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Original Article

M-RAS Regulate CDH1 Function in Blastomere Compaction during Porcine Embryonic Development

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ABSTRACT Cell adhesion plays an important role in the differentiation of the morphogenesis and the trophectoderm epithelium of the blastocyst. In the porcine embryo, CDH1 mediated adhesion initiates at compaction before blastocyst formation, regulated post-translationally via protein kinase C and other signaling molecules. Here we focus on muscle *RAS* oncogene homolog (M-RAS), which is the closest relative to the RAS related proteins and shares most regulatory and effector interactions. To characterize the effects of M-RAS on embryo compaction, we used gain- and loss-of-function strategies in porcine embryos, in which *M-RAS* gene structure and protein sequence are conserved. We showed that knockdown of *M-RAS* in zygotes reduced embryo development abilities and CDH1 expression. Moreover, the phosphorylation of ERK was also decreased in *M-RAS* KD embryos. Overexpression of M-RAS allows *M-RAS* KD embryos to rescue the embryo compaction and blastocyst formation. Collectively, these results highlight novel conserved and multiple effects of M-RAS during porcine embryo development.

Keywords: blastocyst formation, CDH1, embryo compaction, muscle *RAS* oncogene homolog (M-RAS)

INTRODUCTION

M-Ras shares ~50% sequence identity with the canonical Ras proteins (Kimmelman et al., 1997), and, although it can interact with some of the well-established Ras effectors, such as Raf, PI3K, and RalGDS. M-Ras also binds to distinct effectors, including certain RapGEFs and the Shoc2 scaffold (Herrmann et al., 1996; Rebhun et al., 2000; Gao et al., 2001; Rodriguez-Viciana et al., 2006). In contrast to the canonical Ras proteins, constitutively active M-Ras has a low transforming activity and is rarely mutated in human cancer (Quilliam et al., 1999); however, gain-of-function mutations in M-RAS have been iden-

tified in patients with Noonan syndrome (Higgins et al., 2017). Notably, mutations in one of the distinct effectors of M-RAS, the SHOC2 scaffold, have also been detected in Noonan patients (Cordeddu and Di, 2009; Hannig et al., 2014), and, likewise, Shoc2 has been implicated in cell migratory events (Young et al., 2013; Kaduwal et al., 2015; Jeoung et al., 2016).

Subsequent biochemical studies have shown that the direct binding of Shoc2 to active GTP-bound M-Ras allows the Shoc2 scaffold to nucleate a ternary complex consisting of active M-Ras, Shoc2 and the catalytic subunit of PP1 (PP1c) (Rodriguez-Viciana et al., 2006). In RTK-mediated signaling, the M-Ras/Shoc2/PP1c ternary com-

plex functions to dephosphorylate a negative regulatory 14-3-3 binding site on the Raf kinases, which promotes Raf binding to the canonical Ras proteins and facilitates ERK cascade activation (Rodriguez-Viciana et al., 2006; Matsunaga-Udagawa et al., 2010; Galperin et al., 2012). This complex has been implicated in the dynamic regulation of ERK activity and cell polarity in some cancer cell lines (Young et al., 2013). Moreover, M-Ras/Shoc2 signaling contributes to junction turnover by modulating the Cdh1/p120-catenin interaction and, in turn, the junctional expression of Cdh1. The regulatory effect of the M-Ras/Shoc2 complex was mediated at least in part through the phosphor-regulation of p120-catenin and required downstream ERK cascade activation (Kota et al., 2019).

Compaction appears to occur in all mammalian embryos but at different cell cycles. In the pig, compaction and cell polarization occur more gradually than in the mouse and coincide with relocation of CDH1 to contact sites, the process not completing long before blastocyst formation (Reima et al., 1993). In the developing embryo CDH1 is a key player during morula compaction and *CDH1* null embryos die early during embryogenesis as development of the blastocyst and trophectoderm is impaired (Larue et al., 1994; Riethmacher et al., 1995).

