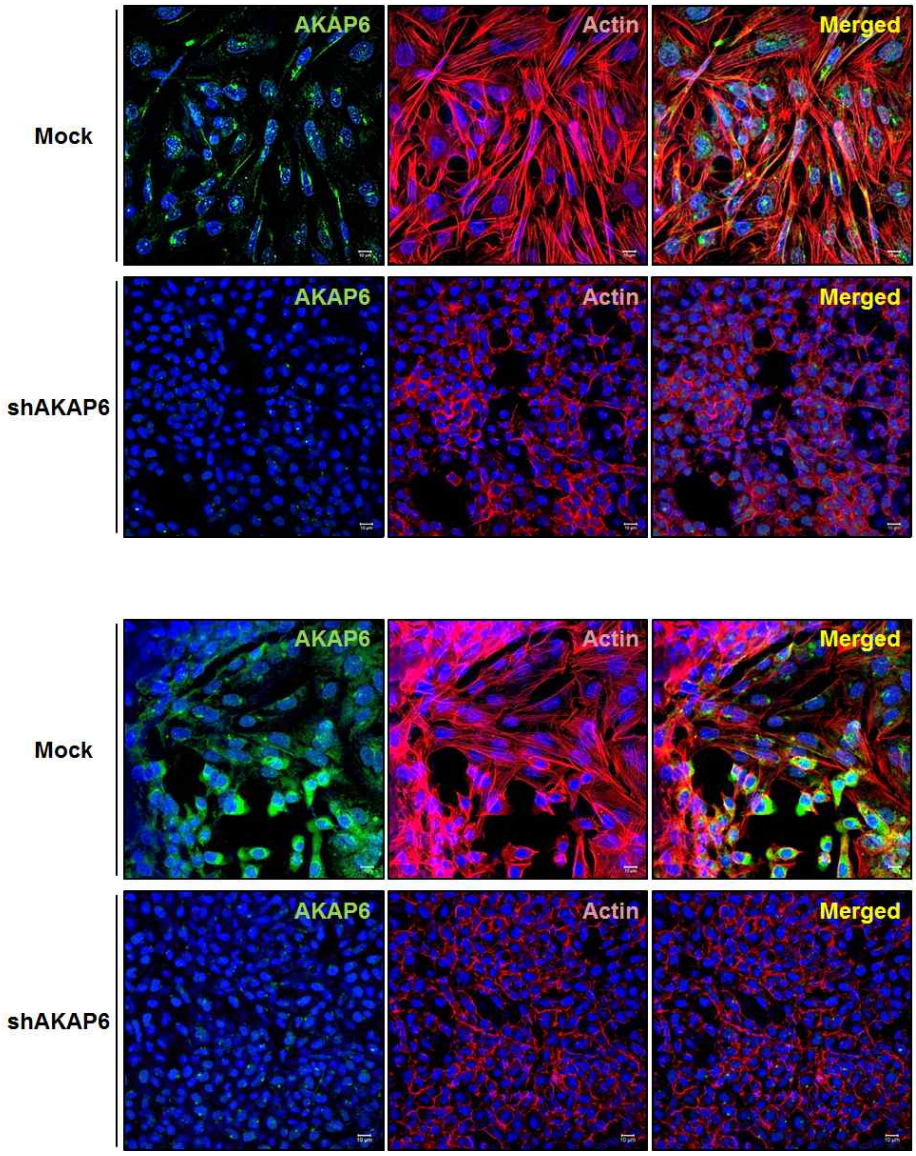
A.


Undifferentiated
ESCs
Differentiated
EB
Mock
shAKAP6

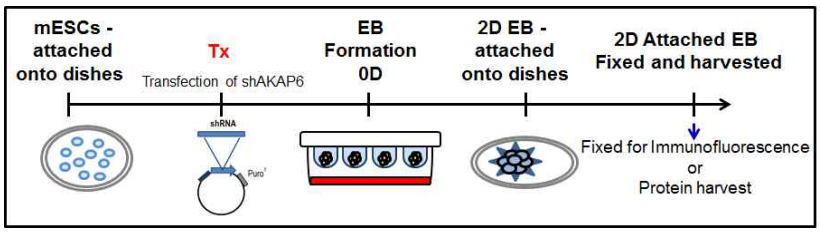
B.


mES
maintaining
EB
formation
EB
Attach
Attached
EB
-2D
0D
3D

C.


Mock
AKAP6
Actin
Merged
shAKAP6
AKAP6
Actin
Merged
Mock
AKAP6
Actin
Merged
shAKAP6
AKAP6
Actin
Merged

D.


mESCs - attached onto dishes
Tx
Transfection of shAKAP6
EB Formation 0D
2D EB - attached onto dishes
2D Attached EB Fixed and harvested
shRNA
Puro
Fixed for Immunofluorescence or Protein harvest

E.

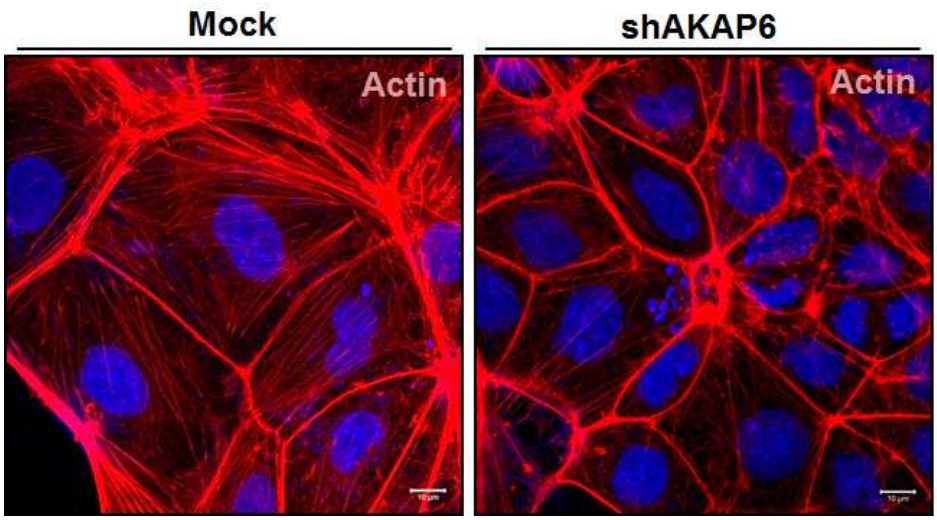

Mock
shAKAP6
Actin
Actin

Figure 3. Disrupted actin arrangement in AKAP6 knock-down cells.

(A) Morphological differences of control and shAKAP6 cells in differentiated EBs. Scale bar: 200μm

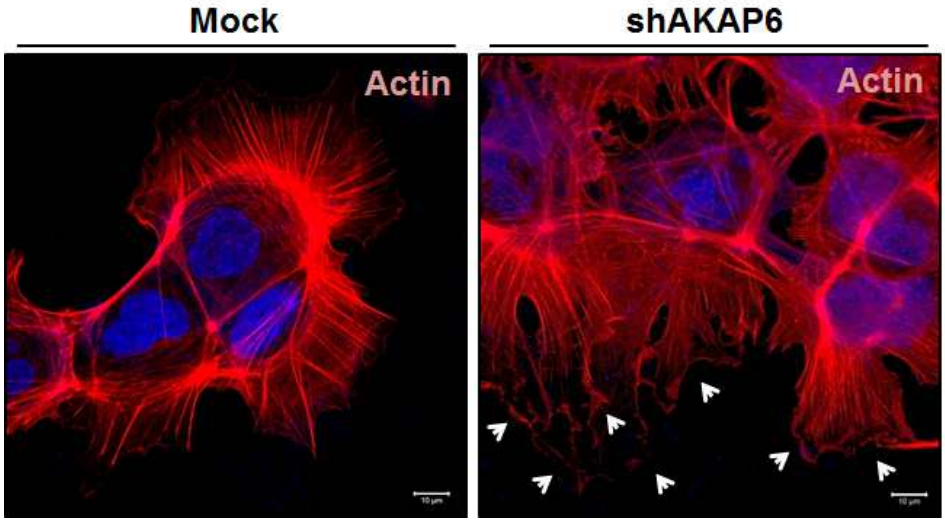
(B) Schematic time table for actin staining of shAKAP6 cells.

(C) Immunofluorescence indicated that actin distribution differed from control and shAKAP6 cells. Scale bars: 10μm.

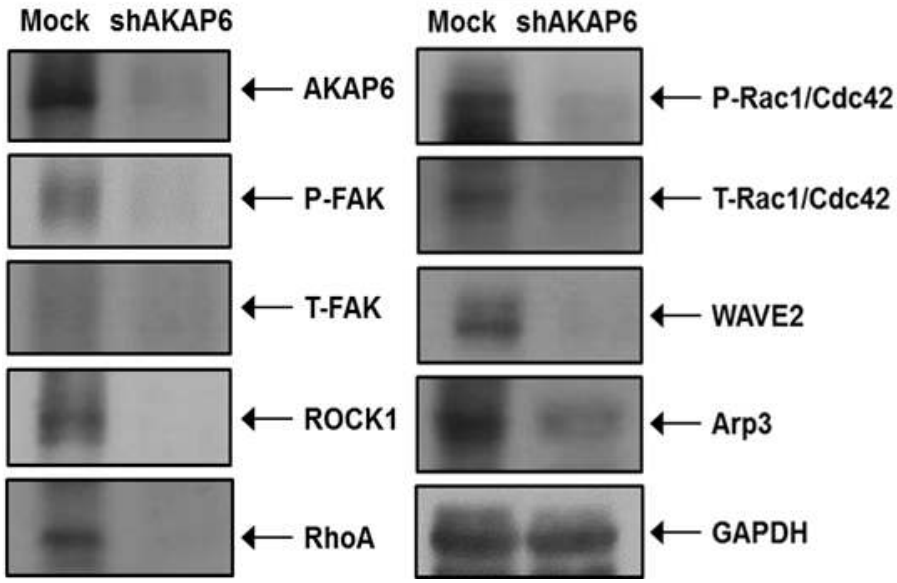
(D) Schematic view for transient knock-down of AKAP6 and actin staining.