To further elucidate the biological functions of the M-RAS on porcine embryonic compaction, we investigated the mechanism by which M-RAS and CDH1 contribute to the regulation of compaction.

MATERIALS AND METHODS

Reagents

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte collection and *in vitro* maturation (IVM)

All animal studies were performed in strict accordance with the institutional guideline and prior approval from the Institutional Animal Care and Use Committee (IACUC) of the Chungbuk National University, Republic of Korea.

Prepubertal porcine ovaries were transported from a local abattoir (Farm Story Hannang, Chungwon, Chungbuk, Republic of Korea) within one hour of harvest. Cumulus-oocyte complexes (COCs) were aspirated from 3-6 mm of ovarian follicles using 18-gauge needles attached to a 10-mL syringe. High-density cumulus oocytes were collected and washed three times with HEPE-PVA medium (HEPES medium supplemented with 0.01% polyvinyl alcohol). After washing, 60-80 COCs were cultured in 500 mL of IVM medium consist of M-199 (Invitrogen, Carlsbad, CA) containing 20 ng/mL epidermal growth factor, 1 g/mL insulin, 75 g/mL kanamycin, 0.91 mM Na pyruvate, 0.57 mM L-cysteine, 10% (v/v) porcine follicular fluid, 0.5 µg/mL follicle stimulating hormone (FSH), and 0.5 µg/mL luteinizing hormone (LH) at 38.5°C in an atmosphere containing 5% CO_2 at 100% humidity.

Parthenogenetic activation and *in vitro* culture (IVC)

For parthenogenetic activation, mature oocytes were denuded by gentle pipetting in 1 mg/mL hyaluronidase until all the cumulus cells were removed from around the oocyte. Oocytes were then washed three times in PBS-BSA (DPBS added in 0.1% BSA) and activated using an Electro Cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA). The electric pulse was stimulated at 110 V/cm twice for 60 µsec in 280 mM mannitol medium supplemented with 0.1 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.01% polyvinyl alcohol (PVA, w/v), and 0.5 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid. Activated oocytes were treated in PZM-5 medium containing 7.5 µg/mL cytochalasin B in an incubator for 3 h to suppress extrusion of the pseudo second polar body. Embryos were thoroughly washed and cultured in bicarbonate buffered PZM-5 supplemented with 4 mg/mL BSA in 4-well plates for 6 d at 38.5°C and 5% CO_2. Two-cell and 4-cell cleavage rate and morula and blastocyst formation rate were examined, respectively, at 24, 48, 96, and 144 h after activation. To determine the total cell number, day-6 blastocysts were randomly collected and stained with 10 mg/mL Hoechst 33342 in PBS for 5 min.

M-RAS double-stranded RNA preparation

To prepare *M-RAS* double-stranded RNA (dsRNA), *M-RAS* was amplified using a pair of primers (Table 1) containing the T7 promoter sequence. The purified PCR products were then used to synthesize dsRNA with the MEGAscript T7 Kit (AM1333; Thermo Fisher Scientific) according to the manufacturer's instructions. After *in vitro* transcription, dsRNA was treated with DNase I and RNase A to remove the DNA template and any single-stranded RNAs, followed by purification with phenol-chloroform extraction and isopropyl alcohol precipitation. The puri-

Table 1. Summary of PCR primers

Gene	Primer sequence, 5'–3'		Product length (bp)	Used for
	Forward	Reverse		
M-RAS	gaattaatacgactcactatagggagaaacctccccacatacaagc	gaattaatacgactcactatagggagacagcaaagccagaccctatc	868	M-RAS knockdown
	gaactcttccagCCCCGCCGTGTCCAGAAC	tcctcgcccttgctcaccatgctagcCAATATCACGCACTGCAGTTTG	624	Active M-RAS^{Q71L}
	cgctcaactttggcagatctgaattcCATGGCAACCAGTGCCGTCC	acggcggggctgGAAGAGTTCAGCGCCATGC		
	ttgcccaccttctctcagag	ggccacctgtgtagacaatg	278	mRNA quantification

fied dsRNA was dissolved in RNase-free water and stored at -80°C until use.

Cloning and *in vitro* mRNA synthesis

The open reading frames of pig *M-RAS* (Table 1) were amplified from cDNA prepared from pig embryos. Amplified PCR products were subcloned into the pRN3 vector, and *in vitro* transcription was performed with a MEGA-script T3 Kit (Thermo Fisher Scientific).

Microinjection

For knockdown or overexpression experiments, *M-RAS* dsRNA or *M-RAS* cRNA was microinjected into the cytoplasm of a parthenogenetically activated oocyte using an Eppendorf Femto-Jet (Eppendorf, Hamburg, Germany) and Nikon Diaphot Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with a Narishige MM0-202N hydraulic 3-dimensional micromanipulator (Narishige, Amityville, NY, USA). After injection, oocytes were cultured in PZM-5 medium. The control group was microinjected with green fluorescent protein dsRNA.

Immunofluorescence and confocal microscopy

After washing 3 times with PBS and PVA, embryos were fixed in 3.7% paraformaldehyde for 30 min at room temperature, permeabilized with PBS and PVA containing 0.5% Triton X-100 at room temperature for 30 min and incubated in PBS and PVA containing 1.0% BSA at room temperature for 1 h. These embryos were incubated overnight at 4°C with anti-M-RAS (1:100,14213-1-AP; Proteintech), anti-CDH1 (1:100, 610182, BD Biosciences), or anti-ZO1 (1:100; 33-9100, Invitrogen) diluted in blocking solution. After washing 3 times with PBS and PVA, the embryos were incubated at room temperature for 1 h with goat anti-rabbit IgG, rabbit anti-goat IgG, or anti-mouse IgG. The oocytes and embryos were stained with 10 mg/mL Hoechst 33342 for 5 min, washed 3 times with PBS and PVA, mounted onto slides, and examined using a confocal microscope (Zeiss LSM 710 Meta). Images were processed using Zen software (v.8.0; Zeiss).

Real-time RT-PCR

Embryos were collected, and mRNA was extracted from a pool of 30 embryos per group using the DynaBeads mRNA Direct Kit (61012; Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was obtained by reverse transcription of mRNA using the Oligo (deoxythymine) 20 primer and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Amplification was conducted as follows: 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 25 s, 72°C for 10 s, and final extension at 72°C for 5 min. Target genes was *M-RAS*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. The primers used to amplify each gene are shown in Table 1. mRNA quantification data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western blot analysis

A total of 100 porcine embryos per group were placed in 1-time SDS sample buffer and heated at 98°C for 10 min. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Next, the membranes were blocked in Tris buffered saline containing 0.1% Tween 20 and 5% nonfat milk for 1 h and then incubated at 4°C overnight with anti-M-RAS, ERK, pERK, CHD1 or β-tubulin, wash three time by TBST. Then incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:20000; Santa Cruz Biotechnology). Membranes were developed by SuperSignal West Femto Trial Kit (34094, Thermo Scientifc, Waltham, MA, USA). Blots

were visualized by a charge-coupled device camera and UviSoft software (Uvitec, Cambridge, United Kingdom).

Statistical analysis

Each experiment was repeated at least 3 times, and representative images are shown in figures. All data were subjected to Student's t test. All percentage data were subjected to arcsine transformation prior to statistical analysis and presented as the means 6 SEM. Significance was set at a value of $p < 0.05$. All calculations were performed using SPSS software v.19 (IBM SPSS, Chicago, IL, USA). All box plots show the median (line), mean (+), and 25th and 75th percentiles (boxes), and the whiskers show the minimum to maximum values.