(E) Actin immunostaining indicated that actin arrangement was also disrupted in transient knock-down of AKAP6.

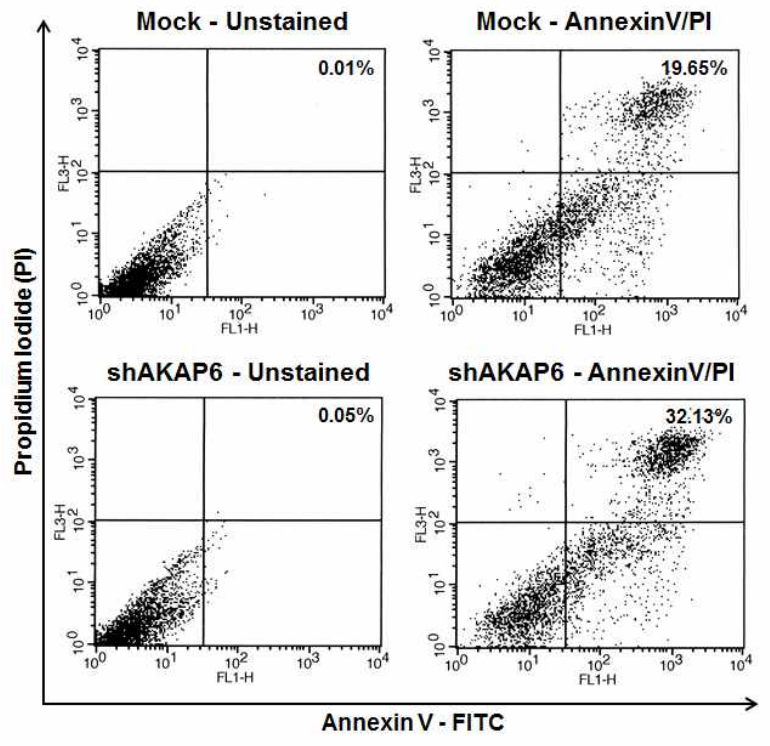
A.

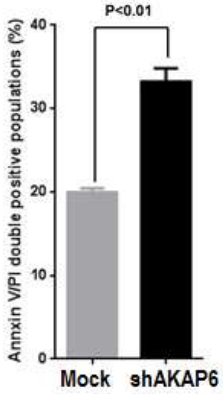

Mock
shAKAP6
Actin
Actin

B.

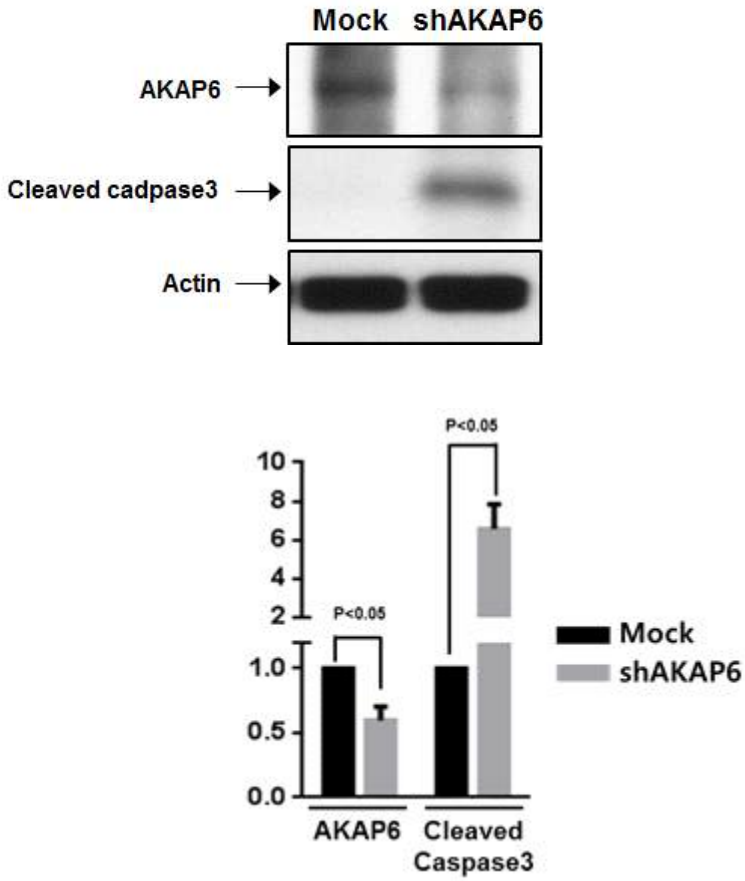

Mock shAKAP6
AKAP6
P-FAK
T-FAK
ROCK1
RhoA
Mock shAKAP6
P-Rac1/Cdc42
T-Rac1/Cdc42
WAVE2
Arp3
GAPDH

C.