RESULTS

Expression patterns of M-RAS during porcine embryo development

We evaluated the expression and subcellular location of M-RAS at different stages during porcine embryo development (Fig. 1). M-RAS was expressed in the cytoplasm at zygote stage and highly expressed on the membrane from 2-cell stage until morula stage. The signal was detected on the nucleus from blastocyst (Fig. 1A). Moreover, the protein expression was decreased during preimplanta-
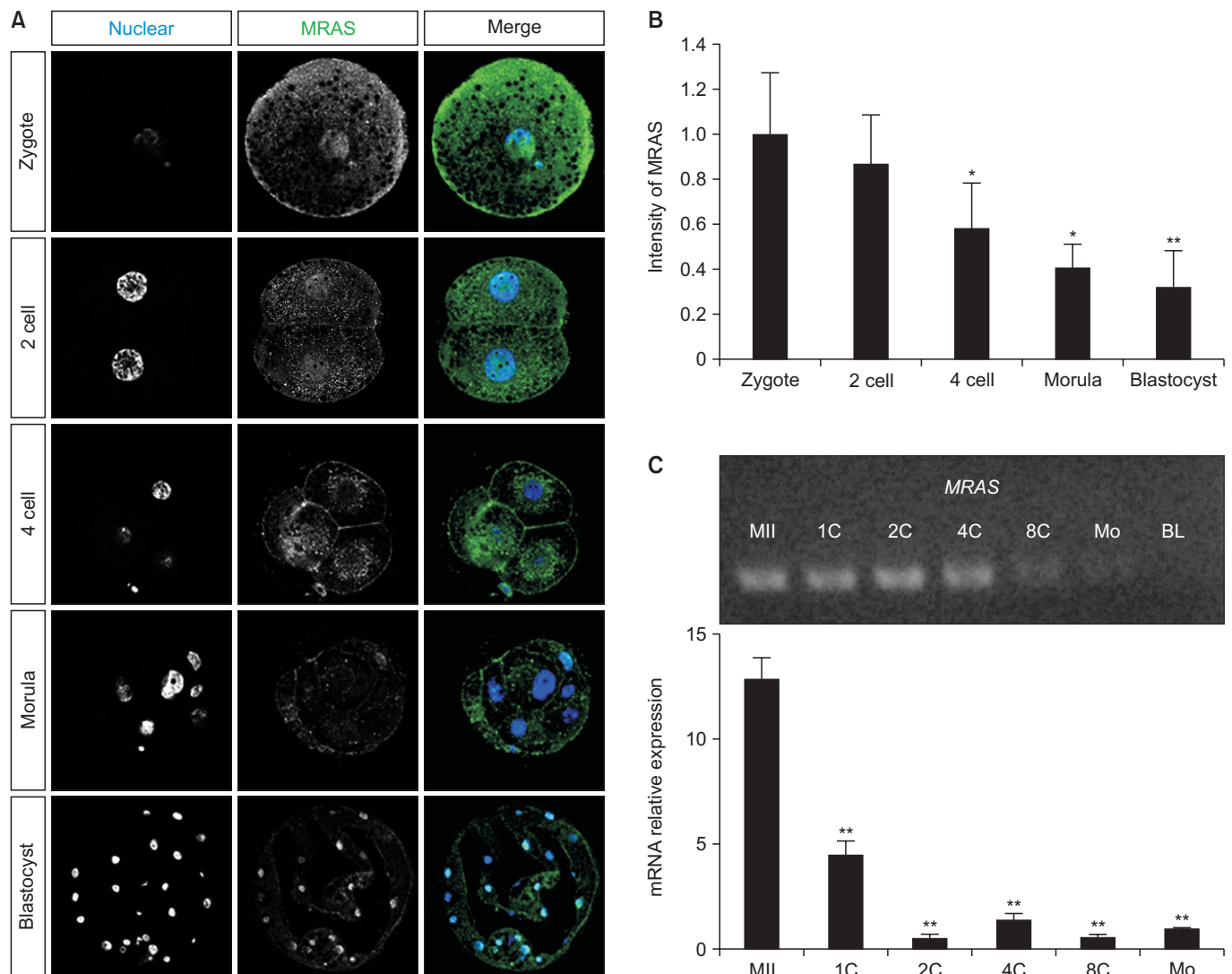
Fig. 1. Expression patterns of M-RAS during porcine embryo development. (A) Immunofluorescence images of M-RAS at different stages of porcine embryos. (B) Intensity of M-RAS at different stages. $*p < 0.05$; $**p < 0.01$. (C) The mRNA expression level of *M-RAS* performed by qPCR at different stages.

tion development (Fig. 1B). The mRNA expression level of *M-RAS* was detected by qPCR. *M-RAS* was maternally expressed in MII oocytes. Thereafter, *M-RAS* expression decreased and remained at a low level until blastocyst (Fig. 1C).