Mock - Unstained
0.01%
Mock - AnnexinV/PI
19.65%
shAKAP6 - Unstained
0.05%
shAKAP6 - AnnexinV/PI
32.13%
FL3-H
FL1-H
Propidium Iodide (PI)
Annexin V - FITC


P<0.01
Annxin V/PI double positive populations (%)
40
30
20
10
0
Mock
shAKAP6

D.


Mock
shAKAP6
AKAP6
Cleaved cadpase3
Actin
P<0.05
P<0.05
10
8
6
4
2
1.0
0.5
0.0
AKAP6
Cleaved Caspase3
Mock
shAKAP6

E.

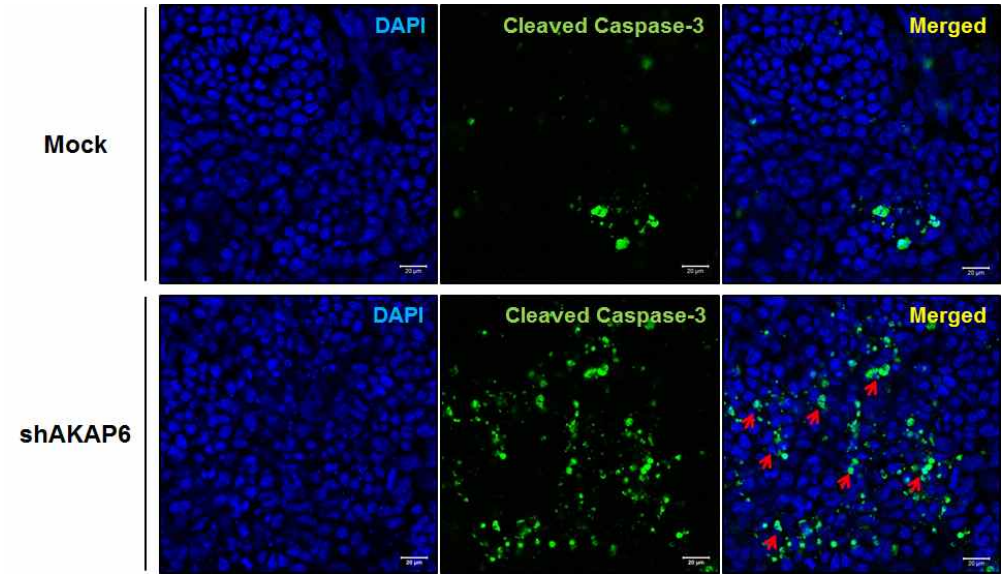

Mock
DAPI
Cleaved Caspase-3
Merged
shAKAP6
DAPI
Cleaved Caspase-3
Merged

Figure 4. Down regulation of actin-related proteins and apoptotic induction in AKAP6 knock-down mouse ESCs.

(A) Immunofluorescence images for membrane ruffling events that frequently occurred in AKAP6 knock-down mESCs. Scale bars: 10μm.

(B) In AKAP6 knock-down mESCs, actin-related proteins were detected by western blot analysis. (n=3)

(C) Annexin V/PI FACS analysis for confirmation of apoptotic characters. (n=2)

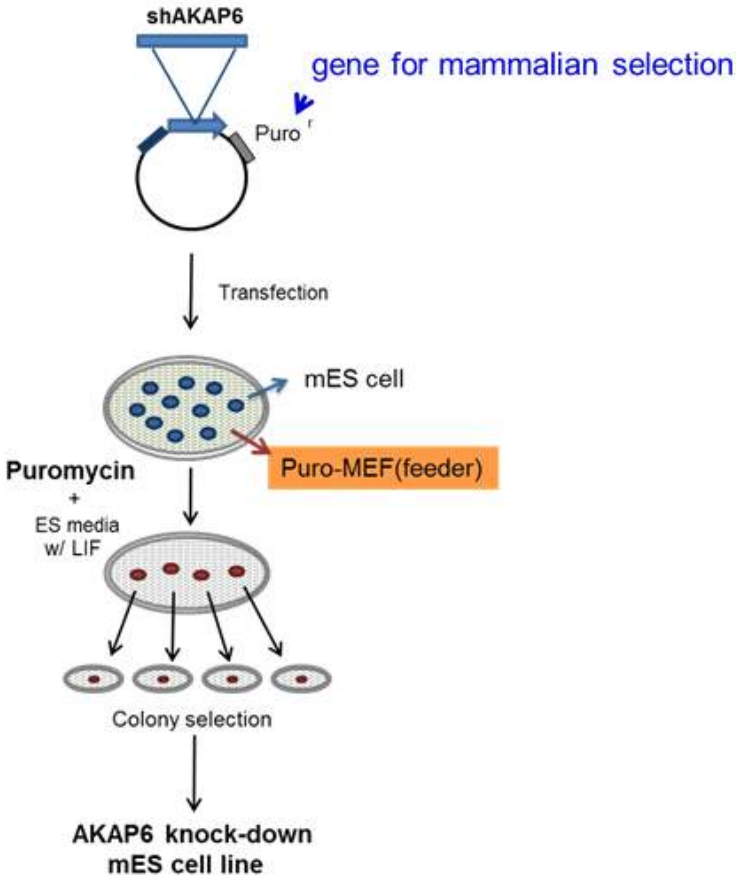
(D) Detection of cleaved caspase 3 expression by Western blot analysis. (n=2)

(E) Detection of cleaved caspase 3 expression by immunostaining. Scale bars: 20μm.