## Effects of *M-RAS* knockdown on early porcine embryonic development

To investigate the role of M-Ras in compaction during porcine embryo development, *M-RAS* double-stranded RNA (*dsM-RAS*) was injected into porcine parthenotes, which were then cultured *in vitro* for 6 days (Fig. 2). The blastocyst rates were calculated. M-RAS knock-down (*M-RAS* KD) embryos displayed a significantly lower developmental rate than control embryos, even though no difference at 2-cell stage (Fig. 2A, 2B). Compared to controls, the mRNA expression level of *M-RAS* KD embryos was decreased at both 2-cell stage and blastocyst stage (Fig. 2C, 2D), and a lower level of M-RAS protein as revealed by western blotting (Fig. 2E).

The active M-RAS regulates cell-cell adhesion by modulating the E-cadherin which correlates with ERK cascade activation

To more directly assess the role of M-RAS in regulating intercellular adhesion, we examined whether the presence or absence of M-RAS had any effects on E-cadherin localization in different stages of embryos (Fig. 3). In *M-RAS* KD embryos, the decrease in cell-cell adhesion coincided with a lower expression of E-cadherin shown by immune fluorescence and western blotting (Fig. 3A, 3B). However, no detectable change in ZO-1 (also known as tight junction protein-1) was present in *M-RAS* KD porcine embryos (Fig. 3C). In addition, the phosphorylation level of ERK was significantly higher than *M-RAS* KD embryos.

Overexpression of M-RAS rescues the disrupt of embryonic compaction after M-RAS knockdown

To confirm that the role of the M-RAS on embryonic compaction, we first produced the cRNA of activated M-
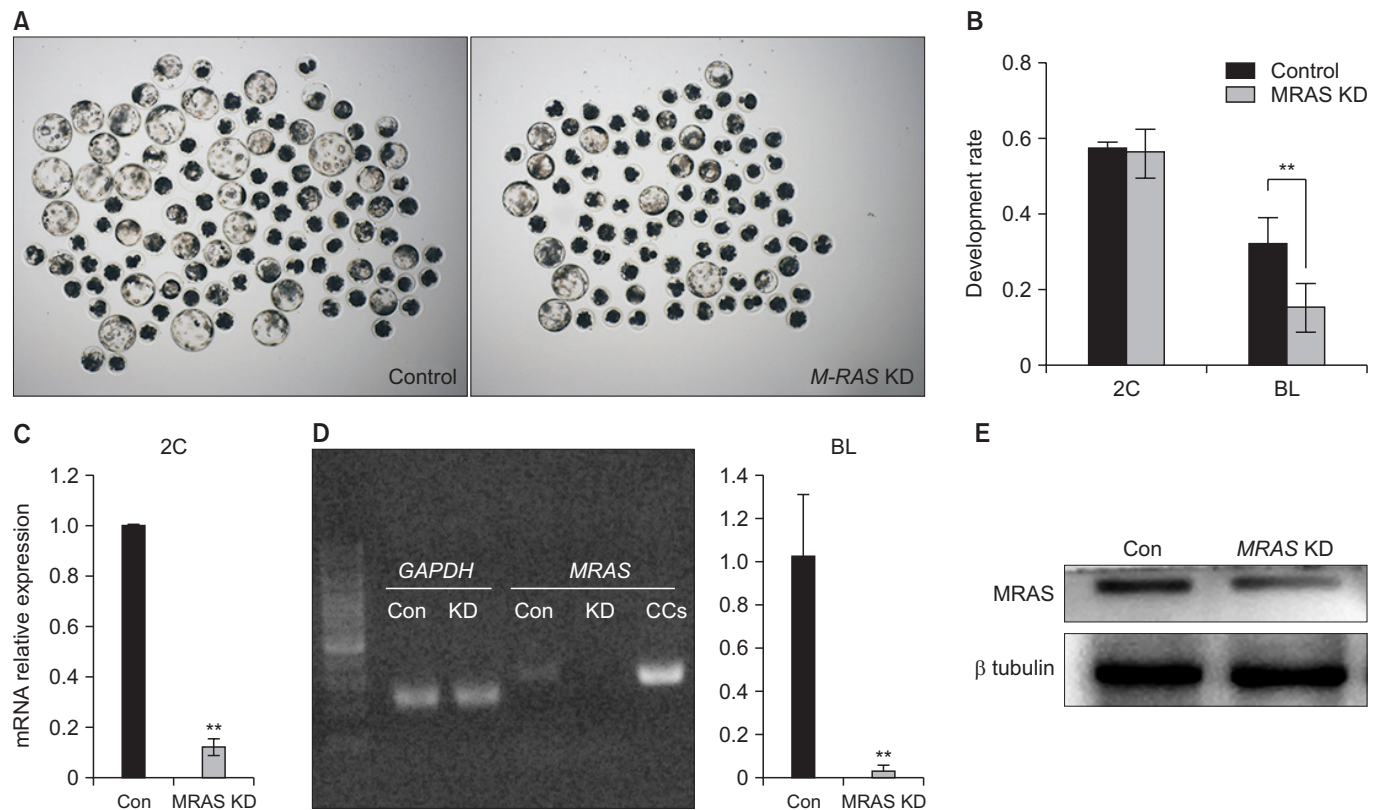
Fig. 2. M-RAS is required for porcine embryo preimplantation development. (A) Image of blastocyst after dsRNAs injection (con, *dseGFP*). (B) Developmental abilities of embryos after dsRNAs injection. (C-D) The mRNA expression level of *M-RAS* performed by qPCR at 2-cell stage (C) and blastocyst stage (D) (con, *dseGFP*; KD, *M-RAS* KD; CCs, cumulus cells). (E) Protein expression of M-RAS after dsRNAs injection performed by western blotting. **$p < 0.01$.