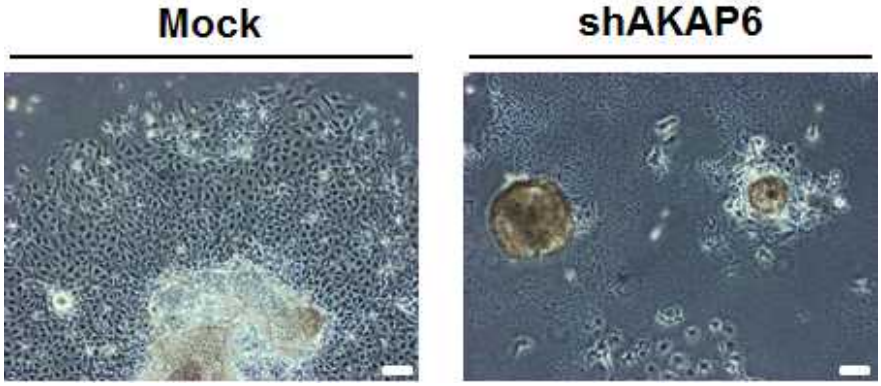
Table Ⅰ. Primer Sequence for Quantitative RT-PCR and Quantitative Real-time PCR.

Name		Sequence	Size (bp)
Oct4	Fw	GAAGCCCTCCCTACAGCAGA	297
	Rv	CAGAGCAGTGACGGGAACAG	
Nanog	Fw	CCCCACAAGCCTTGGAATTA	255
	Rv	CTCAAATCCCAGCAACCACA	
AKAP6	Fw	AAGGAACGAGCGCCGAGAAACA	103
	Rv	TGCTGGCACAACCTCAGAATGG	
AKAP6	Fw	TCTGGGGACATAAGTGTGAG	314
	Rv	CCTGAATGATGCGTTGGACT	
Troma-1	Fw	ATCGAGATCACCACCTACCG	241
	Rv	TCTTCACAACCACAGCCTTC	
Sox17	Fw	CTCGGGGATGTAAAGGTGAA	180
	Rv	TAGCTCTGCGTTGTGCAGAT	
Desmin	Fw	TGACAACCTGATAGACGACC	180
	Rv	TTAAGGAACGCGATCTCCTC	
SMA	Fw	ACTGGGACGACATGGAAAAG	240
	Rv	CATCTCCAGAGTCCAGCACA	
VE-cad	Fw	CGTGGTGGAAACACAAGATG	181
	Rv	TGGGTCCACAACAGTCAGAA	
Nestin	Fw	TAGAGGTGCAGCAGCTGCAG	170
	Rv	AGCGATCTGACTCTGTAGAC	
Ncam	Fw	AGATGGTCAGTTGCTGCCAA	187
	Rv	AGAAGACGGTGTGTCTGCTT	

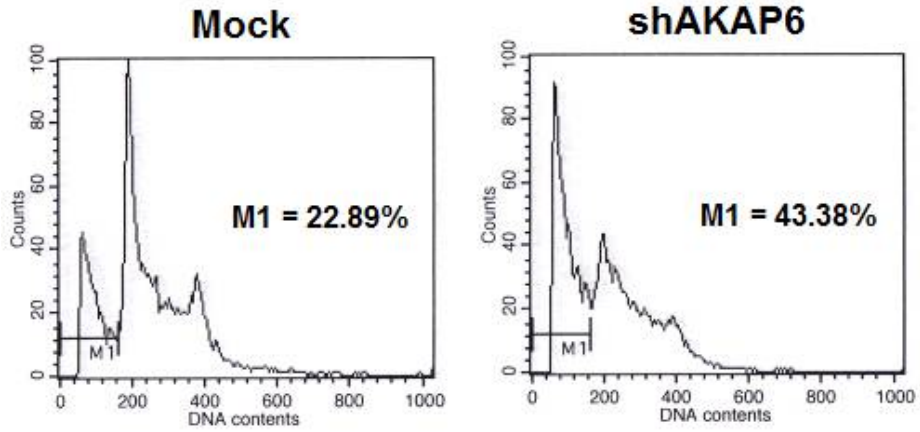
A.


shAKAP6
gene for mammalian selection
Puro r
Transfection
mES cell
Puro-MEF(feeder)
Puromycin
+
ES media
w/ LIF
Colony selection
AKAP6 knock-down
mES cell line

B.


Mock
shAKAP6

C.


Mock
shAKAP6
M1 = 22.89%
M1 = 43.38%
Counts
DNA contents
M1
0
20
40
60
80
100
200
400
600
800
1000

Supplement Figure 1. Stable AKAP6 knock-down cell lines.

(A) Experimental outlines for making stable knock-down cells.

(B) shAKAP6 cells were mostly broken when they were cultured. Phase-contrast microscope. Scale bar: 200μm

(C) PI-FACS cell cycle analysis of shAKAP6 stable cell line.

References

1. Smith AG. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol.* 2001;17:435-62.
2. Sae-Won Lee, Han-Kyul Jeong, Ji-Young Lee, Jimin Yang, et al. Hypoxic priming of mESCs accelerates vascular-lineage differentiation through HIF1-mediated inverse regulation of Oct4 and VEGF. *EMBO Mol Med.* 2012 Sep;4(9):924-38.
3. Rubin CS. A kinase anchor proteins and the intracellular targeting of signals carried by cyclic AMP. *Biochim Biophys Acta.* 1994 Dec 30;1224(3):467-79.
4. Wong W, Scott JD. AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol.* 2004 Dec;5(12):959-70.
5. Kapiloff MS, Jackson N, Airhart N. mAKAP and the ryanodine receptor are part of a multi-component signaling complex on the cardiomyocyte nuclear envelope. *J Cell Sci.* 2001 Sep;114(Pt 17):3167-76.
6. Michel JJ, Townley IK, Dodge-Kafka KL, Zhang F, Kapiloff MS, Scott JD. Spatial restriction of PDK1 activation cascades by anchoring to mAKAPalpha. *Mol Cell.* 2005 Dec 9;20(5):661-72.
7. Rohman MS, Emoto N, Takeshima Y, Yokoyama M, Matsuo M. Decreased mAKAP, ryanodine receptor, and SERCA2a gene expression in mdx hearts. *Biochem Biophys Res Commun.* 2003 Oct 10;310(1):228-35.
8. Chinzei R, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S,

et al. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. *Hepatology*. 2002 Jul;36(1):22-9.

9. Mikiko Koikea, Shujiro Sakakib, Yoshifumi Amanoa, Hiroshi Kurosawa. Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. *J Biosci Bioeng*. 2007 Oct;104(4):294-9.
10. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol.* 2000 Apr;18(4):399-404.
11. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000) Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6: 88-95
12. Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A (2010) Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7: 150-161
13. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol.* 1985 Jun;87:27-45.
14. Kanungo J, Pratt SJ, Marie H, Longmore GD. Ajuba, a cytosolic LIM protein, shuttles into the nucleus and affects embryonal cell proliferation and fate decisions. *Mol Biol Cell.*

2000 Oct;11(10):3299-313.

15. Kemler R, Brûlet P, Schnebelen MT, Gaillard J, Jacob F. Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. *J Embryol Exp Morphol.* 1981 Aug;64:45-60.
16. Liu J, He X, Corbett SA, Lowry SF, Graham AM, Fässler R, Li S. Integrins are required for the differentiation of visceral endoderm. *J Cell Sci.* 2009 Jan 15;122(Pt 2):233-42. doi: 10.1242/jcs.037663.
17. Qu XB, Pan J, Zhang C, Huang SY. Sox17 facilitates the differentiation of mouse embryonic stem cells into primitive and definitive endoderm in vitro. *Dev Growth Differ.* 2008 Sep;50(7):585-93.
18. Wang P, Rodriguez RT, Wang J, Ghodasara A, Kim SK. Targeting SOX17 in human embryonic stem cells creates unique strategies for isolating and analyzing developing endoderm. *Cell Stem Cell.* 2011 Mar 4;8(3):335-46. doi: 10.1016/j.stem.2011.01.017.
19. Schroeder IS, Sulzbacher S, Nolden T, Fuchs J, Czarnota J, Meisterfeld R, Himmelbauer H, Wobus AM. Induction and selection of Sox17-expressing endoderm cells generated from murine embryonic stem cells. *Cells Tissues Organs.* 2012;195(6):507-23. doi: 10.1159/000329864. Epub 2011 Nov 25.
20. Niakan KK, Ji H, Maehr R, Vokes SA, Rodolfa KT, Sherwood RI, Yamaki M, Dimos JT, Chen AE, Melton DA, McMahon AP, Eggan K. Sox17 promotes differentiation in mouse embryonic stem cells by directly regulating

extraembryonic gene expression and indirectly antagonizing self-renewal. *Genes Dev.* 2010 Feb 1;24(3):312-26. doi: 10.1101/gad.1833510.