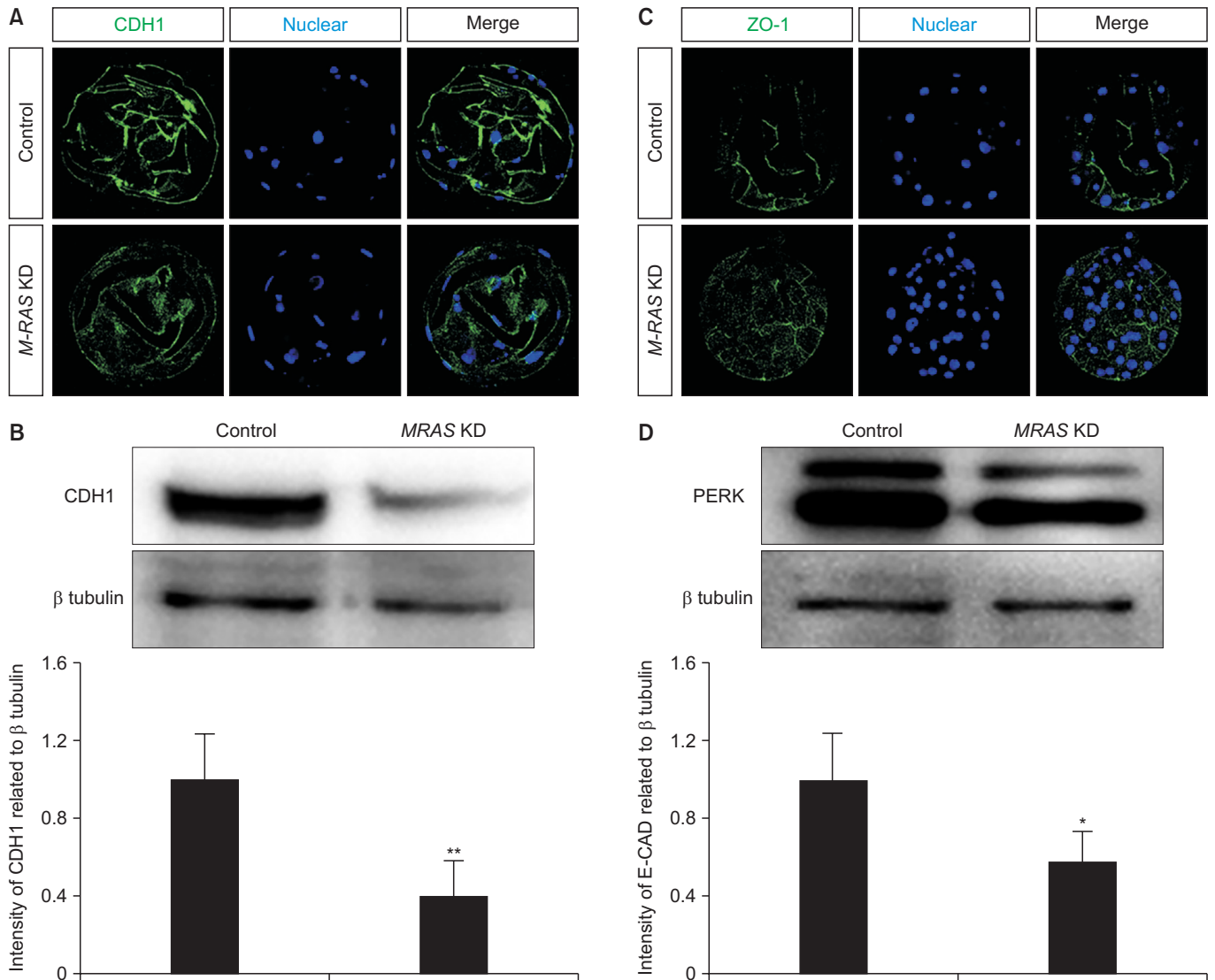
Fig. 3. M-RAS modulates the compaction through the effects on cell-cell junction. (A) Immunofluorescence images of CDH1 at blastocyst stage after dsRNAs injection. (B) Protein expression of CDH1 after dsRNAs injection performed by western blotting. (C) ZO-1 staining was detected on blastocyst embryos after dsRNAs injection. (D) The phosphorylation level of ERK was performed by western blotting. *$p < 0.05$; **$p < 0.01$.