21. Kintner CR, Melton DA. Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. *Development.* 1987 Mar;99(3):311-25.
22. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature.* 2008 Jan 10;451(7175):141-6. Epub 2007 Dec 23.
23. Tat PA, Sumer H, Jones KL, Upton K, Verma PJ. The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. *Cell Transplant.* 2010;19(5):525-36. doi: 10.3727/096368910X491374. Epub 2010 Feb 8.
24. Yang J, Bian W, Gao X, Chen L, Jing N. Nestin expression during mouse eye and lens development. *Mech Dev.* 2000 Jun;94(1-2):287-91.
25. Sidhu KS, Ryan JP, Tuch BE. Derivation of a new human embryonic stem cell line, endeavour-1, and its clonal propagation. *Stem Cells Dev.* 2008 Feb;17(1):41-51. doi: 10.1089/scd.2007.0055.
26. Sakurai K, Shimoji M, Tahimic CG, Aiba K, Kawase E, Hasegawa K, Amagai Y, Suemori H, Nakatsuji N. Efficient integration of transgenes into a defined locus in human embryonic stem cells. *Nucleic Acids Res.* 2010 Apr;38(7):e96. doi: 10.1093/nar/gkp1234. Epub 2010 Jan 13.

27. Roche E, Sepulcre P, Reig JA, Santana A, Soria B. Ectodermal commitment of insulin-producing cells derived from mouse embryonic stem cells. *FASEB J.* 2005 Aug;19(10):1341-3. Epub 2005 May 31.
28. Sritanaudomchai H, Pavasuthipaisit K, Kitiyanant Y, Kupradinun P, Mitalipov S, Kusamran T. Characterization and multilineage differentiation of embryonic stem cells derived from a buffalo parthenogenetic embryo. *Mol Reprod Dev.* 2007 Oct;74(10):1295-302.
29. Hong CS, Saint-Jeannet JP. The activity of Pax3 and Zic1 regulates three distinct cell fates at the neural plate border. *Mol Biol Cell.* 2007 Jun;18(6):2192-202. Epub 2007 Apr 4.
30. Meng XL, Shen JS, Kawagoe S, Ohashi T, Brady RO, Eto Y. Induced pluripotent stem cells derived from mouse models of lysosomal storage disorders. *Proc Natl Acad Sci U S A.* 2010 Apr 27;107(17):7886-91. doi: 10.1073/pnas.1002758107. Epub 2010 Apr 12.
31. Hofner M, Höllrigl A, Puz S, Stary M, Weitzer G. Desmin stimulates differentiation of cardiomyocytes and up-regulation of brachyury and nkx2.5. *Differentiation.* 2007 Sep;75(7):605-15. Epub 2007 Mar 23.
32. Schaart G, Viebahn C, Langmann W, Ramaekers F. Desmin and titin expression in early postimplantation mouse embryos. *Development.* 1989 Nov;107(3):585-96.
33. Oda Y, Yoshimura Y, Ohnishi H, Tadokoro M, Katsube Y, Sasao M, Kubo Y, Hattori K, Saito S, Horimoto K, Yuba S, Ohgushi H. Induction of pluripotent stem cells from human

third molar mesenchymal stromal cells. *J Biol Chem.* 2010 Sep 17;285(38):29270-8. doi: 10.1074/jbc.M109.055889. Epub 2010 Jul 1.

34. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007 Nov 30;131(5):861-72.
35. Torres J, Prieto J, Durupt FC, Broad S, Watt FM. Efficient differentiation of embryonic stem cells into mesodermal precursors by BMP, retinoic acid and Notch signalling. *PLoS One.* 2012;7(4):e36405. doi: 10.1371/journal.pone.0036405. Epub 2012 Apr 30.
36. Liu F, Kang I, Park C, Chang LW, Wang W, Lee D, Lim DS, Vittet D, Nerbonne JM, Choi K. ER71 specifies Flk-1+ hemangiogenic mesoderm by inhibiting cardiac mesoderm and Wnt signaling. *Blood.* 2012 Apr 5;119(14):3295-305. doi: 10.1182/blood-2012-01-403766. Epub 2012 Feb 17.
37. Drukker M, Tang C, Ardehali R, Rinkevich Y, Seita J, Lee AS, Mosley AR, Weissman IL, Soen Y. Isolation of primitive endoderm, mesoderm, vascular endothelial and trophoblast progenitors from human pluripotent stem cells. *Nat Biotechnol.* 2012 May 27;30(6):531-42. doi: 10.1038/nbt.2239.
38. Ben-Nun IF1, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang YC, Charter SJ, Laurent LC, Ryder OA, Loring JF. Induced pluripotent stem cells from highly endangered species. *Nat Methods.* 2011 Sep 4;8(10):829-31. doi: 10.1038/nmeth.1706.

39. Matsui H, Sakabe M, Sakata H, Yanagawa N, Ikeda K, Yamagishi T, Nakajima Y. Induction of initial heart alpha-actin, smooth muscle alpha-actin, in chick pregastrula epiblast: the role of hypoblast and fibroblast growth factor-8. *Dev Growth Differ.* 2008 Mar;50(3):143-57. doi: 10.1111/j.1440-169X.2008.00987.x. Epub 2008 Feb 27.
40. Ema M, Yokomizo T, Wakamatsu A, Terunuma T, Yamamoto M, Takahashi S. Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. *Blood.* 2006 Dec 15;108(13):4018-24. Epub 2006 Aug 22.
41. Iida M, Heike T, Yoshimoto M, Baba S, Doi H, Nakahata T. Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. *FASEB J.* 2005 Mar;19(3):371-8.
42. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development.* 1998 May;125(9):1747-57.
43. Borm B, Requardt RP, Herzog V, Kirfel G. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Exp Cell Res.* 2005 Jan 1;302(1):83-95.
44. Apoptosis in Toxicology edited by R. J. Roberts © 2000 Taylor & Francis
45. Apoptosis in Carcinogenesis and Chemotherapy: Apoptosis in

cancer edited by George G. Chen, Paul B. S. Lai © 2009 Springer Science+Business Media B.V.

46. Kurokawa K, Matsuda M. Localized RhoA activation as a requirement for the induction of membrane ruffling. *Mol Biol Cell.* 2005 Sep;16(9):4294-303. Epub 2005 Jun 29.
47. Suetsugu S, Yamazaki D, Kurisu S, Takenawa T. Differential roles of WAVE1 and WAVE2 in dorsal and peripheral ruffle formation for fibroblast cell migration. *Dev Cell.* 2003 Oct;5(4):595-609.
48. Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem.* 2000 May 26;275(21):16309-15.
49. Takahashi R, Sonoda Y, Ichikawa D, Yoshida N, Eriko AY, Tadashi K. Focal adhesion kinase determines the fate of death or survival of cells in response to TNFalpha in the presence of actinomycin D. *Biochim Biophys Acta.* 2007 Apr;1770(4):518-26. Epub 2006 Nov 30.
50. Vargas MA, Tirnauer JS, Glidden N, Kapiloff MS, Dodge-Kafka KL. Myocyte enhancer factor 2 (MEF2) tethering to muscle selective A-kinase anchoring protein (mAKAP) is necessary for myogenic differentiation. *Cell Signal.* 2012 Aug;24(8):1496-503. doi: 10.1016/j.cellsig.2012.03.017. Epub 2012 Mar 30.

국문초록

배경 - 배아줄기세포를 이용한 연구는 세포 기반의 질병 치료에 많은 가능성을 제시해 주고 있다. 따라서, 많은 연구자들이 배아줄기세포의 세포 내 분자 조절 기전을 밝혀내는 연구들에 집중하고 있다. 여기서 우리는 마우스 배아줄기세포의 apoptosis 유발이 AKAP6 scaffolding protein에 의해 조절되는 새로운 측면을 제시하였다.

방법 및 결과 - 마우스 배아줄기세포를 hanging drop 기법으로 EB 분화를 유도하였다. EB 분화 1, 4, 7일 RNA와 Protein에서 AKAP6 발현을 확인해본 결과, 마우스 배아줄기세포가 미분화일 때 보다 분화된 상태일 때 발현됨을 관찰하였다. AKAP6가 마우스배아줄기세포에 어떤 영향을 미치는지 알아보고자 AKAP6 knock-down stable cell line을 만들었다. 먼저, 분화에 대한 영향을 알아보고자 AKAP6 knock-down stable cell line으로 분화 유도 후 분화 마커들의 발현을 RNA 수준에서 확인해 보았다. 하지만, AKAP6의 발현이 유의하게 감소하였음에도 불구하고 분화 마커에는 유의한 변화가 없었다. 그러나, AKAP6 knock-down stable cell line의 분화 배양 중에 knock-down 세포의 특이적인 세포구조 차이를 발견하였다. 특히 세포막 ruffling 현상이 knock-down 세포에서 빈번하게 발생함을 관찰하였다. Ruffling 현상은 세포 이동 혹은 세포 사멸로 발생된다고 알려져 있으며 따라서 우리는 마우스 배아줄기세포에서 AKAP6의 발현을 억제하였을 때 나타는 ruffling이 어떠한 현상으로 인한 것인지 알아보았다. AKAP6의 발현을 억제 후 분화를 유도하여 세포 이

동시 활성화 되는 분자들의 Protein 수준을 확인 해 본 결과, AKAP6의 발현 억제 시 이동에 관련된 분자들의 발현이 감소됨을 보았다. 더욱이 anti-apoptotic factor로 알려져 있는 Focal Adhesion Kinase의 양적 감소는 ruffing 현상이 세포 사멸에 의한 것이라는 것을 제시해 주었다. 이후 AKAP6의 발현을 억제 하였을 때 세포 사멸 특징을 나타내는 실험들을 진행 하였으며, 마우스 배아줄기세포에서 AKAP6의 발현을 억제하면 세포사멸이 유발된다는 결과들을 얻을 수 있었다.

결론 - 처음으로 우리는 AKAP6의 마우스 배아줄기세포에 대한 영향을 제시하였다. 특히 AKAP6의 knock-down이 마우스 배아줄기세포의 분화가 아닌 세포사멸을 유발 한다는 결론을 도출하였으며, 이러한 결과들은 세포 사멸과 연관되는 질병의 줄기세포를 이용한 새로운 치료 효과를 제안해준다.

주요어 : mouse Embryonic Stem Cells, A-kinase Anchoring Protein 6, Apoptosis

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