RAS^{Q71L} and injected together with dsRNA of *eGFP* or *M-RAS* to the zygote stage embryos. Compared to control embryos which were expressing activated M-RAS^{Q71L}, blastocyst rate was no significantly change in ds*M-RAS* and M-RAS^{Q71L} injected embryos (Fig. 4A, 4B).

DISCUSSION

M-RAS was first identified as an effector of embryo compaction through its function as a positive regulator of growth factor mediated ERK cascade activation. *M-RAS* has variable expression throughout the body, with high expression in the heart, brain, breast, and ovaries. M-RAS encodes a 208-amino acid, membrane-associated protein which participates in Ras/MAPK pathway signaling.

M-RAS is a member of the Ras super family of proteins, which includes HRAS, KRAS, NRAS, Rit1, and RRAS (Aoki et al., 2005; Schubbert et al., 2006; Cirstea and Kdvorsky, 2010; Aoki et al., 2013; Flex et al., 2014). All Ras proteins belong to a class of small GTPases and cycle between an active GTP-bound state and an inactive, GDP-bound state. In the active, GTP-bound state, Ras proteins undergo a conformational change allowing binding to other effector proteins and participation in signaling cascades.

A

B
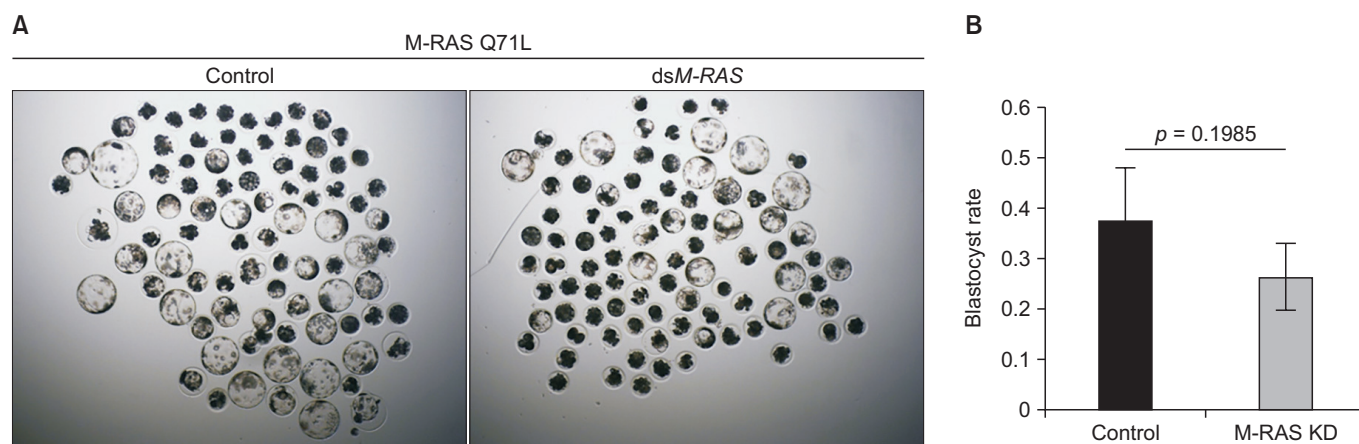
Fig. 4. Activated M-RAS rescued the defect after *M-RAS* KD. (A) Image of blastocyst after RNAs injection. (B) The blastocyst develop-ment rates after RNAs injection (con, *dseGFP + M-RAS*^{Q71L}; *M-RAS* KD, *dsM-RAS + M-RAS*^{Q71L}).

The intrinsic Ras GTPase hydrolyzes Ras-GTP back to Ras-GDP, with the aid of GTPase-activating proteins (GAPs), to terminate activity and signaling.

M-RAS specifically functions within the Ras/MAPK pathway by forming a ternary complex with SHOC2 and PP1. The formation of this complex localizes SHOC2 and PP1 to the cell membrane where SHOC2 acts as a regula-tory subunit of PP1c, targeting it to specifically dephos-phorylate the P-S259 inhibitory site of RAF1 kinase. The removal of this inhibitory phosphate stimulates Raf1 ac-tivity and, thereby, activity of downstream MAPK pathway signaling (Rodriguez-Viciana et al., 2006). M-RAS, there-fore, makes a critical contribution to ERK activation by receptor tyrosine kinases and downstream MAPK signal-ing (Young et al., 2013). Our results suggest that M-RAS regulate the cell adhesion proteins through the activity of this M-RAS/SHOC2/PP1C complex.

Elevated expression of E-cadherin at adhesion junctions is known to increase cell-cell adhesiveness, and bind-ing of p120-catenin to the juxta membrane domain of E-cadherin promotes the surface stability of E-cadherin by preventing its endocytosis and degradation. In particu-lar, we found that E-CADHEREN protein level of *M-RAS* KD embryos was reduced whereas interaction between E-cadherin and p120-catenin previously reported to in-crease cell-cell adhesiveness was increased (Kota et al., 2019). In this study, we demonstrate that M-RAS trans-duces signal specific to the embryonic program including preimplantation development and compaction.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Xiang-Shun Cui, Dongjie Zhou designed the experi-ment. Dongjie Zhou conducted the experiments, analyzed the results, and wrote the article. Yingjie Niu helped with the analyses of the results and figures. Xiang-Shun Cui revised the manuscript.

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